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MINIREVIEWS

Non-celiac gluten sensitivity: All wheat attack is not celiac

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Abstract

Currently, 1% of the United States population holds a diagnosis for celiac disease (CD), however, a more recently recognized and possibly related condition, "non-celiac gluten sensitivity" (NCGS) has been suggested to affect up to 6% of the United States public. While reliable clinical tests for CD exist, diagnosing individuals affected by NCGS is still complicated by the lack of reliable biomarkers and reliance upon a broad set of intestinal and extra intestinal symptoms possibly provoked by gluten. NCGS has been proposed to exhibit an innate immune response activated by gluten and several other wheat proteins. At present, an enormous food industry has developed to supply gluten-free products (GFP) with GFP sales in 2014 approaching $1 billion, with estimations projecting sales to reach $2 billion in the year 2020. The enormous demand for GFP also reflects a popular misconception among consumers that gluten avoidance is part of a healthy lifestyle choice. Features of NCGS and other gluten related disorders (*e.g.*, irritable bowel syndrome) call for a review of current distinctive diagnostic criteria that distinguish each, and identification of biomarkers selective or specific for NCGS. The aim of this paper is to review our current understanding of NCGS, highlighting the remaining challenges and questions which may improve its diagnosis and treatment.

Key words: Non-celiac gluten sensitivity; Celiac disease; Gluten; Wheat; Gluten related disorder; Gluten free diet

Core tip: Non-celiac gluten sensitivity, a less known clinical entity has been estimated to have a prevalence of up to 6% in the United States. This review identifies the pathophysiology of the disease delineating clearly the important components of wheat which play a role in its innate immune response. The updated guidelines on the diagnosis of this disease is discussed here with a bridge to other management strategies apart from the gluten free diet that are now being investigated.

Igbinedion SO, Ansari J, Vasikaran A, Gavins FN, Jordan P, Boktor M, Alexander JS. Non-celiac gluten sensitivity: All wheat attack is not celiac. *World J Gastroenterol* 2017; 23(40): 7201-7210 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i40/7201.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i40.7201

INTRODUCTION

In the past decade, the consumption of gluten free food has become increasingly popular in the Western world. A Gallup poll conducted in July 2015 showed that 20% of Americans opt for a gluten free diet (GFD) while 17% say they avoid gluten free foods[1]. Estimates by the nutrition industry indicate that sales of gluten-free products (GFPs) had a compound annual growth rate of 34% over the five year period ending in 2014, with annual sales totaling close to $1 billion[2]. Current market projections predict sales will again soar to approach $2 billion in the year 2020[3]. Market research by the Mintel group gives a liberal estimation of the sales of products in the gluten-free industry to be $11.6 billion in the year ending 2015, an estimated 136% increase from 2013[4]. The significant increase in the retail sales of GFPs is alarming. However, currently only approxiately 1% of the United States population is diagnosed with celiac disease[5]. Despite this, gluten-free food consumption is clearly on an exponential rise. This enormous allocation of resources reflects self-diagnosis for gluten sensitivity and may reflect an additional condition described as non-celiac gluten sensitivity (NCGS).

The first comprehensively documented case of "gluten sensitivity" (in a non-celiac disease/wheat allergy patient) was reported in 1980[6]. That report described eight adult women suffering from incapacitating abdominal pain and chronic diarrhea which rapidly remitted when a gluten-free diet was initiated, with symptomatic relapse after re-exposure to a gluten containing diet (GCD). Jejunal biopsies performed on these patients, however, failed to identify CD-like histopathology, leading to a consensus description of these patients as having "gluten-sensitive diarrhea without evidence of celiac disease". This was the beginning of the current era of what we now consider NCGS. Since then, many subsequent studies have attempted more extensive descriptions of NCGS to create effective diagnostic criteria and management strategies.

In 2011, an international consensus on NCGS reached an agreement on the definition of NCGS, defining it as a "non-allergic and non-autoimmune condition in which the consumption of gluten can lead to symptoms similar to those seen in CD." NCGS is defined as gluten sensitivity because symptoms are relieved by gluten withdrawal, and re-appear upon introduction to gluten[7]. At least part of the difficulty in handling NCGS patients is that essentially, it is often a diagnosis by exclusion. First, CD and WA need to be excluded as possible diagnoses. Beyond this, NCGS often carries an extensive and relatively broad set of symptoms which affects diverse organ systems[8]. Symptoms of NCGS could be very disabling presenting as gastrointestinal and/or extra-intestinal symptoms[9]. The onset of NCGS symptoms after gluten consumption can also range widely, appearing hours to even days following exposure to a GCD; the timing of resolution in NCGS symptoms may also vary widely.

Given that NCGS presentation could resemble CD or WA, further research into highlighting their hallmark findings is necessary. It is essential now that individuals with gluten-related disorders are identified accurately and managed appropriately. This is important in order to curtail the significant rise in the retail sales of GFPs in the food industry as millions of dollars could be salvaged given the appropriate diagnosis amongst other important reasons. This current review discusses our present understanding of NCGS, its differences and similarities to CD and other gluten-related disorders, etiopathogenesis and management strategies. It is important to recognize that NCGS is a distinct disorder from CD and it is imperative that it is accurately distinguished.

EPIDEMIOLOGY

Despite becoming a more common diagnosis, there is currently a paucity of information on NCGS especially regarding its actual prevalence in the general population. This lack of information reflects the decision of many patients to start GFD after self-diagnosis without any formal clinical testing or management recommendation by their physician. As a result of this ambiguity, the prevalence of NCGS has been reported to vary enormously from 0.6%-6% in Western populations[10-12]. It is unclear how much of the rise in consumption of gluten free foods actually reflect a higher prevalence of NCGS in the population or the choice to adhere to a GFD based on patient's preferences.

Despite the lack of accurate epidemiologic measurements of the actual US prevalence of NCGS, data in 2009-2010 from the National Health and Nutrition Examination Survey (NHANES) showed that of the

7762 NHANES participants who were free of celiac disease, 49 individuals reported a strict adherence to a GFD. This reflects a weighted prevalence of NCGS of only 0.55%[11]. Another more recent evaluation of the NHANES participants from 2009-2012 separated patients into those with CD and those without CD consuming a GFD. This study estimated a prevalence of NCGS of 0.8% in patients without CD who were consuming a GFD[10]. The prevalence in these NHANES studies were estimated based on the assessment that the individuals without CD avoiding gluten fall under the classification of NCGS.

During the period 2004 and 2010, 5896 patients were seen at the Center for Celiac Research at the University of Maryland. Of these, 347 participants met the criteria for diagnosis of "Gluten Sensitivity" reflecting about 6% of the patients seen[12]. These gluten triggered symptoms included abdominal pain (68%), eczema and/or rash (40%); headache (35%); "foggy mind" or difficulty focusing (34%); fatigue (33%); diarrhea (33%); depression (22%); anemia (20%); numbness in the legs, arms or fingers (20%); and joint pain (11%)[12]. The proportion of patients with NCGS in this study extrapolated to the general population estimated an enormous prevalence of NCGS compared with that previously seen in prior studies. Based on our review, the estimation of the prevalence of NCGS ranges widely from 0.6% to 6%, a ten-fold difference. As identified, the prevalence of NCGS in the general population may exceed that of CD - now estimated at 1%[5]. However this appears to be a high estimate based solely on symptomatology.

As NCGS is a relatively "new" entity compared to CD, it is also unclear whether or to what extent genetic or environmental risks might predispose individuals to this disorder. Studies have suggested gender contributions with the female to male prevalence ratio between 3:1[13,14] to 5.4:1[15], indicating a female predominance in NCGS. This may however reflect the observation that female patients are more prone to reporting gluten-related symptoms thus leading to referrals for further work-up as seen in a United Kingdom study in 2014[16]. There has also been a heightened interest in the association of NCGS with other medical conditions. An overlap between irritable bowel syndrome (IBS) and NCGS has been suggested since most of the gastrointestinal symptoms in NCGS resemble IBS (similar Rome III criteria), including abdominal pain/discomfort, bloating, diarrhea and constipation[15]. There is also a debate as to whether a GFD can help symptom resolution in IBS after excluding CD, as clinical trials have shown that GFD can reduce symptoms in patients with diarrhea-predominant IBS (IBS-D)[17]. Although recent research has suggested that a low fermentable oligo-, di-, mono-saccharides and polyols (FODMAPs) diet, regardless of gluten content, improves symptoms in IBS[18]. Given the close symptomatic resemblance between NCGS and IBS, prevalence estimates may be unclear as patients with NCGS could be mislabeled as IBS.

PATHOGENESIS

The pathobiology of NCGS is poorly understood, hotly debated and under intense research (Figure 1). NCGS potentially involves many triggers as are seen in CD and IBS. The initiating event in NCGS mainly involves exposure of gut epithelium to gluten containing foods leading to immune-mediated and/or non-immune mediated responses. Whereas CD shows increased gut permeability, NCGS shows a decreased gut solute permeability with increased epithelial barrier and elevated expression of the tight junctional component claudin-4[19-21]. Due to the lack of evidence for T-cell involvement and the apparent contribution from toll-like receptors (*e.g.,* TLR-2, TLR-1)[21], NCGS may be more of an innate rather than adaptive immune response. Since specific triggers are not clear in NCGS, this could make NCGS a relatively different pathobiological entity from other gluten related conditions like CD and WA[20,22]. Changes in the gut microbiome (dysbiosis) produced by gluten consumption[23] may also influence NCGS. This predominantly innate TLR based systemic response potentially incited by changes in microbiota was further supported by the increased levels of LBP (lipopolysaccharide binding protein) and soluble CD14 proteins[24]. Additionally there is evidence for NCGS developing in individuals with some genetic predispositions (*e.g.,* HLA-DR4, HLA-DR2)[21], which is higher than general population but lower than patients with CD who have a strong genetic component. However, currently this association does not appear to be as clear[21,22].

Gluten, the main inciting agent in CD also has a significant role in NCGS pathology[25]. In addition to gluten, several other food-derived stimuli have been shown to also be important in the etiopathogenesis of NCGS include alpha amylase/trypsin inhibitors (ATIs)[26], FODMAPS[18] and other short chain fructans. Gliadin is the alcohol soluble fraction of gluten, and contains the bulk of the "immunogenic" components[27]. Gliadin has the ability to promote an inflammatory response primarily in the upper gastrointestinal tract. Gliadin has shown to trigger both innate and adaptive response via recruitment of CD4+ T cells and increasing expression of interleukin-15 by enterocytes[28]. The effects of gliadin-induced enteric responses are not however completely seen in NCGS with innate immune response apparently playing a bigger role than adaptive response[21,29]. The heterogeneous behavior of NCGS in comparison to CD can be explained by different motifs of gliadin protein exerting different responses with alpha gliadin peptides having a significant role in innate adaptive response, and probably expressed more in NCGS individuals[30]. NCGS individuals also have increased levels of TLR2 and to a lesser extent TLR1 expression, increased

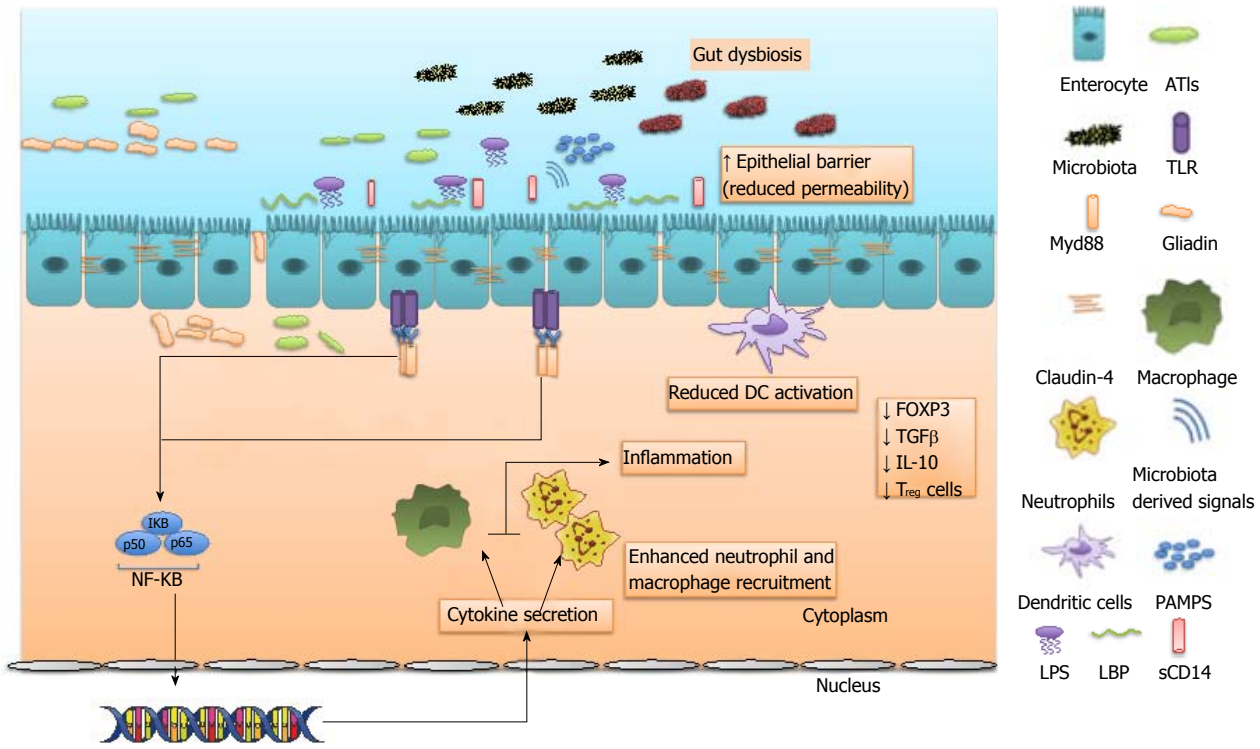

Figure 1 Toll like receptors act as primary sensors to gliadin and non-gluten proteins like alpha amylase/trypsin inhibitors, and microbiota derived signals in non-celiac gluten sensitivity. Dysregulated microbiota also release lipopolysaccharide (LPS) in circulation which result in production of LPS-binding protein (LBP) from gastrointestinal and hepatic epithelial cells and soluble CD14 (sCD14) from monocytes/macrophages. This leads to activation of transcription factor nuclear factor-kappaB (NF-κB), which controls the expression of an array of inflammatory cytokine genes resulting in recruitment of neutrophils and macrophages. In contrast to CD, there is limited dendritic cell activation, resulting in reduced expression of Treg cells, reduced FOXP3, TGFβ, IL-10. All these factors with increased expression of claudin-4 culminate in reduced intestinal permeability and increased epithelial barrier. Gut dysbiosis can also promote inflammation through expansion of pro-inflammatory pathobionts. TLRs: Toll like receptors.

number of α and β intraepithelial lymphocytes, and reduced number of T-regulatory cells[21]. Solute barrier function remains largely unchanged in NCGS, which is consistent with fairly normal adaptive immune response, normal intestinal permeability and expression of tight junction proteins[14,21]. Gluten's opioid like effect on the intestinal transit time and nocebo effect has also been studied and may have an important role on the overall pathology and clinical presentation of the disease[31].

In the recent years, other non-gluten components of wheat have also been shown to play a major role. ATIs in particular, have been implicated in NCGS pathology. The role of ATIs in mounting an immunological response has been shown in animal and human research models and is believed to be an important oral antigen both in CD as well as in NCGS[26]. This predominantly innate immune response involves macrophages, neutrophils and intestinal dendritic cells *via* activation of the toll like receptor complexes[21,26]. Their less tendency to affect the overall gut morphology falls well in line with NCGS pathology.

The gut microbiota has been shown to play a significant role in the pathogenesis of CD, in which gut dysbiosis precedes the activation of inflammatory and immune mediated pathways[32]. The role of gut dysbiosis has also recently been emphasized in NCGS pathobiology and might help to explain both intestinal and non-intestinal manifestations in the disease by upregulating gut and systemic inflammation[33].

CLINICAL PRESENTATION

The symptoms of NCGS can occur within hours to days following exposure to gluten-containing diet and can then dissipate upon withdrawal of gluten. Most of the presentation resembles CD, the main similarities and differences are presented in Table 1. Frequent symptoms reported in NCGS include bloating (87%), abdominal pain (83%), epigastric pain (52%), diarrhea (50%), and constipation (24%)[8]. Extra-intestinal manifestations are also reported which include; lack of well-being (68%), tiredness (64%), headache (54%), anxiety (39%), "foggy mind" or difficulty focusing (38%)[8].

Some of the other less commonly reported symptoms include weight loss, depression and skin rash[8]. Unlike CD, nutrient deficiencies such as iron, vitamin D and vitamin B 12 deficiencies are not significantly seen in NCGS[34]. NCGS also has a lesser association with autoimmune disorders when compared with CD[14,35].

Table 1 Comparison of gluten related disorders[34,51-72]

	NCGS	CD	IBS	WA
Colonic manifestations	Diarrhea Abdominal pain Bloating Constipation Nausea Vomiting	Diarrhea Abdominal pain Bloating Constipation Nausea Vomiting	Diarrhea Abdominal pain Bloating Constipation Mucous Discharge Dyspepsia Early satiety	Diarrhea Abdominal pain Bloating Constipation Nausea Vomiting
Extra-colonic manifestations	Headache Migraine Foggy mind Fatigue Eczema like rash Myositis Numbness Psychological changes	Anemia Osteoporosis Neurological disturbances Pubertal delay Dermatitis herpetiformis Foggy mind Lymphoma	Major depression Anxiety Somatoform disorder Fibromyalgia Temporomandibular disorder Dyspareunia	Hives Angioedema Asthma Cough Post Nasal Drip Eczema
Symptom Onset	Hours to Days	Hours to months	Unclear relation to gluten ingestion	Minutes to Hours
Cytomorphology	Small bowel intraepithelial lymphocytosis (Marsh 0-1)	Villous atrophy with crypt hyperplasia	Normal	Normal
Biomarkers	IgG-AGA Zonulin LBP sCD14	IL-17(A) TCR-γδ IELs IgA tTGA IgA EMA CD3 + IELS Zonulin	TNF- α IL-6 IL-8	IgE antibodies to wheat protein
Immunophenotype	HLA- DQ2 and DQ8 genotypes in 50% patients	HLA- DQ2 and DQ8 genotypes in 80% patients	Increase in: B cells expressing IgG or co-stimulatory molecules CD80 or CD86 T cells expressing b7 + HLADR+ and CD69+	Transforming growth factor-b (TGFb) mutations have been associated with higher rates of allergic disease
Diagnosis	See figure 2	*Serologic testing followed by small bowel biopsy	Rome III diagnostic criteria	Skin prick test Presence of serum IGE antibodies to wheat protein
Management	GFD Probiotics AN-PEP	GFD	Symptomatic treatment and elimination of stressors	GFD Subcutaneous epinephrine for any acute episodes
ICD codes	K90.41	K90.0	K58	K52.29

*4 out of 5 of the following: Typical symptoms of CD, Serum CD IgA class auto antibodies at high titer, HLA-DQ2 and/or HLA-DQ8 genotypes, Celiac enteropathy found on small bowel biopsy, Response to a GFD(14).

DIAGNOSIS

NCGS should be considered in patients with the history and physical examination indicative of a gluten related disorder. The clinical presentation of NCGS, as outlined earlier, should include the remission of symptoms upon withdrawal of gluten. The proposed diagnostic work-up includes 3 vital steps.

The 1st step in an NCGS diagnosis is the exclusion of CD and WA. The patient needs to be on a GCD for a period of 6-wk. Several tests should be performed during this period to exclude WA; wheat specific IgE and skin prick test, and CD; IgA-tTG, IgG-DGP and IgA-EMA. If highly suspicious of CD, the physician can proceed with upper endoscopy for duodenal biopsy, although this should not be routine testing for every patient. If the biopsy results indicate less CD probability (Marsh 0-1) then the clinician can proceed to the next step[5]. Of note, these tests can be tailored by the clinician based on the patients' presenting symptoms. The physician can bypass testing of CD or WA and proceed with the work-up for NCGS if patients' history and physical is not suggestive of the condition.

The 2nd step consists of starting the patient on a GFD for a period of at least 6 wk and monitoring for symptom response. This symptom response is evaluated using the gastrointestinal symptom rating scale (GSRS) and a numerical rating scale (NRS). GSRS is used to identify the symptoms while the NRS is used to quantify the symptoms, weekly from week 0 (baseline) till week 6[36]. A symptomatic response to the GFD is defined as a decrease in 30% of the baseline score with no worsening of other symptoms in at least 50% of the observation time (3 wk)[36]. If the patient fails to show an improvement in symptoms in 6 wk upon resumption of GFD, the diagnosis of NCGS is ruled out and other diagnoses such as IBS and other functional bowel disorders need to be explored.

The 3rd step required to confirm the diagnosis

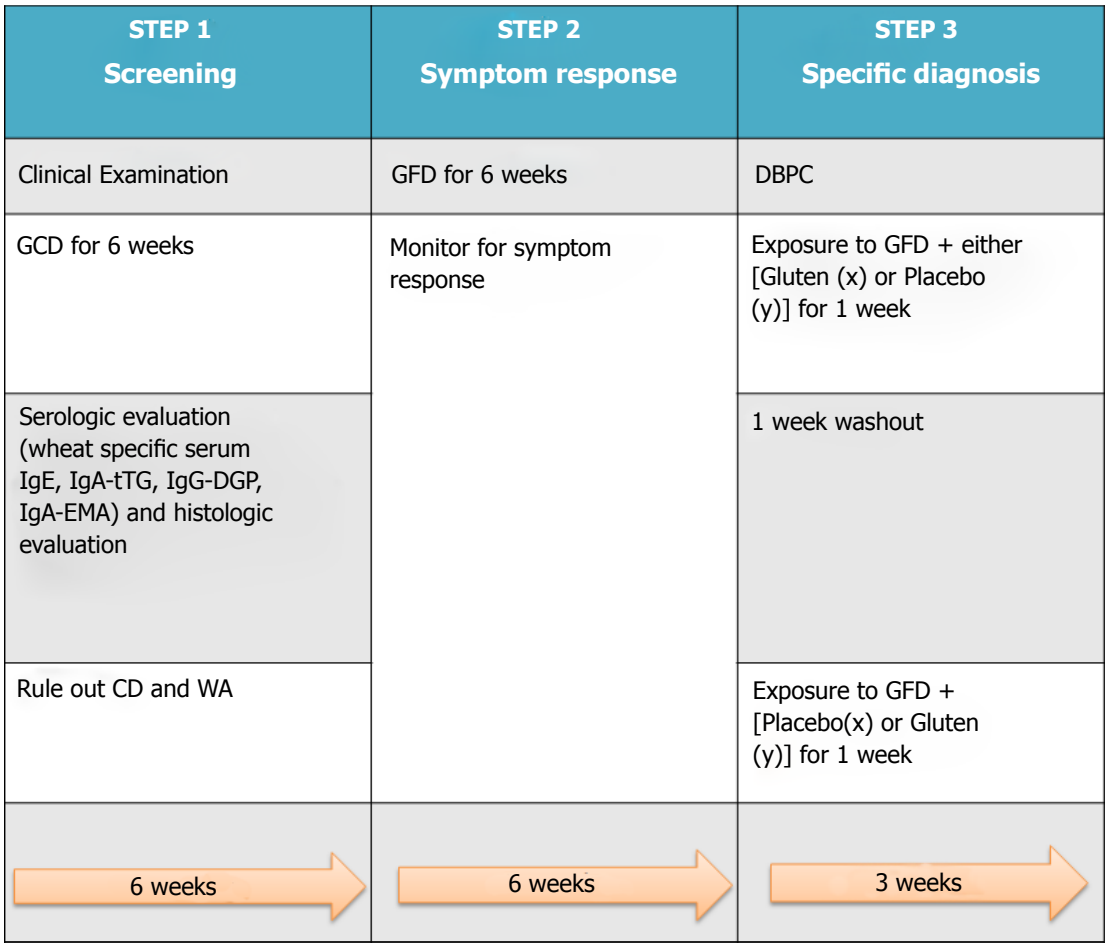

Figure 2 Proposed diagnostic algorithm for non-celiac gluten sensitivity. GCD: Gluten containing diet; GFD: Gluten free diet; CD: Celiac disease.

of NCGS in patients who respond to treatment with the GFD involves the re-introduction of the GCD. This is performed because a potential nocebo effect (test subjects believe there is a side effect and they experience them as a result of this) secondary to the exposure to gluten in step 1 cannot be ruled out[37]. In this step, the patient is randomly assigned into test group x or y. The patient is exposed to either GFD + placebo (x) or GFD + gluten (y) for a week. A 1-week washout period of strict GFD is observed, followed by the crossover to GFD + gluten (x) or GFD + placebo (y)[36]. A variation of a 30% symptomatic improvement on introduction of the diet free from gluten in test group y or a variation of 30% symptomatic appearance on introduction of the GCD in test group x (assessed by the modified GSRS and NRS scales) indicates a positive result. Below this 30% value is considered a negative result[36]. This 30% mark is not a scientific estimate but is rather a fair estimate of an appropriate response[36]. This threshold although used in the Salerno experts' criteria needs scientific validation.

In step 3, the placebo added should be gluten-free and must look very similar to the gluten content so that both the clinician and the patient are unaware of the difference between the gluten and the placebo. The dose of gluten to be used for the challenge should be close to the average daily intake of gluten (10-15 g)[38]. The dose should also be easy to mix with the gluten carrying package. The package carrying gluten can be in any form; bread, muffin. This package should also consist of ATIs as well. The suggested prepared package should contain 10-15 *g* of gluten and at least 0.3 g of ATIs. The package must also be free of FODMAPs[36]. The proposed diagnostic algorithm for NCGS is shown in Figure 2.

MANAGEMENT

Currently, the management of NCGS involves a multi-disciplinary approach. The patient is started on a GFD with the help of a registered dietician[14]. A gluten free diet usually helps resolve the intestinal and extra-intestinal symptoms of NCGS. The recommendation is to continue adherence to GFD for lifetime. There have been few if any studies showing whether or not reintroduction of gluten into the diet after a prolonged period of being asymptomatic will cause reversion into previous symptoms[39].

The importance of gluten has been outlined in recent research. Gluten consumption has been associated with a decreased risk of developing type 2 diabetes Mellitus and also a decreased risk of coronary heart disease[40,41]. Understanding these benefits of gluten beyond nutrition reflects the need for caution in the use of GFDs in patients without a proven diagnosis of NCGS. Although GFD seems to be the most important management strategy, it should be suggested only after careful examination and a definite diagnosis of NCGS.

Several ongoing clinical trials are now examining other possible treatment methods for NCGS besides the GFD. One study looks at the use of probiotics for alleviation of NCGS symptoms while maintaining a gluten free diet with introduction of gluten in a controlled environment. This randomized DBPC study evaluates NCGS patients' response to a gluten source (two slices of bread each day for 7 d) whilst getting probiotics (Bifidobacterium longum ES1 - a patented probiotic bacterial strain) or a placebo during that time[42]. Theoretically the use of probiotics could result in resolving gut dysbiosis by reintroducing gut flora, with reduction in both gut and systemic inflammation but this needs to be clinically examined. Probiotic use has long been a source of debate amongst the medical community regarding its exact role as far as therapeutic option, it's essential to see if it can be proven to have an ultimate benefit.

Another treatment option under investigation is the use of the enzyme Aspergillus niger prolyl endoprotease (AN-PEP). It has been reported in previous studies that the AN-PEP enzyme significantly enhanced gluten digestion in the stomach of healthy volunteers[43]. A randomized placebo controlled clinical trial is ongoing looking at the effect of AN-PEP on gluten degradation in our target population; gluten sensitive individuals[44]. If we can apply AN-PEP as a means of degrading gluten before it can affect the human gut, it would achieve a major goal as patients can enjoy foods without worrying about diet restriction, worrisome symptoms or soaring food costs.

The use of ancient diploid wheat species (*e.g.,* Triticum monococcum ssp.) as compared with common wheat as a new treatment strategy is gaining ground[45]. Gianfrani *et al*[45]. demonstrated the low toxicity of these wheat proteins in celiac disease patients following in vitro gastrointestinal digestion. Newer studies have shown the distinction between these older wheat variants and modern wheat[46]. Older wheat variants were shown to have lower bioactivity and a lower concentration of ATIs in comparison with modern wheat[46]. Mechanistically, the modern wheats were observed to have high levels of TLR-4-activating ATIs which are highly resistant to intestinal proteolysis[46]. The application of these studies to favor the use of ancient wheat variants in the NCGS population would be a major step in the advancement of treatment strategies in this disease.

Mayer and Tillisch highlighted the role of the brain-gut axis in abdominal pain syndromes[47]. Recent literature has attempted to further describe how various clinical manifestations seen in IBS and also syndromes like NCGS could be viewed as a dysregulation in the signaling pathway that involves the gut, the enteric nervous system and the central nervous system[33,47,48]. Newer therapeutic options currently being hypothesized in NCGS include the use of vagus nerve stimulation and corticotropin releasing factor (CRF) antagonists to normalize any possible gut brain dysregulation[33,48]. The use of vagus nerve stimulation is based on its positive effects on many inflammatory diseases of vagus-innervated organs (including the GI tract) which thus suggest a case for its application in NCGS[33]. The use of CRF antagonists (seen to induce significant signal reductions in emotional control centers of the brain[23]) is based on the theory that NCGS often has a chronic anxiety or stress related component associated with the anticipation of abdominal pain. Consequently controlling the emotional impact of NCGS may improve its symptoms. The several on-going studies/ theories in this area hopefully can shed light on the neuropsychiatric therapeutic components of NCGS.

With the release of the updated ICD 10 coding system, NCGS, along with WA, IBS and CD each have their own codes despite lingering ambiguities about NCGS diagnosis/management. NCGS's code is K90.41 and it falls under the categories "enteropathy" (code K63.9) and gluten sensitivity (Code K90.41). Although treatment strategies in NCGS are still evolving, it is important to note that NCGS is a specific and billable diagnosis for reimbursement purposes[49].

CONCLUSION

A recent study involving gastroenterologists showed that although most of these specialist physicians were able to "identify" NCGS and prescribe GFD for its treatment, 44% of them were unable to define or agree on a uniform definition[50]. This highlights the difficulty in making the diagnosis of NCGS based on a specific clinical criterion. NCGS warrants further investigation and updated diagnostic and therapeutic strategies.

As we currently stand, the NCGS diagnostic process is arduous for the patient, requiring them to be on a GCD diet through several parts of the process. This could cause distress by eliciting unpleasant symptoms. Thus, an alternative diagnostic approach without subjecting the patient to the stressor would be a significant clinical advancement. Some biomarkers identified to aid in the diagnosis of NCGS are IgG-AGA[15] and Zonulin[51]. However these markers are evidently increased in CD as well[15,51]. Establishing NCGS specific biomarkers is imminent to also monitor for both the resolution of the disease and compliance

of the patient to the GFD. Whether NCGS is a transient disease or a chronic disease warranting lifetime diet restriction still needs to be investigated.

The lack of information with regards to the prevalence of NCGS in the developing world is outstanding. Most of the studies being done on this condition are focused on the population, which includes the affluent communities of Europe and North America. Adhering to the GFD is expensive and not easily affordable, the likelihood of a low-income patient self-diagnosing as NCGS and going on a GFD is very minimal. This warrants the question as to if NCGS is really a condition more prevalent in the affluent as they are much more conscious of their diet intake as well as informed.

New and evolving science around gluten related disorders has led to better understanding of the disease process, making it easier for the clinicians to set specific management guidelines. Prospective clinical trials in a diverse population is warranted to better investigate the etiology and progression of this disease. There is still a role of further research to better understand NCGS as a medical condition.

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ORIGINAL ARTICLE

Basic Study

Glucagon-like peptide-2 modulates the nitrergic neurotransmission in strips from the mouse gastric fundus

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Abstract

AIM

To investigate whether glucagon-like peptide-2 (GLP-2) influences the neurally-induced responses in gastric strips from mice, since no data are available.

METHODS

For functional experiments, gastric fundal strips were mounted in organ baths containing Krebs-Henseleit solution. Mechanical responses were recorded *via* force-displacement transducers, which were coupled to a polygraph for continuous recording of isometric tension. Electrical field stimulation (EFS) was applied via two platinum wire rings through which the preparation

was threaded. The effects of GLP-2 (2 and 20 nmol/L) were evaluated on the neurally-induced contractile and relaxant responses elicited by EFS. Neuronal nitric oxide synthase (nNOS) enzyme was evaluated by immunohistochemistry.

RESULTS

In the functional experiments, electrical field stimulation (EFS, 4-16 Hz) induced tetrodotoxin (TTX)-sensitive contractile responses, which were reduced in amplitude by GLP-2 ($P < 0.05$). In the presence of the nitric oxide (NO) synthesis inhibitor L-NNA, GLP-2 no longer influenced the neurally-evoked contractile responses ($P > 0.05$). The direct smooth muscle response to methacholine was not influenced by GLP-2 ($P > 0.05$). In the presence of guanethidine and carbachol, the addition of GLP-2 to the bath medium evoked TTX-sensitive relaxant responses that were unaffected by L-NNA ($P > 0.05$). EFS induced a fast NO-mediated relaxation, whose amplitude was enhanced in the presence of the hormone ($P < 0.05$). Immunohistochemical experiments showed a significant increase ($P < 0.05$) in nNOS immunoreactivity in the nerve structures after GLP-2 exposure.

CONCLUSION

The results demonstrate that in gastric fundal strips, GLP-2 influences the amplitude of neurally-induced responses through the modulation of the nitrergic neurotransmission and increases nNOS expression.

Key words: Immunohistochemistry; Gastric motility; Glucagon-like peptide-2; Neuronal nitric oxide synthase; Non-adrenergic non-cholinergic neurotransmission

Core tip: The results of the present study demonstrate for the first time that, in strips from the mouse gastric fundus, glucagon-like peptide-2 (GLP-2) depresses the amplitude of the neurally-induced contractile responses and enhances the amplitude of the relaxant ones through the modulation of the nitrergic neurotransmission. GLP-2 also increases neuronal nitric oxide synthase immunoreactivity in the nerve structures. All these inhibitory effects might contribute to gastric relaxation, thus increasing the organ capacity. Since gastric distension represents a peripheral satiety signal from a physiological point of view, it could be speculated that the relaxant effects of GLP-2 might concur to suppress feeding behavior in rodents.

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INTRODUCTION

Glucagon-like peptide-2 (GLP-2), produced by enteroendocrine "L" cells[1,2], plays a role in the regulation of metabolism and energy homeostasis[3,4], and has been reported to influence many gastrointestinal functions[5,6]. GLP-2 acts on G protein-coupled receptors (GLP-2r)[7-9] largely distributed in the gastrointestinal tract[2,10]. In the gut, GLP-2r has been revealed in several cell types, including excitatory and inhibitory neurons[10-13]. In this view, GLP-2 has been demonstrated to exert a neuromodulatory action either by inhibiting intestinal transit *in vivo*[14] *or* by reducing colonic and duodenal cholinergic muscle contractions *in vitro*[10,11].

Concerning gastric motility, contrasting results have been reported in humans regarding the ability of GLP-2 to slow emptying[15-17]. *In vivo* experiments in pigs have shown that the hormone reduced antral motility through central nervous system mechanisms[18]. In addition to the central actions, experiments carried out *in vitro* have demonstrated that GLP-2 exerts peripheral effects on either gastric strips or isolated whole stomach from mice[19]. Particularly, the hormone has been shown to relax gastric smooth muscle acting indirectly through the stimulation of vasoactive intestinal polypeptide (VIP) release from myenteric neurons with site-related effects, exerting its action in the fundal region only[19]. However, to our knowledge, no data have been reported about the influence of GLP-2 on the neurally-induced gastric responses elicited by electrical stimulation in *in vitro* preparations. For the above reason, we presently investigated whether GLP-2 influences the neurally-induced contractile and relaxant responses in gastric fundal strips from mice. Along with VIP[20], nitric oxide (NO)[21] is considered one of the major non-adrenergic, non-cholinergic (NANC) inhibitory neurotransmitters responsible for the proximal stomach relaxation; thus, we also evaluated the effects of GLP-2 on both the nitrergic neurotransmission and the neuronal nitric oxide (nNOS) expression.

MATERIALS AND METHODS

Animals

Experiments were performed on 8- to 12-week-old female mice (CD1 Swiss strain; Envigo, Udine, Italy). The animals were kept under the following conditions: 12-h light/12-h dark photoperiod, constant temperature (21 ± 1 ℃), and standard laboratory feed. The mice were killed by prompt cervical dislocation to minimize animal suffering.

Mechanical experiments

As formerly reported[22-25], the stomach was promptly removed, and two full-thickness longitudinal strips (2 mm × 10 mm) were cut from each gastric fundal region. One end of each strip was tied to a platinum

rod, while the other was connected to a force displacement transducer (Grass model FT03, Quincy, MA, United States) by a silk thread for continuous recording of isometric tension. The transducer was coupled to a polygraph (Grass model 7K, Quincy, MA, United States). Preparations were mounted in double-jacketed organ baths (5 mL) containing Krebs-Henseleit solution, gassed with a 95% O_2-5% CO_2 mixture, of the following composition (mmol/L): NaCl 118, KCl 4.7, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25, $CaCl_2$ 2.5 and glucose 10 (pH 7.4). The temperature of the Krebs-Henseleit solution in the organ baths was maintained at 37 ± 0.5 ℃.

Electrical field stimulation (EFS) was applied by means of two platinum wire rings (2 mm diameter, 5 mm apart) through which the strip was threaded. Electrical pulses (rectangular waves, 80 V, 4-16 Hz, 0.5 ms, for 15 s) were delivered by a Grass model S8 stimulator. Preparations were allowed to equilibrate for 1 h under an initial load of 0.8 g: during this period, prolonged washes with Krebs-Henseleit solution were performed to avoid the accumulation of metabolites in the organ baths.

Experimental protocol

The influence of GLP-2 (2 nmol/L and 20 nmol/L) on either neurally-induced or direct smooth muscle contractile responses was firstly investigated. EFS (4-16Hz) was also performed in the presence of 1 μmol/L tetrodotoxin (TTX) or 200 μmol/L N^G-nitro-L-arginine (L-NNA). Elapsed time between two subsequent electrical stimulations depended on the time taken by the strip tension to regain baseline. The interval between two subsequent direct muscular responses to methacholine (2 μmol/L) was at least 30 min. During this period prolonged washes with Krebs-Henseleit solution were performed. To verify the viability of the strips, contractions to methacholine (2 μmol/L) were evaluated at the beginning and at the end of each experiment.

In a second series of experiments, the effects of GLP-2 (2 nmol/L and 20 nmol/L) on the EFS-induced NANC relaxant responses were investigated. For this purpose, functional experiments were carried out in the presence of 1 μmol/L guanethidine sulphate and 1 μmol/L carbachol (CCh) to rule out adrenergic and cholinergic influences, respectively. Elapsed time between two subsequent additions of CCh was at least 15 min, during which prolonged washes with Krebs-Henseleit solution were performed. When a stable plateau phase of the contraction evoked by CCh was reached, EFS or drugs were applied. The response to GLP-2 (2 nmol/L and 20 nmol/L) was also tested 20 min following the addition of TTX or the NO synthesis inhibitor L-NNA to the bath medium. Immunohistochemical experiments were performed in parallel.

Tissue sampling for morphological studies

Two gastric fundus specimens per animal (6 mice) were taken, which were prepared as reported above and handled as follows. The specimens were stabilized in 5 mL organ baths containing Krebs-Henseleit solution; at the end of the steadying period, one half of the specimens was exposed to GLP-2 for 30 min, which was added directly to the bath reaching 20 nmol/L concentration. The second half was maintained in Krebs solution and used as a control. At the end of the exposure time, the specimens were transferred into a solution of paraformaldehyde (4%) dissolved in phosphate-buffered saline (0.1 mol/L, PBS, pH 7.4), maintained overnight (ON) at 4 ℃. The day after, the specimens were dehydrated, cleared in xylene and embedded in paraffin. The rotary microtome (MR2, Boeckeler Instruments Inc. Tucson, Arizona, United States) allowed to cut 5 μm thick sections, which were collected on slides suitable to histological and immunofluorescent staining.

Histological and immunofluorescent staining

The sections were deparaffinized through consecutive passages in xylene and in decreasing ethanol concentration solutions up to the final step in distilled water. Some sections were H&E stained to visualize the integrity of the muscle wall. Some others underwent the immunofluorescent procedure. For antigen retrieval the sections were transferred for 20 min in a EDTA 1 mmol/L, pH 9.0 + tris buffer 10 mmol/L, maintained at the temperature of 90-92 ℃; then they were allowed to cool in the same solution. After few washes in 0.1 mol/L PBS, the section were first incubated for 20 min with bovine serum albumin (1.5%, Sigma Aldrich, Milan, Italy) diluted in PBS and then ON at 4 ℃ in the presence of the nNOS antibody (rabbit polyclonal; 1:1500; Chemicon, Temecula, CA, United States). After several washes, the appropriate fluorochrome-conjugated secondary antibody (goat anti-rabbit, AlexaFluor 488; 1:333; Jackson ImmunoResearch, West Grove, PA, United States) diluted in PBS were applied for 2 h at RT. Finally, the sections were counterstained with Bisbenzimide Hoechst Trihydrochloride (BHT; Sigma Aldrich srl, Milan, Italy) to visualize the cell nuclei, and set in the aqueous medium (Immu-Mount, Thermo Scientific, Waltham, MA, United States). To verify the specificity of the primary antibody, the procedure has been performed omitting the antibody. An epifluorescence microscope (Zeiss Axioskop microscope, Oberkochen, Germany) allowed to visualize the fluorescent labelling. A digital camera (Leica DFC310 FX 1.4-megapixel camera, Leica Microsystems, Mannheim, Germany) coupled to an image acquisition software (LAS V3.8, Leica Microsystems, Germany) allowed to capture and archive the related images.

Quantitative analysis

The quantitative analysis of the labelled structures was done acquiring digitized images for the whole length of the section (1 section/animal; 6 animals/group) using 20 x objective and the acquisition of overlapping images was avoided. The total number of images obtained was comparable between control and GLP-2 treated sections as confirmed by the similar length of the fundal specimens. ImageJ (NIH, Bethesda, MD, United States) was used to analyze the fluorescent labeling in the acquired images. The area of the labelled structures in the muscle wall was quantified and expressed as fraction of the total image area x 100. The labelling was converted to a binary image; the threshold value was set in control images and maintained in treated ones. Two researchers counted in blind the myenteric labelled neurons in each section (1 section/mouse; 6 mice/group) and the result was proposed as neuron number per mm.

Drugs

The following drugs were used: guanethidine sulphate, CCh, GLP-2, methacholine, L-NNA, TTX. All of the above drugs were purchased from Sigma Chemical (St. Louis, MO, United States) with the exception of GLP-2 that was obtained from Tocris Bioscience (Bristol, United Kingdom). Solutions were freshly prepared, except for TTX, for which a stock solution was stored at -20 ℃. Drug concentrations are referred as final bath concentrations and are in the range of those previously reported to be effective in *in vitro* gastric preparations[26-28]. Particularly, the chosen doses of GLP-2 employed are those reported able to cause gastric relaxation in mice[19].

Data analysis and statistical tests

Amplitude of contractions is given as percentage of the muscular contraction elicited by methacholine (2 μmol/L) taken as 100% or as absolute values (g). Amplitude of responses to methacholine was measured 30 s after a stable plateau phase was reached. Relaxant responses are calculated as percentage decrease relative to the muscular tension induced by CCh (1 μmol/L) just before obtaining relaxations. With respect to pre-stimulus level, amplitude values of EFS-induced fast relaxations refer to the maximal peak obtained during the stimulation period and amplitude values of EFS-induced sustained relaxations refer to the maximal peak, obtained following the stimulation period. The statistical significance was evaluated by paired or unpaired Student *t*-test to compare two experimental groups or one-way ANOVA followed by Newman-Keuls posttest when more than two groups were compared (Prism 3.0; GraphPad Software, San Diego, CA, United States). Differences were considered significant when $P \leq 0.05$. Results are mean ± SE. The number of muscle strip preparations is indicated by *n* in the results.

RESULTS

Functional experiments

At basal tension ($n = 28$), EFS (4-16 Hz) induced contractile responses whose amplitude increased by increasing the stimulation frequency (Figure 1A and B). These responses were abolished by 1 μmol/L TTX ($n = 4$) indicating their nervous nature. The addition of GLP-2 (2 nmol/L or 20 nmol/L) to the bath medium ($n = 16$), other than causing a long-lasting decay of the basal tension (0.15 ± 0.02 and 0.20 ± 0.03 g at 2 nmol/L and 20 nmol/L, respectively), decreased the amplitude of the excitatory responses elicited by EFS (Figure 1A and B).

In the presence of the NO synthesis inhibitor L-NNA (200 μmol/L) the amplitude of the EFS-induced contractions was enhanced (Figure 2A and B). The effects of L-NNA, already appreciable 10 min after its addition to the bath medium, persisted up to 1 h (longer time not observed). In the presence of L-NNA (200 μmol/L), GLP-2 (2 nmol/L or 20 nmol/L) still evoked the decay of the basal tension (Figure 2A and B) but no longer depressed the amplitude of the EFS-induced contractile responses even at the highest concentration employed (Figure 2A and B).

Methacholine (2 μmol/L) caused a sustained contraction that was TTX-insensitive and reached a plateau phase (mean amplitude 0.98 ± 0.3 g) that persisted until washout. GLP-2 (2 nmol/L or 20 nmol/L) did not influence the response to methacholine (mean amplitude 1.02 ± 0.2 g, $P > 0.05$). No significant differences ($P > 0.05$) in amplitude of the contractile response to methacholine (2 μmol/L) were observed between that obtained at the end or at the beginning of the experiments, thus indicating that the viability of the preparations was not compromised.

The effects of GLP-2 were also tested on the neurally-induced relaxant responses. In the presence of guanethidine, addition of CCh (1 μmol/L) to the bath medium ($n = 28$) caused a rapidly arising contraction (mean amplitude 1.2 ± 0.2 g), which persisted until washout. In CCh precontracted strips, GLP-2 (2 nmol/L or 20 nmol/L) elicited ($n = 12$) dose-dependent slow and long-lasting relaxations (Figure 3) that were abolished by 1 μmol/L TTX ($n = 4$; data not shown) and unaffected ($P > 0.05$) by L-NNA (200 μmol/L) ($n = 4$) (mean amplitude 27 ± 1.5% *vs* 25 ± 1.9% and 47 ± 1.7% *vs* 50 ± 1.6% at 2 nmol/L and 20 nmol/L, respectively).

In CCh precontracted strips, EFS (4-16 Hz) elicited ($n = 16$) a fast relaxant response followed, at the higher stimulation frequencies, by a slow relaxation (Figure 4A). As previously observed in the mouse gastric fundus[23], the fast component of the response was abolished by 200 μmol/L L-NNA ($n = 4$), indicating its nitrergic nature. In the presence of GLP-2 (2 nmol/L or 20 nmol/L), the amplitude of the EFS-induced fast nitrergic relaxation was increased ($P < 0.05$; Figure

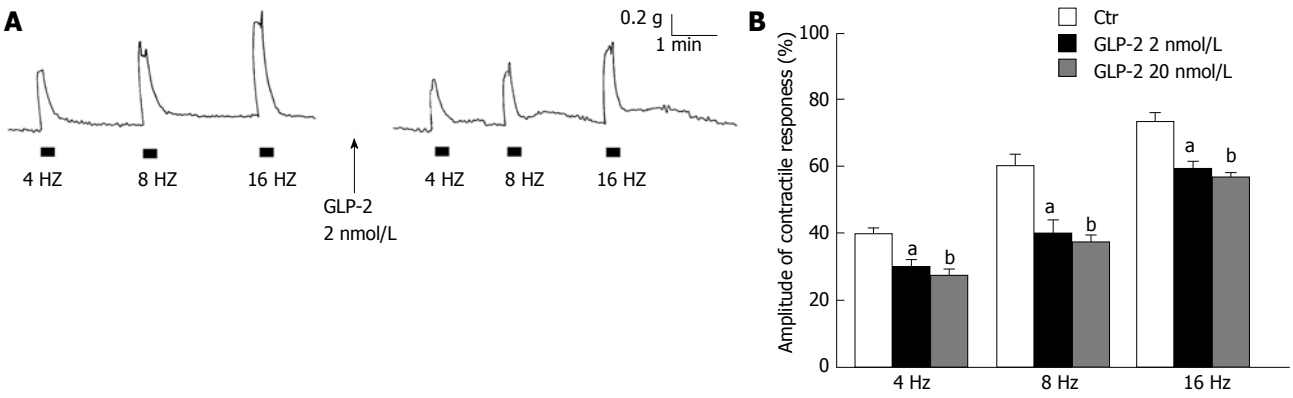


Figure 1 Influence of glucagon-like peptide-2 on the neurally-induced contractile responses. A: Typical tracing showing the electrical field stimulation (EFS)-induced contractile responses at different stimulation frequencies (left panel). Ten min after the addition of 2 nmol/L glucagon-like peptide-2 (GLP-2) to the bath medium (right panel), the amplitude of the EFS-induced excitatory responses is decreased in the whole range of stimulation frequencies employed; B: Bar chart of the effects of GLP-2 (2 and 20 nmol/L) on the mean amplitude of the EFS-induced contractile responses elicited at different stimulation frequencies. Excitatory responses are expressed as percentage of the muscular contraction evoked by 2 μmol/L methacholine, taken as 100%. All values are mean ± SE of six preparations. [a]*P* < 0.05 *vs* the ctr [b]*P* < 0.05 and *P* > 0.05 *vs* the ctr and *vs* 2 nmol/L GLP-2, respectively, ANOVA and Newman-Keuls post-test).

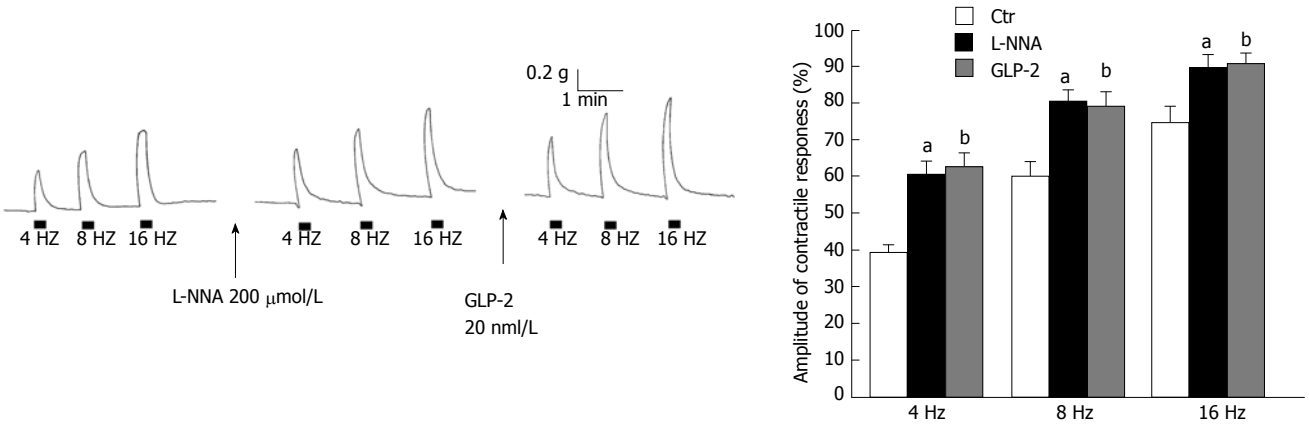


Figure 2 Lack of effects of glucagon-like peptide-2 on the neurally-induced contractile responses in the presence of the nitric oxide synthesis inhibitor L-NNA. A: Typical tracing showing the electrical field stimulation (EFS)-induced contractile responses at different stimulation frequencies (left panel). Following the addition of L-NNA (200 μmol/L) to the bath medium, the amplitude of the EFS-induced excitatory responses is enhanced (middle panel). In the presence of L-NNA, glucagon-like peptide-2 (GLP-2, 20 nmol/L) no longer depresses the amplitude of the neurally-induced contractile responses (right panel); B: Bar chart of the effects of GLP-2 (20 nmol/L) on the mean amplitude of the EFS-induced contractile responses in the presence of L-NNA (200 μmol/L). Excitatory responses are expressed as percentage of the muscular contraction evoked by 2 μmol/L methacholine, taken as 100%. All values are mean ± SE of six preparations. [a]*P* < 0.05 *vs* the ctr [b]*P* < 0.05 and *P* > 0.05 *vs* the ctr and *vs* GLP-2, respectively (ANOVA and Newman-Keuls post-test).

4A and B), whereas that of the slow relaxation was reduced (6.1 ± 0.6% *vs* 12.75 ± 2.3% and 19.68 ± 1.6% *vs* 30.46 ± 2.2%, at 8 and 16 Hz, respectively; *P* < 0.05) (Figure 4A) or even abolished at the highest dose employed.

Morphological experiments

Anatomical and histological evaluation: At the end of the tissue sampling procedure, the control and GLP-2 treated specimens appeared anatomically intact and the H&E staining confirmed the integrity of the muscle wall (Figure 5).

Immunofluorescence: In the gastric fundus of both control (Figure 6A-C) and GLP-2 (20 nmol/L) treated specimens (Figure 6D-F), the nNOS-immunoreactivity (IR) was observed in the soma of the myenteric neurons and in the fibers located in the longitudinal and circular muscle layers. No IR was detected when the primary antibody was omitted (Figure 6G-H). Quantitation of the IR showed a statistically significant increase in nNOS positive neuron number and nerve fibers (Figure 6I). The length of gastric fundal strips subjected to quantitative analysis was not different between control and GLP-2 treated specimens (4.17 ± 0.60 mm controls *vs* 4.07 ± 0.48 mm GLP-2 treated).

DISCUSSION

The present study demonstrates for the first time that GLP-2 influences the neurally-induced contractile and relaxant responses in mouse fundal strips through a modulatory action on nitrergic neurotransmission and increases nNOS expression. Particularly, the functional

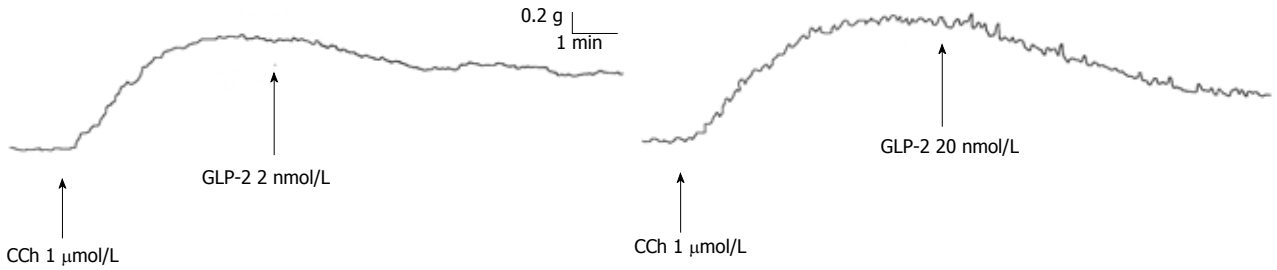
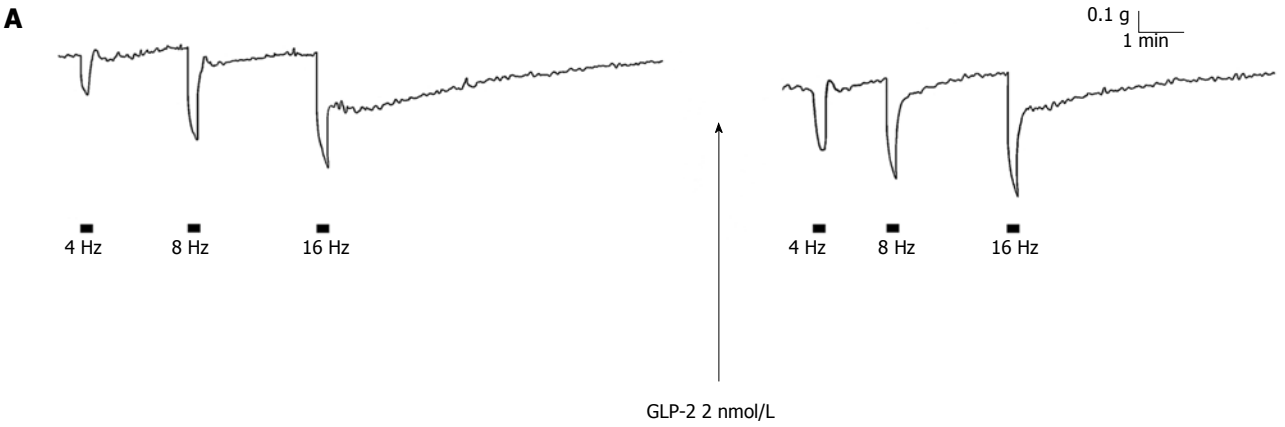


Figure 3 Effects of glucagon-like peptide-2 on gastric strips precontracted with carbachol. Typical tracings showing the effect of the addition of glucagon-like peptide-2 (GLP-2, 2 and 20 nmol/L) to the bath medium. GLP-2 causes a dose-dependent slow and long-lasting relaxation. CCh: Carbachol .



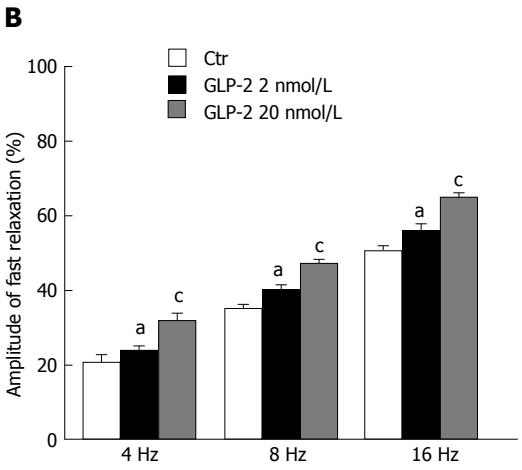


Figure 4 Influence of glucagon-like peptide-2 on the neurally-induced relaxant responses. A: Typical tracing showing the electrical field stimulation (EFS)-induced relaxant responses at different stimulation frequencies (left panel). EFS evokes fast relaxant responses, followed by a slow component at the higher stimulation frequencies (≥ 8 Hz). GLP-2 at 2 nmol/L causes (right panel) an increase in amplitude of the EFS-induced fast relaxation in whole range of stimulation frequencies employed and a decrease of the slow one; B: Bar chart of the effects of GLP-2 (glucagon-like peptide-2, 2 and 20 nmol/L) on the mean amplitude of the EFS-induced fast relaxation. Note that GLP-2 increases the amplitude of the EFS-induced fast relaxant responses in a dose-related manner. Relaxant responses are expressed as percentage decrease relative to the muscular tension induced by 1 μmol/L CCh taken as 100%. Amplitude values of EFS-induced fast relaxations refer to the maximal peak obtained during the stimulation period. All values are mean ± SE of six preparations. $^{a}P < 0.05$ *vs* the ctr; $^{c}P < 0.05$ *vs* the ctr and *vs* 2 nmol/L GLP-2 (ANOVA and Newman-Keuls post-test).

experiments show that GLP-2 is able to depress the amplitude of the EFS-induced contractile responses at each stimulation frequency applied.

It is known that gastric motor responses are a balance between nervous excitatory (mainly cholinergic) and inhibitory (NANC) influences exerted on the smooth muscle. Since during EFS both excitatory and inhibitory nervous fibers are simultaneously activated, the reduction in amplitude of the neurally-induced contractions caused by GLP-2 might be ascribable either to a minor activation of the excitatory component or to a major nervous inhibitory influence exerted on the smooth muscle. In this view, the increase in amplitude of the EFS-induced contractile responses by the NO synthesis inhibitor L-NNA indicates the removal of a nitrergic inhibitory nervous influence. Notably, in

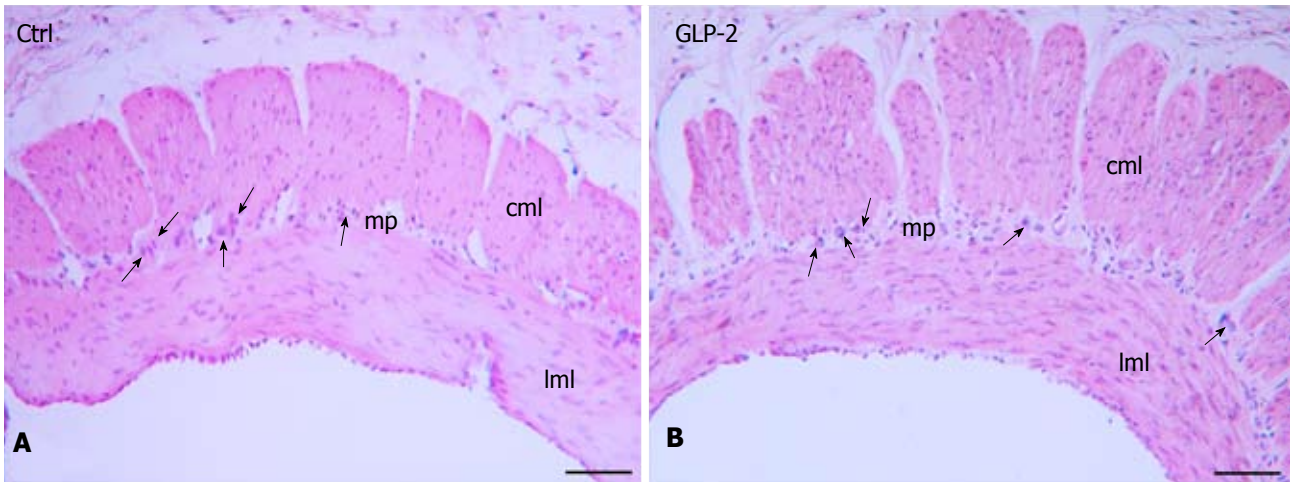


Figure 5 H&E staining of gastric fundus strip. The longitudinal and circular muscle layers (lml, cml) and the myenteric plexus (mp) are shown both in control (A) and in GLP-2 treated (B) specimens. The arrows indicated some myenteric neurons. Bar: A, B = 10 μm.

the presence of L-NNA, GLP-2 no longer depressed the amplitude of the neurally-induced excitatory responses. These data demonstrate that the hormone effects on the EFS-induced contractions occur through the modulation of the nitrergic neurotransmission and, in addition, exclude its inhibitory action on the cholinergic neurotransmission.

The lack of effects of GLP-2 on the direct smooth muscle responses elicited by methacholine further supports its neuromodulatory action. This observation also proves that the depressant actions of GLP-2 on the EFS-induced contractile responses are not ascribable to aspecific effects such as the decay of the basal tension. Moreover, the similar amplitude of the contraction to methacholine at the beginning and at the end of the experiments demonstrates that muscle responsiveness is not compromised.

In keeping with the above observations, the results of the experiments performed in the presence of guanethidine and CCh demonstrate, for the first time, that the hormone influences the amplitude of the neurally-induced relaxant responses in gastric fundal strips through a modulatory action on the nitrergic neurotransmission. As previously observed in strips from the mouse gastric fundus in the presence of guanethidine and CCh[23,26,28], EFS induced a fast NO-mediated relaxation, followed by a slow and sustained relaxant response at the highest stimulation frequencies. In the present experiments, the observation that GLP-2 increased the amplitude of the EFS-induced fast nitrergic relaxation in the whole range of stimulation frequencies in a dose-related manner further supports the involvement of the NO pathway in the hormone effects. To ascertain whether the effects of GLP-2 on both the neurally-induced contractile and relaxant responses were ascribable to an increased NO production, nNOS expression was evaluated in gastric preparations by immunohistochemistry. The results show that GLP-2 induces an increase in the nNOS labeling in the nerve structures of the gastric fundus muscle coat. Accordingly, an increased NO production in neurons induced by exogenous GLP-2 has been suggested to occur in the mouse duodenum in which the hormone inhibited the spontaneous mechanical activity[11]. In the present experiments, the increased nNOS expression in the nerve structures of the muscle wall fits well with the progressive decrease in amplitude of the EFS-induced contractile responses as well as with the increase of the fast relaxant responses caused by GLP-2. Thus, these hormone effects occur only during EFS, *i.e.*, when the nitrergic pathway is engaged, also explaining the ineffectiveness of L-NNA to influence the relaxant response induced by the addition of GLP-2 to bath medium. Indeed, gastric relaxation to GLP-2 in mice has been reported to occur through the release of VIP from myenteric neurons[19]. In this view, immunohistochemical studies in mouse, human and porcine intestine reported the presence of the GLP-2 receptor in the enteric neurons[10-12,29] and showed that some of them co-localized with VIP[11,12]. We previously observed[26] that, in strips from the mouse gastric fundus, the EFS-induced slow relaxation was abolished following VIP receptors desensitization, suggesting the involvement of the peptide in this kind of response. On this basis, it could be speculated that the reduction/abolition of the neurally-induced slow relaxation by GLP-2 observed in the present experiments occurred because VIP, released earlier from the nerve terminal by the hormone, has already occupied the muscular receptors engaged in the EFS-induced vipergic relaxant response. However, even if the validation of this hypothesis was beyond the aim of the present work, the observation that GLP-2 influences in an opposite manner the two components of the neurally-induced relaxant responses excludes an aspecific effect.

The present results demonstrate that the hormone depresses the amplitude of the neurally-induced

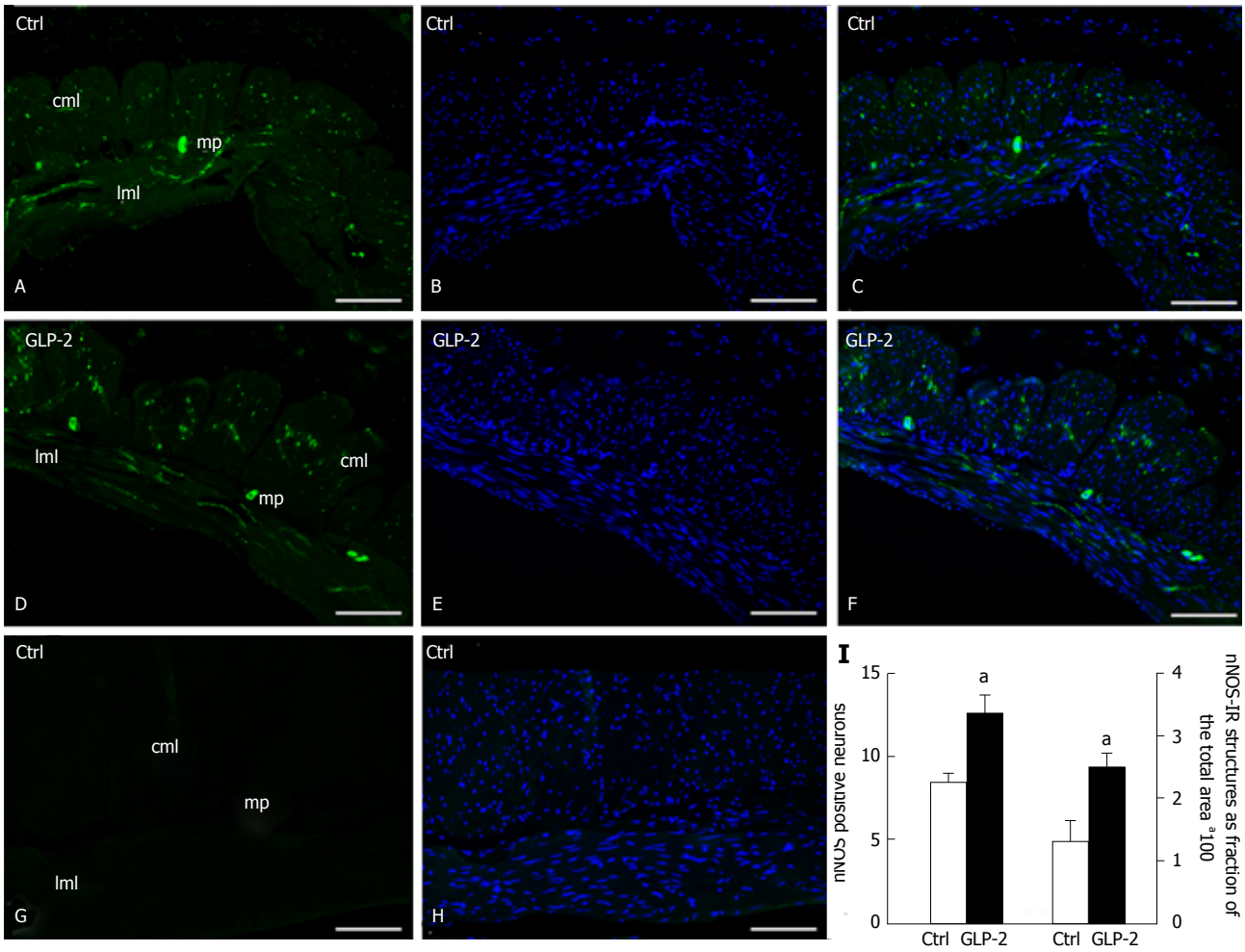


Figure 6 Neuronal nitric oxide synthase-immunoreactivity in control and glucagon-like peptide-2 treated specimens. In control (A) and glucagon-like peptide-2 (GLP-2) treated (D) specimens, neuronal nitric oxide synthase-immunoreactivity (nNOS-IR) (green) is present in the soma of some myenteric neurons and in several nerve fibers. DAPI fluorescent staining for DNA (blue) identified the nuclei of both smooth muscle and neural cells (B-E). In C and F, merged images of nNOS and Bisbenzimide Hoechst Trihydrochloride (BHT) fluorescent staining are shown. When the primary antibody is omitted (G), only the BHT labelling is observed (H). The quantitative analysis showed a significant increase (I) in nNOS-IR myenteric neurons (left side) and in IR fibers in the entire muscle coat (right side). $^{a}P < 0.05$ *vs* controls (unpaired Student's t-test). mp: Myenteric plexus; cml: Circular muscle layer; lml: Longitudinal muscle layer. Bar: A-H = 10 μm.

contractile responses, enhances the amplitude of the NO-mediated fast relaxant responses and increases the nNOS expression in the nerve fibers. All of these effects of GLP-2 might contribute to gastric relaxation, thus increasing the organ capacity. Since gastric distension represents a peripheral satiety signal[30] from a physiological point of view, it could be hypothesized that the relaxant effects of GLP-2 might contribute to suppress feeding behavior in rodents[31,32].

In conclusion, this study shows for the first time that GLP-2 influences the neurally-induced responses in gastric strips from mice through a modulatory action on the nitrergic neurotransmission and increases nNOS expression.

COMMENTS

Background

Glucagon-like peptide-2 (GLP-2) has been shown to relax gastric smooth muscle acting on myenteric neurons. However, to our knowledge, no data are present in the literature on the effects of GLP-2 on the neurally-induced gastric responses elicited by electrical stimulation in *in vitro* preparations. For the above reason, they presently investigated whether GLP-2 influences the neurally-induced contractile and relaxant responses in strips from the mouse gastric fundus. Moreover, since nitric oxide (NO) is considered one of the major NANC inhibitory neurotransmitters responsible for the proximal stomach relaxation, they also evaluated the effects of GLP-2 on both the nitrergic neurotransmission and the neuronal nitric oxide (nNOS) expression.

Research frontiers

Previous studies have indicated that GLP-2 might contribute to suppress feeding behavior in rodents. Thus, the relaxant effects of GLP-2 on gastric preparations, leading to organ distension, might represent peripheral satiety signals.

Innovations and breakthroughs

This is the first study showing that GLP-2 is able to influence the neurally-induced responses in gastric strips from mice through a modulatory action on nitrergic neurotransmission and to increase nNOS expression.

Applications

The evidence that GLP-2 is able to induce gastric relaxation, through the modulation of the nitrergic neurotransmission, contributes to the knowledge of

its peripheral effects. The results highlight an additional site of action that might be implicated in the hormone control of feeding behavior.

Terminology

Gastric motor responses are the result of a balance between nervous excitatory, mainly cholinergic, and NANC inhibitory influences exerted on smooth muscle. During EFS, both excitatory and NANC inhibitory nervous fibers are simultaneously activated. The release of neurotransmitters from enteric neurons may be modulated by a variety of substances, including hormones.

Peer-review

This is a very interesting study investigating the influence of GLP2 on neurally-induced responses in gastric fundal strips.

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ORIGINAL ARTICLE

Basic Study

Hypothermic machine perfusion with metformin-University of Wisconsin solution for *ex vivo* preservation of standard and marginal liver grafts in a rat model

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Author contributions: Chai YC and Dang GX contribute equally to this study; Dang GX and Hu LS conceived and designed the experimental study; Chai YC and Dang GX performed the surgical procedure; Chai YC and Zhang HK collected the data; Zhang HK provided statistical analysis; Dang GX and Zhang RT contributed to data interpretation; He HQ and Shi JH reviewed all histopathological specimens and performed morphometric measurements; Chai YC wrote the article; Hu LS, LvY and Wang B critically revised the article; all authors participated in the revision of the manuscript, read and approved the final manuscript.

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Abstract

AIM

To compare the effect of University of Wisconsin (UW) solution with or without metformin, an AMP-activated

protein kinase (AMPK) activator, for preserving standard and marginal liver grafts of young and aged rats *ex vivo* by hypothermic machine perfusion (HMP).

METHODS

Eighteen young (4 mo old) and 18 aged (17 mo old) healthy male SD rats were selected and randomly divided into three groups: control group, UW solution perfusion group (UWP), and UW solution with metformin perfusion group (MUWP). Aspartate aminotransferase (AST), alanine aminotransferase (ALT), lactate dehydrogenase (LDH), interleukin-18 (IL-18), and tumor necrosis factor-alpha (TNF-α) in the perfused liquid were tested. The expression levels of AMPK and endothelial nitric oxide synthase (eNOS) in liver sinusoidal endothelial cells were also examined. Additionally, microscopic evaluation of the harvested perfused liver tissue samples was done.

RESULTS

AST, ALT, LDH, IL-18 and TNF-α levels in the young and aged liver-perfused liquid were, respectively, significantly lower in the MUWP group than in the UWP group ($P < 0.05$), but no significant differences were found between the young and aged MUWP groups. Metformin increased the expression of AMPK and eNOS protein levels, and promoted the extracellular release of nitric oxide through activation of the AMPK-eNOS mediated pathway. Histological examination revealed that in the MUWP group, the extent of liver cells and tissue damage was significantly reduced compared with the UWP group.

CONCLUSION

The addition of metformin to the UW preservative solution for *ex vivo* HMP can reduce rat liver injury during cold ischemia, with significant protective effects on livers, especially of aged rats.

Key words: Metformin; AMP-activated protein kinase; Cold ischemia injury; Hypothermic machine perfusion; Liver Grafts

Core tip: Metformin can activate the AMP-activated protein kinase pathway that could enhance the activity of endothelial nitric oxide synthase and finally increase the generation of nitric oxide, which plays an important role in the protection of liver sinusoidal endothelial cells. Hence, our study was designed to evaluate the protective effect of University of Wisconsin storage solution with metformin for preserving standard and marginal liver grafts of young and aged rats *ex vivo* by hypothermic machine perfusion (HMP). According to the results, HMP with metformin plays a significant protective role for liver grafts during cold ischemia, with significant effects especially for aged-marginal donors.

Chai YC, Dang GX, He HQ, Shi JH, Zhang HK, Zhang RT, Wang B, Hu LS, Lv Y. Hypothermic machine perfusion with metformin-University of Wisconsin solution for *ex vivo* preservation of standard and marginal liver grafts in a rat model. *World J Gastroenterol* 2017; 23(40): 7221-7231 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i40/7221.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i40.7221

INTRODUCTION

Currently, liver transplantation is the only effective therapy for end-stage liver disease[1]. Both preservation of donor organs and post-transplant ischemic reperfusion injury (IRI) are important factors affecting the prognosis of transplantation[2]. At present, due to the shortage of liver donation, marginal donation, which includes aged donation, adipo-hepatic donation, and donation after cardiac death (DCD), increases the risk for more severe IRI because of suboptimal function and long-term warm and cold ischemia[3-5]. Cold ischemia injury plays an important role in the IRI mechanism after revascularization of transplants. In this period, the liver sinusoidal endothelial cells are the first to be injured in a donor liver, causing damage of the stable hepatic microenvironment, hepatic microcirculation disturbance, and exacerbation of IRI[6]. Therefore, there is a current pressing need to explore and improve methods of organ preservation and minimize IRI of donor livers during transplantation[7,8].

In recent years, machine perfusion (MP) has been explored as an alternate method of organ preservation to static cold storage. Clinically, hypothermic machine perfusion (HMP, 4-6 ℃) has been effective for kidney transplantation, but MP methods have not been widely used in liver transplantation. According to the latest research, MP has been meaningful for the preservation and repair of marginal liver donation[9], but this still needs further clinical verification[10,11].

Another important direction of research on donor liver cold preservation is the auxiliary protective intervention of donor livers against IRI factors of microcirculation[12] and hepatocyte metabolism[13] through drugs. Activation of adenosine 5′-monophosphate-activated protein kinase (AMPK) signaling pathways increases the activity of endothelial nitric oxide synthase (eNOS) to generate nitric oxide. This provides a cytoprotective effect to the hepatic sinusoidal endothelium of the donor liver and has been considered for preconditioning of the donor liver to reduce IRI. In addition, AMPK signaling is also known to regulate glucose metabolism and prevent cell death, thus extending the cytoprotective effect on hepatocytes[14]. Therefore, it plays an important role in protecting hepatic sinusoidal endothelium and reducing injury of donor livers[15]. As an agonist of AMPK, metformin additionally lowers the blood glucose by reducing hepatic gluconeogenesis and strengthening

glucose uptake of peripheral tissue[16].

Hence, we hypothesized that liver sinusoidal endothelial cells can be protected from injury by activating AMPK signaling pathways with the addition of metformin perfused *in vitro*, which could ultimately lead to an improvement of liver donor organ preservation. Consequently, in this study, we added metformin to University of Wisconsin (UW) solution, *ex vivo*, in HMP models of livers of young and aged rats and investigated the effects on biochemical indicators and sinusoidal cell morphology.

MATERIALS AND METHODS

A total of 18 young healthy male SD rats (4 mo old, weighing 250–300 g) and 18 aged (17 mo old, weighing 600–630 g) SD rats were randomly selected for the study. The experimental animals were provided by Animal Experiment Center of Xi'an Jiaotong University.

LongerPump DG-2-B/D Precise Miniature Peristaltic Pump (Longer Precision Pump Co., Ltd., Baoding, China) with a rotation speed of 0-100 rpm and a flow rate of 0-48 mL/min; SPS-1 Static Preservation Solution (UW solution, Organ Recovery Systems, Inc.); and metformin (1,1-dimethylbiguanide hydrochloride, CAS # 1115-70-4, Biomol GmbH) were used. A 165 mg/L stock solution of metformin was prepared by adding 2.5 mL of metformin solution at a concentration of 1 g/10 mL to 1000 mL of sterile water, stirring until dissolved, and then adding this (0.66 mL) to 1000 mL of UW solution. A final metformin concentration of 0.165 mg/L or 1 mmol/L was used. Serum interleukin-18 (IL-18) and tumor necrosis-alpha (TNF-α) levels were measured by enzyme-linked immunosorbent assay (ELISA) using commercial kits (Rat Interleukin 18 ELISA kit, CLOUD-CLONE CORP., TX, United States; Rat TNF-alpha ELISA kit, MultiSciences Biotech Co., Ltd, Shanghai, China).

Study design

Young and aged rats were respectively randomly divided into three groups, with six rats in each group. The groups were: control groups A (young rats) and D (aged rats), UW solution perfusion (UWP) groups B (young rats) and E (aged rats), and experimental groups C (young rats) and F (aged rats) that were perfused with metformin-UW solution (MUWP).

Step one - Establishing the experimental model

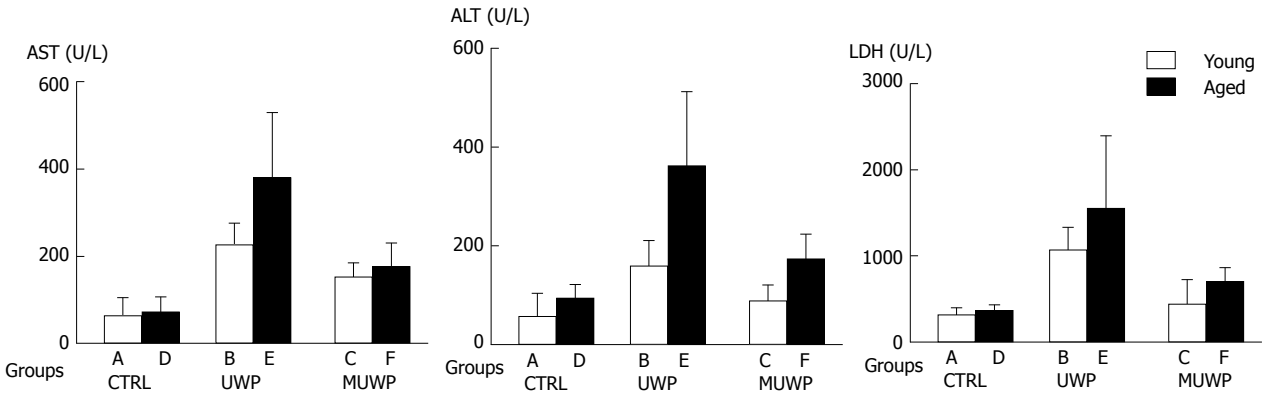
Rats were fasted for 8 h and general anesthesia was induced in all rats by intraperitoneal injection of 5% pentobarbital sodium at 20 mg/kg. Fixation, skin preparation, and disinfection were completed, and then a large median and transverse abdominal incision for laparotomy was made. The liver was isolated, heparinized, perfused *in situ* with cold UW solution until the liver turned into a khaki color, and rapidly harvested at room temperature. The *ex vivo* liver was then placed into a basin filled with cold UW solution and made to lie in the basin on an ice pad. All *ex vivo* livers were grouped and underwent HMP with circulating UW solution at 4 ℃ at a flow rate of 4 mL/min maintained at 80 mL of the total circulation volume with the help of a peristaltic pump. Groups A and D did not require extended period of HMP (only 2 h); groups B and E were perfused with UW solution for 12 h; and groups C and F were perfused mechanically with UW solution with 0.165 mg/L of metformin for 12 h. After HMP, 6 mL of the perfused liquid was collected from every group and stored at -20 ℃.

Step two - Examination of the expression levels of p-eNOS, t-eNOS, p-AMPK, and t-AMPK in liver sinusoidal endothelial cells of young rats

Extraction of liver sinusoidal endothelial cells from young rats: After step one, the *ex vivo* livers of every group were perfused with Gey's balanced salt solution (150 mL free of Ca^{2+} and Mg^{2+}, mixed with pronase 400 mg and collagenase 40 mg) for 7 min at 37 ℃ at a flow rate of 20 mL/min. Then, the resulting mixture was centrifuged at 2500 rpm with Krebs-Henseleit solution (with 10% fetal calf serum and 0.002% DNase I) perfused at a flow rate of 10 mL/min. Cell pellet was then resuspended in DMEM supplemented with 20% fetal calf serum and 10 mL of 100 U/L penicillin G and loaded carefully in the centrifuge for 3 min at a flow perfusion rate of 15 mL/min, and accelerated at 18 mL/min for 1 min. The input flow rate was increased again by 20 mL/min, while 50 mL of the cell suspension of the effluent was collected. Once more, a 50 mL cell suspension was collected by 25 mL/min. The 100 mL homogenate was centrifuged at 2000 rpm for 5 min. The supernatant liquid was extracted out, and the liver sinusoidal endothelial cell-debris pellet was collected in the bottom of collection tube.

Extraction of total protein and examination of the expression levels of target proteins: The cell debris was lysed in Cell Lysis Buffer (Cell Signaling Technology; Beverly, MA, United States; carefully chilling 1 × stock buffer on ice and add 1 mmol/L PMSF immediately just prior to use) for 30 min on ice, and then centrifuged at 12000 rpm for 5 min at 4 ℃. The supernatant with total cellular proteins was collected from every group, determined, transferred to a sterile tube (1.5 mL), and stored at -20 ℃. Then, the two-dimensional electrophoresis (2-DE) maps of target proteins were obtained by Western blot to examine the expression levels, and the amounts of expression of AMPK and eNOS were analyzed with gray scale from the maps.

Tests included: (1) Biochemical indicator tests: the levels of aspartate transaminase (AST), alanine transaminase (ALT), lactate dehydrogenase (LDH),



Groups	A / D	B / E	C / F
AST (U/L)	66.40 ± 40.59 / 75.47 ± 31.11	223.50 ± 55.07 / 378.15 ± 150.13	152.53 ± 35.58 / 176.93 ± 55.10
ALT (U/L)	59.97 ± 46.58 / 95.78 ± 30.62	161.20 ± 45.53 / 363.25 ± 150.12	88.25 ± 36.22 / 173.6 ± 51.72
LDH (U/L)	322.13 ± 93.48 / 385.17 ± 45.28	1039.99 ± 308.78 / 1543.05 ± 855.93	450.72 ± 287.96 / 709.3 ± 164.20

Figure 1 Analysis of biochemical indicators in the perfused liquid. Aspartate aminotransferase (AST), alanine transaminase (ALT), and lactate dehydrogenase (LDH) levels (mean ± SD) in the perfused liquid of the ex vivo livers of the three groups: CTRL (control groups A and D), UWP (UW solution perfusion groups B and E), and MUWP (metformin perfusion groups C and F). Data are presented as mean ± SD (n = 6) and compared by two-way analysis of variance and the Sidak's multiple comparisons test.

IL-8, and TNF-α in the perfused liquid were examined; (2) Histo-morphological by light microscopy: the liver tissues after perfusion were fixed with 10% formalin and immersed in the wax for sections and hematoxylin-eosin (HE) dye. A scoring system was used to grade the degree of histological damage quantitatively (by blinding) at the Department of Pathology, based on the following histological features: hydropic degeneration of hepatocytes and liver sinusoidal endothelial cells, stenosis of the hepatic sinusoid, and number of Kupffer cells. Each feature was graded as absent, mild, moderate, or massive, with a score of 0-3, respectively; and (3) Electron microscopic examination: The specimens of perfused hepatic tissue were fixed to observe by transmission electron microscopy.

Statistical analysis

All calculations were performed with SPSS 22.0 software. The grayscale of 2-DE maps of target proteins was analyzed with ImageProPlus 6.0 software. Quantitative data are expressed as mean ± SD and assessed by one- or two-way analysis of variance, and multiple comparisons between groups were performed using Student-Newman-Keuls method. Scoring data were analyzed by Kruskal-Wallis one-way analysis of variance on ranks and compared between any two groups using Mann-Whitney U test. $P < 0.05$ was considered statistically significant.

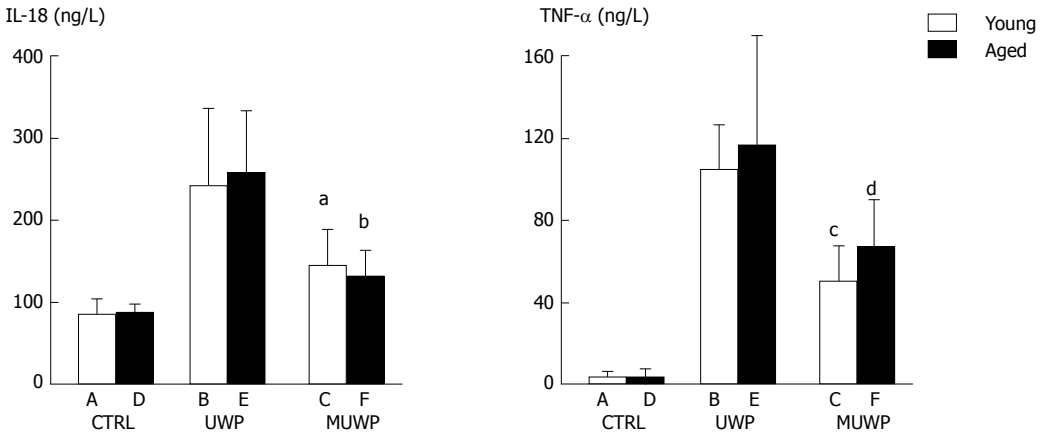
RESULTS

Analysis of biochemical indicators

The control group (groups A and D) was subject to HMP for only 2 h, while the UWP (groups B and E) and MUWP groups (groups C and F) underwent HMP for 12 h.

After completing the perfusion of each group, AST, ALT, and LDH levels in the perfused liquid were determined (Figure 1). There were no significant differences in AST, ALT, or LDH between the young rat control group (group A) and the aged rat control group (group D). The AST, ALT, and LDH in the UWP group (groups B and E) were significantly higher than those in groups A and D ($P < 0.05$), Besides, the AST and ALT in the aged group E were significantly higher than those in the young group B ($P < 0.05$). The AST, ALT, and LDH in the MUWP group, which consisted of the young group C and aged group F, were, respectively, significantly lower than those in the young group B and aged group E of UWP ($P < 0.05$), although no significant differences between the young and the aged group were found.

IL-18 and TNF-α levels in the perfused liquid were measured by ELISA (Figure 2). There were no significant differences in the levels of IL-18 or TNF-α between the young rat control group (group A) and the aged rat control group (group D). IL-18 and TNF-α levels in the UWP group were significantly higher than those in the control group ($P < 0.05$). Nevertheless, there were no significant differences between the young group B and the aged group E of UWP. IL-18 and TNF-α levels in the young group C and the aged group F of MUWP were, respectively, appreciably lower than those in the young group B and the aged group E of UWP ($P < 0.05$), but no significant differences between the young group C and the aged group F of MUWP were found.



Groups	A / D	B / E	C / F
IL-18 (ng/L)	87.9 ± 14.97 / 88.5 ± 8.63	243.50 ± 91.50 / 260.37 ± 72.73	145.05 ± 44.42 / 131.38 ± 32.53
TNF-a (ng/L)	3.60 ± 2.80 / 3.76 ± 3.70	91.65 ± 18.63 / 101.43 ± 47.37	43.70 ± 15.28 / 57.80 ± 20.77

Figure 2 Analysis of inflammatory factors in the perfused liquid. The IL-18 and TNF-α levels in the perfused liquid (mean ± SD) of the *ex vivo* livers of the three groups: CTRL (control groups A and D), UWP (UW solution perfusion groups B and E), and MUWP (metformin perfusion groups C and F). Data are presented as mean ± SD (n = 6) and compared by two-way analysis of variance and the Tukey's and Sidak's multiple comparisons tests. [a]P < 0.05 *vs* group B; [b]P < 0.05 *vs* group E; [c]P < 0.05 *vs* group B; [d]P < 0.05 *vs* group E.

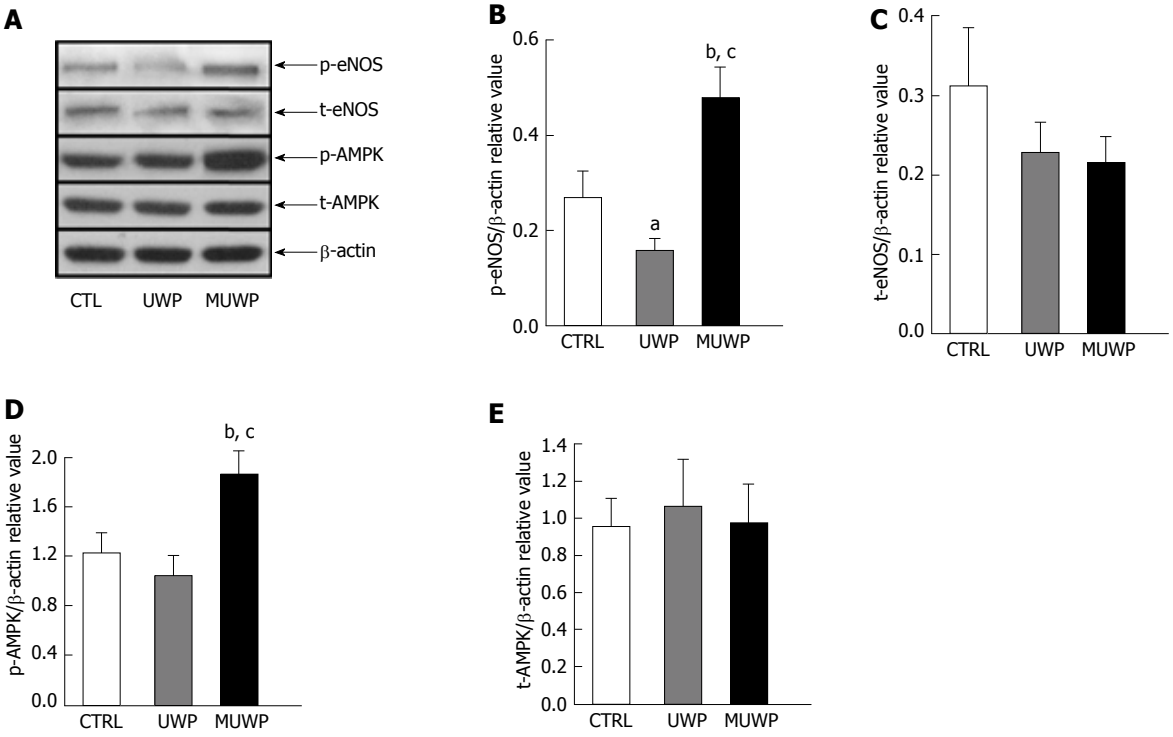


Figure 3 Histograms of relative gray values of the expression levels of p-eNOS, t-eNOS, p-AMPK, and t-AMPK in the liver sinusoidal endothelial cells of young rats. AMPK/eNOS pathway was activated *ex vivo* by metformin. A: Phosphorylation of AMPK and eNOS was increased in the MUWP group at 12 h after HMP; B-E: Quantitative analysis of total AMPK, total NOS, phosphorylated AMPK, and eNOS expression in CTRL (control groups A and D), UWP (UW solution perfusion groups B and E), and MUWP (metformin perfusion groups C and F) groups. Data are presented as mean ± SD (n = 3) and compared by ordinary one-way analysis of variance and the Dunnett's multiple comparisons test. [a]P < 0.05 *vs* CTRL group; [b]P < 0.05 *vs* CTRL group; [c]P < 0.05 *vs* UWP group.

Expression levels of target proteins

Discriminant analysis of relative gray values was used to determine the expression levels of p-eNOS, t-eNOS, p-AMPK, and t-AMPK in liver sinusoidal endothelial cells of young rats (Figure 3). The expression level of p-eNOS in the MUWP group was significantly higher (80.1%) than that in the control group (P = 0.036) and 205.88% higher than that in the UWP group (P = 0.008), which was significantly (41.1%) lower than that in the control group (P = 0.045) (Figure 3B). The expression level of t-eNOS in the control group exhibited no significant difference compared with the

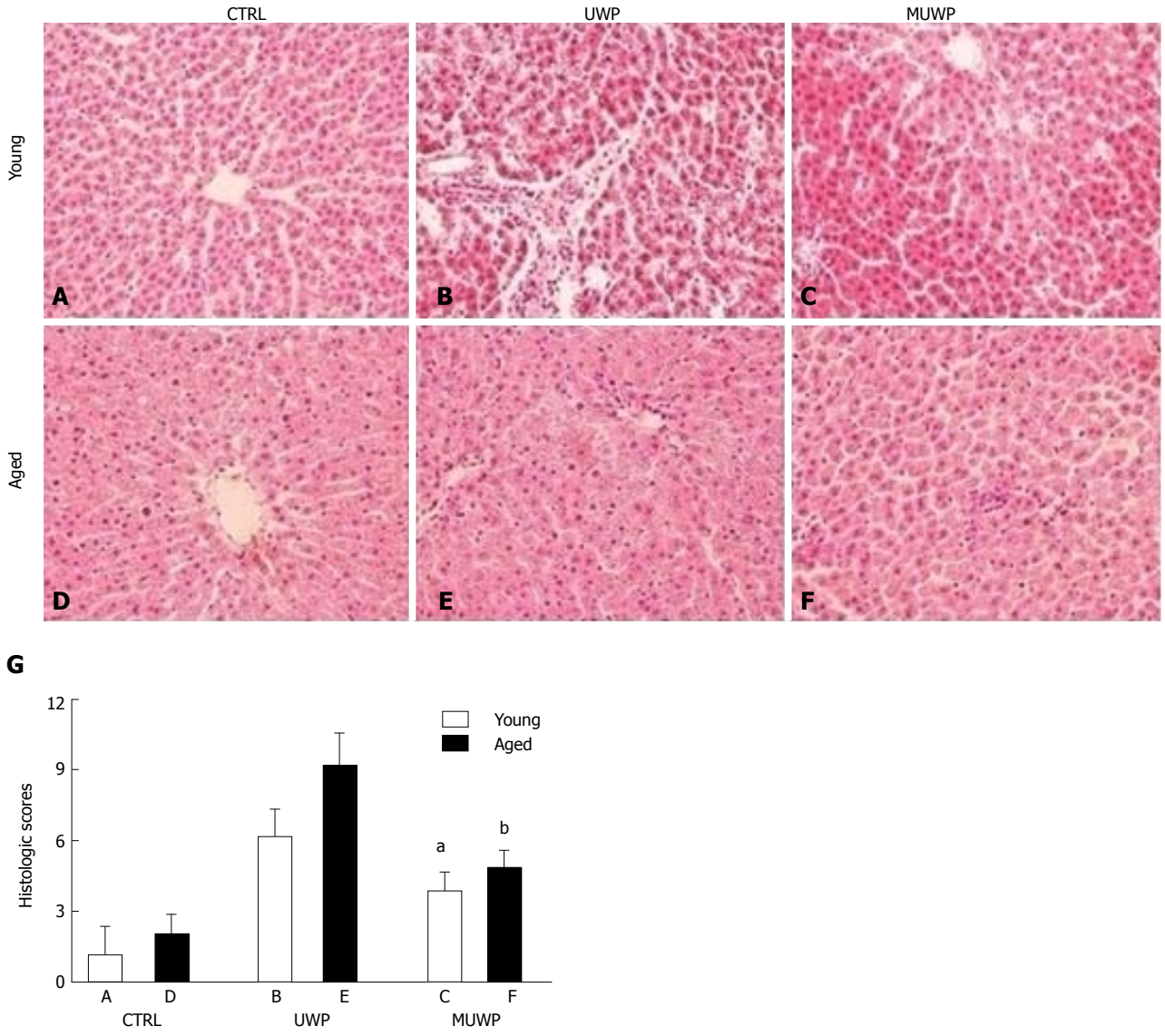


Figure 4 Staining of the slices of *ex vivo* rat liver tissue. A and D: The young control group A and the aged control group D, respectively; B and E: The young group B and aged group E of UWP, respectively; C and F: The young group C and the aged group F of MUWP, respectively; G: Histopathologic scoring of structural injury in each group. Data are presented as mean ± SD (n = 6) and compared by Kruskal-Wallis one-way analysis of variance on ranks and Mann-Whitney U test. [a]P < 0.05 *vs* group B of UWP; [b]P < 0.05 *vs* group E of UWP. Original magnification (A-F): × 400. UWP: University of Wisconsin solution perfusion; MUWP: Metformin perfusion solution.

UWP and MUWP groups (P = 0.251) (Figure 3C). The expression level of p-AMPK in the MUWP group was significantly higher (51.7%) than that in the control group (P = 0.038) and 78.5% higher than that in the UWP group (P = 0.018), even though there were no significant differences between the control and UWP groups (P = 0.217) (Figure 3D). The expression level of t-AMPK in the control group demonstrated no significant differences compared with the UWP and the MUWP groups (P = 0.868) (Figure 3E).

Histo-morphological examination

The HE-stained sections were observed under a microscope. The hepatocytes in the young rat control group (group A) and the aged rat control group (group D) were shaped normally, with no stenosis observed in the hepatic sinusoid and no swelling in the sinusoidal endothelial cells. The size of hepatocytes in group D was slightly larger than that in group A (Figure 4A-D). Cellular swelling was obvious, accompanied by narrowing of the hepatic sinusoid, in the UWP group (groups B and E), particularly, more severe in the aged group E. The swelling of the sinusoidal endothelial cells, as well as Kupffer cells was observed in both groups B and E (Figure 4B-E). Hepatocytes in the MUWP group showed mild edema, which was slightly more severe in group F than in group C, although there were no obvious differences in the hepatic sinusoid between them. Swelling of sinusoidal endothelial cells was inconspicuous in both groups; however, a small number of Kupffer cells were observed (Figure 4C-F). There were no significant differences in the histologic

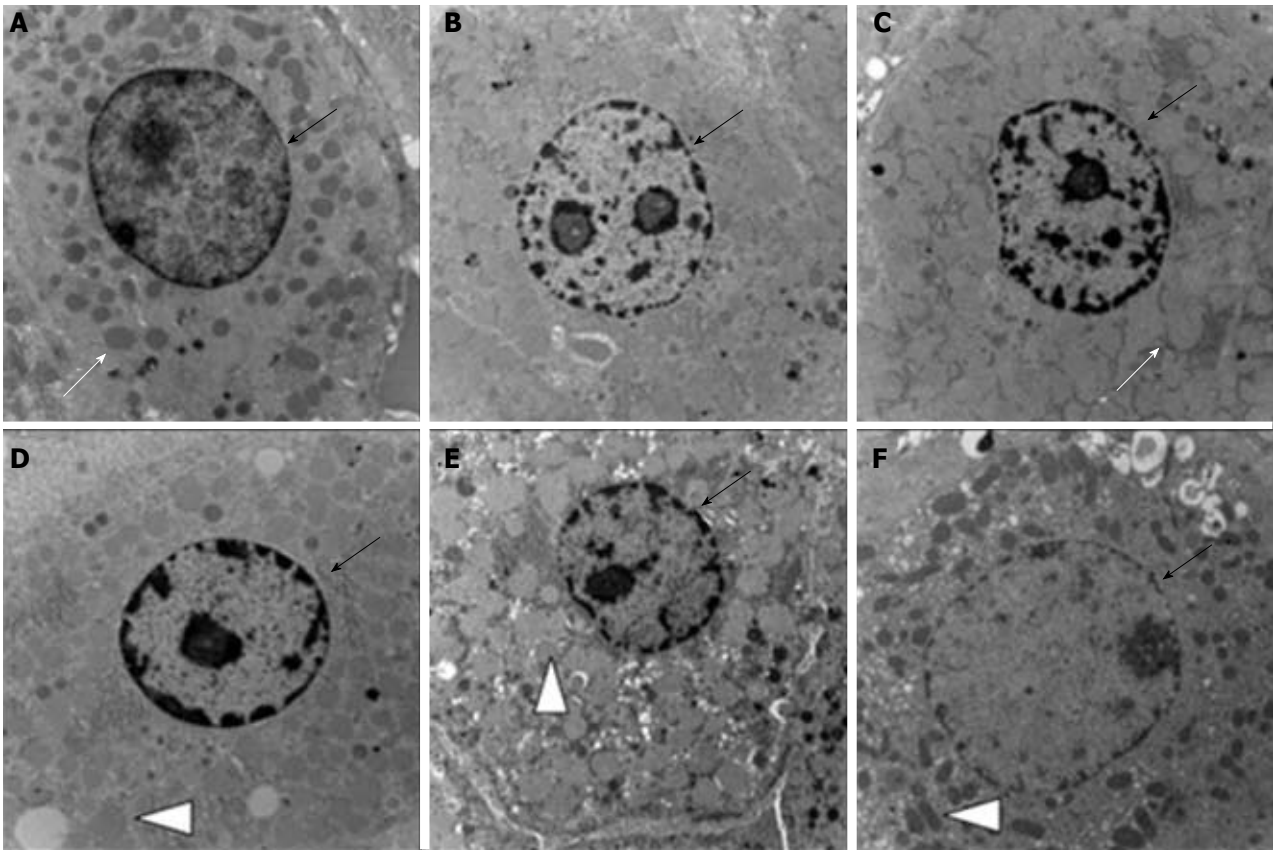


Figure 5 Ultrastructural changes of hepatocytes. A and D: Hepatocytes of the young control group A and the aged control group D, respectively; B and E: Hepatocytes of the young group B and aged group E of UWP without metformin, respectively; C and F: Hepatocytes of the young group C and aged group F of MUWP, respectively. The black arrows indicate the nuclei, the white arrows indicate the intracellular mitochondria of the young groups, and the white triangles indicate the intracellular mitochondria of the aged groups. UWP: University of Wisconsin solution perfusion; MUWP: Metformin perfusion solution. Original magnification (A-F): ×10000.

scores between the young rat control group (group A) and the aged rat control group (group D) ($P < 0.05$). The histologic scores of the young rat group and the aged rat group of UWP and MUWP were, respectively, appreciably higher than those of the young rat control group (group A) and the aged rat control group (group D), and that histologic scores of the young group C and the aged group F of MUWP were, respectively, appreciably lower than those of the young group B and the aged group E of UWP ($P < 0.05$). Besides, the histologic scores of aged group E were significantly higher than those of young group B of UWP ($P < 0.05$), but no significant differences were found between the young group C and the aged group F of MUWP (Figure 4G).

Histological observation by electron microscopy

The ultrastructural changes of hepatocytes were observed under an electron microscope. Structure of hepatocytes in the young control group (group A) and aged control group (group D) was generally normal with a round and clear nucleus located in the center of the cells, while lipid droplets in the cytoplasm increased in the aged control group (group D), and fat-storing cells were seen in the Disse's space with irregular shapes, and the cytoplasm in it contained a large amount of lipid droplets. In contrast, lipid droplets and fat-storing cells were not found in the young control group (group A), but the cells contained rich cytoplasmic organelles (Figure 5A-D). Hepatocyte swelling, nuclear chromatin condensation, extensive mitochondrial swelling, and crista fragmentation were observed in groups B and E of UWP, more severe in the aged group E. Likewise, fat-storing cells in the Disse's space increased in the aged group E, but not found in the young group E (Figure 5B-E). In contrast, hepatocytes of the MUWP group showed mild edema with round nucleus, slightly more evident in the young group C than in the aged group F, and also the mitochondrial swelling degree was slightly higher in the young group C. In addition, the increase in lipid droplets in the cytoplasm and the number of fat-storing cells, dilation of the smooth endoplasmic reticulum, and intercellular collagenous fibrosis were only seen in the aged group F (Figure 5C-F).

The ultrastructural changes of the hepatic sinusoid were also observed under an electron microscope. Structure of the hepatic sinusoidal endothelial cells in the young control group A and the aged control group D remained generally intact with protrusion into the sinusoid, being clear in the young group A. Kupffer cells

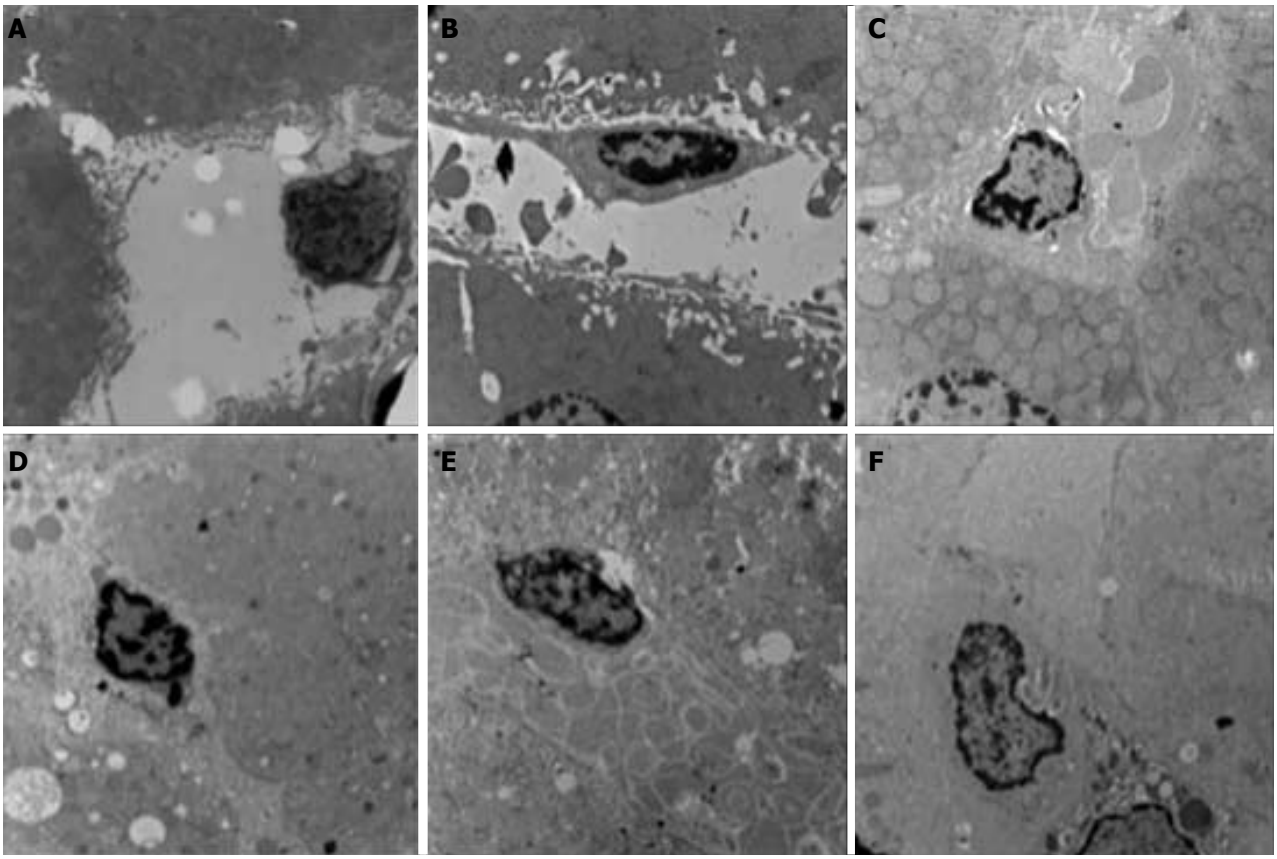


Figure 6 Ultrastructural changes of liver sinusoidal endothelial cells. A and D: Sinusoidal endothelial cells of the young control group A and the aged control group D, respectively; B and E: Sinusoidal endothelial cells of the young group B and aged group E of UWP, respectively; C and F: Sinusoidal endothelial cells of the young group C and aged group F of MUWP, respectively. UWP: University of Wisconsin solution perfusion; MUWP: Metformin perfusion solution. Original magnification (A–F): ×10000.

were seen in the aged group D, though (Figure 6A-D). Sinusoidal endothelial cells were kept meristematic and flat-shaped, with chromatin showing mild margination, and cell debris-like structure together with Kupffer cells in the sinusoids was increased in groups B and E of UWP, particularly, more severely in aged group E. Likewise, fat-storing cells increased in the aged group E, while they were not found in the young group E (Figure 6B-E). The structure of the hepatic sinusoidal endothelial cells in the MUWP group remained generally normal, while a slightly larger cell volume was seen in the aged group F, and cubic-shaped sinusoidal endothelial cells with protrusion into the sinusoid were seen in the young group C. In addition, the low-electron-density protein substance could be seen in the sinusoid in the aged group F.

DISCUSSION

An increasing number of patients with liver disease await liver transplantation; the acute shortage of donor livers is only likely to continue. It was believed that aged livers from donors above 60 years old were characterized by small sizes, atherosclerosis, steatosis, and decreased function of metabolism, and were not suitable for donation[17]. However, to cope with the demand for donor livers, marginal liver donation has been applied clinically after following strict screening protocols with good transplantation outcomes and breaking the myth for the age limit of liver donors[18,19]. Unlike hearts, lungs, and kidneys, livers are much less influenced by age with respect of pathophysiological changes. However, aged livers also show morphologic changes, such as hepatic fibrosis, hepatocyte swelling, trend for multinucleation, increase of lipofuscin, and reduction in the size of the hepatocyte. Compared with young donors, aged donors show reduced and shrunken fenestra of liver sinusoidal endothelial cells, thickened hepatic sinusoidal endothelium, and pseudo capillarization formed by discontinuous basement membranes, leading to potential microcirculatory disturbance and a risk of aggravating IRI[20]. Thus, easier apoptosis and detachment of liver sinusoidal endothelial cells of aged livers further aggravate microcirculatory disturbance, seriously influence post-transplant effects, and even cause failure of some liver transplantation[21]. According to the results of this study, aged groups appeared to be more sensitive to the possible protective effect of metformin. Furthermore, the histological observations by light and

electron microscopy also support this conclusion.

In order to prevent cold ischemia injury, auxiliary protective intervention by drugs with different mechanisms of action, including the MAPK agonist, is being explored[22]. Scientists from different academic institutions have published research articles in which they revealed the molecular mechanism by which metformin could activate AMPK and inhibit the mTORC1 signaling pathway in livers *via* the AXIN/LKB1-v-ATPase-Ragulator pathway[23,24], protect the integrity of epithelial cells in multiple stressed conditions (such as inflammation, infection, and anoxia), and even demonstrate an anticancer effect[25-27]. Before revealing this molecular mechanism, it was known that AMPK activation plays a critical role in reducing liver IRI. However, how AMPK and eNOS phosphorylation direct their effects on the endothelial function is still elusive[28], even if there is plenty of evidence to show that AMPK activation can enhance the activity of eNOS, resulting in generation of nitric oxide[15,29,30]. Extension of cold preservation by maintaining a supercooled state can make donor liver cells remain viable up to 96 h, and human livers showed improvement in endothelial function with 2 h of HMP[31]. In the current study, the AMPK activator metformin was added to e*x vivo* HMP models of rat livers in UW solution after HMP for 12 h. Our results showed that all the biochemical indexes and inflammatory factors in the UWP group were significantly higher than those in the control group, but all the indexes in both the young and aged groups of MUWP, respectively, were appreciably lower than those in the young and aged groups of UWP. Histological observation by light and electron microscopy showed that injury of the related microstructure was milder in the MUWP group than in the UWP group. To a certain extent, it can be deduced that metformin activated protective mechanisms. Detection of expression levels of eNOS and AMPK in the liver sinusoidal endothelial cells of young rats showed that phosphorylation of AMPK and eNOS was increased in the MUWP group at 12 h after HMP, and this can be inferred as the AMPK/eNOS pathway was activated *ex vivo* by metformin.

This study confirmed that the addition of metformin to organ preservation solution can activate the AMPK/eNOS pathway, and this can not only significantly decrease the inevitable injury to donor livers caused by long-term HMP, but can reduce the difference between aged and young livers after HMP, protecting livers of aged rats, which should probably improve the utilization of marginal liver donor tissues. However, whether metformin can sequentially improve hepatic injury during reperfusion-ischemia requires further investigation. But at least, we provided a novel idea, which is also a simple procedure for drug auxiliary intervention with HMP in *ex vivo* rat donor liver and deserves further research with a promising prospect.

In conclusion, this study confirmed that metformin can activate the AMPK/eNOS pathway and reduce injury to *ex vivo* rat liver during cold ischemia in MP. The combination of metformin with the organ preservation solution effectively enhanced the quality of donor livers, with significant protective effects on livers especially of aged rats, which could be used to improve the utilization of marginal livers.

ARTICLE HIGHLIGHTS

Background

Liver transplantation is the only effective therapy for end-stage liver disease. At present, due to the shortage of liver donation, marginal donation, which includes aged donation, adipo-hepatic donation, and donation after cardiac death, increases the risk for more severe ischemic reperfusion injury (IRI) because of suboptimal function and long-term warm and cold ischemia. Therefore, there is a current pressing need to explore and improve methods of organ preservation and minimize IRI of donor livers during transplantation.

Research frontiers

According to the latest research, machine perfusion has been meaningful for the preservation and repair of marginal liver donation. Another important direction of research on donor liver cold preservation is the auxiliary protective intervention of donor livers against IRI factors of microcirculation and hepatocyte metabolism through drugs. Activation of adenosine 5'-monophosphate-activated protein kinase (AMPK) signaling pathways increases eNOS activity to generate nitric oxide (NO), which plays an important role in the protection of liver sinusoidal endothelial cells. Metformin is an agonist of AMPK. Hence, we assumed that liver sinusoidal endothelial cells can be protected from injury by activating AMPK signaling pathways with the addition of metformin perfused *in vitro*, which could ultimately cause an improvement of liver donor organ preservation.

Research objectives

In this study, we added metformin to University of Wisconsin (UW) solution, to compare the effect of UW solution with or without metformin, an AMPK activator, for preserving standard and marginal criteria liver grafts of young and aged rats *ex vivo* by hypothermic machine perfusion (HMP).

Research methods

Eighteen young (4-mo-old) and 18 aged (17-mo-old) healthy male SD rats were selected and randomly divided into three groups: control group, UW solution perfusion group (UWP), and UW solution with metformin perfusion group (MUWP). Aspartate aminotransferase (AST), alanine aminotransferase (ALT), *lactate dehydrogenase* (LDH), interleukin-18 (IL-18), and tumor necrosis factor-alpha (TNF-α) in the perfused liquid were tested. The expression levels of AMPK and eNOS in liver sinusoidal endothelial cells were also examined. Additionally, microscopic evaluation of the harvested perfused liver tissue samples was done.

Research results

AST, ALT, LDH, IL-18 and TNF-α levels in the young and aged liver-perfused liquid in the MUWP group were, respectively, significantly lower than those in the UWP group ($P < 0.05$), but no significant differences between the young and aged MUWP groups were found. Metformin increased the expression of AMPK and eNOS protein levels, and promoted the extracellular release of nitric oxide through activation of the AMPK-eNOS mediated pathway. Histological examination revealed that in the MUWP group, the extent of liver cells and tissue damage was significantly reduced compared with the UWP group.

Research conclusions

This experiment confirmed that the addition of metformin to organ preservation solution can activate AMPK/eNOS pathway, which can not only reduce injury to *ex vivo* rat livers during cold ischemia, but can reduce the difference between aged and young livers after HMP, with especially significant effects of protecting livers of aged rats, which should probably improve the utilization of marginal liver donor tissues. However, whether metformin can sequentially improve hepatic injury during reperfusion-

ischemia requires further investigation. But at least, we provided a novel idea, which is also a simple procedure for drug auxiliary intervention with HMP in *ex vivo* rat donor livers and deserves further research with a promising prospect.

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ORIGINAL ARTICLE

Basic Study

Relationship between autophagy and perineural invasion, clinicopathological features, and prognosis in pancreatic cancer

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Author contributions: Yang YH and Liu JB contributed equally to this work; Yang YH and Liu JB performed the majority of the experiments and critically revised the manuscript; Gui Y and Lei LL assisted with various experiments and helped to analyze the data; Zhang SJ and Liu JB drafted and edited the manuscript.

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Abstract

AIM

To investigate the relationship between autophagy

and perineural invasion (PNI), clinical features, and prognosis in patients with pancreatic cancer.

METHODS

Clinical and pathological data were retrospectively collected from 109 patients with pancreatic ductal adenocarcinoma who underwent radical resection at the First Affiliated Hospital of Zhengzhou University from January 2011 to August 2016. Expression levels of the autophagy-related protein microtubule-associated protein 1A/1B-light chain 3 (LC3) and PNI marker ubiquitin carboxy-terminal hydrolase (UCH) in pancreatic cancer tissues were detected by immunohistochemistry. The correlations among LC3 expression, PNI, and clinical pathological features in pancreatic cancer were analyzed. The patients were followed for further survival analysis.

RESULTS

In 109 cases of pancreatic cancer, 68.8% (75/109) had evidence of PNI and 61.5% (67/109) had high LC3 expression. PNI was associated with lymph node metastasis, pancreatitis, and CA19-9 levels ($P < 0.05$). LC3 expression was related to lymph node metastasis ($P < 0.05$) and was positively correlated with neural invasion ($P < 0.05$, $r = 0.227$). Multivariate logistic regression analysis indicated that LC3 expression, lymph node metastasis, pancreatitis, and CA19-9 level were factors that influenced neural invasion, whereas only neural invasion itself was an independent factor for high LC3 expression. Univariate analysis showed that LC3 expression, neural invasion, and CA19-9 level were related to the overall survival of pancreatic cancer patients ($P < 0.05$). Multivariate COX regression analysis indicated that PNI and LC3 expression were independent risk factors for poor prognosis in pancreatic cancer ($P < 0.05$).

CONCLUSION

PNI in patients with pancreatic cancer is positively related to autophagy. Neural invasion and LC3 expression are independent risk factors for pancreatic cancer with a poor prognosis.

Key words: Pancreatic cancer; Perineural invasion; Autophagy; Clinical pathological features; Prognosis

Core tip: The relationship between autophagy and perineural invasion (PNI) was explored for the first time in pancreatic cancer. Pancreatic cancer PNI is related to microtubule-associated protein 1A/1B-light chain 3 (LC3) expression-determined autophagy. PNI and LC3 expression were independent prognostic factors in pancreatic cancer. There might be a special association between autophagy and PNI, which contributes to pancreatic cancer progression. This study might provide a new insight for the mechanism of PNI in pancreatic cancer.

Yang YH, Liu JB, Gui Y, Lei LL, Zhang SJ. Relationship between autophagy and perineural invasion, clinicopathological features, and prognosis in pancreatic cancer. *World J Gastroenterol* 2017; 23(40): 7232-7241 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i40/7232.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i40.7232

INTRODUCTION

Pancreatic cancer, also known as "the king of cancer", is a malignant tumor with a poor prognosis that has almost equal mortality and morbidity in patients. The incidence of pancreatic cancer is increasing yearly[1]. Surgical resection is the only possible cure of pancreatic cancer, although less than 20% of patients are eligible for radical surgery[2]. At the time of diagnosis, most pancreatic cancer patients have distant metastasis due to early occult symptoms, a lack of effective screening, and perineural growth characteristics. The incidence of perineural invasion (PNI) in pancreatic cancer is up to 80%-100% and is an important factor leading to postoperative pancreatic cancer recurrence. Previous studies have shown a higher recurrence rate after surgery and shorter disease-free and overall survival rates in cases of pancreatic cancer with PNI compared with those of cases without PNI. PNI evaluation of pancreatic cancer can predict disease recurrence and prognosis after surgery[3,4]. However, the pathogenesis of PNI has not yet been defined.

Autophagy has a dual role in promoting and inhibiting tumor growth[5,6]. Autophagy, as a mechanism of avoidance of anoikis in pancreatic cancer, is closely related to the survival of pancreatic cancer cells. Microtubule-associated protein 1A/1B-light chain 3 (LC3) is a typical marker of autophagy. LC3 labeling has been used to evaluate autophagy, and high levels of LC3 expression have been found in pancreatic cancer cells[7]. In addition, a previous study also showed that pancreatic cancer cells with PNI have higher levels of autophagy[7].

No study has examined the relationship between autophagy and PNI in pancreatic cancer cells. However, it can be inferred that only those pancreatic cancer cells that can survive within nerve tissues can eventually develop into a clinically visible form of pancreatic cancer PNI. Autophagy is likely one of the mechanisms involved in cancer cell survival. Therefore, this study focused on the relationship between pancreatic cancer cell autophagy and PNI, clinicopathological features, and prognosis, with an aim to provide a clinical basis for further study of the autophagy mechanisms affecting

the pathogenesis of pancreatic cancer PNI.

MATERIALS AND METHODS

General data

Retrospective data were collected from 109 cases of pathologically confirmed pancreatic ductal adenocarcinoma patients who underwent radical surgery for pancreatic cancer from January 2011 to August 2016 at the First Affiliated Hospital of Zhengzhou University. The included pancreatic cancer patients were not treated with radiation or chemotherapy prior to surgery, but received postoperative adjuvant gemcitabine- or non-gemcitabine-based chemotherapy, and/or radiotherapy. Tissue specimens were fixed in formalin and paraffin-embedded for histological study. Clinical and pathological data were collected, and all cases were followed. The date of surgical resection was considered as the starting time, and August 2016 was the deadline. The primary endpoint was death due to pancreatic cancer. Eighty cases were followed for more than 12 mo. All patients provided informed consent for the collection of biological samples, and this study was approved by the Institutional Review Board of the First Affiliated Hospital of Zhengzhou University (Scientific Research No. 5, 2015).

Out of the 109 patients studied, 61 were male, and 48 were female. Their ages ranged from 19 to 81 years, and the median age was 59 years. Tumor diameters ranged from 0.7-12 cm with a median value of 4.0 cm. The survival time of 80 patients who were followed for more than 12 mo was 1 to 54 mo, and the median survival time was 20 mo. By the end of the follow-up period, 38 patients had died. The clinical stages were classified according to the AJCC 2011 standard.

Immunohistochemistry

Expression levels of LC3 and the nerve fiber marker ubiquitin carboxy-terminal hydrolase (UCH) were detected by immunohistochemistry using a standardized streptavidin-peroxidase (SP) method and the SP immunohistochemical Kit (ZSGB-Bio, Beijing, China) according to the manufacturer's instructions. The pancreatic cancer tissues were routinely embedded in paraffin and sliced into 4-μm-thick continuous sections. The sections were then warmed in an autoclave to remove residual wax and were hydrated before antigen retrieval. Endogenous peroxidase activity was eliminated by incubating the tissue sections at room temperature with 3% H_2O_2 for 10 min. The tissue sections were washed three times with PBS continuously and incubated with a small amount of goat serum at room temperature in a closed chamber for 15 min. The goat serum was then poured off (not washed), and anti-LC3 (Proteintech Group, diluted 1:120) and anti-UCH antibodies (Proteintech, diluted 1:100) were added and incubated overnight at 4 ℃. The next day, the slides were thoroughly washed with PBS, and a biotinylated secondary antibody in a working liquid was added and incubated at room temperature for 15 min. After the slides were washed carefully with PBS again, horseradish peroxidase was added, and the slides were incubated for 5 min at room temperature. The slides were then rinsed with PBS three times, and a drop of DAB buffer was placed on the tissue slice and rinsed after 1 min to stop the color reaction. Then, the nuclei were stained with hematoxylin, and the samples were dehydrated in gradient alcohol, covered with transparent balata, and observed by microscopy. PBS was used in place of the primary antibody to serve as the negative control, and a known positive slice served as the positive control. The immunohistochemical results were evaluated in a double-blind manner by two pathologists. If the results were inconsistent, a third pathologist reviewed the data to reach a consensus.

LC 3 immunohistochemical score

LC3-positive cells detected by immunohistochemistry required the following characteristics: (1) clear cell structure; (2) accurate localization of the positive particle; and (3) obviously higher pigmentation than that of the background and clear contrast. Positive LC3 expression was mainly localized in pancreatic cancer cell cytoplasm. Five random power fields (400 × magnification) were observed for each case using an optical microscope. One hundred homogeneous cells were counted, and the staining intensity and the proportion of positive cells were observed. A semi-quantitative analysis was conducted using the product method. Dye intensity was scored as follows: no yellow, 0 points; light yellow, 1 point; yellow or deep yellow, 2 points; and brown or tan, 3 points. The expression range was scored as follows: <10%, 0 points; 10% to 25%, 1 point; 26% to 50%, 2 points; 51% to 75%, 3 points; and >75%, 4 points. The value from dye intensity was then multiplied by the value from the expression range to obtain the overall score: 0 points, negative (-); 1 to 3 points, weakly positive (+); 4 to 6 points, moderately positive (+ +); and 7 to 12 points, strongly positive (+ + +). More than 3 points indicated high expression, and 3 points or fewer indicated low expression.

PNI evaluation

UCH was expressed in the cytoplasm of all nerve fibers. The location of nerve fibers can be clearly defined by UCH to determine the invasion of cancer cells to the nerve tissue. According to previous reports, positive PNI status was determined as cancer cells being found in the perineural spaces, perineurium or nerve tract[8].

Survival analysis

The effects of clinicopathological factors such as LC3 expression and PNI on the overall survival in pancreatic cancer were analyzed in 80 patients with

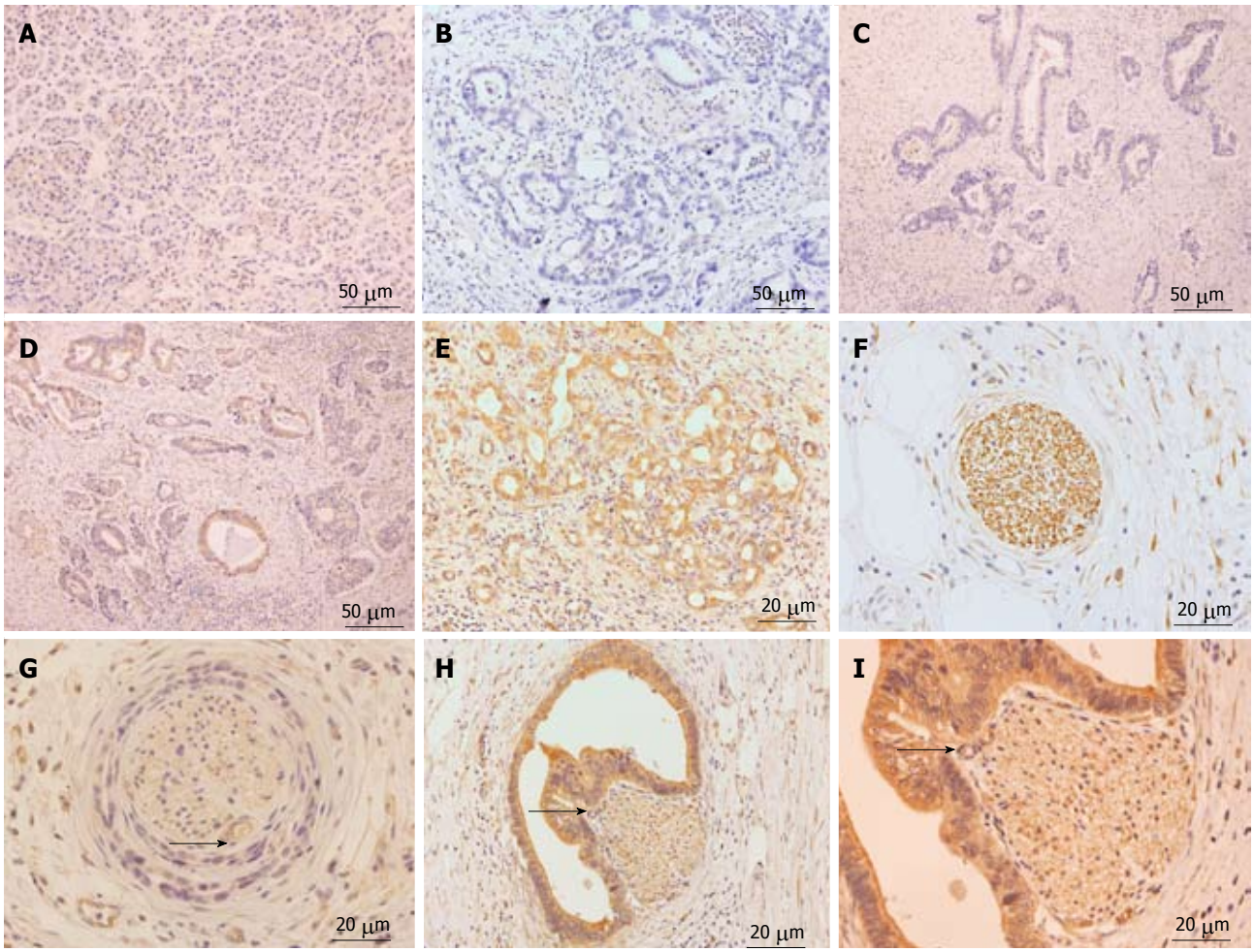

Figure 1 Representative immunohistochemical results of microtubule-associated protein 1A/1B-light chain 3 and perineural invasion. A: Negative expression of LC3 in normal paraneoplastic pancreatic tissue (× 200); B: Negative expression of LC3 in pancreatic cancer tissue (× 200); C: Weakly positive expression of LC3 in pancreatic cancer tissue (× 200); D: Moderately positive expression of LC3 in pancreatic cancer tissue (× 200); E: Strongly positive expression of LC3 in pancreatic cancer tissue (× 200); F and G: Perineural invasion in pancreatic cancer tissues (× 400, arrow represents cancer cells infiltrating into nerve tissue); H: Pancreatic cancer cells with high LC3 expression enclosing and invading into nerve tissue (× 200, arrow represents cancer cells infiltrating into nerve tissue); I: Pancreatic cancer cells with high LC3 expression enclosing and invading into nerve tissue (× 400, arrow represents cancer cells infiltrating into nerve tissue). LC3: Microtubule-associated protein 1A/1B-light chain 3.

more than 12 mo of follow-up data. The following factors were included in the survival analysis: LC3 expression, age, gender, tumor location, tumor size, histological grade, clinical stage, vascular invasion, lymph node metastasis, pancreatitis, diabetes status, and preoperative CA19-9 level.

Data analysis

Statistical analyses were performed using SPSS 19.0 and GraphPad Prism 5.0 statistical software. Enumeration data were checked by the Chi-square test or the four-grid table Fisher exact probability method. Correlations between clinicopathological factors such as LC3 expression and PNI were analyzed using the Spearman correlation method. LC3 expression and the factors that independently influenced neural infiltration were analyzed using two categories and unconditional logistic regression. Univariate and multivariate analyses were performed on factors that might affect the prognosis according to a COX risk regression model. The survival curve was plotted according to the Kaplan-Meier method. Results were considered significant when $P < 0.05$.

RESULTS

Evaluation of LC3 expression and PNI in pancreatic cancer

LC3 expression was mainly localized to the cytoplasm. In contrast to normal paraneoplastic pancreatic tissues (Figure 1A), the expression of LC3 in pancreatic cancer tissues ranged from low to high in four grades: negative, weakly positive, moderately positive, and strongly positive (Figure 1B-E). The immunohistochemical results indicated that LC3 protein expression was observed in pancreatic ducts, acinar epithelial cells, islet cells, and pancreatic cancer tissues. There was significantly increased LC3 protein expression in pancreatic cancer tissues and peritumoral tissues. In 109 pancreatic cancer tissues, 42 had

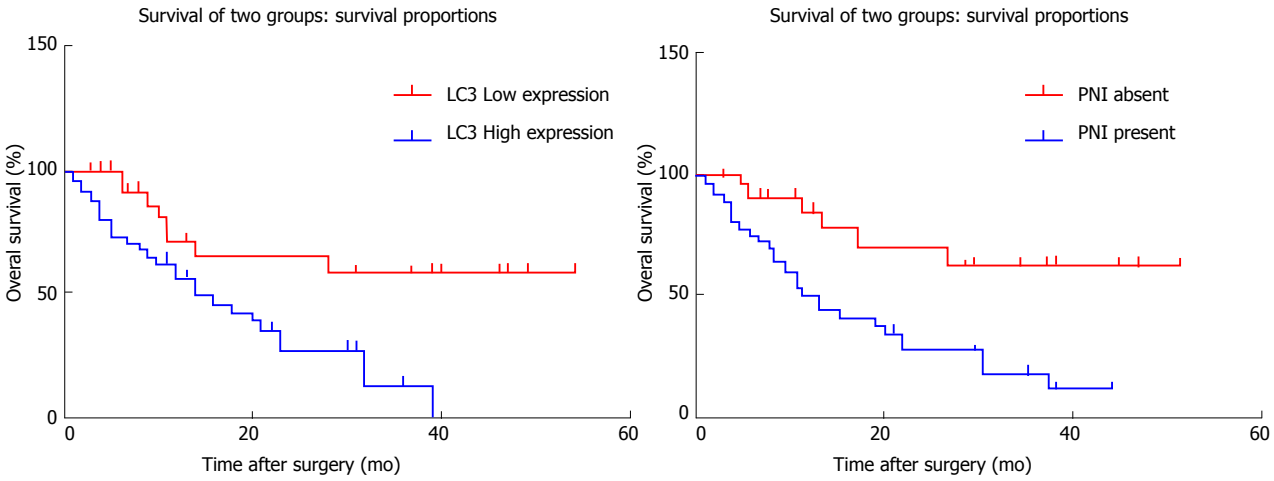

Figure 2 Kaplan-Meier estimates of overall survival in patients who underwent radical surgery. A: The overall survival rate of the LC3 low-expression group was better than that of the high-expression group ($P < 0.05$); B: The overall survival rate of the patients without nerve invasion group was better than that of those with nerve infiltration ($P < 0.05$).

Table 1 Relationship between microtubule-associated protein 1A/1B-light chain 3 expression and perineural invasion in pancreatic cancer

LC3	PNI		*P* value	*r*
	Absent	Present		
Low expression	18	22	0.018	0.227
High expression	16	53		

$P < 0.05$, PNI absent *vs* present. PNI: Perineural invasion

low LC3 expression (termed "low autophagy level"), including 6 (5.5%) negative cases and 36 (33%) weakly positive cases (Figure 1B and C); while 67 had high LC3 expression (termed "high autophagy level"), including 50 (45.9%) that were moderately positive and 17 (15.6%) that were strongly positive (Figure 1D and E). PNI by pancreatic cancer cells occurred mainly in the pancreatic cancer stroma. According to a previous study[9], four types of relationships exist between PNI and cancer nests: no PNI, perineurium invasion, perineural space invasion, and invasion to the nerve fiber tracts. In 109 cases of pancreatic cancer, 75 were positive (Figure 1F-I), and 34 were negative for nerve invasion. The positive rate of nerve invasion was 68.8%. High LC3 expression was also found in the nests surrounding the PNI (Figure 1I).

Relationship between LC3 expression and PNI in pancreatic cancer

The analysis of LC3 expression and PNI of pancreatic cancer demonstrated a significant positive correlation between these two parameters ($P = 0.018$, $r = 0.227$). LC3 expression in pancreatic cancer tissues with PNI was significantly higher than that in pancreatic cancer tissues without PNI (Table 1).

Relationship between pancreatic cancer cell autophagy and PNI and clinicopathological features

LC3 expression was associated with lymph node metastasis ($P < 0.05$). LC3 expression was not related to sex, gender, tumor location, tumor size, histological grade, clinical stage, vascular invasion, or diabetes mellitus status. PNI was related to lymph node metastasis, pancreatitis, and CA19-9 levels ($P < 0.05$) and was not related to sex, age, tumor location, tumor size, histological grade, clinical stage, vascular invasion, or diabetes mellitus status (Table 2).

Multivariate logistic regression analysis of LC3 expression and PNI

The clinicopathological factors possibly associated with LC3 expression and PNI were evaluated by multivariate analysis using a logistic regression model. The clinicopathological factors included LC3 expression and nerve infiltration, age, tumor site, tumor size, histological grade, clinical stage, vascular invasion, lymph node metastasis, diabetes, pancreatitis, and preoperative CA19-9 level. The results showed that LC3 expression, lymph node metastasis, pancreatitis, and CA19-9 level were the factors that influenced the occurrence of PNI, which was an independent factor affecting LC3 expression (Tables 3 and 4).

Survival analysis

According to LC3 expression, patients were divided into a high-expression or a low-expression group. The overall survival rate of the low-expression group was better than that of the high-expression group, and the risk of death was 2.78-times higher in the LC3 high-expression group than that in the low-expression group. This difference between the two groups was significant (Figure 2A). The patients were also

Table 2 Relationships of perineural invasion and microtubule-associated protein 1A/1B-light chain 3 expression with clinicopathological features

Parameter	*n*	PNI		*P* value	LC3		*P* value
		Absent	Present		Low expression	High expression	
Age (yr)							
≤ 58	54	19	35	0.373	23	31	0.206
> 58	55	15	40		17	38	
Gender							
Male	61	15	46	0.093	26	35	0.148
Female	48	19	29		14	34	
Tumor location							
Head	66	17	49	0.129	28	38	0.124
Body/tail	43	17	26		12	31	
Tumor size (cm)							
≤ 2	18	6	12	0.83	8	10	0.455
> 2	91	28	63		32	59	
Histologic grade							
Well or moderate	74	24	50	0.685	23	51	0.077
Poor	35	10	25		17	18	
Vascular invasion							
Negative	83	26	57	0.957	31	52	0.801
Positive	26	8	18		9	17	
Lymph node metastasis							
Negative	36	17	19	0.011[a]	18	18	0.043[a]
Positive	73	17	56		22	51	
AJCC stage							
I + II	69	22	47	0.838	25	44	0.895
III + IV	40	12	28		15	25	
Pancreatitis							
Negative	30	15	15	0.009[a]	12	18	0.659
Positive	79	19	60		28	51	
Diabetes							
Negative	89	27	62	0.684	33	56	0.862
Positive	20	7	13		7	13	
CA19-9 level (U/mL)							
≤ 37	37	19	19	0.001[a]	13	24	0.808
> 37	72	15	57		27	45	

[a]*P* < 0.05, LC3 low expression *vs* high expression; PNI absent *vs* present. LC3: Microtubule-associated protein 1A/1B-light chain 3; PNI: Perineural invasion.

Table 3 Multivariate logistic regression analysis of perineural invasion with clinicopathological features in pancreatic cancer

Parameter	Estimate, B	Standard error	Wald statistic	*P* value	Odds ratio	95%CI
Lymph node metastasis (positive *vs* negative)	1.068	0.499	4.581	0.032[a]	2.911	1.094-7.743
CA199 (> 37 U/mL *vs* ≤ 37 U/mL)	1.508	0.493	9.368	0.002[a]	4.520	1.720-11.874
Pancreatitis (present *vs* absent)	1.301	0.514	6.419	0.011[a]	3.673	1.343-10.049
LC3 (high *vs* low)	1.032	0.491	4.406	0.036[a]	2.806	1.071-7.351
Constant	-7.209	1.799	16.058	0	0.001	

[a]*P* < 0.05. CI: Confidence interval; LC3: Microtubule-associated protein 1A/1B-light chain 3; PNI: Perineural invasion.

divided into a nerve-invasion group and a no-nerve-invasion group according to whether there was nerve infiltration. The overall survival rate of the patients without nerve invasion was better than that of the nerve-invasion group. The risk of death was 2.93-times greater in the PNI-positive group than in the PNI-negative group, and the difference between the two groups was significant (Figure 2B). Univariate analysis showed that CA19-9 level, PNI, and LC3 expression influenced the prognosis (Table 5). A factor of *P* < 0.2 was added into the COX risk regression model. Multivariate analysis was performed using the stepwise conditional method. The results showed that PNI and LC3 expression were independent prognostic factors that influenced pancreatic cancer (Table 6).

DISCUSSION

Pancreatic cancer has a poor treatment outcome because of a low resection rate, early invasion and metastasis, and insensitivity to radiotherapy and chemotherapy[10-13]. PNI is common in pancreatic cancer, and also found in breast, prostate, and rectal cancers[14]. Many studies have suggested that PNI is the main cause of abdominal pain in patients and is also one of the important causes of local recurrence of pancreatic

Table 4 Multivariate logistic regression analysis of microtubule-associated protein 1A/1B-light chain 3 expression with clinicopathological features in pancreatic cancer

Parameter	Estimate, B	Standard error	Wald statistic	*P* value	Odds ratio	95%CI
PNI (present *vs* absent)	0.997	0.427	5.451	0.02[a]	2.71	1.174-6.259
Constant	-1.115	0.732	2.316	0.128	0.328	

[a]$P < 0.05$. PNI: Perineural invasion.

Table 5 Univariate analysis of survival in patients who underwent radical surgery

Parameter	Hazard ratio	95%CI	*P* value
PNI (present *vs* absent)	3.701	1.539-8.903	0.003[a]
LC3 (high *vs* low)	3.196	1.433-7.126	0.005[a]
Sex (male *vs* female)	1.154	0.590-2.260	0.676
Age (> 58 yr *vs* ≤ 58 yr)	1.176	0.621-2.225	0.619
Tumor location (body/tail *vs* head)	1.102	0.570-2.131	0.773
Histologic grade (poorly *vs* well or moderate)	1.287	0.636-2.604	0.484
Tumor size (> 2 cm *vs* ≤ 2 cm)	0.940	0.444-1.991	0.871
Vascular invasion (positive *vs* negative)	1.821	0.883-3.755	0.105
Lymph node metastasis (positive *vs* negative)	0.871	0.449-1.688	0.682
AJCC stage (III + IV *vs* I + II)	1.473	0.752-2.889	0.259
Diabetes (present *vs* absent)	1.105	0.522-2.337	0.795
Pancreatitis (present *vs* absent)	1.075	0.520-2.222	0.845
CA19-9 level (> 37 U/mL *vs* ≤ 37 U/mL)	2.648	1.286-5.454	0.008[a]

[a]$P < 0.05$. LC3: Microtubule-associated protein 1A/1B-light chain 3; PNI: Perineural invasion.

cancer[15-19]. PNI is a continuous process involving multiple molecular factors and tumor microenvironment, but it is unclarified how cancer cells maintain their survival and proliferation from pancreatic cancer tissues to the external pancreatic plexus[20]. Autophagy is the process of degrading cytoplasmic proteins or organelles through lysosomes. Under physiological conditions, autophagy plays a major role in maintaining the intracellular environment stability[21,22]. Autophagy is an important mechanism of escaping apoptosis for tumor cells. Moreover, autophagy may mediate resistance to chemotherapy in pancreatic cancer[23-25]. Therefore, this study was designed and completed in a retrospective manner to evaluate the relationship between pancreatic cancer cell autophagy and PNI and patient survival.

This study found that high expression of LC3 was present in the cancer nests around the nerve infiltration, consistent with the discovery of Yang *et al*[7]. In histology terms, it has been suggested that high LC3 expression is related to PNI. Further analysis of immunohistochemical data showed that there was a significant positive correlation between LC3 expression and PNI in pancreatic cancer tissues. Therefore, we speculated that, in the PNI process, a high autophagy level may assist cancer cells in escaping apoptosis, avoiding the damage of adverse stress and providing energy for the invasion and metastasis of pancreatic cancer.

There is dissidence in the correlation between PNI and lymph node metastasis[26-28]. This study showed that PNI was associated with lymph node metastasis, pancreatitis, and CA19-9 levels ($P < 0.05$), while it had no association with sex, age, tumor location, tumor size, histological grade, clinical stage, vascular invasion, or diabetes mellitus. LC3 was related to lymph node metastasis but no other clinicopathological features. Multivariate logistic regression analysis also showed that LC3, lymph node metastasis, pancreatitis, and CA19-9 levels were the factors that influenced PNI, while PNI was an independent factor affecting LC3 expression. Tanaka *et al*[26] and Ozaki *et al*[28] believe that lymph node metastasis can promote PNI. The cancer cells in lymph node metastases can form a lymphatic satellite around the nerve and break through the nerve membrane; then, nerve invasion occurs. This study is consistent with results suggesting the need for regional lymph node dissection during surgical treatment[27]. Through this study, we can conclude that high LC3 expression, lymph node metastasis, pancreatitis, and CA19-9 levels usually indicate the possibility of nerve invasion. Gender, age, tumor site, tumor size, histological grade, clinical stage, vascular invasion, and diabetes are not effective indicators of neural invasion and autophagy and cannot be used as a determinant of resection of the peripancreatic nerve

Table 6 Multivariable analysis of survival in patients who underwent radical surgery

Parameter	Hazard ratio	95%CI	*P* value
PNI (present *vs* absent)	2.962	1.212-7.238	0.017[a]
LC3 (high *vs* low)	2.491	1.107-5.608	0.027[a]

[a]$P < 0.05$. LC3: Microtubule-associated protein 1A/1B-light chain 3; PNI: Perineural invasion.

during surgery.

The overall survival rates of the LC3 high-expression group and the nerve-invasion group were significantly lower than those of the low-expression group and the no-nerve-invasion group. Univariate analysis showed that the level of CA19-9, PNI, and autophagy were associated with prognostic factors. Multivariate analysis showed that PNI and high LC3 expression were independent prognostic factors in pancreatic cancer patients. Autophagy is very complex and often plays an important role in tumor progression[6,29]. Interestingly, this study found that high autophagy level is closely related to PNI, while both of which are independent risk factors for pancreatic cancer with a poor prognosis. The autophagy associated with poor survival in pancreatic cancer could be explained by the properties of autophagy assisting cancer cells to evade stress-induced apoptosis in PNI environment and undoubtedly promote tumor cell survival[30,31]. Therefore, the high autophagy of cancer cells may promote the malignant progression of pancreatic cancer, resulting in PNI and the poor treatment outcome in patients with the disease.

In summary, autophagy and PNI of pancreatic cancer cells are independent risk factors for adverse prognosis. There is a significant correlation between them, and there may be a pathway between them through which they interact with each other to promote the malignant progression of pancreatic cancer. How to control the role of autophagy in PNI of pancreatic cancer and then improve cancer prognosis requires further studies into the molecular mechanisms involved.

ARTICLE HIGHLIGHTS

Research background

Pancreatic cancer is a malignant tumor with a poor prognosis that has almost equal mortality and morbidity in patients. At the time of diagnosis, most pancreatic cancer patients have distant metastasis due to early occult symptoms, a lack of effective screening, and perineural growth characteristics. The incidence of perineural invasion (PNI) in pancreatic cancer is 80%-100% and is an important factor leading to postoperative pancreatic cancer recurrence. PNI evaluation of pancreatic cancer can predict disease recurrence and prognosis after surgery. However, the pathogenesis of PNI has not yet been defined. Autophagy, as a mechanism of avoidance of anoikis in pancreatic cancer, is closely related to the survival of pancreatic cancer cells. Microtubule-associated protein 1A/1B-light chain 3 (LC3) is a typical marker of autophagy. LC3 labeling has been used to evaluate autophagy, and high levels of LC3 expression have been found in pancreatic cancer cells. In addition, a previous study showed that pancreatic cancer cells with PNI have higher levels of autophagy. No study has examined the relationship between autophagy and PNI in pancreatic cancer cells.

Research motivation

The relationship between autophagy and PNI was explored for the first time in pancreatic cancer. Pancreatic cancer PNI is related to LC3 expression-determined autophagy. PNI and LC3 expression were independent prognostic factors in pancreatic cancer. There might be a special association between autophagy and PNI, which contributes to pancreatic cancer progression. This study might provide new insight into the mechanism of PNI in pancreatic cancer.

Research objectives

This study focused on the relationship between pancreatic cancer cell autophagy and PNI, clinicopathological features, and prognosis. The authors found that autophagy and PNI of pancreatic cancer cells are independent risk factors for adverse prognosis. There is a significant correlation between them, and there may be a pathway between them through which they interact with each other to promote the malignant progression of pancreatic cancer. Controlling the role of autophagy in PNI of pancreatic cancer may improve cancer prognosis, which requires further studies into the molecular mechanisms involved.

Research methods

Clinical and pathological data were retrospectively collected from 109 patients with pancreatic ductal adenocarcinoma who underwent radical resection at the First Affiliated Hospital of Zhengzhou University from January 2011 to August 2016. Expression levels of the autophagy-related protein LC3 and perineural invasion marker ubiquitin carboxy-terminal hydrolase in pancreatic cancer tissues were detected by immunohistochemistry. The correlations among LC3 expression, perineural invasion, and clinical pathological features in pancreatic cancer were analyzed. The patients were followed for further survival analysis.

Research results

In this study, they found that LC3, lymph node metastasis, pancreatitis, and CA199 level were factors that influenced neural invasion, whereas only neural invasion itself was an independent factor of high LC3 expression. Perineural invasion and LC3 expression were independent risk factors for poor prognosis in pancreatic cancer. There is a significant correlation between them.

Research conclusions

This study focused on the relationship between pancreatic cancer cell autophagy and PNI, clinicopathological features and prognosis. The authors found that autophagy and PNI of pancreatic cancer cells are independent risk factors for adverse prognosis. There is a significant correlation between them, and there must be a pathway between them through which they interact with each other to promote the malignant progression of pancreatic cancer. Controlling the role of autophagy in PNI of pancreatic cancer may improve cancer prognosis, which requires further studies into the molecular mechanisms involved.

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ORIGINAL ARTICLE

Basic Study

Dachaihu decoction ameliorates pancreatic fibrosis by inhibiting macrophage infiltration in chronic pancreatitis

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Abstract

AIM

To explore the role of macrophages in chronic pancreatitis (CP) and the effect of Dachaihu decoction (DCHD) on pancreatic fibrosis in mice.

METHODS

KunMing mice were randomly divided into a control group, CP group, and DCHD group. In the CP and DCHD groups, mice were intraperitoneally injected with 20% L-arginine (3 g/kg twice 1 d/wk for 6 wk). Mice in the DCHD group were administered DCHD intragastrically at a dose of 14 g/kg/d 1 wk after CP induction. At 2 wk, 4 wk and 6 wk post-modeling, the morphology of the pancreas was observed using hematoxylin

and eosin, and Masson staining. Interleukin-6 (IL-6) serum levels were assayed using an enzyme-linked immunosorbent assay. Double immunofluorescence staining was performed to observe the co-expression of F4/80 and IL-6 in the pancreas. Inflammatory factors including monocyte chemoattractant protein-1 (MCP-1), macrophage inflammatory protein-1α (MIP-1α) and IL-6 were determined using real time-polymerase chain reaction. Western blot analysis was used to detect fibronectin levels in the pancreas.

RESULTS

Compared with the control group, mice with 20% L-arginine-induced CP had obvious macrophage infiltration and a higher level of fibrosis. IL-6 serum concentrations were significantly increased. Double immunofluorescence staining showed that IL-6 and F4/80 were co-expressed in the pancreas. With the administration of DCHD, the infiltration of macrophages and degree of fibrosis in the pancreas were significantly attenuated; IL-6, MCP-1 and MIP-1α mRNA, and fibronectin levels were reduced.

CONCLUSION

The dominant role of macrophages in the development of CP was mainly related to IL-6 production. DCHD was effective in ameliorating pancreatic fibrosis by inhibiting macrophage infiltration and inflammatory factor secretion in the pancreas.

Key words: Dachaihu decoction; Pancreatic fibrosis; Macrophages; Interleukin-6

Core tip: Macrophages, important inflammatory cells, can also promote fibrogenesis by interfering with the synthesis and degradation of the extracellular matrix, as confirmed in both liver fibrosis and renal fibrosis models. Our study suggested that macrophages also play an important role in the development of pancreatic fibrosis. We found that macrophages are an important source of interleukin-6, which is involved in the progression of chronic pancreatitis (CP). Dachaihu decoction (DCHD), a traditional Chinese medicinal formula, effectively improves the clinical symptoms of CP patients, but the underlying mechanism remains unclear. We found that DCHD ameliorates pancreatic fibrosis by inhibiting macrophage infiltration and inflammatory factor secretion.

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INTRODUCTION

Chronic pancreatitis (CP) is a progressive inflammatory disease that is characterized by irreversible injury of the pancreas leading to endocrine and exocrine dysfunction[1]. It has been reported that pancreatic fibrosis is a common pathological feature and characterizes the end-stage of CP of different etiologies[2]. The progressive fibrotic cascade can eventually lead to loss of pancreatic function as well as systemic complications, including malabsorption, diabetes mellitus, and others. However, the underlying molecular mechanisms of CP remain unclear, and the available therapeutic strategies are very few in number[3].

Based on the necrosis-fibrosis sequence playing a key role in the underlying pathogenesis of CP, pancreatic fibrosis commonly arises from inflammation or tissue injury[4]. Recent studies have highlighted the role of macrophages in promoting wound healing and fibrosis[5,6]. Large numbers of macrophages have been observed around areas of pancreatic injury in rat models of CP[7,8]. Detlefsen and colleagues[9] demonstrated that many infiltration macrophages are also found in the pancreas of alcoholic CP patients, which facilitate fibrosis formation by producing transforming growth factor (TGF)-β and platelet derived growth factor (PDGF)-β. Thus, we propose that macrophages may play a critical role in the progression of pancreatic fibrosis, but its definite mechanism remains unclear.

Interleukin-6 (IL-6) is a key pro-inflammatory cytokine that is involved in inflammation and the immune response. It is mainly released by macrophages, fibroblasts, endothelial cells, and myeloid cells[10,11]. In a previous study, Zhang *et al*[12] found that acinar injury results in the infiltration of myeloid cells (*i.e.*, macrophages and neutrophils) into the pancreas during severe acute pancreatitis, and infiltrating macrophages further release IL-6, which can lead to pancreatitis-associated acute lung injury. A study by Lesina *et al*[13] demonstrated that tumor-associated macrophages appear to be the major source of IL-6 in murine and human pancreatic ductal adenocarcinoma. Several reports have confirmed that serum level of IL-6 in patients with CP was significantly increased compared with that in control subjects[12,14]. However, the contribution of IL-6 and the relationship between IL-6 and macrophages in pancreatic fibrosis have not been analyzed in detail.

Dachaihu decoction (DCHD; major Radix bupleuri decoction) is a traditional Chinese medicine formula that was first described by Zhongjing Zhang in "Treatise on Febrile Disease Caused by Cold (Shanghan Lun)". DCHD has been widely used in the clinical treatment of acute pancreatitis (AP)[15]. Recently, it was used to treat patients with CP, and it was found that the effects included improving the clinical symptoms of CP

patients. But whether it can inhibit pancreatic fibrosis and its underlying mechanism remain uncertain. The aim of the present study was to ascertain the role of macrophages in pancreatic fibrosis induced by 20% L-arginine, as well as to determine the effects and mechanism of DCHD on inhibiting pancreatic fibrosis.

MATERIALS AND METHODS

Animals and materials

KunMing mice (20-28 g) were obtained from the Experimental Animal Center of Xi'an Jiaotong University (Certificate No. 0011744) and housed under specific-pathogen-free conditions. The mice received humane care in accordance with the Shaanxi University of Chinese Medicine Animal Care Committee guidelines. Antibodies against fibronectin (FN), F4/80, and IL-6 were provided by Santa Cruz Biotechnology Inc. (Santa Cruz, CA, United States). All secondary antibodies and immunofluorescence and immunohistochemistry reagents were purchased from Boster Biotechnology Inc. (Wuhan, China). L-arginine was obtained from Sigma-Aldrich Co. (St. Louis, MO, United States). Crude DCHD, including Radix bupleuri, Radix scutellariae, Fructus aurantii immaturus, Paeonia lactiflora, Rhizomapinelliae, Rheum palmatum, Rhizoma zingiberis recens and Fructus jujubae (Table 1), was purchased from the Department of Pharmaceutical Preparation of Chinese Medicine of Shaanxi University. The components of DCHD were extracted twice by boiling in distilled water for 1 h, and the drug was subsequently filtered and reduced by boiling to a volume of 70 mL (1 g crude drug/mL) and stored at 4 ℃ for later use. Masson staining and amylase enzyme-linked immunosorbent assay (ELISA) kits were purchased from the Nanjing Jiancheng Biotechnology Company (Nanjing, China). Reverse transcriptase polymerase chain reaction (PCR) kits and primers were provided by Transgen Biotech Co. (Beijing, China).

L-arginine-induced chronic pancreatitis

The KunMing mice were randomly divided into three groups (n = 36): the control group, the CP group, and the DCHD group. Mice in the CP and DCHC groups were injected intraperitoneally with 20% L-arginine (two 3 g/kg injections separated by 1 h, weekly for 6 wk), while the mice in the control group were injected with saline. Mice in the DCHD group were treated with DCHD (14 g/kg per day) intragastrically 1 wk after the 20% L-arginine injections.

Serum and tissue sample collection

Mice were anesthetized and sacrificed at 2 wk, 4 wk and 6 wk after 20% L-arginine or saline injection (n = 9 at each time point). Blood samples were obtained from the inferior vena cava, centrifuged at 1500 rpm for 10 min, and serum was collected and stored at -80 ℃ for IL-6 analysis. The pancreas was removed, weighed, and mixed well. One part of the pancreas was fixed in 4% formaldehyde solution for hematoxylin and eosin (HE) and immunofluorescence staining, while the other part was frozen in liquid nitrogen and stored at -80 ℃ for RNA and protein extraction.

ELISA test

Plasma was collected at 2 wk, 4 wk and 6 wk after modeling and serum was obtained by centrifuging samples at 1500 rpm for 10 min. IL-6 levels were evaluated using an ELISA kit (Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's instructions.

Histology and HE and Masson staining

To observe morphological and fibrosis changes in the pancreas, pancreatic sections were subjected to HE or Masson staining. Samples were dehydrated and embedded in paraffin, sectioned (3 μm) after fixation in 4% formaldehyde solution for 12 h. Images were collected using an optical microscope.

Immunohistochemistry and immunofluorescence staining

Immunohistochemical staining and immunofluorescence staining were performed. For immunohistochemistry, antibodies were applied at the following concentrations: F4/80, 1:150 dilution, overnight at 4 ℃; and biotinylated secondary antibody, 1:400 dilution, for 1 h. Bound peroxidase was visualized after a 3,3-diaminobenzidine reaction for 2-5 min and after light counterstaining with hematoxylin, dehydration, and mounting. The slides were examined using a Zeiss Axio1 imager (Carl-Zeiss-Promenade, Jena, Germany). For immunofluorescence, antibodies were applied at the following concentrations: F4/80 and IL-6 antibodies, 1:150 dilution, overnight at 4 ℃ in the dark; FITC-labeled and PE-labeled secondary antibody, 1:400 dilution, for 1 h. DAPI (1:1000) was used to counterstain the nuclei for 5-15 s, and the slides were mounted on coverslips with anti-fade mounting medium. Images were collected using a fluorescent microscope (Olympus IX51, Shinjuku-ku, Tokyo, Japan).

RNA isolation and real-time PCR

Total RNA of pancreatic tissue was extracted using the Trizol reagent (RNeasy Minicolumns, Transgen, Beijing, China) and reverse- transcribed into cDNA using reverse transcriptase kits. RT-PCR was performed using SYBR Green reaction mix and the ABI-7500 Sequence Detection System (Thermo Fisher Scientific, Shanghai, China). The primers, number of cycles, and annealing temperatures are shown in Table 2.

Protein extraction and western blotting

Protein was extracted from pancreas tissue using standard methods. The protein concentrations were measured using a BCA protein assay kit (Boster

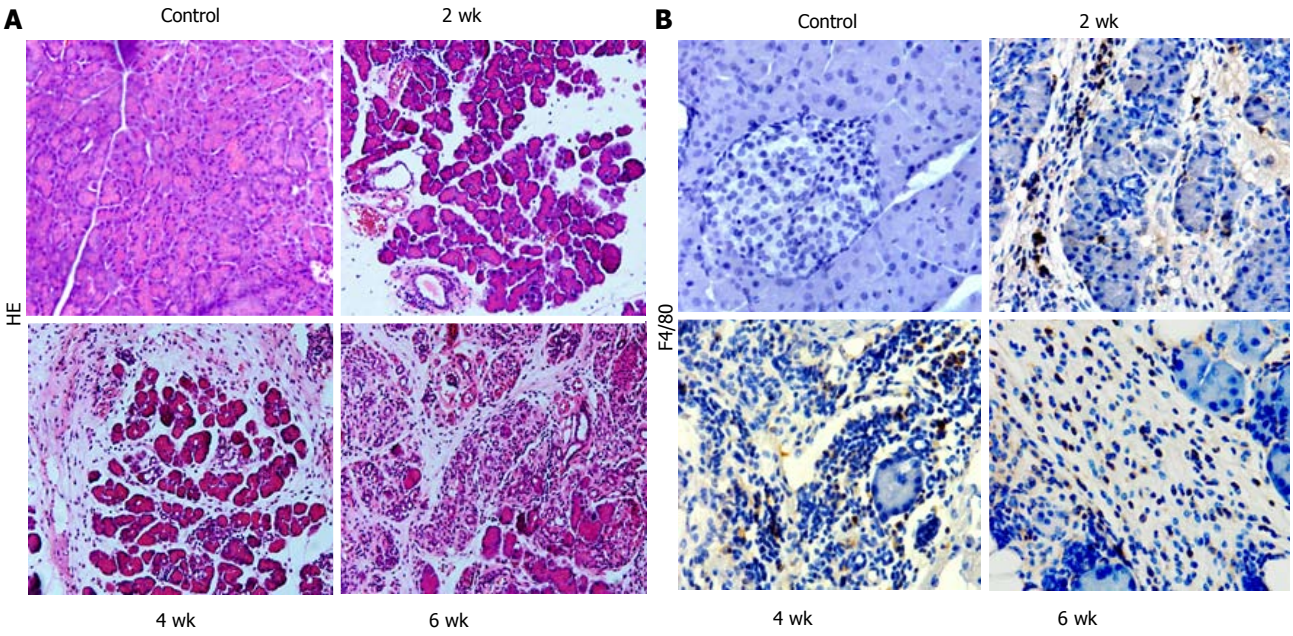

Figure 1 Morphological changes and macrophage infiltration in pancreatic sections obtained from mice with chronic pancreatitis induced by L-arginine. A: Pancreatic morphology at different time points of L-arginine induction using hematoxylin and eosin staining (original magnification, × 100); B: Macrophage infiltration in pancreas was detected using immunohistochemistry. Macrophages were stained using an anti-mouse F4/80 antibody (original magnification, × 200).

Table 1 Contents of Dachaihu decoction

Chinese name	Botanical name	Common name	Genus	Family	Weight, g	Part used
Chai hu	*Bupleurumabchasicum Manden*	Radix bupleuri	Bupleurum	umbelliferae	15	Root
Huang qin	*Scutellaria baicalensis Georgi baicalensis*	Radix scutellariae	Scutellaria	Labiatae	9	Root
Zhishi	*Citrus × aurantium L.*	Fructus aurantii immaturus	Citrus	Rutaceae	9	Fruit
Shaoyao	*Paeonia × suffruticosa Andrews*	Paeonia lactiflora	Paeonia	Paeoniaceae	9	Root
Ban xia	*Pinelliaternate (Thunb.) Makino*	Rhizomapinelliae	pinellia	Araceae	9	Root and rhizoma
Da huang	*Rheum palmatum L.*	Rheum palmatum	Rheum	polygonaceae	6	Root and rhizome
Sheng jiang	*Zingiber officinale Roscoe*	Rhizoma zingiberis recens	Zingiber Adans .	zingiberaceae	15	Root and rhizome
Da zao	*Ziziphus jujube MIll*	Fructus jujuba	Ziziphus	Rhamnales	20	fruit

Biotechnology Inc.) and adjusted to 4 μg/μL. Before loading, the samples were boiled at 95 ℃ for 5 min, subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Bio-Rad, Hercules, CA, United States), and transferred onto PVDF membranes (EMD Millipore, Darmstadt, Germany). The membranes were blocked with 5% non-fat milk followed by incubation with anti-actin (Boster Biotechnology Inc.) and anti-FN antibodies (sc-101759; Santa Cruz Biotechnology Inc.). The sizes of the detected bands were determined by reference to a 10-180 kDa protein marker ladder.

Statistical analysis

Values are presented as the mean ± SD and were analyzed using the unpaired Student's *t*-test and one-way analysis of variance (commonly known as ANOVA). A value of $P < 0.05$ was considered to indicate statistical significance.

RESULTS

Macrophage infiltration is increased in CP

We induced CP in mice using repeated 20% L-arginine injections over 6 wk. Two weeks after modeling, morphological signs of CP were found, including leukocyte infiltration, acinar cell necrosis, and a small amount of connective tissue deposition. At 4 wk and 6 wk, loss of acinar cells, collagen deposition, and aggravated fibrosis in the pancreas were observed (Figure 1A).

Immunohistochemistry using the macrophage marker F4/80 was performed. Two weeks after L-arginine injection, F4/80-positive cells were predominant in mice with CP compared with the normal group (Figure 1B). The positive macrophage ratio was highest at week 4, and levels were sustained until week 6. Thus, macrophages were abundant during CP and

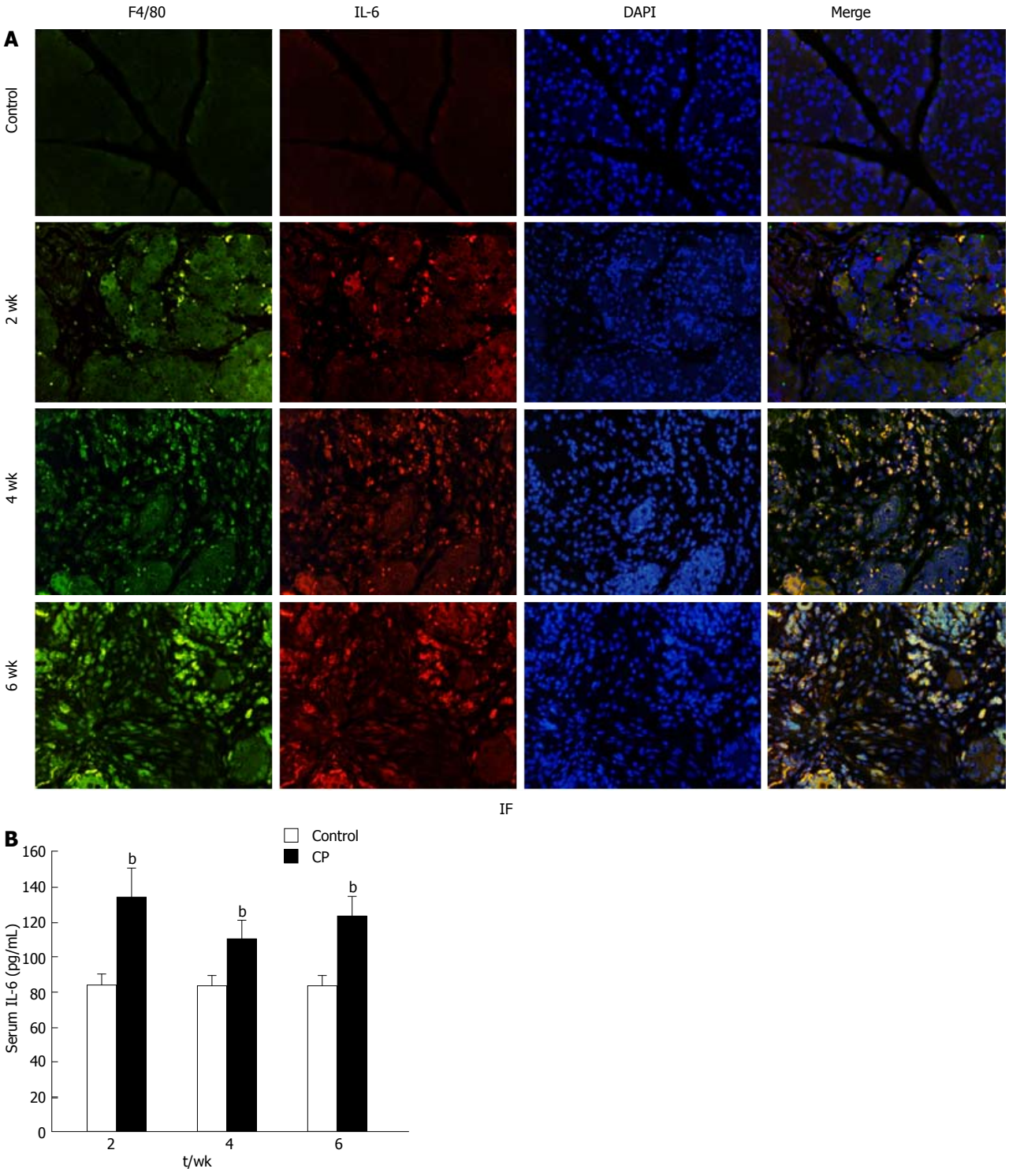

Figure 2 Interleukin-6 production is related to macrophage infiltration. A: Double immunofluorescent signals from F4/80 and interleukin-6 in L-arginine-induced chronic pancreatitis tissue at different time points (original magnification, × 200); B: Interleukin-6 levels in the sera of mice with chronic pancreatitis. Values are shown as mean ± SD (n = 36). $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* control.

were involved in the development of fibrosis (Figure 1B).

Macrophage infiltration in the pancreas results in IL-6 production

The serum concentration of IL-6 was significantly increased 2 wk after 20% L-arginine injection. At 4 wk, the IL-6 level was somewhat decreased but remained elevated to week 6 (Figure 2A). These findings demonstrate that high IL-6 levels contribute to the development of CP.

While macrophage infiltration and IL-6 levels were increased in CP, their relationship remains unclear. To determine whether IL-6 is mainly secreted by

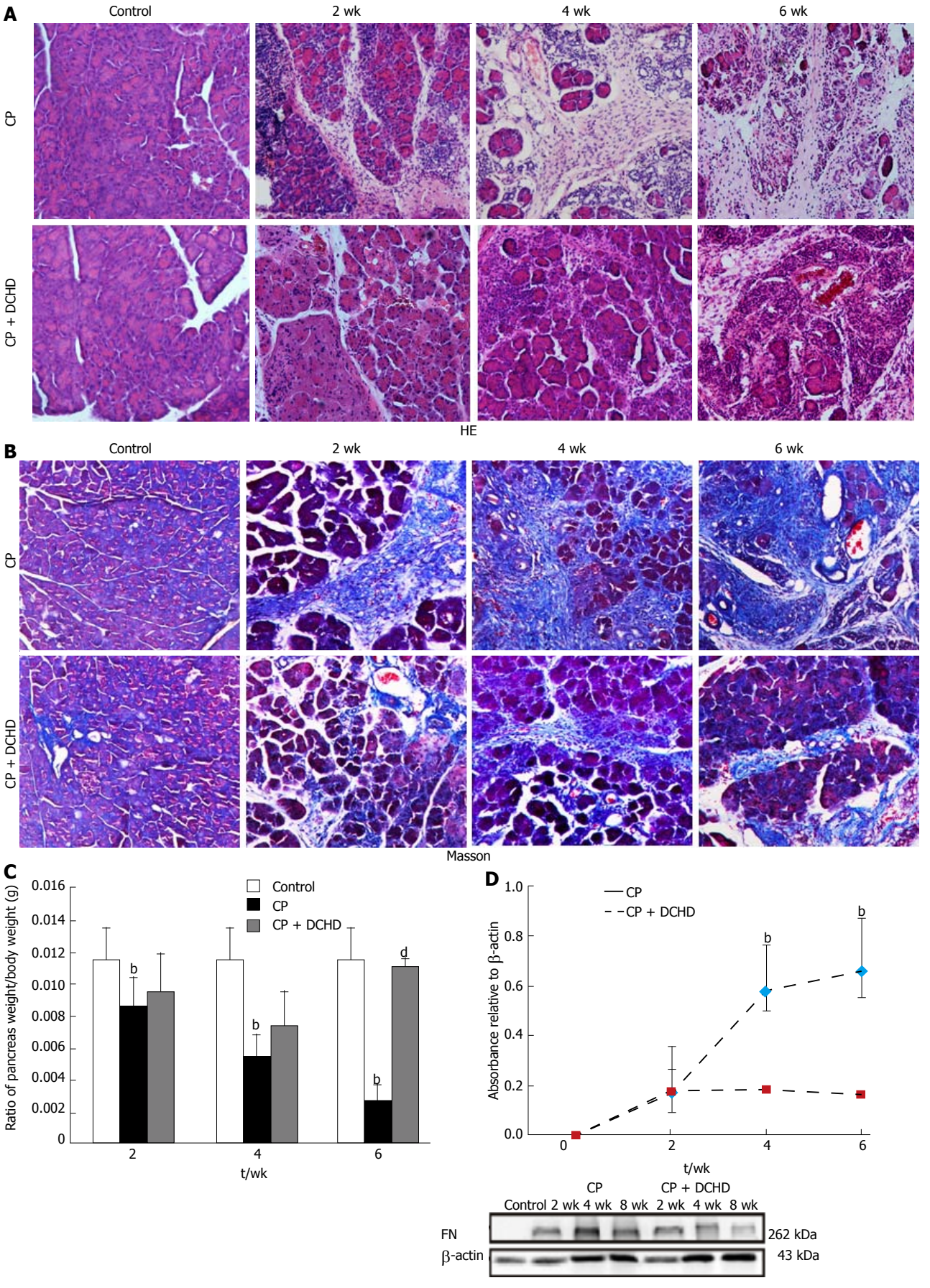

Figure 3 Dachaihu decoction protects against pancreatic injury and pancreatic fibrosis in mice with chronic pancreatitis. A: Changes in pancreatic morphology at different time points were evaluated by hematoxylin and eosin staining (original magnification, × 100); B: Collagen deposition was detected by Masson staining (original magnification, × 100); C: Ratio of pancreas weight/body weight changes in the different groups; D: The expression of fibronectin in the pancreas following the development of chronic pancreatitis. Values are shown as mean ± SD (n = 9). $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* control; $^{c}P < 0.05$, $^{d}P < 0.01$ *vs* CP group.

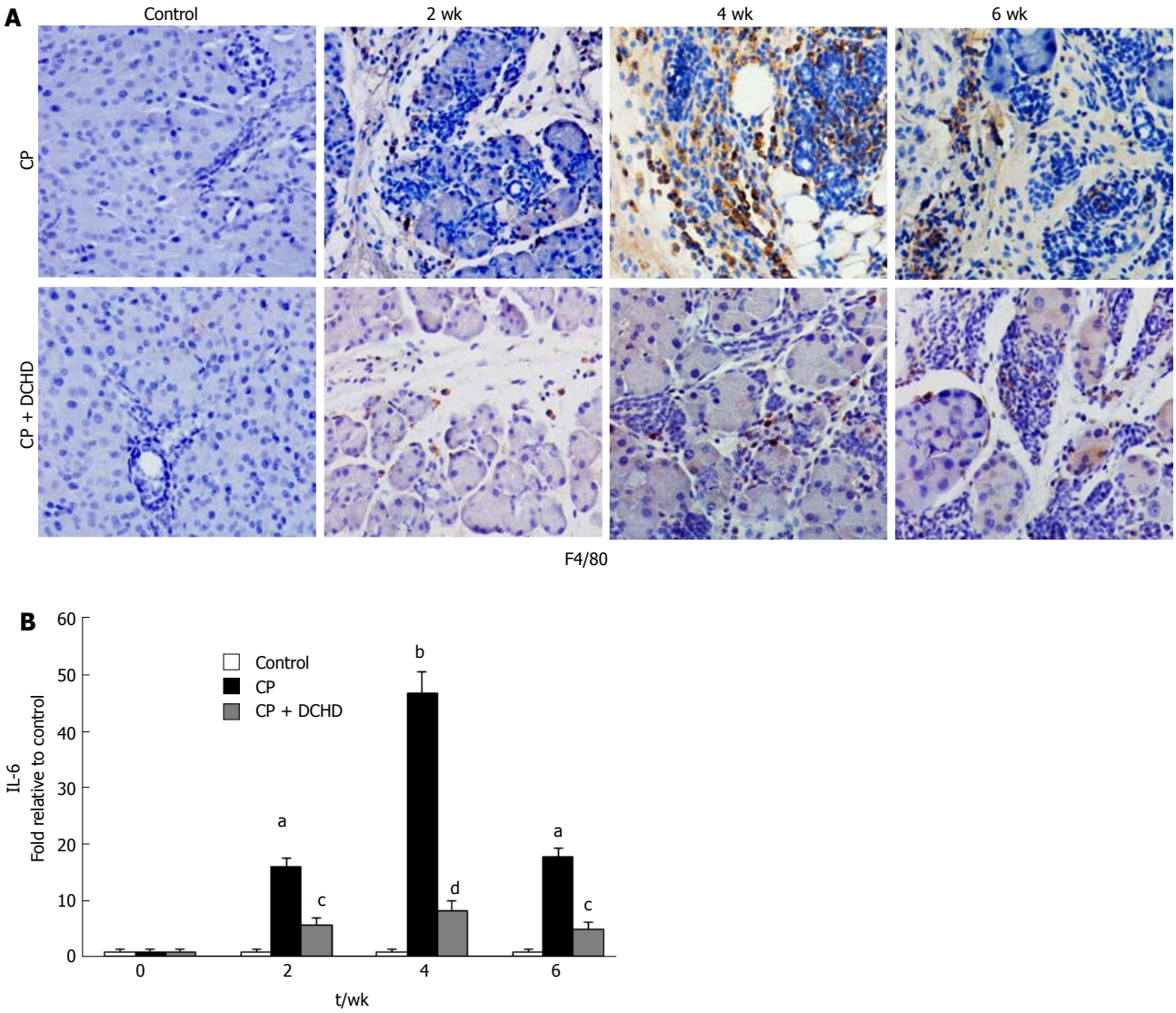

Figure 4 Dachaihu decoction attenuated macrophage infiltration and interleukin-6 expression in the pancreas. A: Immunohistochemistry of macrophages associated with chronic pancreatitis (original magnification, × 200); B: Interleukin-6 mRNA levels in the pancreas determined by RT-PCR. Values are shown as mean ± SD (n = 9). $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* control; $^{c}P < 0.05$, $^{d}P < 0.01$ *vs* CP group.

Table 2 Primer sequences used for real time-polymerase chain reaction

Gene	Forward primer	Reverse primer	Cycles	Temperature, ℃
IL-6	5′-GAAGTAGGGAAGGCCGTGG-3′	5′-CTCTGCAAGAGACTTCCATCCAGT-3′	40	60
MCP-1	5′-GTTGGCCTCAGCCAGATGCA-3′	5′-AGCGTACTCATTGGGATCATCTTG-3′	40	60
MIP-1α	5′-TGCCCTTGCTHTTCTTCTCTGCACCATGGC-3′	5′-GGCAAATTCCACGAAAATTCATTGCTGACT-3′	40	60
β-actin	5′-CATCCTGCGTCTGGACCT-3′	5′-TCAGGAGGAGCAATGATCTTG-3′	40	60

macrophages, double staining of F4/80 and IL-6 labeled with different fluorescence markers was performed on pancreas paraffin sections. As shown in Figure 2B, no positive F4/80 and IL-6 cells were found in the pancreas of the control group. However, L-arginine administration resulted in a significant increase in the expression of F4/80 and IL-6 in the pancreas. While there was only weak expression in the samples at 2 wk, co-expression of F4/80 and IL-6 increased markedly at 4 wk and 6 wk. These results demonstrate that IL-6 is mainly secreted by macrophages in CP.

DCHD alleviates pancreatic injury and pancreatic fibrosis in mice with CP

We next sought to investigate whether pancreatic injury and subsequent fibrosis were alleviated by DCHD treatment. DCHD administration resulted in

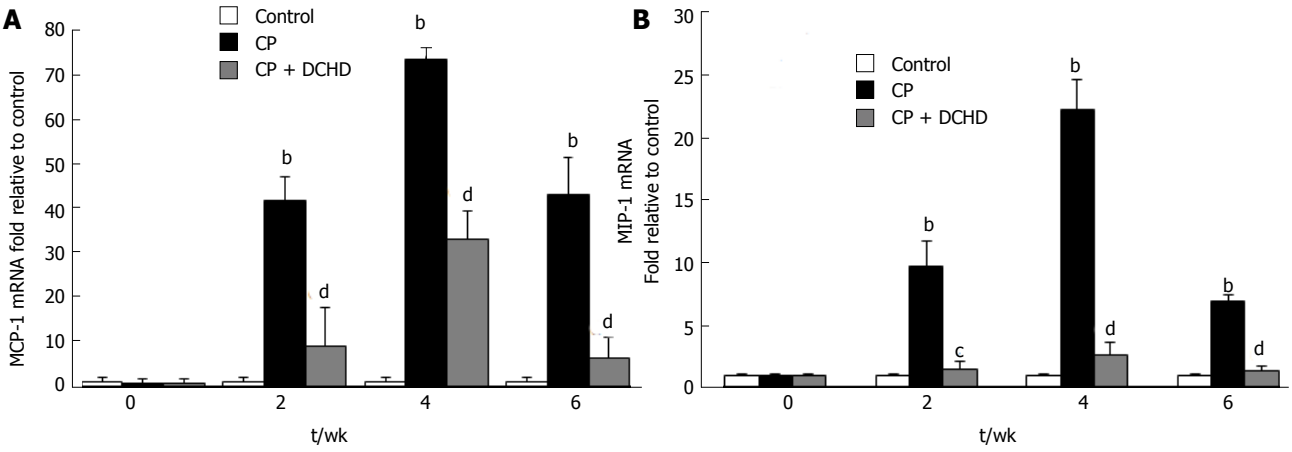

Figure 5 Effects of Dachaihu decoction on MCP-1 and MIP-1α mRNA levels in the pancreas of mice with chronic pancreatitis. A: MCP-1 mRNA levels in the pancreas as determined by real time-polymerase chain reaction; B: MIP-1α levels in the pancreas as determined by real time-polymerase chain reaction. Values are shown as mean ± SD (n = 9). $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* control; $^{c}P < 0.05$, $^{d}P < 0.01$ *vs* CP group.

significantly improved tissue damage, pancreatic fibrosis, and ratio of pancreas weight/body weight (Figure 3A-C). Moreover, Masson staining revealed that the area of pancreas fibrosis markedly decreased in the DCHD group (Figure 3B).

FN is a major indicator of extracellular matrix deposition and the degree of fibrosis. Western blotting revealed no FN expression in the pancreas of control mice; however, 20% L-arginine treatment resulted in increased FN expression in the pancreas at 2 wk, 4 wk, and (maximally) 6 wk. Interestingly, DCHD treatment attenuated FN levels compared with those in the CP group at the same time point, illustrating that DCHD can suppress the deposition of FN in pancreatic fibrosis (Figure 3D).

DCHD attenuated macrophage infiltration and IL-6 mRNA expression in the pancreas

To understand the mechanism underlying the effects of DCHD treatment in CP, we used immunohistochemistry to detect macrophage infiltration. Compared with the CP group, DCHD treatment significantly decreased pancreatic macrophage infiltration at 2 wk. Accompanied with evident and an improvement in pancreatic fibrosis at 4 wk and 6 wk, macrophage infiltration was also significantly reduced (Figure 4A). Thus, limiting macrophage accumulation in the pancreas may be an underlying mechanism of DCHD in inhibiting the development of pancreatic fibrosis.

Considering the association between IL-6 and macrophages, the expression of IL-6 mRNA in the pancreas was assessed using RT-PCR. IL-6 expression levels were increased in CP and were highest at week 4. Compared with those in the CP group, IL-6 levels in the DCHD group were significantly decreased (Figure 4B). This finding demonstrates that IL-6 is produced by macrophages and thus may be a potential treatment target for DCHD.

DCHD decreased chemokine mRNA levels were associated with macrophage infiltration

To further explore the mechanism underlying macrophage accumulation, pancreatic mRNA levels of the macrophage-associated chemokines MCP-1 and MIP-1α were detected using RT-PCR. MCP-1 and MIP-1α were up-regulated in the CP group compared with the control group at 2 wk, 4 wk and 6 wk (Figure 5A-B), while they were significantly decreased in the DCHD group at the same time points.

DISCUSSION

Generally, pancreatic fibrosis is a common pathological feature of CP. Recurrent acute pancreatic insults trigger necro-inflammation, and inflammatory cells, once in their active states, release cytokines that in turn promote fibrogenesis, which is involved in the progression of pancreatic fibrosis[16]. Recent studies have confirmed the dominant role played by activated macrophages in liver fibrosis by interfering with the synthesis and degradation of the extracellular matrix[17]. In a rat model of liver cirrhosis, activated macrophages were shown to accelerate fibrogenesis by activating hepatic myofibroblasts, while the suppression of macrophage infiltration inhibited liver fibrogenesis[17]. Treiber *et al*[18] found large numbers of macrophages among inflammatory cells infiltrating the pancreas of a mouse model of CP. In our study, the development of pancreatic fibrosis was accompanied by a significant increase in F4/80-positive cells in pancreatic tissue, suggesting that activated macrophages may play a role in the progression of pancreatic fibrosis.

A study by Saeki *et al*[19] revealed that infiltration of macrophages in AP originates mainly from peripheral blood mononuclear cells (PBMCs) due to chemokines. Our CP model was based on repetitively inducing AP, and we speculated that infiltration of macrophages

in CP was attributable to PBMCs' recruitment. MCP-1 and MIP-1α are potent C-C type chemokines that are mainly secreted by activated macrophages and fibroblasts. They recognize the C-C chemokine receptor type (CCR)-2 or CCR-3 on PBMCs and recruit macrophages to injured tissues[20,21]. Therefore, we measured MCP-1 and MIP-1α mRNA levels and found that they were up-regulated in mice with CP, which may contribute to macrophage accumulation in CP.

To obtain a deeper understanding of the role of macrophages in pancreatic fibrosis, other researchers previously stimulated PBMCs with lipopolysaccharide, and found that TGF-β1 and IL-6 were significantly elevated in culture supernatant. Moreover, further co-culture of macrophages with pancreatic stellate cells resulted in increased FN and collagen type I levels[22]. TGF-β1 is considered one of the strongest fibrosis factors[9], but the role of IL-6 in CP has not been recognized. Numerous studies have shown that IL-6 was related to fibrosis progress in the liver and lung[23,24]. Recent experiments also demonstrated that the serum level of IL-6 in CP patients was significantly increased[14].

In our present study, IL-6 levels in serum and IL-6 mRNA levels in the pancreas were also significantly increased in the progression of CP mice. These results indicated that IL-6 participates in the development of pancreatic fibrosis. To determine the source of IL-6 production, we preformed immunofluorescent double-staining of F4/80 and IL-6 to evaluate F4/80 and IL-6 expression in the pancreas and found large numbers of doubly positive cells in the pancreas of the CP group. We can thus infer that macrophage infiltration is the main source of IL-6 secretion, and an excess of IL-6 might aggravate inflammation and further accelerate pancreatic fibrogenesis.

The traditional Chinese herbal compound DCHD is composed mainly of Radix bupleuri, Radix scutellariae, Fructus aurantii immaturus, Paeonia lactiflora, Rhizomapinelliae, Rheum palmatum, Rhizoma zingiberis recens, and Fructus jujubae[25]. In the clinic, DCHD has been effective in treating the characteristics of the "shao-yang signs", such as fever, chills and rib-side fullness, and "yang-ming" signs, such as abdominal pain and fullness[15]. Therefore, DCHD is used mainly to treat acute and chronic digestive diseases, such as AP, inflammatory bowel disease, hepatitis and disorders of the gallbladder[15].

Clearly, CP, a common disease of the digestive system, is associated with the shao-yang and yang-ming signs of abdominal pain and rib-side distention. According to the traditional Chinese medical theory, many doctors consider that the mechanism underlying CP involves "liver-spleen disharmony, qi and blood stasis, and dampness-heat obstruction"[26], while the main actions of DCHD are removal of heat and toxic materials and elimination of blood stasis[27]. DCHD may be effective in treating CP. Modern medical studies have shown that petunidin and quercetin from *Radix bupleuri* exert anti-inflammatory effects and act as antioxidants[15,26,28]. Therefore, DCHD has also been used to treat patients of CP, and some related clinical symptoms such as abdominal pain, abdominal distension and loss of appetite, with significant improvement[15], but the underlying molecular mechanisms remain unclear.

In this study, the effects of DCHD on the development of pancreatic fibrosis in CP were evaluated systematically using an L-arginine-induced CP mouse model. Following DCHD administration, morphological changes indicative of CP, including pancreatic acinar cell injury, inflammatory cells infiltration, and pancreatic fibrosis were improved. This suggests that DCHD can attenuate inflammatory lesions and intervene in the progression of pancreatic fibrosis, and led us to perform further study on its underlying mechanism of action.

We next found that DCHD treatment significantly attenuated macrophage infiltration and IL-6 expression in the pancreas. Combined with our previous results, DCHD may suppress pancreatic fibrosis *via* the inhibition of macrophage infiltration, which in turn reduces the release of IL-6. In addition, MCP-1 and MIP-1α expression was also significantly decreased, suggesting that the DCHD-induced decrease in macrophage infiltration was due to a reduction in the release of macrophage-associated chemokines.

The present study suggests that macrophages play a critical role in CP and pancreatic fibrosis, and their dominant role in CP may be related to the release of cytokines, specifically IL-6. We demonstrated that macrophages are the main source of IL-6 production in CP. In addition, DCHD can ameliorate pancreatic fibrosis by inhibiting the macrophage infiltration of CP, which provides a new experimental basis for the clinical promotion of DCHD.

However, our work had certain limitations. We confirmed that the effect of macrophages on CP is related to IL-6, but the detailed mechanism of IL-6 on CP progression was not further explored. In addition, DCHD is effective in CP treatment, but its in-depth mechanism is not further studied, and these are our next steps.

ARTICLE HIGHLIGHTS

Research background

Pancreatic fibrosis is a common pathological feature characteristic of chronic pancreatitis (CP). As the necrosis-fibrosis sequence plays an important role in the underlying pathogenesis of CP, pancreatic fibrosis commonly develops after repeated inflammation. Macrophages are important inflammatory cells that can also promote fibrogenesis by interfering with the synthesis and degradation of the extracellular matrix. This has been confirmed in models of liver fibrosis and renal fibrosis, but any role of macrophages in pancreatic fibrosis has remained unclear. A traditional Chinese medicinal formula, Dachaihu decoction (DCHD), has been widely used in the clinical treatment of AP, and recently, DCHD was

used to treat CP, which significantly improved the patient's clinical symptoms, but, again, the underlying mechanism has been unclear.

Research motivation

CP is a progressive inflammatory disease characterized by irreversible injury to the pancreas leading to endocrine and exocrine dysfunctions that pose serious threats to human health, because the pathogenesis is unclear, and few treatments are available. Recent studies suggested that DCHD can significantly improve the clinical symptoms of CP patients, such as abdominal pain, abdominal distension, loss of appetite and others. However, DCHD cannot be widely used clinically because the therapeutic mechanism of action remains unclear.

Research objectives

The aim was to explore the role played by macrophages in the pathogenesis of CP to further elucidate the mechanisms of CP. In addition, they assessed the efficacy of DCHD in treating CP in animal experiments to determine the mechanism involved, providing an experimental basis for promoting the use of DCHD in clinical practice.

Research methods

KunMing mice were randomly divided into a control group, CP group, and DCHD group. In the CP and DCHD groups, mice were intraperitoneally injected with 20% L-arginine (3 g/kg twice a day, 1 d a week). Mice in the DCHD group were given DCHD (14 g/kg per day) intragastrically for 1 wk after CP induction. The animals were anesthetized and sacrificed at 2 wk, 4 wk and 6 wk after study commencement. Pancreatic morphology and the extent of fibrosis were assessed using hematoxylin and eosin and Masson staining. Serum interleukin-6 (IL-6) levels were assessed by enzyme-linked immunosorbent assay. Double immunofluorescence staining was performed to assess the co-expression of F4/80 and IL-6 in the pancreas. The mRNA levels of inflammatory factors including monocyte chemoattractant protein-1 (MCP-1), macrophage inflammatory protein-1α (MIP-1α) and IL-6 were determined by real time-polymerase chain reaction. Western blotting was used to measure fibronectin (FN) levels in the pancreas.

Research results

We found that macrophage infiltration was significantly increased in the CP group, and the IL-6 levels in serum and IL-6 mRNA levels in the pancreas were also increased in the CP group. Further, immunofluorescent double-staining of F4/80 and IL-6 revealed that macrophages were the main source of the IL-6. In addition, DCHD ameliorated pancreatic fibrosis, inhibited the pancreatic infiltration of macrophages in the CP group, and reduced the release of cytokines IL-6, MCP-1 and MIP-1α mRNA, and FN levels. However, our work had certain limitations. We confirmed that the effect of macrophages on CP is related to IL-6, but the detailed mechanism of IL-6 on CP progression was not further explored. In addition, DCHD is effective in CP treatment, but its in-depth mechanism was not studied further.

Research conclusions

The authors confirmed that macrophages are involved in the development of pancreatic fibrosis and may constitute a new therapeutic target. In addition, DCHD can ameliorate pancreatic fibrosis by inhibiting macrophage infiltration and inflammatory factor secretion in the pancreas.

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ORIGINAL ARTICLE

Basic Study

Prostaglandin E1 protects hepatocytes against endoplasmic reticulum stress-induced apoptosis *via* protein kinase A-dependent induction of glucose-regulated protein 78 expression

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Abstract

AIM

To investigate the protective effect of prostaglandin E1 (PGE1) against endoplasmic reticulum (ER) stress-induced hepatocyte apoptosis, and to explore its underlying mechanisms.

METHODS

Thapsigargin (TG) was used to induce ER stress in the human hepatic cell line L02 and hepatocarcinoma-derived cell line HepG2. To evaluate the effects of PGE1 on TG-induced apoptosis, PGE1 was used an hour prior to TG treatment. Activation of unfolded protein response signaling pathways were detected by western blotting and quantitative real-time RT-PCR. Apoptotic index and cell viability of L02 cells and HepG2 cells were determined with flow cytometry and MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-

tetrazolium] assay.

RESULTS

Pretreatment with 1 μmol/L PGE1 protected against TG-induced apoptosis in both L02 cells and HepG2 cells. PGE1 enhanced the TG-induced expression of C/EBP homologous protein (CHOP), glucose-regulated protein (GRP) 78 and spliced X box-binding protein 1 at 6 h. However, it attenuated their expressions after 24 h. PGE1 alone induced protein and mRNA expressions of GRP78; PGE1 also induced protein expression of DNA damage-inducible gene 34 and inhibited the expressions of phospho-PKR-like ER kinase, phospho-eukaryotic initiation factor 2α and CHOP. Treatment with protein kinase A (PKA)-inhibitor H89 or KT5720 blocked PGE1-induced up-regulation of GRP78. Further, the cytoprotective effect of PGE1 on hepatocytes was not observed after blockade of GRP78 expression by H89 or small interfering RNA specifically targeted against human GRP78.

CONCLUSION

Our study demonstrates that PGE1 protects against ER stress-induced hepatocyte apoptosis *via* PKA pathway-dependent induction of GRP78 expression.

Key words: Hepatocytes; Endoplasmic reticulum stress; Thapsigargin; Glucose-regulated protein 78; Protein kinase A; Apoptosis

Core tip: The mechanism underlying the hepatoprotective effect of prostaglandin E1 (PGE1) remains unclear. In this study, we found that pretreatment with PGE1 protected hepatocytes against thapsigargin-induced apoptosis. PGE1 alone induced protein and mRNA expressions of glucose-regulated protein (GRP)78. Treatment with protein kinase A (PKA)-inhibitor H89, KT5720 or small interfering (si)RNA specifically targeted against human GRP78 blocked PGE1-induced up-regulation of GRP78. The hepatoprotective effect of PGE1 was lost by blocking GRP78 expression with either H89 or siRNA. Our study demonstrates for the first time that PGE1 protects against endoplasmic reticulum stress-induced hepatocyte apoptosis *via* PKA pathway-dependent induction of GRP78 expression.

Yang FW, Fu Y, Li Y, He YH, Mu MY, Liu QC, Long J, Lin SD. Prostaglandin E1 protects hepatocytes against endoplasmic reticulum stress-induced apoptosis *via* protein kinase A-dependent induction of glucose-regulated protein 78 expression. *World J Gastroenterol* 2017; 23(40): 7253-7264 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i40/7253.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i40.7253

INTRODUCTION

Hepatocyte apoptosis can be triggered by intra- or extracellular signals[1]. The intracellular signals for hepatocyte apoptosis are induced by DNA damage, oxidative stress, growth factor deprivation, mitochondrial dysfunction, ATP depletion and endoplasmic reticulum (ER) stress[2,3]. A complex interaction occurs among these intracellular apoptotic signaling pathways. ER stress is known to induce hepatocyte apoptosis under various pathological conditions[4]. ER stress is implicated in the pathogenesis of various liver diseases, such as obesity-associated fatty liver disease[5,6], viral hepatitis[7], alcohol-induced liver injury[8], drug-induced liver injury[9] and ischemia/reperfusion injury of the liver[10,11]. Devising a treatment strategy to protect hepatocytes from ER stress-induced apoptosis will benefit most patients with liver diseases.

ER is a multifunctional intracellular organelle responsible for the synthesis, processing and trafficking of proteins that are essential for cell growth and survival. ER also serves as a storage organelle for calcium[12]. When the homeostasis of ER is disturbed under various pathophysiological conditions, ER stress is induced and the unfolded protein response (UPR) is activated. The UPR activates three ER transmembrane transducers: inositol-requiring enzyme (IRE) 1a, PKR-like ER kinase (PERK), and activating transcription factor (ATF) 6α[12-14]. Activation of these three UPR pathways enhances the ER's protein folding *via* up-regulation of the synthesis of glucose-regulated protein (GRP) 78. UPR signals also accelerate the degradation of misfolded proteins and reduce the synthesis of new proteins. Therefore, UPR during ER stress facilitates restoration of homeostasis. However, sustained or unresolved ER stress can activate a cascade of apoptotic signals that eventually result in cell death[15,16].

In several experimental models of liver injury, prostaglandin (PG) E1 has been shown to protect against hepatocyte apoptosis[17-19]. PGE1 is also effective in the treatment of patients with fulminant hepatitis and those with primary graft non-function after liver transplantation[20,21]. Thus, PGE1 appears to protect hepatocytes against apoptosis through various mechanisms[22-24]. However, the underlying mechanism of its hepatoprotective effect is not well understood.

In two recent studies, PGE1 was shown to induce expressions of heat-shock protein (HSP) and GRP78 in animal models of liver injury caused by ischemia reperfusion and hepatectomy[25,26]. These findings suggest that modulation of UPR may mediate the hepatoprotective effects of PGE1. However, the role of PGE1 in ER stress-induced apoptosis of hepatocytes is largely unknown.

In this study, we evaluated the protective effect of PGE1 against ER stress-induced hepatocyte apoptosis in both the normal human hepatocyte cell line L02 and

the hepatocarcinoma-derived cell line HepG2[27].

MATERIALS AND METHODS

Chemical reagents

RPMI 1640 was obtained from Thermo-Fisher Biochemical Products Co. Ltd (Beijing, China). Fetal bovine serum (FBS), thapsigargin (TG), protein kinase A (PKA) inhibitor H89 and KT5720, 4-phenylbutyric acid (PBA) and PGE1 were purchased from Sigma (St. Louis, MO, United States). Antibodies against GRP78, PERK, eukaryotic translation initiation factor-2α (eIF-2α), phospho-PERK (p-PERK) and phospho-eIF2α (p-eIF2α), C/EBP homologous protein (CHOP), spliced X box-binding protein 1 (sXBP1), growth arrest and DNA damage-inducible gene 34 (GADD34) and β-actin were purchased from Santa Cruz Biotechnology (Dallas, TX, United States). Annexin V-FITC/propidium iodide (PI) apoptosis detection kit was purchased from Dojindo Laboratories (Kumamoto, Japan). Small interfering (si)RNA scramble control and validated human GRP78-siRNA were purchased from Santa Cruz Biotechnology. All other chemicals and reagents were obtained from Sigma, unless stated otherwise.

Cell culture

The human hepatocyte cell line L02 and hepatocarcinoma-derived cell line HepG2 were obtained from the cell bank of the Type Culture Collection at the Chinese Academy of Sciences (Shanghai, China). The L02 and HepG2 cells were propagated at 37 ℃ in 5% CO_2 in RPMI 1640 medium containing 10% (v/v) FBS and 100 units/mL penicillin, and were passaged every 5-7 d. The L02 and HepG2 cells were cultured until they acquired 80%-100% confluence. Thereafter, the cells were rinsed three times with 10 mL of phosphate-buffered saline (PBS) and cultured in a medium lacking FBS for 24-36 h. To evaluate the effects of PGE1 or PBA on TG-induced apoptosis, PGE1 or PBA was used an hour prior to TG treatment and, thereafter, the medium was not changed. TG and H89 were dissolved in dimethyl sulfoxide (DMSO) at concentrations of 40 μmol/L and 20 μmol/L, respectively, as stock solution. PGE1 was dissolved in ethanol at a concentration of 2.82 mmol/L as stock solution. PBA was dissolved in RPMI 1640 medium. All control conditions included corresponding vehicles at the appropriate concentrations (ethanol for PGE1 and DMSO for TG and H89).

Cell apoptosis analysis

Apoptosis was determined using the annexin V-FITC/PI apoptosis detection kit, according to the manufacturer's instructions. Briefly, 2×10^6 cells were harvested using 0.05% trypsin with 0.5% mmol/L EDTA. To analyze the whole apoptotic cell population, non-adherent cells present in the culture medium were added to the harvested cells. The cells were then washed twice with pre-chilled PBS and resuspended in 500 μL annexin binding buffer. Then, 5 μL of annexin V-FITC and 5 μL of PI were added to each sample and incubated in dark at room temperature for 10 min. Flow cytometry (Gallios; Beckman Coulter, Brea, CA, United States) was performed according to the manufacturer's specifications. The apoptotic index was calculated as the percentage of annexin V^+ cells divided by the total number of cells in the gated region.

Cell viability assay

Cell viability was assessed with MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] method using the CellTiter 96® AQueous One Solution Cell Proliferation Assay kit (Promega Corporation, Madison, WI, United States) according to the manufacturer's instructions. In brief, early passage of L02 or HepG2 cells were plated in triplicate on 96-well plates (10000 cells/well) and cultured in modified RPMI 1640 for 24 h. Thereafter, the cells were rinsed three times in 200 μL of PBS and cultured in a medium lacking fetal calf serum. Cell viability was determined by replacing the medium with 20 μL of MTS. After incubation of the cells at 37 ℃ for 3 h, the absorbance was measured at 490 nm using a microplate reader (Bio-Rad model 680; Bio-Rad, Hercules, CA, United States). Cell viability was normalized as a percentage of control. This experiment was performed five times.

Western blotting

Cell lysates containing 40 μg of protein were resolved by SDS-PAGE using 4%-20% polyacrylamide gradient gel, and the fractioned proteins were subsequently transferred to nitrocellulose membranes. After blocking with Tris-based saline buffer containing 5% dry milk and 0.1% Tween 20 for 1 h, the membranes were blotted with the corresponding antibodies. The primary and secondary antibodies used were: rabbit anti-human GRP78 (1:500 dilution), PERK (1:1000 dilution), phospho (p)-PERK (1:500 dilution), eIF-2α (1:500 dilution), p-eIF-2α (1:250 dilution), sXBP1 (1:500 dilution), CHOP (1:1000 dilution), GADD34 (1:1000 dilution), mouse anti-human β-actin, goat anti-rabbit IgG conjugated with horseradish peroxidase (HRP), and goat anti-mouse IgG conjugated with HRP. The membranes were developed using a chemiluminescence detection system and thereafter exposed to BioMax Light Film (Kodak, Rochester, NY, United States). The band intensity for each protein was measured using ImagePro Plus analysis software (MediaCybernetics, Silver Spring, MD, United States) and the expression normalized to that of β-actin.

Quantitative real-time RT-PCR

Total RNA was isolated from L02 cells using TRIzol reagent (Invitrogen, Carlsbad, CA, United States) according to the manufacturer's instructions. RNase-free DNase I (TaKaRa, Dalian, China) was applied before cDNA synthesis to remove any genomic contamination. A total of 1 μg RNA from each sample was used for cDNA synthesis with a reverse transcription kit (TaKaRa). Reverse transcription (final volume of 20 μL) was performed at 42 ℃ for 10 min, followed by 75 ℃ for 5 min. The real-time PCR reaction (25 μL) containing 12.5 μL of 2 × SYBR Premix Ex*Taq* II (Tli RNaseH Plus; TaKaRa), 1 μL of each 10 μmol/L primers, 2 μL of cDNA template, and 8.5 μL RNAse/DNAse-free water was performed on a CFX96 PCR system (Bio-Rad). The reaction process was as follows: denaturation at 95 ℃ for 3 min, followed by 40 cycles of amplification (95 ℃ for 10 s and 60 ℃ for 30 s), ending with a melt curve ranging from 60 ℃ to 95 ℃ with a heating rate of 0.3 ℃/15 s. All samples were run in triplicate. Relative expression of GRP78 was calculated using the delta-delta-Ct method with β-actin as the reference control.

Primers used in the PCR were: GRP78 forward, 5′-AAATAAGCCTCAGCGGTTTCTT-3′ and reverse, 5′-TCAAGTTCTTGCCGTTCAAGG-3′; β-actin forward, 5′- CGGGAAATCGTGCGTGAC-3′ and reverse, 5′-CAGGAAGGAAGGCTGGAAG-3′ (TaKaRa). Quantitative real-time PCR was performed according to the MIQE guidelines[28].

GRP78-siRNA transfection

Briefly, cell culture plates containing 6-wells were seeded with 2 × 10^5 cells/well and cultured in RPMI 1640 medium containing 10% (v/v) FBS and 100 units/mL penicillin. siRNA scramble control and validated human GRP78-siRNA were used. GRP78 inhibition was performed using a commercially available siRNA kit (Santa Cruz Biotechnology). Knock-down of the target molecule, GRP78, was monitored by western blotting and real-time PCR.

Statistical analysis

Results of cell apoptosis and cell viability are expressed as mean ± SD. One-way analysis of variance (ANOVA) with Bonferroni's post hoc analysis was performed to compare multiple groups. The Student's *t*-test was used to assess between-group differences. The level of significance was set at $P < 0.05$.

RESULTS

TG-induced ER stress and apoptosis in L02 cells and PGE1 protected L02 and HepG2 cells against ER stress-induced apoptosis

We confirmed TG-induced ER stress in L02 cells (Figure 1). At 48 h, TG (1 μmol/L) caused significant

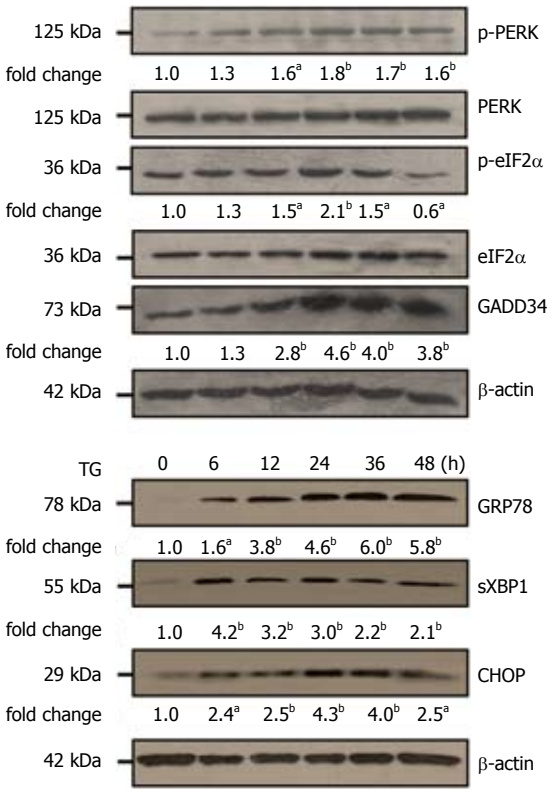

Figure 1 TG induced endoplasmic reticulum stress in L02 cells. L02 cells were treated with 1 μmol/L TG for 6, 12, 24, 36 and 48 h. Expressions of p-PERK, p-eIF2α, GADD34, GRP78, sXBP1 and CHOP were assessed by western blotting. Representative blots from three independent experiments are presented. The results of densitometric analysis are presented as a fold-change compared to that at 0 h ($^aP < 0.05$; $^bP < 0.01$). CHOP: C/EBP homologous protein; ER: Endoplasmic reticulum; GADD34: Growth arrest and DNA damage-inducible gene34; GRP78: Glucose-regulated protein 78; p-eIF2α: Phospho-eukaryotic translation initiation factor-2α; p-PERK: Phospho-PKR-like ER kinase; sXBP1: Spliced X box-binding protein1; TG: Thapsigargin.

enhancement in phosphorylation of PERK and GADD34 proteins, as compared to that observed at 6 h. Further, phosphorylation of eIF2α at 24 h was also up-regulated, as compared to that at 6 h. TG also induced a significant increase in the expressions of GRP78, CHOP and sXBP1 proteins.

We also confirmed TG-induced apoptosis in both L02 and HepG2 cells by means of flow cytometry and MTS assay. As shown in Figure 2A, the apoptotic index of L02 cells after TG treatment was significantly higher than that of control from 6 h to 48 h ($P < 0.01$). At 48 h, the apoptotic index reached 32.66%. TG also showed a dose-dependent increase in apoptotic index with increase in the concentration of TG (1 μmol/L, 2 μmol/L and 3 μmol/L; data not shown). Cell viability of both L02 and HepG2 cells significantly decreased from 24 h to 48 h after TG treatment (Figure 2B and C).

We assessed the effect of PGE1 on ER stress-induced apoptosis in L02 cells (Figure 2A). From 6 h to 48 h, 1 μmol/L PGE1 pretreatment significantly decreased TG-induced apoptotic index. At 48 h, 0.5 μmol/L and 1 μmol/L PGE1 showed dose-dependent

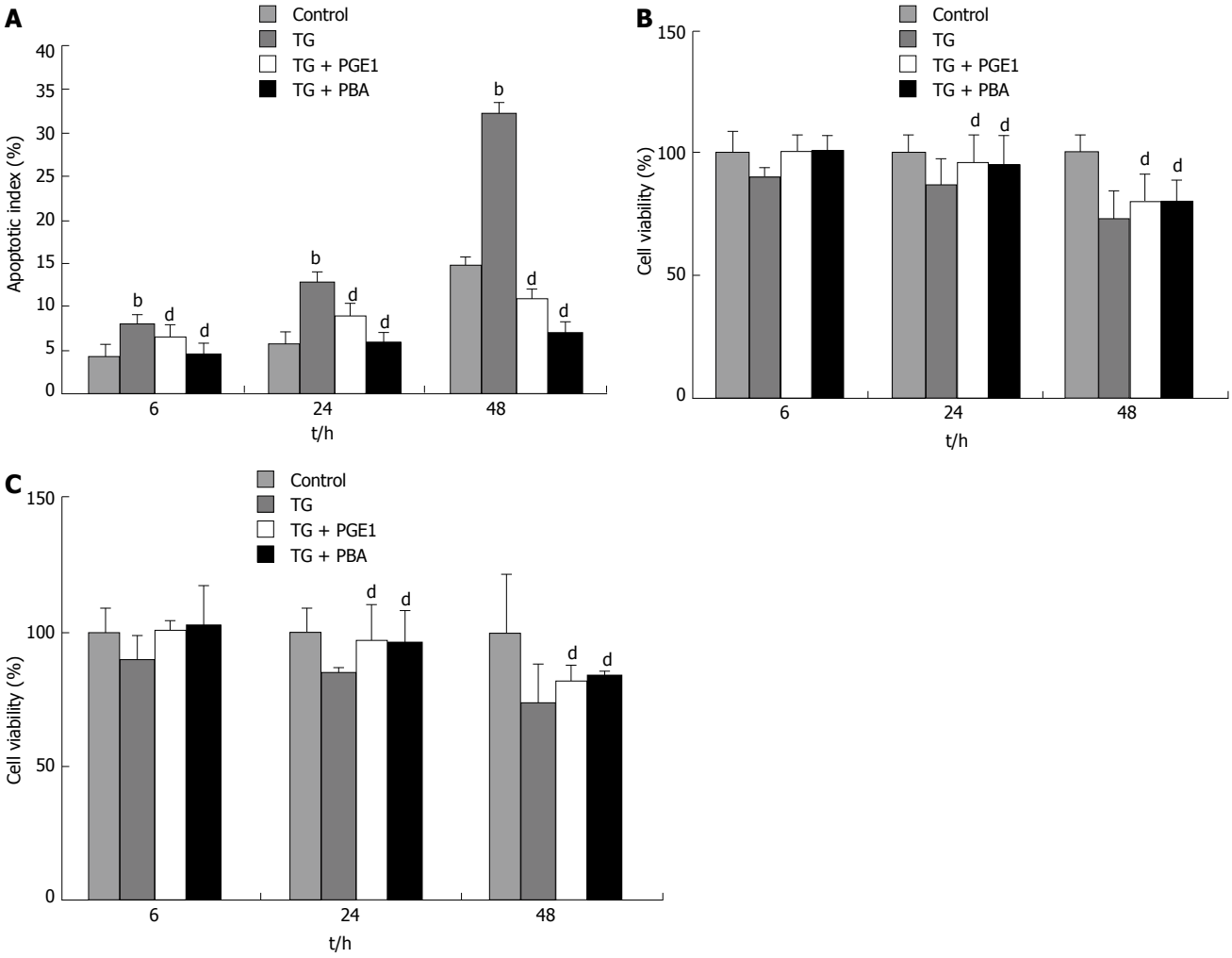

Figure 2 Prostaglandin E1 protected against thapsigargin-induced apoptosis in both L02 cells and HepG2 cells. L02 cells and HepG2 cells were pretreated with PGE1 or PBA for 1 h and treated with a final concentration of 1 μmol/L TG for 6, 24 and 48 h. A: Apoptotic index of L02 cells was determined by flow cytometry. Histograms represent mean ± SD of five separate experiments, each of which was performed in triplicate. $^{b}P < 0.01$ *vs* control at the same time point; $^{d}P < 0.01$ *vs* TG at the same time point; B and C: Cell viability of L02 cells and HepG2 cells was determined by MTS assay. The absorbance was measured at 490 nm and cell viability was normalized as a percentage of control. Histograms represent mean ± SD of five separate experiments, each of which was performed in triplicate. $^{d}P < 0.01$ *vs* TG at the same time point. MTS: [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium]; PBA: 4-phenylbutyric acid; PGE1: Prostaglandin E1; TG: Thapsigargin.

protection against TG-induced apoptosis (data not shown). PBA is a low molecular weight chemical chaperone and an ER stress inhibitor. PBA at 10 nmol/L significantly inhibited TG induced apoptosis (Figure 2A). MTS assay revealed that PGE1 significantly increased the viability of TG-treated L02 cells and HepG2 cells (Figure 2B and C). These results demonstrate the protective effect of PGE1 on ER stress-induced apoptosis in L02 cells and in HepG2 cells.

PGE1 promoted rapid recovery of ER stress

After demonstration of the cytoprotective effect of PGE1, we investigated the effects of PGE1 on UPR signals induced by TG. We selected three indicators from UPR, GRP78, CHOP and sXBP1, as GRP78 is a common downstream target of the three UPR signal pathways. CHOP is the major protein involved in apoptotic signals induced by ER stress, and sXBP1 is activated through the oligomerization of IRE1a[29]. As shown in Figure 3, 1 μmol/L PGE1 appeared to increase TG-induced GRP78, CHOP and sXBP1 expressions till 6 h post-treatment. However, from 24 h to 48 h, PGE1 suppressed TG-induced GRP78 and CHOP expressions. These results indicate that although PGE1 increased the early expression of UPR signals, it promoted the rapid recovery of ER stress.

PGE1 induced GRP78 expression via the PKA pathway

The observation that PGE1 attenuated the ER stress-induced apoptosis and promoted the rapid recovery from ER stress prompted us to investigate the effect of PGE1 on GRP78, CHOP and GADD34 expressions. GRP78 is a critical chaperone which determines the outcome of ER stress. GADD34 is involved in both recovery and resumption of protein synthesis as well as in the ER stress-induced apoptosis[14,30]. We also investigated the effects of PGE1 on phosphorylation of eIF2α and PERK, since the activation of PERK and

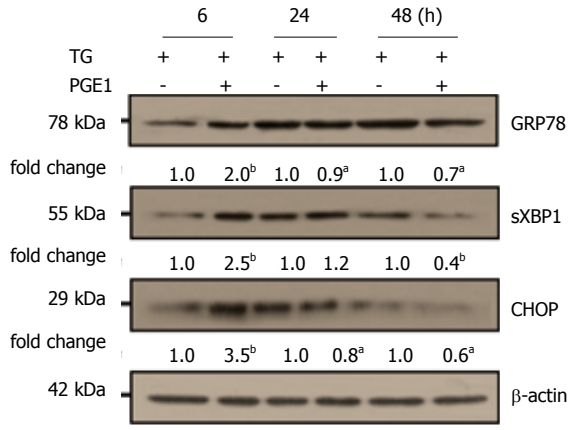

Figure 3 Prostaglandin E1 promoted rapid recovery of endoplasmic reticulum stress. L02 cells were pretreated with 1 μmol/L PGE1 for 1 h and at a final concentration of 1 μmol/L TG for 6, 24 and 48 h. Expressions of GRP78, CHOP and sXBP1) were assessed on western blotting. One representative blot each of three individual experiments is presented. The results of densitometric analysis are presented as a fold-change compared to those of TG at the same time point ($^{a}P < 0.05$, $^{b}P < 0.01$). CHOP: C/EBP homologous protein; ER: Endoplasmic reticulum; GRP78: Glucose-regulated protein 78; PGE1: Prostaglandin E1; sXBP1: Spliced X box-binding protein 1; TG: Thapsigargin.

eIF2α is known to induce CHOP and GADD34. From 3 h to 24 h after treatment with 1 μmol/L PGE1, a significant increase in GRP78 expression was observed in both L02 cells and HepG2 cells (Figure 4A and B). PGE1 also decreased CHOP expression in L02 cells and induced the expression of GADD34; however, it suppressed the expression of p-PERK and p-eIF2α proteins (Figure 4A). Results of quantitative real-time RT-PCR also demonstrated that PGE1 induced the mRNA expression of GRP78 from 3 h to 24 h (Figure 4C).

To explore the signal pathways that may mediate the effect of PGE1 on GRP78 expression, we inhibited the PKA pathway by H89 or KT5720. Treatment with 10 μmol/L of H89 or 1 μmol/L KT5720 appeared to counteract the effect of PGE1 on GRP78 expression (Figure 5A and B). These results indicate that PGE1 increased GRP78 expression *via* a PKA-dependent pathway.

PGE1 protected L02 and HepG2 cells against ER stress-induced apoptosis via PKA-dependent induction of GRP78 expression

H89 at 10 μmol/L appeared to inhibit the protective effect of PGE1 on hepatocyte apoptosis, which suggests that the cytoprotective effect of PGE1 was mediated *via* the PKA signaling pathway (Figure 5C). To further test whether the protective effect of PGE1 was dependent on the induction of GRP78 expression, we inhibited GRP78 expression by transfection with siRNA specifically targeted against human GRP78. On blocking the PGE1-induced up-regulation of GRP78 expression by siRNA (Figure 6A-C), PGE1 appeared to lose its cytoprotective effect in both L02 cells and HepG2 cells (Figure 6D-G). These findings imply that PGE1 protected against hepatocyte apoptosis *via* PKA-dependent induction of GRP78 expression.

DISCUSSION

The key findings of this study are that PGE1 protected hepatocytes from ER stress-induced apoptosis, that PGE1 enhanced the expression of GRP78 *via* the PKA pathway, and that the cytoprotective effect of PGE1 on hepatocytes was mediated *via* PKA-dependent GRP78 induction.

TG is known to induce ER stress by blocking ER Ca^{2+} uptake, which leads to depletion of ER Ca^{2+} stores[31]. PGE1 can also increase intracellular Ca^{2+} level by promoting the influx of Ca^{2+} from the external medium as well as by mobilization of Ca^{2+} from intracellular stores[32]. As intracellular Ca^{2+} level is an important factor in hepatocyte apoptosis and necrosis, cotreatment with TG and PGE1 may have modulated apoptotic signals of L02 cells, which eventually led to cell death by necrosis. Therefore, we assessed cell apoptosis and viability on flow cytometry and by MTS assay. Our results strongly suggest that PGE1 protects hepatocytes from ER stress-induced apoptosis.

PGE1 is known to have a direct vasodilator as well as an anti-platelet effect[22,33]. Several *in vivo* studies have indicated that PGE1 protects against hepatocyte apoptosis and promotes hepatocyte proliferation *via* inducing down-regulation of proinflammatory cytokine levels[24,26], suppression of tumor necrosis factor-α receptor and adhesion molecule expression[23,24], and by up-regulating cyclin C and cyclin D1 expressions[34]. PGE1 also inhibits oxidative stress and nitrosative stress-induced hepatocyte death by inhibiting production of superoxide anion, by enhancing nitric oxide synthase expression and by inhibiting nuclear factor-κB activation *in vitro*[19,35,36]. In this study, we demonstrated that PGE1 protected hepatocytes from ER stress-induced apoptosis. Our findings suggest that modulation of UPR may mediate the cytoprotective effect of PGE1 in various pathological conditions.

The mechanisms involved in ER stress-induced apoptosis are yet to be elucidated. The adaptive capacity of cells to ER stress is an important determinant of the outcomes of ER stress. Apoptosis results from sustained or strong ER stress when UPR fails to restore ER homeostasis[37,38]. In the present study, PGE1 treatment enhanced TG-induced CHOP, GRP78 and sXBP1 expressions at 6 h, and significantly attenuated TG-induced CHOP, GRP78 and sXBP1 expressions, as assessed at 48 h. These results suggest that PGE1 boosted the initial expression of UPR and promoted restoration of ER homeostasis during ER stress.

During ER stress, UPR may have been activated to restore ER homeostasis. UPR is precisely regulated by three ER stress transducers and their downstream signals. GRP78 is considered as a master regulator of response to ER stress[39,40]. GRP78 binds with all three major UPR sensors (PERK, IRE1a and ATF6) and

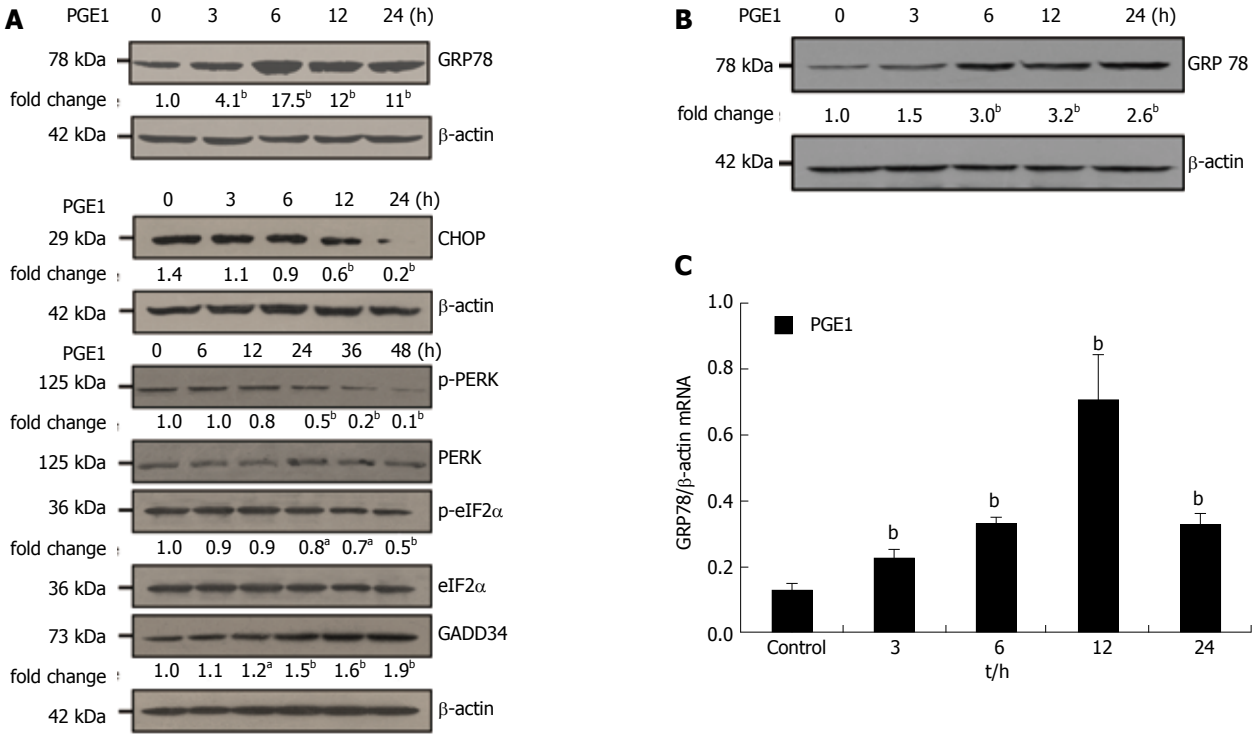

Figure 4 Prostaglandin E1 induced glucose-regulated protein 78 protein and mRNA expressions. To determine GRP78 and CHOP protein or mRNA expressions, L02 cells and HepG2 cells were treated with 1 μmol/L PGE1 for 3, 6, 12 and 24 h; for detecting GADD34 and the p-PERK and p-eIF2α, L02 cells were treated with 1 μmol/L PGE1 for 6, 12, 24, 36 and 48 h. A: Expressions of GRP78, CHOP, GADD34, p-PERK and p-eIF2α in L02 cells were assessed by western blotting. One representative blot each from the three individual experiments is presented. The results of densitometric analysis are presented as a fold-change compared to those at 0 h ($^aP < 0.05$, $^bP < 0.01$); B: Expression of GRP78 in HepG2 cells was assessed by western blotting. One representative blot each from the three individual experiments is presented. The results of densitometric analysis are presented as a fold-change compared to those at 0 h ($^bP < 0.01$); C: mRNA expression of GRP78 in L02 cells was assessed by quantitative real-time PCR (E). Histograms represent mean ± SD of three experiments ($^bP < 0.01$ *vs* those at 0 h). CHOP: C/EBP homologous protein; ER: Endoplasmic reticulum; GADD34: Growth arrest and DNA damage-inducible gene 34; GRP78: Glucose-regulated protein 78; p-eIF2α: Phospho-eukaryotic translation initiation factor-2α; PGE1: Prostaglandin E1; p-PERK: Phospho-PKR-like ER kinase; TG: Thapsigargin.

keeps them inactivated while the homeostasis of ER is maintained. During ER stress, GRP78 dissociates from PERK, ATF6 and IRE1a and binds to unfolded or misfolded proteins, promotes their proper folding or directs them to degradation. The dissociation of GRP78 from PERK, ATF6 and IRE1a, triggers the UPR response and further enhances the expression of GRP78. Increased GRP78 augments the folding capacity of the ER, inactivates the three ER sensors and promotes restoration of ER homeostasis[29].

The protective role of GRP78 against apoptosis during ER stress has been demonstrated both *in vivo* and *in vitro*[41-44]. The insulin signaling pathway has been found to promote cell proliferation and improve cell survival *via* up-regulation of GRP78 expression[45]. In this study, PGE1 significantly inhibited TG-induced apoptosis. PGE1 induced protein and mRNA expressions of GRP78. Further, inhibition of GRP78 expression *via* either H89 or siRNA hindered the protective role of PGE1 on TG-induced apoptosis. These results demonstrate that the cytoprotective effect of PGE1 on hepatocytes was mediated *via* induction of GRP78 during ER stress. The protein expression of GRP78 induced by PGE1 peaked at 6 h; however, mRNA expression of GRP78 induced by PGE1 peaked at 12 h. It is difficult to explain the difference in the time frame for attainment of peak levels of mRNA and protein expressions of GRP78. One explanation is that PGE1 regulated the GRP78 expression not only at the transcriptional level but also at the translational or posttranslational level. Whether PGE1 regulates the expression of GRP78 at the translational or posttranslational level warrants further studies.

The other important signal pathways in ER stress-induced apoptosis are mediated *via* induction of CHOP and GADD34. During UPR, phosphorylation of eIF2α *via* the PERK pathway results in inhibition of mRNA translation and general protein synthesis. However, mRNA for activating transcription factor (ATF) 4 is selectively up-regulated. Activation of ATF4 results in induction of CHOP and GADD34 expressions. GADD34 and protein phosphatase 1 were shown to promote dephosphorylation of eIF2α and allow protein synthesis[46]. However, recent studies have shown that induction of GADD34 exacerbates the disturbance of ER homeostasis and leads to cell apoptosis by increasing oxidative stress[47]. CHOP has been identified as one of the most important mediators of ER stress-induced apoptosis; it induces apoptosis through various signal pathways[48].

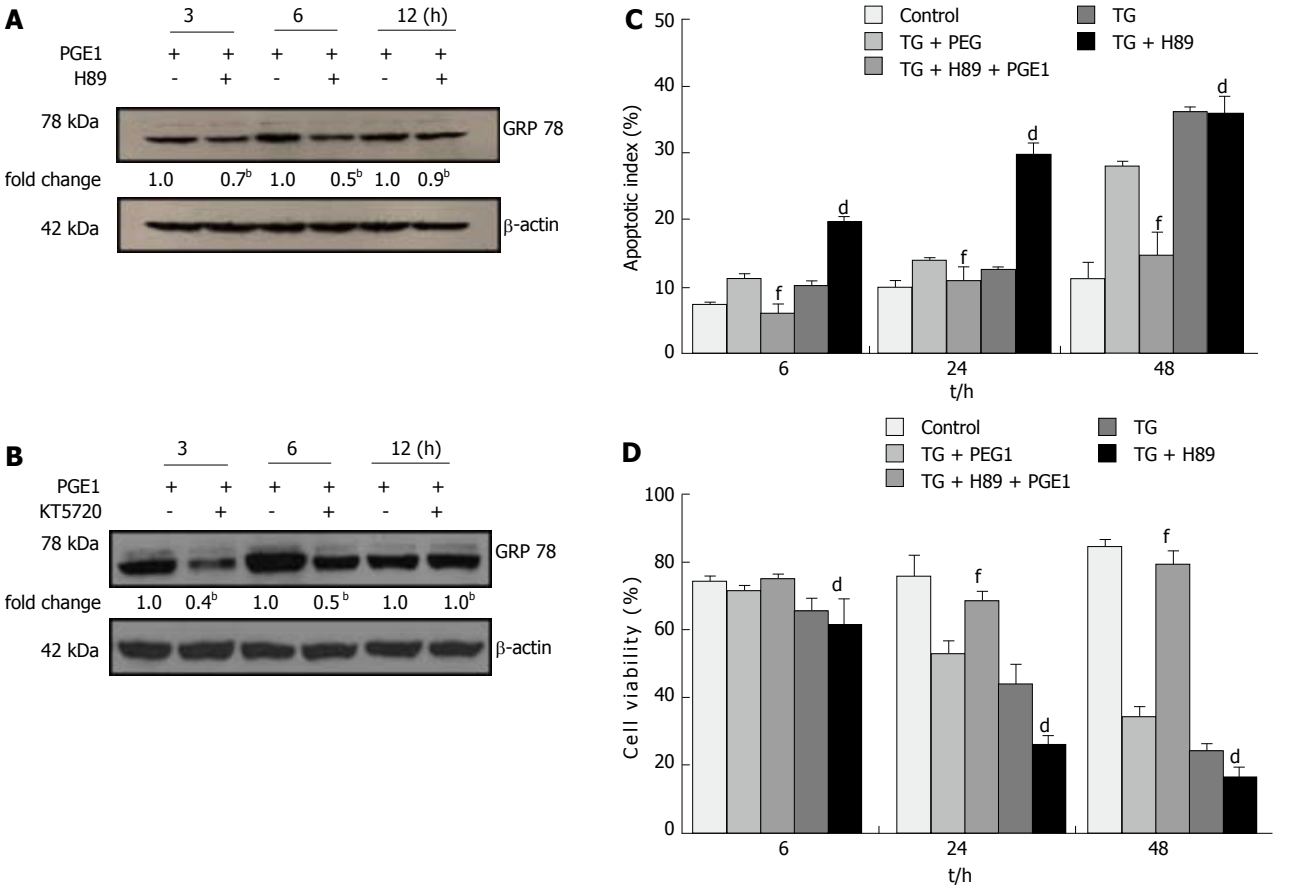

Figure 5 Prostaglandin E1 induced glucose-regulated protein 78 expression and protected L02 cells against endoplasmic reticulum stress-induced apoptosis *via* a protein kinase a-dependent pathway. A and B: L02 cells were pretreated with or without PKA inhibitor H89 (10 μmol/L) or KT5720 (1 μmol/L), and then 1 μmol/L PGE1 for 3, 6 and 12 h. The expressions of GRP78 were detected by western blotting. One representative blot each from three independent experiments is presented. The results of densitometric analysis are presented as a fold-change compared to those of PGE1 at the same time points ($^bP < 0.01$); C: L02 cells were pretreated with or without 10 μmol/L H89, and then 1 μmol/L PGE1 and a final concentration of 1 μmol/L TG for 6, 24 and 48 h. The apoptotic index was determined by flow cytometry. Histograms represent mean ± SD of five separate experiments, each of which was performed in triplicate. ($^dP < 0.01$ *vs* those of TG + PGE1 at the same time point; $^fP < 0.01$ *vs* those of TG at the same time point); D: L02 cells were pretreated with or without 10 μmol/L H89, and then 1 μmol/L PGE1 and a final concentration of 1 μmol/L TG for 6, 24 and 48 h. Cell viability of L02 cells was determined by MTS assay. Histograms represent mean ± SD of five separate experiments, each of which was performed in triplicate ($^dP < 0.01$ *vs* those of TG + PGE1 at the same time point; $^fP < 0.01$ *vs* those of TG at the same time point). GRP78: Glucose-regulated protein 78; MTS: [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium]; PGE1: Prostaglandin E1; PKA: Protein kinase A; TG: Thapsigargin.

In the current study, we had anticipated inhibition of GADD34 by PGE1. However, we observed an enhanced expression of GADD34 and significant inhibition of p-PERK, CHOP and p-eIF2α expressions in L02 cells. Our results indicate that the cytoprotective effect of PGE1 against hepatocyte apoptosis does not depend on the inhibition of GADD34. Since PGE1 lost its protective effects after inhibition of GRP78 expression in this study, it is possible that the increased expression of GADD34 in our study was the result of induction of GRP78 by PGE1 and that this represented restoration of ER homeostasis. Further study is needed to clarify the role of GADD34 in ER stress-induced apoptosis.

The biological effects of PGE result from its binding to its receptors, EP1 to EP4, and previous studies have shown that L02 cells express EP1 receptors[49]. Binding of PGE1 with its receptors stimulates production of the second messenger cyclic 3, 5 adenosine monophosphate (cAMP). cAMP may act *via* distinct intracellular signaling effectors, such as PKA and the exchange proteins activated by cAMP[50]. A previous study has shown that cAMP has PKA-independent interaction with Ca^{2+} stored in lymphocytes[51]. To test whether the hepatoprotective effect of PGE1 is mediated *via* PKA-independent interaction with Ca^{2+} stores in L02 cells, we used H89 to block the PKA pathway. The results showed that the protective effects of PGE1 were largely dependent on the PKA pathway. However, H89 inhibited GRP78 expression most effectively at 6 h, and then at 12 h the inhibitory effect was alleviated; the increased apoptotic index lasted from 6 h to 48 h. It is difficult to explain this result. One explanation is that H89 may also have inhibited other kinases in addition to PKA. H89 has been shown to inhibit at least 8 kinases beside PKA[52]. The role of PKA and other kinases in ER stress-induced apoptosis remains to be studied in the future.

It is known that GRP78 expression is regulated at the transcriptional level by ER stress. Previous

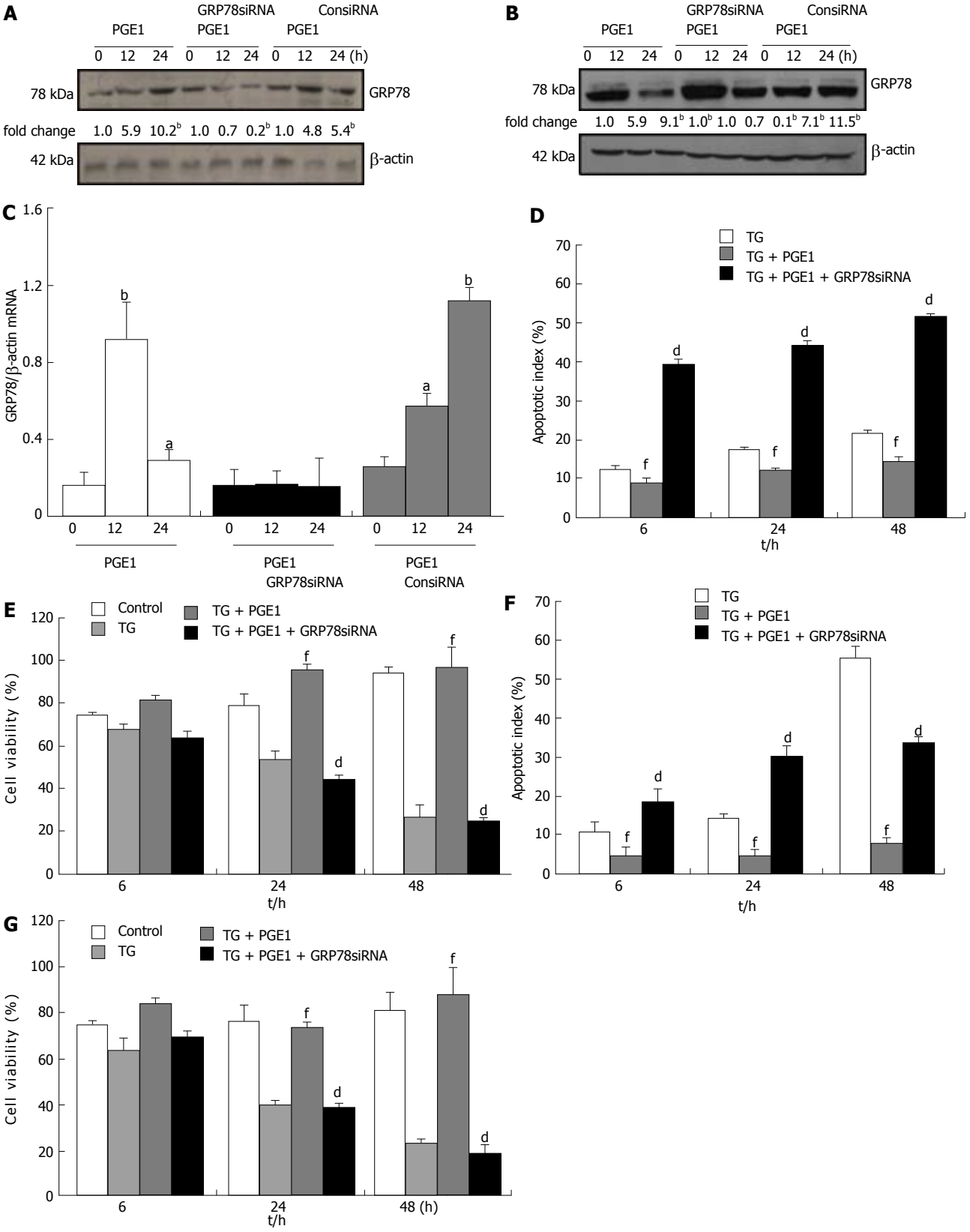

Figure 6 Prostaglandin E1 protected against endoplasmic reticulum stress-induced apoptosis *via* induction of glucose-regulated protein 78 expression in both L02 and HepG2 cells. L02 and HepG2 cells were transfected with either siRNA scramble control (ConsiRNA) or siRNA against human GRP78 (GRP78 siRNA) for 48 h. The cells were treated with 1 μmol/L PGE1 for 12 h and 24 h. A and B: Expressions of GRP78 in L02 cells and in HepG2 cells were detected by western blotting. One representative blot each from the three individual experiments is presented. The results of densitometric analysis are presented as a fold-change compared to those at 0 h ($^bP < 0.01$); C: mRNA expression of GRP78 in L02 cells was detected by real-time PCR. Histograms represent mean ± SD of three experiments ($^aP < 0.05$; $^bP < 0.01$ *vs* those at 0 h). D and F: Apoptotic indices of L02 cells and HepG2 cells were determined by flow cytometry. Histograms represent mean ± SD of five independent experiments, each of which was performed in triplicate ($^dP < 0.01$ *vs* those of TG + PGE1 at the same time point; $^fP < 0.01$ *vs* those of TG at the same time point); E and G: Cell viability of L02 cells and HepG2 cells was determined by MTS assay. Histograms represent mean ± SD of five independent experiments, each of which was performed in triplicate ($^dP < 0.01$ *vs* those of TG + PGE1 and $^fP < 0.01$ *vs* those of TG at the same time point). GRP78: Glucose-regulated protein 78; PGE1: Prostaglandin E1; MTS: [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium]; siRNA: Small interfering RNA; TG: Thapsigargin.

studies have shown that preconditioning to ER stress protects against cell death *via* induction of GRP78 or autophagy[53]. In this study, although PGE1 pretreatment significantly induced GRP78 expression and enhanced TG-induced early CHOP and sXBP1 expressions, PGE1 alone inhibited the expressions of p-PERK and p-eIF2α and CHOP. Therefore, whether PGE1 induced GRP78 expression *via* induction of ER stress is not known. In previous studies, leptin was shown to induce GRP78 expression in neuronal cells through the PI3K-mTOR pathway[54]; further, oncostatin M was also shown to induce GRP78 expression without triggering ER stress[55]. The mechanisms involved in the induction of GRP78 expression by PGE1 warrant further investigation.

In conclusion, this is the first study to demonstrate that the cytoprotective effect of PGE1 against ER stress-induced apoptosis is mediated *via* PKA-dependent induction of GRP78 expression in hepatocytes. Further studies are required for devising treatment strategies to protect hepatocytes against ER stress-induced apoptosis, which will be of much clinical relevance in the context of liver diseases.

COMMENTS

Background

Prostaglandin (PG) E1 has been shown to protect against hepatocyte apoptosis; however, the role of Prostaglandin E1 (PGE1) in endoplasmic reticulum (ER) stress-induced apoptosis of hepatocytes is largely unknown.

Research frontiers

ER stress has been implicated in the pathogenesis of various liver diseases. Understanding the mechanisms underlying ER stress-induced apoptosis and devising a treatment strategy to protect hepatocytes from ER stress-induced apoptosis will benefit most patients with liver diseases.

Innovations and breakthroughs

Pretreatment with PGE1 protected hepatocytes against thapsigargin-induced apoptosis. PGE1 alone induced protein and mRNA expressions of glucose-regulated protein (GRP) 78. Treatment with protein kinase A (PKA)-inhibitor H89, KT5720 or small interfering (si)RNA specifically targeted against human GRP78 blocked PGE1-induced up-regulation of GRP78. The hepatoprotective effect of PGE1 was lost by blocking GRP78 expression by either H89 or siRNA. Our study demonstrates, for the first time, that PGE1 protects against ER stress-induced hepatocyte apoptosis *via* PKA pathway-dependent induction of GRP78 expression.

Applications

PKA and GRP78 may be new targets for pharmacological treatment in patients with liver diseases.

Peer-review

The strengths of this study lie in the experimental design, methodology and that the authors describe the caveats and potential pitfalls in great detail. Overall, the study was well-designed and the paper is interesting and sound.

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ORIGINAL ARTICLE

Retrospective Study

Genetic associations with adverse events from anti-tumor necrosis factor therapy in inflammatory bowel disease patients

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Abstract

AIM

To study the type and frequency of adverse events associated with anti-tumor necrosis factor (TNF)

therapy and evaluate for any serologic and genetic associations.

METHODS

This study was a retrospective review of patients attending the inflammatory bowel disease (IBD) centers at Cedars-Sinai IBD Center from 2005-2016. Adverse events were identified *via* chart review. IBD serologies were measured by ELISA. DNA samples were genotyped at Cedars-Sinai using Illumina Infinium Immunochipv1 array per manufacturer's protocol. SNPs underwent methodological review and were evaluated using several SNP statistic parameters to ensure optimal allele-calling. Standard and rigorous QC criteria were applied to the genetic data, which was generated using immunochip. Genetic association was assessed by logistic regression after correcting for population structure.

RESULTS

Altogether we identified 1258 IBD subjects exposed to anti-TNF agents in whom Immunochip data were available. 269/1258 patients (21%) were found to have adverse events to an anti-TNF-α agent that required the therapy to be discontinued. 25% of women compared to 17% of men experienced an adverse event. All adverse events resolved after discontinuing the anti-TNF agent. In total: *n* = 66 (5%) infusion reactions; n = 49 (4%) allergic/serum sickness reactions; *n* = 19 (1.5%) lupus-like reactions, *n* = 52 (4%) rash, *n* = 18 (1.4%) infections. In Crohn's disease, IgA ASCA (*P* = 0.04) and IgG-ASCA (*P* = 0.02) levels were also lower in patients with any adverse events, and anti-I2 level in ulcerative colitis was significantly associated with infusion reactions (*P* = 0.008). The logistic regression/human annotation and network analyses performed on the Immunochip data implicated the following five signaling pathways: JAK-STAT (Janus Kinase-signal transducer and activator of transcription), measles, IBD, cytokine-cytokine receptor interaction, and toxoplasmosis for any adverse event.

CONCLUSION

Our study shows 1 in 5 IBD patients experience an adverse event to anti-TNF therapy with novel serologic, genetic , and pathways associations.

Key words: Genetic associations; Inflammatory bowel disease; Anti-tumor necrosis factor; Adverse events

Core tip: Tumor necrosis factor-α (TNF-α) plays a key role in the development and progression of inflammatory bowel disease (IBD). Anti-TNF therapy is highly efficacious in treating IBD patients, but many experience adverse events. Few studies have evaluated factors associated with adverse events to anti-TNF therapy. In this study, we found some genetic associations and pathways that are enriched for genes associated with the development of adverse events. Future studies will need to confirm these findings as the ability to identify subjects at high risk may help clinicians anticipate and therefore prevent or avoid these adverse events in the future.

Lew D, Yoon SM, Yan X, Robbins L, Haritunians T, Liu Z, Li D, McGovern DPB. Genetic associations with adverse events from anti-tumor necrosis factor therapy in inflammatory bowel disease patients. *World J Gastroenterol* 2017; 23(40): 7265-7273 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i40/7265.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i40.7265

INTRODUCTION

Tumor necrosis factor-α (TNF-α) plays a key role in the development and progression of inflammatory bowel disease (IBD)[1-3]. While anti-TNF therapy is an effective therapeutic option for IBD patients[1,4,5], response to these agents is highly heterogeneous and a high proportion of patients either fail initial induction therapy or lose response during maintenance therapy[6-8]. Predicting response to these agents has been studied extensively by evaluating a multitude of factors including potential genetic components[7], but an important addition would be the ability to predict the development of adverse events associated with these agents including: infusion reactions; infections; rash; allergic reactions; serum sickness like reactions; and lupus-like reactions[9-11]. Minimizing the risks is important to increase patient compliance and improve response to therapy, and will also become more important as physicians struggle with decisions around where to position biologic therapies as therapeutics targeting novel mechanisms become available[12,13].

Currently, there are few studies designed to determine factors associated with adverse events to anti-TNF-α therapy. The objectives of this study were to describe the type and frequency of adverse events associated with anti-TNF-α therapy in a large cohort and evaluate for any serologic and genetic associations.

MATERIALS AND METHODS

Study population

This study was a retrospective review of patients attending the IBD centers at Cedars-Sinai IBD Center from 2005-2016. Patients included in the study were

those that had given consent, had available genotype data, carried a diagnosis of IBD (Crohn's disease, ulcerative colitis, or IBDU), and who had been treated with anti-TNF-α agents (infliximab, adalimumab, certolizumab pegol). All research-related activities were approved by the Cedars-Sinai Medical Center Institutional Review Board (IRB #3358).

Data gathering

Detailed clinical information for each patient was obtained *via* chart review. Clinical information included age at disease diagnosis, type of IBD (CD, UC, or IBDU), gender, and type of anti-TNF-α agent used. All patients were seen by gastroenterologists, experienced in managing patients with IBD treated with anti-TNF agents, at the IBD centers at Cedars-Sinai Medical Center, Los Angeles.

Adverse events

Adverse events were identified *via* chart review by evaluating the "Allergies" section and the progress notes written by the gastroenterologist. Potential adverse events include infusion reactions, serum sickness-like reactions, drug-induced lupus, rash, infections, and non-specific symptoms (arthralgias, shortness of breath, rash, *etc*). An infusion reaction was defined as any significant adverse experience that occurred during or within two hours of infusion[14]. An allergic or a serum sickness-like reaction was defined clinically as the occurrence of myalgias, arthralgias, fever, or rash within 1-14 d after reinfusion of infliximab[15].

The likelihood of a causal relationship for each adverse event was determined based on the assessment of the gastroenterologist as documented in the progress note and as evidenced by the following: time elapsed between a dose and adverse event, resolution of the adverse event when the therapy was discontinued, and return of the adverse event if the therapy was resumed[16].

Serological analysis

IBD serologies (ANCA, anti-nuclear cytoplasmic antibodies; anti-CBir1, anti-flagellin; anti-I2, anti-*Pseudomonas fluorescens*-associated sequence I2; anti-OmpC, anti-outer membrane porin C; ASCA, anti-*Saccharomyces cerevisiae* antibodies) were measured by enzyme-linked immunosorbent assay (ELISA) as previously described[17]. Results were expressed as ELISA units (EU/mL) relative to Cedars-Sinai Medical Center laboratory or a Prometheus laboratory standard derived from a pool of patient sera with well-characterized disease found to have reactivity to these antigens. All assays were performed in a blinded fashion.

Genotype data

DNA samples were genotyped at Cedars-Sinai using Illumina Infinium Immunochipv1 array per manufacturer's protocol (Illumina, San Diego, CA, United States). Average genotyping call rate for samples that passed quality control was 99.8%; average replicate concordance and average heritability rates were > 99.99% and 99.94%, respectively. Single-nucleotide polymorphisms (SNPs) underwent methodological review and were evaluated using several SNP statistic parameters to ensure optimal allele-calling[18].

Statistical analysis

χ^2 test and logistic regression were performed to identify demographic and clinical characteristics associated with development of adverse events. For continuous variables with skewed distribution (*e.g.*, serology levels), Wilcox test signed rank test was performed. SNPs association with adverse events was evaluated using PLINK. Principal components (PCs) from population stratification analysis were included in the PLINK analysis to control for potential confounding[19]. Two-sided *P*-value of 0.05 was considered statistically significant.

Genetic pathway, and network analyses

For any reaction, infusion reactions, and allergic reactions, the logistic regression/human annotation and network analysis was performed with statistically significant SNPs ($P < 0.001$). These SNPs were first annotated into corresponding genes, and the genes were further analyzed with multiple biological functional databases including human protein reference databases (http://www.hprd.org), Reactome, NCI/Nature pathway interaction database and others. The final networks were then constructed form the known interactions from any of these databases. Pathways and gene set enrichment analysis was performed with STRING (http://string-db.org/) and cytoscape (http://www.cytoscape.org).

RESULTS

Patient demographics and characteristics

1258 IBD (954 CD patients, 260 UC, 44 IBDU) patients qualified for this study. The average age of onset was 25.7 years and, and the overwhelming majority were of European ancestry.

Adverse events

A total of 269/1258 patients (21%) were found to have experienced an adverse event. The different

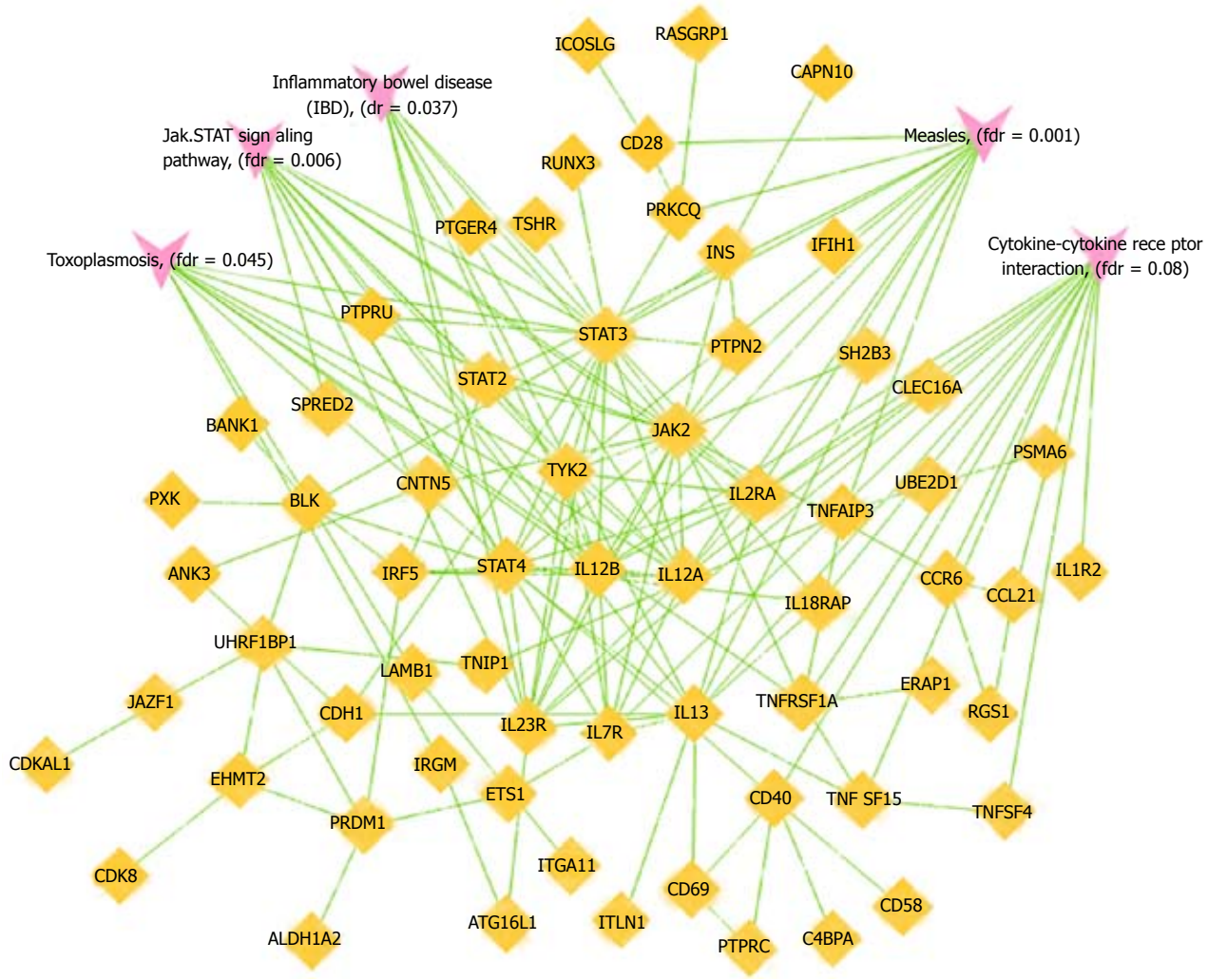

Figure 1 Network analyses of allergic reactions to anti-tumor necrosis factor agents.

types of adverse events were similar among women and men, except for lupus-like reactions and rashes, which were both more commonly seen in women (Table 1).

Serology

In CD patients we observed that IgA ASCA+/- was associated with a lower risk of developing any adverse event (OR = 0.68, *P* = 0.047), while IgG ASCA+/- and total ASCA+/- show a similar trend and borderline association respectively (OR = 0.72 and 0.70, *P* = 0.09 and 0.05, respectively). IgA ASCA (*P* = 0.04) and IgG ASCA (*P* = 0.02) levels were also lower in patients with any adverse events (Table 2). Anti-I2 level in UC was significantly associated with infusion reactions (*P* = 0.008) (Table 2). No other associations were seen with IBD-associated serologies (data not shown).

Genetic analysis

IBD-associated SNPs that achieved a nominal level of significance with adverse events are shown in Table 3. For any reaction, infusion reactions, and allergic reactions, the logistic regression/human annotation and network analyses performed on the Immunochip data implicated the following five signaling pathways: JAK-STAT (Janus Kinase-signal transducer and activator of transcription), Measles, IBD, Cytokine-cytokine receptor interaction, and Toxoplasmosis (Table 4 and Figure 1). After False Discovery Rate correction, all associations remained statistically significant except for the Cytokine-cytokine receptor interaction for infusion and allergic reactions.

DISCUSSION

In our cohort approximately 1 in 5 IBD patients experienced adverse events to anti-TNFs that eventually led to the discontinuation of the therapy in keeping with the current literature, although most studies only report the presence or not of adverse events and do not comment on whether patients had to

Table 1 Adverse events based on type of inflammatory bowel disease and gender *n* (%)

Clinical traits	All adverse events	Infusion reactions	Allergic reactions	Lupus-like reactions	Rash	Other
Type of IBD						
Crohn's disease (*n* = 954)	220 (23)	52 (5)	45 (5)	14 (1)	40 (4)	69 (7)
Ulcerative colitis (*n* = 260)	42 (16)	14 (5)	4 (2)	4 (2)	10 (4)	10 (4)
IBDU (*n* = 44)	7 (16)	0 (0)	0 (0)	1 (2)	2 (5)	4 (9)
Total	269 (21)	66 (5)	49 (4)	19 (1.5)	52 (4)	83 (7)
Gender						
Male (*n* = 624)	108 (17)	28 (4)	24 (4)	3 (0.5)	17 (3)	36 (6)
Female (*n* = 634)	161 (25)	38 (6)	25 (4)	16 (3)	35 (6)	47 (7)

All values expressed as *n* (%). IBD: Inflammatory bowel disease.

Table 2 Serological associations with anti-tumor necrosis factor adverse reactions in patients with ulcerative colitis and Crohn's disease (anti-I2, anti-*Pseudomonas fluorescens*-associated sequence I2; ASCA, anti-Saccharomyces cerevisiae antibodies)

IBD type	Adverse event	Serological marker	Serology levels in positive (U/mL)	Serology levels in negative (U/mL)	*P* value
CD	Any	IgA ASCA	7	10	0.040
CD	Any	IgG ASCA	18	26.5	0.020
UC	Infusion	Anti-I2	0	7	0.008

IBD: Inflammatory bowel disease; CD: Crohn's disease; UC: Ulcerative colitis.

Table 3 Inflammatory bowel disease-associated single nucleotide polymorphisms associated with different type of adverse events

Adverse event	Risk allele	SNP	Gene (s) of interest	*P* value	OR (95%CI)
Infusion	C	rs6740462	*SPRED2*	0.003	0.4 (0.2-0.7)
Infusion	G	rs1182188	*GNA12*	0.007	1.8 (1.2-2.6)
Infusion	G	rs10061469	*TMEM174, FOXD1*	0.010	0.5 (0.3-0.9)
Infusion	A	rs477515	*HLA-DRB1*	0.010	1.7 (1.1-2.5)
Allergic	A	rs4692386	*SMIM20, RBPJ*	0.010	0.5 (0.3-0.9)
Allergic	A	rs10761659	*ZNF365*	0.010	0.6 (0.3-0.9)
Lupus-like	G	rs13407913	*ADCY3*	0.003	3.5 (1.6-7.9)
Lupus-like	C	rs10051722	*CHSY2, HINT1*	0.010	2.7 (1.2-5.7)
Rash	A	rs1363907	*ERAP2*	0.003	3.0 (1.5-6.2)
Rash	G	rs11010067	*PARD3*	0.003	0.2 (0.1-0.6)
Rash	C	rs7746082	*PREP*	0.005	2.7 (1.3-5.3)
Any	C	rs6740462	*SPRED2*	0.0007	0.6 (0.5-0.8)
Any	C	rs10774482	*ERC1*	0.003	1.4 (1.1-1.8)

SNP: Single nucleotide polymorphisms.

discontinue[20]. The incidence of serious lupus-like reactions requiring the discontinuation of anti-TNFs was found to be 1.1%[21], which was comparable to that seen in our population of 1.5%. To our knowledge, this is the largest study examining adverse events with anti-TNF agents.

We identified a number of genetic associations with known IBD loci including two (*HLA-DRB1* and *ERAP2* [endoplasmic reticulum aminopeptidase 2]) that are associated with a number of immune-mediated diseases as well as IBD and also, in the case of *HLA-DRB1*, with the development of extra-intestinal manifestations in IBD[22-24]. Furthermore, both are involved in peptide presentation by HLA molecules[25,26]. We also observed associations at other IBD genes including *ZNF365,* a transcription factor in maintaining genomic stability during DNA replication in the brain, heart, lung, pancreas, small intestine and colon[27,28]. Our genetic findings also implicated genes that maintain colonic wall permeability including *SPRED* (Sprouty-related EVH1 domain-containing protein) and *PARD3* (Partitioning defective 3 homolog)[29,30]. In addition, *GNA12* (Guanine nucleotide-binding protein alpha-12), a modulator of different transmembrane signaling systems, has also been implicated in the loss of barrier integrity[31]. Our pathway analyses strongly implicated

Table 4 Pathway analyses from genetic associations with adverse events from immunochip analyses

Type of adverse event	Pathway	Number of genes	*P* value	*P* value (Bonferroni)
Any	JAK-STAT signaling pathway	13	3.98×10^{-6}	7.96×10^{-4}
Any	Measles	12	8.87×10^{-6}	0.0018
Any	IBD	8	3.05×10^{-5}	0.0061
Any	Cytokine-cytokine receptor interaction	16	5.03×10^{-5}	0.0100
Any	Toxoplasmosis	10	5.49×10^{-5}	0.0110
Infusion	JAK-STAT signaling pathway	12	3.68×10^{-5}	0.0074
Infusion	Measles	12	8.24×10^{-6}	0.0016
Infusion	IBD	7	2.11×10^{-4}	0.0420
Infusion	Cytokine-cytokine receptor interaction	14	4.98×10^{-4}	0.0990
Infusion	Toxoplasmosis	11	9.02×10^{-6}	0.0018
Allergic	JAK-STAT signaling pathway	12	2.96×10^{-5}	0.0060
Allergic	Measles	12	6.6×10^{-6}	0.0010
Allergic	IBD	7	1.84×10^{-4}	0.0370
Allergic	Cytokine-cytokine receptor interaction	14	4.02×10^{-4}	0.0800
Allergic	Toxoplasmosis	9	2.25×10^{-4}	0.0450

IBD: Inflammatory bowel disease.

five signaling pathways including JAK-STAT signaling pathway, Cytokine-cytokine receptor interaction pathway, Measles signaling pathway, Toxoplasmosis signaling pathway, and the IBD signaling pathway. The network analyses for allergic reactions (Figure 1) show a number of key nodes including *TYK2, BLK* and *IL13*, which have previously been shown to be associated with allergic susceptibility[32-34].

IBD serologies (ANCA, anti-CBir1, anti-I2, anti-OmpC, and ASCA) can distinguish CD from UC, risk stratify IBD patients, and also predict postoperative complications and occur as a result of an aberrant or exaggerated response to commensal flora[35]. The association with ASCA and I2 are interesting. Perhaps these markers identify patients with a predilection towards small bowel involvement. Patients with colonic disease tend to respond less to anti-TNFs or require higher doses[8,36] and, perhaps therefore, these patients are more likely to develop antibodies or reactions to anti-TNFs. Further studies will be needed to confirm these borderline associations.

There are several potential limitations of this study including, the relatively small sample size and the retrospective nature of the study (despite it being the largest of its kind to date). Additionally, we did not have information on anti-drug antibody formation as the majority of these patients developed adverse events prior to the widespread use of these parameters in clinical practice. It is also important to note that our study population was predominantly of European ancestry. While IBD is rising in non-Europeans, the highest prevalence is still seen in European ancestry populations. For this reason, and the location of Cedars-Sinai Medical Center in west Los Angeles, the majority of our patients are "European". Previous work have shown ethnic differences in genetic associations with adverse events[37], and a study similar to this one should be performed for other ethnic groups.

In conclusion, our study revealed that approximately 1 in 5 IBD patients experienced an adverse event to anti-TNF therapies that required cessation of therapy. The majority of these were infusion/allergic reactions but approximately 1 in 30 women will develop a lupus-like reaction and we also observed other serious adverse events including pancreatitis and vascultitis but these were rare. We have demonstrated some genetic associations and pathways that are enriched for genes associated with development adverse events. Future studies will need to confirm these findings as the ability to identify subjects at high risk may help clinicians anticipate and therefore prevent or avoid these adverse events in the future.

ATRICLE HIGHLIGHTS

Research Background

Tumor necrosis factor (TNF) inhibitors are highly efficacious in treating inflammatory bowel disease (IBD). Response to these agents is highly heterogenous, and there have been a multitude of studies aimed at predicting the response to these agents. An important addition is the ability to predict the development of adverse events associated with these agents such as infusion reactions, infections, or rash. Minimizing the risk is important to increase patient compliance and improve response to therapy.

Research motivation

Recognizing the type and frequency of adverse events to anti-TNF therapy, and the potential genetic and serologic associations can help identify subjects at high risk, and may help clinicians anticipate and therefore prevent or avoid these adverse events in the future.

Research objectives

The objectives of this study were to describe the type and frequency of adverse

events associated with anti-TNF-α therapy in a large cohort and evaluate for any serologic and genetic associations. The significance of realizing these objectives is that it can identify subjects at high risk for developing adverse events and can help clinicians anticipate and therefore prevent or avoid these adverse events in the future.

Research methods

This study was a retrospective review, and detailed clinical information was collected *via* manual chart review. χ^2 test and logistic regression were performed to identify demographic and clinical characteristics associated with development of adverse events.

The serological data was measured by ELISA assay at Cedars-Sinai, which was performed in a blinded fashion, and analyzed with Wilcox test signed rank test.

DNA samples were genotyped at Cedars-Sinai using Illumina Infinium Immunochipv1 array per manufacturer's protocol (Illumina, San Diego, CA, United States). Average genotyping call rate for samples that passed quality control was 99.8%; average replicate concordance and average heritability rates were > 99.99% and 99.94%, respectively. Single-nucleotide polymorphisms (SNPs) underwent methodological review and were evaluated using several SNP statistic parameters to ensure optimal allele-calling. SNPs association with adverse events was evaluated using PLINK, with two-sided p-value of 0.05 was considered statistically significant.

For any reaction, infusion reactions, and allergic reactions, the logistic regression/human annotation and network analysis was performed with statistically significant SNPs ($P < 0.001$). These SNPs were first annotated into corresponding genes, and the genes were further analyzed with multiple biological functional databases. The final networks were then constructed form the known interactions from any of these databases.

The research methods described above are standard for a retrospective review analyzing genetic and serologic data.

Research results

About 1 in 5 patients were found to have adverse events to an anti-TNF-α agent that required the therapy to be discontinued. All adverse events resolved after discontinuing the anti-TNF agent. The majority of patients developed infusion reactions. In CD patients we observed that IgA ASCA +/- was associated with a lower risk of developing any adverse event. IgA ASCA and IgG ASCA levels were also lower in patients with any adverse events. Anti-I2 level in UC was significantly associated with infusion reactions. The authors identified a number of genetic associations with known IBD loci including *HLA-DRB1, ERAP2, ZNF365*. Their pathway analyses strongly implicated JAK-STAT signaling pathway, Cytokine-cytokine receptor interaction pathway, Measles signaling pathway, Toxoplasmosis signaling pathway, and the IBD signaling pathway. The network analyses for allergic reactions showed a number of key nodes including *TYK2, BLK* and *IL13*, which have previously been shown to be associated with allergic susceptibility.

They have demonstrated some novel genetic associations and pathways that are enriched for genes associated with development adverse events. Future studies should be performed to confirm our results, and incorporate other ethnic groups besides European ancestry, and include data on anti-drug antibody formation.

Research conclusions

The genetic and serologic associations found in concordance with adverse events to anti-TNF therapy. This is the first study to evaluate and describe these associations. There are potentially genetic and serologic associations with adverse events to anti-TNF therapy that can help clinicians anticipate and therefore prevent or avoid these adverse events in the future.

Current studies describe in great detail the efficacy of anti-TNF therapy and the ability to predict response to therapy. However, current studies are lacking in evaluating the ability to predict the development of adverse events. The results from this study reveal that there indeed are genetic and serologic associations with anti-TNF therapy that can potentially be targeted to prevent or avoid these adverse events in the future. The new hypothesis proposed by this study is that there serologic and genetic associations with anti-TNF therapy.

The methods used in this study were similar to other retrospective studies analyzing genetic and serologic data. Manual chart review was performed to generate detailed clinical information; ELISA assay was performed to gather serologic data; and genetic data was generated using Illumina Infinium Immunochipv1 array. χ^2 test and logistic regression were performed to identify demographic and clinical characteristics associated with development of adverse events; serological data were analyzed with Wilcox test signed rank test; and SNPs association with adverse events were evaluated using PLINK, with two-sided *P*-value of 0.05 considered statistically significant.

The genetic and serologic associations found in concordance with adverse events to anti-TNF therapy are novel and have not been described elsewhere. This study confirmed that there are genetic and serologic associations with adverse events from anti-TNF therapy. Identifying the potential factors associated with adverse events from anti-TNF therapy can help clinicians anticipate and therefore prevent or avoid these adverse events in the future.

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ORIGINAL ARTICLE

Retrospective Study

Clinical features of alcoholic hepatitis in latinos and caucasians: A single center experience

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Abstract

AIM

To study differences of presentation, management, and prognosis of alcoholic hepatitis in Latinos compared to Caucasians.

METHODS

We retrospectively screened 876 charts of Caucasian and Latino patients who were evaluated at University of California Davis Medical Center between 1/1/2002-12/31/2014 with the diagnosis of alcoholic liver disease. We identified and collected data on 137 Caucasians and 64 Latinos who met criteria for alcoholic hepatitis, including chronic history of heavy alcohol use, at least one episode of jaundice with bilirubin ⩾ 3.0 or

coagulopathy, new onset of liver decompensation or acute liver decompensation in known cirrhosis within 12 wk of last drink.

RESULTS

The mean age at presentation of alcoholic hepatitis was not significantly different between Latinos and Caucasians. There was significant lower rate of overall substance abuse in Caucasians compared to Latinos and Latinos had a higher rate of methamphetamine abuse (12.5% *vs* 0.7%) compared to Caucasians. Latinos had a higher mean number of hospitalizations (5.3 ± 5.6 *vs* 2.7 ± 2.7, $P = 0.001$) and mean Emergency Department visits (9.5 ± 10.8 *vs* 4.5 ± 4.1, $P = 0.017$) for alcohol related issues and complications compared to Caucasians. There was significantly higher rate of complications of portal hypertension including gastrointestinal bleeding (79.7% *vs* 45.3%, $P < 0.001$), spontaneous bacterial peritonitis (26.6% *vs* 9.5%, $P = 0.003$), and encephalopathy (81.2% *vs* 55.5%, $P = 0.001$) in Latinos compared to Caucasians.

CONCLUSION

Latinos have significant higher rates of utilization of acute care services for manifestations alcoholic hepatitis and complications suggesting poor access to outpatient care.

Key words: Alcoholic hepatitis; Latino; Hispanic; Caucasian; Alcoholic liver disease

Core tip: We conducted a retrospective chart review on Caucasian and Latino patients with alcoholic hepatitis. We showed that Latinos had significantly higher rates of gastrointestinal bleeding, encephalopathy, spontaneous bacterial peritonitis, recurrence of alcoholic hepatitis, and utilization of acute care services for alcohol related issues compared to Caucasians. However, the survival rates were not significantly different between Latinos and Caucasians. Our findings suggest that Latino patients have poor access to outpatient care and management of complications of portal hypertension.

Pinon-Gutierrez R, Durbin-Johnson B, Halsted CH, Medici V. Clinical features of alcoholic hepatitis in latinos and caucasians: A single center experience. *World J Gastroenterol* 2017; 23(40): 7274-7282 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i40/7274.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i40.7274

INTRODUCTION

Alcoholism is estimated to cause 2.5 million deaths annually worldwide, and it represents 4% of all deaths[1]. Forty percent of all mortality from cirrhosis is attributed to alcoholic liver disease (ALD). ALD includes different clinical entities with different severity and prognosis, ranging from alcoholic fatty liver disease characterized by hepatic steatosis, to severe alcoholic hepatitis (AH), and ultimately alcoholic cirrhosis[1] with portal hypertension and its complications. Disparities exist in the incidence and severity of ALD among various racial and ethnic groups in the United States.

Ethnicity data from the National Center for Health Statistics for 1991-1997 showed greatest liver cirrhosis mortality among Latino men compared to Black and Caucasian men followed by Latina females[2]. A comprehensive discharge study of all patients with ALD in non-federal hospitals in Los Angeles County in 1999 showed that ALD accounted for 1.2% of all deaths and was most prevalent in middle aged Latino men as compared to other ethnicities[3]. A more recent study showed that the Latino population in the United States has a higher incidence and more aggressive pattern of chronic liver diseases than the Caucasian population[4]. Longevity data from a multicenter Veteran Affairs cooperative study showed that the 5-year survival from active ALD was only 28% among Latino men compared to 40% in Caucasian men[5]. Among heavy alcohol drinkers, Latinos were 9.1 times more likely to have a 2-fold elevation in AST and GGT levels when compared to Caucasians[6], and Mexican American men are more likely to be heavy binge drinkers than Caucasians and African American men or Mexican American women[7]. A recent review highlights the linkage of excessive alcohol intake and dependence among Mexicans to the presence of functional SNPs in genes that regulate both alcohol craving mechanisms in the brain and alcohol metabolism in the liver[8]. In our own experience[9], Latino patients treated at UC Davis for ALD were significantly younger than Caucasians at the time of presentation and were more likely than Caucasian patients to present with AH or cirrhosis. Despite these well documented differences, there are no ethnic-specific data on the various manifestations of ALD. AH is the most severe manifestation of ALD is characterized by an inflammatory process of the liver with neutrophilic infiltration leading to ballooning degeneration of hepatocytes, hepatocyte necrosis, steatosis and presence of Mallory bodies. AH clinic presentation is associated with jaundice, fever, hepatomegaly, liver tenderness, encephalopathy, and gastrointestinal bleeding (GIB)[1]. The goal of the present study was to determine the clinical features of AH in Latino patients compared to Caucasian patients.

MATERIALS AND METHODS

We performed a single-center retrospective chart review of 137 Caucasian patients and 64 Latino patients older than 18 years old diagnosed with AH and who were seen at the University of California Davis

Hepatology Clinic or hospitalized at the University of California Davis Medical Center in Sacramento, California between 1/1/2002-12/31/2014. Of note, it is estimated that 83% of Latinos living in the Sacramento area are Mexican Americans (http://quickfacts.census.gov/qfd/states/06/06067.html). We performed a chart review of 876 Caucasian and Latino patients with ICD9 codes for AH, alcoholic cirrhosis, ALD, and unspecified liver damage identify subjects with AH of each group. When possible, we applied the NIAAA Alcoholic Hepatitis Consortia criteria[10] with few modifications given the retrospective nature of the study: (1) chronic history of heavy alcohol use; (2) at least one episode of jaundice with bilirubin ⩾ 3 or coagulopathy with INR ⩾ 1.2 (modification); (3) new onset of liver decompensation; (4) diagnosis acute AH per ICD-9 code on known cirrhosis; and (5) within 12 wk (modification) of last drink and where no other causes of liver decompensation were identified. Otherwise, ICD9 codes for AH were applied to ensure the highest number of subjects was included in the study. We excluded patients with known active hepatitis B (positive HBsAg and Hepatitis B viral load > 20,000 IU/mL) and active hepatitis C (positive HCV Ab and HCV viral load > 615 IU/mL).

We used the demographics section of the electronic medical record and/or from history and physical examination notes to identify our research population, ethnicity, gender, and age of onset of AH. We defined the drinking patterns as follow: moderate drinking was defined as no more than four drinks per day, or 14 drinks per week for men, and no more than three drinks per day, or 7 drinks per week for women, and heavy drinking defined as 15 or more drinks per week, or four drinks in a day, for men or, more than seven drinks per week, or three drinks a day for women. We defined binge drinking as five or more drinks for male or 4 or more drinks in female in about 2 h on multiple occasions. We also gathered drinking duration occurred in our patient population. We included data pertaining to body mass index (BMI) and whether subjects had a diagnosis of metabolic syndrome and diabetes mellitus (DM). We recorded the development of complications associated with AH and/or portal hypertension including GIB, encephalopathy, spontaneous bacterial peritonitis (SBP), and presence of other infections, pancreatitis, development of acute kidney injury (AKI) and/or hepatorenal syndrome, disseminated intravascular coagulopathy (DIC), and hepatocellular carcinoma. We recorded intensive care unit (ICU) admission, length of ICU stay, development of respiratory failure, and length of hospital stay, and we determined the number of emergency room visits and hospitalizations for alcohol related morbidity.

We made the distinction of AH without known history of cirrhosis and AH with known cirrhosis and recorded the number of times a patient met our criteria for AH. To assess severity of AH, we calculated the Discriminant Function (DF) [4.6 × (Pt's PT - Control PT) + TBili]. If ⩾ 32, it is considered severe AH[11] with related increased risk of mortality[12]. We determined Glasgow score for AH, Model for End-Stage Liver Disease (MELD) score, and Child-Turcotte-Pugh class. We collected data on modality of medical treatment of severe AH (supportive care, steroids, pentoxifylline including their duration and doses). Finally, we collected survival data that included total survival days after episode of AH (total number of days since date of AH to last day to be alive or seen at the UC Davis Medical Center or clinic).

Statistical analyses

Categorical variables were compared between ethnicities using χ^2 tests, followed by examination of adjusted residuals in the case of a significant test. Continuous variables were compared between ethnicities using *t*-tests, with duration data and lab data log-transformed prior to testing. Survival was compared between ethnicities using log rank tests.

The effects of subject disease characteristics and treatment on overall survival were analyzed using Cox proportional hazards models. Analyses were conducted for all subjects and separately for each ethnicity. All analyses were adjusted for age, sex, BMI, and, for analyses in all subjects, ethnicity. Analyses were conducted using R, version 3.3.1 (R Core Team, 2016).

RESULTS

The mean age at presentation for all patients was 48.6 ± 10.4 years and was not significantly different between Latinos and Caucasians. Among men, there was a marginally significant higher proportion of male Latino subjects with AH compared to Caucasians subjects (71.9% *vs* 56.2%, $P = 0.049$). There were no significant differences between Latinos and Caucasians in BMI, presence of DM, or metabolic syndrome (Table 1). The mean duration of drinking was longer in Latinos compared to Caucasians (24.3 ± 11.0 years *vs* 20.5 ± 12.7 years, $P = 0.027$). There were no significant differences between Latinos and Caucasians regarding pattern of drinking or daily mean average number of alcoholic drinks (Table 1). Latinos and Caucasians presented different patterns of non-alcoholic substance abuse. Caucasians had significant higher rates of having no history of substance abuse (73.0% *vs* 57.8%), whereas more Latinos had a history of methamphetamine abuse (12.5% *vs* 0.7%) and a remote history of substance abuse (9.4% *vs* 4.4%) than would be expected under homogeneity of variance ($P = 0.003$) (Table 1).

Latinos had about twice the number of emergency department (ED) and hospitalizations visits due to alcohol intoxication and alcohol related issues compared to Caucasians (Figures 1 and 2). There was no significant difference in the mean length of hospital stay between Latinos (52 subjects admitted out of 64)

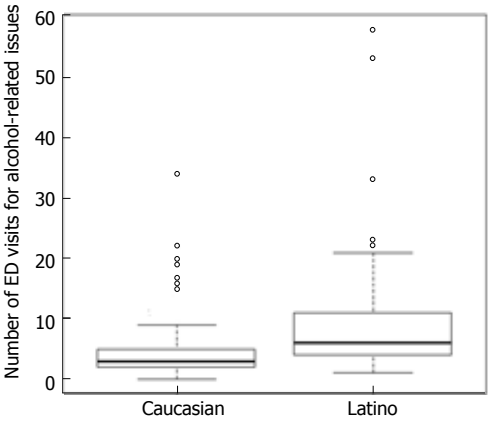

Figure 1 Emergency department visits for alcohol-related issues. There was a significant difference in the mean number of ED visits for alcohol related issues between ethnicities. Latinos had a significant higher mean number of ED visits for alcohol related issues compared to Caucasians (9.5 ± 10.8 *vs* 4.1 ± 4.1 ED visits, $P \leqslant 0.001$). ED: Emergency department.

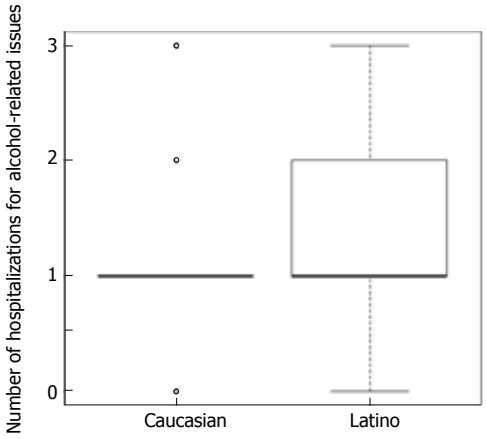

Figure 2 Hospitalizations for alcohol-related issues. There was a significant difference in the mean number of hospital admissions for alcohol related issues between ethnicities. Latinos had a higher mean number of hospital admissions for alcohol related issues compared to Caucasians (5.3 ± 5.6 *vs* 2.7 ± 2.7 admissions, $P = 0.001$).

Table 1 Patient Characteristics *n* (%)

	Caucasian (*n* = 137)	Latino (*n* = 64)	All Patients (*n* = 201)	*P* value
Age of presentation with AH (yr)				0.082
mean ± SD	49.5 (10.4)	46.8 (10.1)	48.6 (10.4)	
Sex				0.049
Male	77 (56.2)	46 (71.9)	123 (61.2)	
Female	60 (43.8)	18 (28.1)	78 (38.8)	
BMI				0.167
mean ± SD	28.1 (5.9)	29.4 (6.2)	28.5 (6)	
DM				0.061
No	105 (76.6)	41 (64.1)	146 (72.6)	
Yes	25 (18.2)	20 (31.2)	45 (22.4)	
Unknown	7 (5.1)	3 (4.7)	10 (5)	
Metabolic syndrome				0.405
No	37 (27)	13 (20.3)	50 (24.9)	
Yes	53 (38.7)	28 (43.8)	81 (40.3)	
Unknown	47 (34.3)	23 (35.9)	70 (34.8)	
Duration of drinking (yr)				0.027
mean ± SD	20 (12.7)	24.3 (11)	21.6 (12.3)	
Pattern of drinking				0.283
Heavy drinking	5 (3.6)	0	5 (2.5)	
Binge drinking	130 (94.9)	64 (100.0)	194 (96.5)	
Unknown	2 (1.5)	0	2 (1.0)	
Number of drinks/d				0.093
mean ± SD	13.9 (10.9)	16.1 (7.2)	14.6 (9.9)	
None	100 (73.0)	37 (57.8)	137 (68.2)	
Cocaine	2 (1.5)	0	2 (1.0)	
Heroin	1 (0.7)	0	1 (0.5)	
Methamphetamine	1 (0.7)	8 (12.5)	9 (4.5)	
Marijuana	9 (6.6)	3 (4.7)	12 (6.0)	
Other	2 (1.5)	0	2 (1.0)	
Polysubstance abuse	10 (7.3)	9 (14.1)	19 (9.5)	
Remote history	6 (4.4)	6 (9.4)	12 (6.0)	
Unknown	6 (4.4)	1 (1.6)	7 (3.5)	

AH: Alcoholic hepatitis; BMI: Body mass index; DM: Diabetes mellitus.

and Caucasians (111 admitted out of 137) admitted to the hospital with AH and there was no significant difference between Latinos and Caucasians requiring ICU admission and length of ICU stay in days (Table 2).

There were no significant differences in the proportions of Latinos and Caucasians who presented with AH without history of cirrhosis (76.6% *vs* 68.6%) and with history of cirrhosis (23.4% *vs* 31.4%) ($P = 0.321$). The mean DF score of the whole group was 46.7 ± 33.9 and there was no significant difference in prevalence of severe AH, as determined by DF > 32, between Latinos and Caucasians, mean MELD score, and

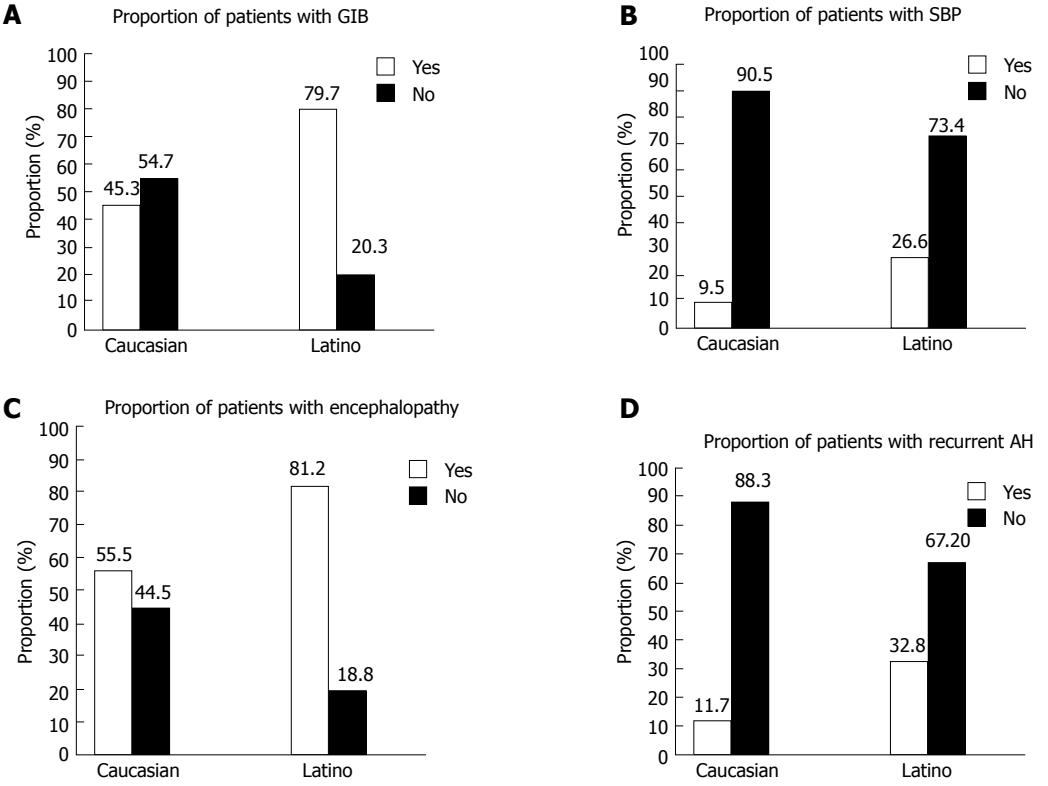

Figure 3 Clinical manifestations of alcoholic hepatitis. There was a significant difference in the proportion of patients who experienced GIB, SBP, encephalopathy, and recurrence of AH between ethnicities. A: A significant higher proportion of Latinos experienced GIB compared to Caucasians (79.7% *vs* 45.3%, $P < 0.001$); B: A significantly higher proportion of Latinos experienced SBP compared to Caucasians (26.6% *vs* 9.5%, $P = 0.003$); C: A significant higher proportion of Latinos experienced encephalopathy compared to Caucasians (81.2% *vs* 55.5%, $P = 0.001$); D: A higher proportion of Latinos had recurrent AH compared to Caucasians (32.8% *vs* 11.7%, $P = 0.005$). AH: Alcoholic hepatitis; GIB: Gastrointestinal bleeding; SBP: Spontaneous bacterial peritonitis.

Table 2 Level of care *n* (%)

	Caucasian	Latino	All patients	*P* value
Hospitalization duration (in days)	111	52	163	0.239
mean ± SD	14.4 (12.8)	13.8 (15.9)	14.2 (13.8)	
ICU admission	111	52	163	0.756
Yes	39 (35.1)	17 (32.7)	56 (34.4)	
No	72 (64.9)	35 (67.3)	107 (65.6)	
ICU stay (in days)	39	15	54	0.587
mean ± SD	7.7 (9.9)	12.2 (16.6)	8.9 (12.1)	

111 out of the 137 Caucasians and 52 out of 64 Latino Subjects were admitted to the hospital for management of alcoholic hepatitis. The other Caucasians and Latino patients were seen in the ED or outpatient setting. ICU: Intensive care unit.

initial mean Glasgow score for AH (Table 3). Among patients who received medical treatment for severe AH (Latinos $n = 15$ and Caucasians $n = 37$), patients from both ethnicities were treated similarly with similar proportions of patients treated with steroids, pentoxyfilline or combination of both (Table 3). There was no significant difference in the Lille score after 7 d of treatment between Latinos ($n = 14$) and Caucasians ($n = 35$) (Table 3).

Latinos and Caucasians admitted with AH presented a similar rate of complications including respiratory failure, acute pancreatitis, AKI, hepatorenal syndrome, and DIC (Table 4). However Latinos had higher rates of portal hypertension complications including overall GIB (Figure 3A), SBP (Figure 3B), encephalopathy (Figure 3C), and recurrence of AH (Figure 3D) compared to Caucasians. Despite these higher rates of complications, Latinos presented a longer median survival compared to Caucasians but the difference was not significant (12.1 *vs* 4.6 years, $P = 0.055$) (Figure 4).

Table 5 shows results of Cox proportional hazards analysis of overall survival. In the analysis of all subjects, after adjusting for age, BMI, sex, and ethnicity, a higher MELD score, a Child-Turcotte-Pugh score of C, an initial Maddrey score > 32, abnormal renal function, and presence of hepatorenal syndrome were all associated with significantly higher hazards of death.

Table 3 Type of alcoholic hepatitis, parameters of liver injury and treatment *n* (%)

	Caucasian	Latino	All patients	*P* value
Type of AH	137	64	201	0.321
Acute AH with no history of cirrhosis	94 (68.6)	49 (76.6)	143 (71.1)	
AH on chronic cirrhosis	43 (31.4)	15 (23.4)	58 (28.9)	
DF score at day 0	137	64	201	0.794
mean ± SD	47.7 (37.2)	46.6 (22.1)	47.3 (33.1)	
DF < 32	44 (32.1)	12 (18.8)	56 (27.9)	
DF > 32	93 (67.9)	52 (81.2)	145 (72.1)	
Initial MELD score	137	64	201	0.661
mean ± SD	21.1 (7)	21.6 (6.7)	21.3 (6.9)	
< 9	2 (1.5)	0	2 (1)	
10-19	61 (44.5)	30 (46.9)	91 (45.3)	
20-29	53 (38.7)	25 (39.1)	78 (38.8)	
30-39	19 (13.9)	9 (14.1)	28 (13.9)	
> 40	2 (1.5)	0	2 (1.0)	
Glasgow AH score at day 0				0.991
mean ± SD	7.5 (1.6)	7.4 (1.5)	7.5 (1.6)	
< 9	109 (71.7)	53 (69.7)	162 (71.1)	
> 9	43 (28.3)	23 (30.3)	66 (28.9)	
Type of treatment	37	15	52	0.862
Steroids	16 (11.7)	8 (12.5)	24 (11.9)	
Pentoxifylline	13 (9.5)	4 (6.2)	17 (8.5)	
Both	8 (5.8)	3 (4.7)	11 (5.5)	
Lille score after 7 d				0.829
< 0.45	18 (13.1)	8 (12.5)	26 12.9)	
> 0.45	17 (12.4)	6 (9.4)	23 (11.4)	

AH: Alcoholic hepatitis; MELD: Model for end stage liver disease; DF: Maddrey discriminant function is defined as DF = 4.6 x [prothrombin time (s) - control prothrombin time (s)] + (serum bilirubin in mg/dL)[11].

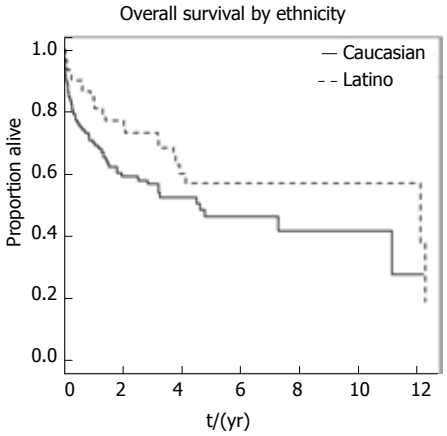

Figure 4 Survival by ethnicity. There was no significant difference in the median survival between ethnicities as shown on Kaplan-Meier Plot. The median survival in years between Latinos and Caucasians was not significantly different (12.1 yr *vs* 4.6 yr, *P* = 0.055).

In Latinos, after adjusting for age, BMI, and sex, a higher MELD score, a Child-Turcotte-Pugh score of C, an initial Glasgow score above 9, and the presence of hepatorenal syndrome were associated with significantly higher hazards of death. In Caucasian subjects, after adjusting for age, BMI, and sex, a higher MELD score, a Child-Turcotte-Pugh score of C, abnormal renal function, and presence of hepatorenal syndrome were associated with significantly higher hazards of death.

DISCUSSION

AH is an acute hepatic inflammation that usually occurs in heavy drinkers and is associated with significant morbidity and mortality in severe cases of up to 30%-50% at 6 mo[13]. Several previous studies indicated that Latino patients with ALD had earlier onset of disease and poorer prognosis compared to Caucasians[9] but specific data on ALD subtypes are lacking. The goal of the present study was to focus on a sample of Latino patients with AH followed at UC Davis Medical Center in Sacramento, California and describe any similarities and differences of AH in comparison to Caucasian patients, with the ultimate goal to identify specific needs and potential gaps in the care of this population. First, Latinos and Caucasians appeared to differ primarily in the duration of alcohol drinking, which was longer for the Latinos, and methamphetamine use, which was also more prevalent in Latinos. These findings are corroborated by previous large epidemiological studies showing that Mexican migrants in California presented a high prevalence of methamphetamine use associated with alcohol use and other psychiatric issues[14]. The two groups received similar medical treatment once admitted for severe AH. However, Latinos appear to present more frequently with manifestations of severe portal hypertension in association with more frequent utilization of the acute services but not ICU. Overall, these disparities did not seem to affect the survival of our studied Latino population. Several clinical studies

Table 4 Complications associated with alcoholic hepatitis *n* (%)

	Caucasian	Latino	All patients	*P* value
Development of respiratory failure	137	64	201	0.687
Yes	13 (9.5)	8 (12.5)	21 (10.4)	
No	124 (90.5)	56 (87.5)	180 (89.6)	
Development of acute pancreatitis				0.413
No	110 (80.3)	52 (81.2)	162 (80.6)	
Yes	13 (9.5)	3 (4.7)	16 (8.0)	
Unknown	14 (10.2)	9 (14.1)	23 (11.4)	
Development of AKI				0.520
Yes	33 (24.1)	19 (29.7)	52 (25.9)	
No	104 (75.9)	45 (70.3)	149 (74.1)	
Hepatorenal syndrome				0.328
No	119 (86.9)	60 (93.8)	179 (89.1)	
Yes, type 1	15 (10.9)	3 (4.7)	18 (9.0)	
Yes, type 2	3 (2.2)	1 (1.6)	4 (2.0)	
Development of DIC				0.497
Yes	3 (2.2)	0	3 (1.5)	
No	89 (65)	50 (78.1)	139 (69.2)	
Unknown	45 (32.8)	14 (21.9)	59 (29.4)	

AKI: Acute kidney injury; DIC: Disseminated intravascular coagulation.

Table 5 Cox proportional hazards analyses of overall survival by subject characteristics[1]

	All subjects		Caucasian subjects		Latino subjects	
	HR (95%CI)	*P* value	HR (95%CI)	*P* value	HR (95%CI)	*P* value
History of GI bleeding: yes *vs* no	0.79 (0.49, 1.28)	0.339	0.91 (0.53, 1.54)	0.718	0.39 (0.09, 1.72)	0.213
History of SBP: yes *vs* no	0.95 (0.50, 1.80)	0.874	0.86 (0.37, 2.01)	0.727	0.98 (0.37, 2.62)	0.97
ICU Admission: yes *vs* No	1.32 (0.80, 2.19)	0.278	1.42 (0.79, 2.56)	0.237	1.31 (0.45, 3.81)	0.618
MELD score[2]	1.88 (1.39, 2.55)	< 0.001	1.86 (1.29, 2.69)	0.001	2.15 (1.19, 3.87)	0.011
Child-turcotte-pugh score: C *vs* A or B	2.85 (1.62, 5.00)	< 0.001	2.62 (1.36, 5.07)	0.004	4.3 (1.24, 14.9)	0.021
Initial glasgow score: > 9 *vs* < 9	1.86 (1.18, 2.93)	0.008	1.6 (0.93, 2.74)	0.091	2.91 (1.21, 6.97)	0.017
Initial maddrey score: > 32 *vs* < 32	2.41 (1.29, 4.49)	0.006	1.82 (0.95, 3.50)	0.072	[3]	[3]
Treatment with steroids: yes *vs* no	1.63 (0.89, 2.98)	0.113	1.68 (0.81, 3.51)	0.164	2.05 (0.61, 6.88)	0.247
Duration of treatment with steroids: ≥ 28 d *vs* < 28 d	2.23 (0.66, 7.58)	0.198	5.28 (0.69, 40.5)	0.110	1.03 (0.01, 83.3)	0.988
Renal function: CKD 1 or higher *vs* CKD 0	7.43 (2.74, 20.1)	< 0.001	7.74 (2.8, 21.4)	< 0.001	[3]	[3]
Hepatorenal syndrome: yes *vs* no	7.07 (3.95, 12.7)	< 0.001	7.77 (3.92, 15.4)	< 0.001	5.65 (1.66, 19.2)	0.006

[1]A separate analysis was conducted for each risk factor shown. All analyses were adjusted for age, sex, BMI, and, for analyses in all subjects, ethnicity; [2]Change in hazard of death for 10 point increase in actual MELD score; [3]Not enough data to analyze. GI bleeding: Gastrointestinal bleeding; SBP: Spontaneous bacterial peritonitis; ICU: Intensive care unit; MELD: Model for end stage liver disease; CKD: Chronic kidney disease.

indicate that Latinos develop more severe liver diseases than other ethnicities and this has been confirmed in common conditions including ALD[9,15], non-alcoholic steatohepatitis[16], and chronic hepatitis C[17,18] or more rare diseases including primary biliary cholangitis[19]. In addition, Latinos might have an increased risk of hepatocellular carcinoma[20]. Ethnic matching between patients with alcohol-related injury or alcohol problems improved the effectiveness of the brief alcohol intervention on drinking outcomes of Latino patients, especially the foreign-born and less acculturated[21]. Our data indicate that, at least in our center, Latinos with AH are receiving similar inpatient medical management compared to Caucasians. It should be noted that only a small number of patients received treatment for severe AH during the studied period. This could be

related to the presence of contraindications to the use of steroids (for example active infections or GIB) or to the lack of proper diagnosis of severe AH by providers. The main issue regards the long term management of the complications of portal hypertension. Previous published data have shown that Latinos are more likely to visit the ED and are more often admitted through the ED for alcohol related issues[22]. Similarly, our study showed that Latinos present more frequently at the ED and are hospitalized more often compared to Caucasians for alcohol related issues, but also present more frequently severe complications of portal hypertension. This suggests a poor outpatient management of chronic complications of liver disease and inadequate access or use of outpatient clinics to receive preventive measures, including GIB prophylaxis with upper endoscopies and beta blockers, encephalopathy prophylaxis with lactulose, or prophylaxis with SBP when indicated. We did not observe any difference in survival between the two subpopulations and this may be in contradiction with the data showing worse outcome for Latinos with ALD. It is also interesting to note that the overall Latino population has better survival rate compared to Caucasians[23]. Our data reflect a small population in a highly diverse urban area but this initial data should help in identifying some of the major challenges in the care of underserved populations. Given the retrospective nature of the data collection and given the fact that is based on single center experience, we could not identify the actual background of the Latino population, which could be very heterogeneous.

This single retrospective study did not confirm previous findings that Latinos developed AH at earlier age compared to Caucasians[9]. In fact, there was no significant difference between the ages of presentation of AH between Latinos and Caucasians. Our study also suggested that there is no difference in the severity of AH at presentation and management between the ethnicities. On the other hand, this study showed Latinos had higher rates of complications associated with AH which include GIB, encephalopathy, and SBP. Latinos also had higher rates of AH recurrence. Interestingly, there was no significant difference in the overall mortality between ethnicities. In fact, Latinos may have better survival than Caucasians even though this is was not statistically significant in our study. Future research is needed to elucidate the effects of alcohol on Latinos and the predisposing factor that lead to the development of AH as well as the study of measures that can improve access to care and adequate outpatient follow-up for this underserved population.

ATRICLE HIGHLIGHTS

Research background

There are well known differences in the presentation of alcoholic liver disease (ALD) between Caucasian and Mexican-American patients. However, there are no ethnic-specific data on alcoholic hepatitis.

Research motivation

Alcoholic hepatitis is the most severe manifestation of ALD and is characterized by an inflammatory process of the liver with neutrophilic infiltration leading to ballooning degeneration of hepatocytes, hepatocyte necrosis, steatosis and presence of Mallory bodies. Alcoholic hepatitis clinic presentation is associated with jaundice, fever, hepatomegaly, liver tenderness, encephalopathy, and gastrointestinal bleeding. Ultimately, alcoholic hepatitis is characterized by a high risk of mortality. Latino and Caucasian patients with ALD and alcoholic hepatitis may have different progression and prognosis compared to other ethnicities.

Research objectives

The goal of the present study was to determine the clinical features of alcoholic hepatitis in Latino patients compared to Caucasian patients. The overarching goal is to optimize the care of patients of different ethnicities and identify their challenges when accessing medical care in the United States.

Research methods

The authors conducted a single-center retrospective chart review of 137 Caucasian patients and 64 Latino patients older than 18 years old diagnosed with alcoholic hepatitis. We collected extensive clinical data on their diagnosis, treatment, and outcome.

Research results

The major finding was a significantly higher rate of complications of portal hypertension including gastrointestinal bleeding, spontaneous bacterial peritonitis, and encephalopathy in Latino compared to Caucasian patients with alcoholic hepatitis. Despite these differences, the two ethnic groups did not present differences in survival. Future studies should prospectively confirm these findings in larger populations.

Research conclusions

According to our results, Latinos with alcoholic hepatitis presents with more severe complications of alcoholic hepatitis and appear to have limitations in access to medical treatment of long term complications of portal hypertension. Efforts should be made to ensure improved access to care and compliance.

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ORIGINAL ARTICLE

Retrospective Study

Predictive factors associated with carcinoid syndrome in patients with gastrointestinal neuroendocrine tumors

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Author contributions: All authors were equally involved in the design of the study; Chang conducted the statistical analyses; and all authors contributed equally in the interpretation of results and writing of the manuscript.

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Institutional review board statement: We conducted a retrospective case-control study using the Truven Health Analytics MarketScan Database and the IMS Health PharMetrics Database, both commercial health insurance claims database for employer-insured beneficiaries in the United States. The databases are fully compliant with the Health Insurance Portability and Accountability Act and meet the criteria for a limited-use dataset. Since the patient and provider data included in this analysis were fully de-identified, this study was exempt from the Institutional Review Board review.

Informed consent statement: This study involved analyses of Health Insurance Portability and Accountability Act-compliant secondary databases, MarketScan and PharMetrics, thus no informed consent was feasible or necessary.

Conflict-of-interest statement: Cai is an employee of Novartis Pharmaceuticals Corporation. Broder, Chang, and Yan are employees of the Partnership for Health Analytic Research, LLC, which received funding from Novartis to conduct the researchdescribed in this manuscript. Metz is an employee of Northwestern University and was paid by Novartis to consult as a subject matter expert. Metz is Chair of the North American Neuroendocrine Tumor Society (NANETS) and also a consultant for Ipsen. Metz has received commercial research grants from Lexicon and Advanced Accelerator Applications (AAA), and is a consultant/on the advisory board for AAA.

Data sharing statement: The study statistician, Eunice Chang, conducted all statistical analysis for this study using Health Insurance Portability and Accountability Act-compliant commercial-insurance secondary databases MarketScan and PharMetrics.

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Abstract

AIM

To discover unknown factors associated with carcinoid syndrome (CS) with the goal of earlier diagnosis of CS.

METHODS

In this retrospective case-control study using United States administrative claims, patients (⩾ 18 years) newly-diagnosed with gastrointestinal neuroendocrine tumors (GI NETs) without CS (controls) were exactly matched to patients with CS (cases) based on NET diagnosis date at a 3-to-1 ratio. Study index date was first CS diagnosis (controls: same distance from NET diagnosis as cases). The most observed conditions, excluding CS-associated symptoms/diagnoses, during the year before index date were assessed. Forward-stepwise logistic regression models were used to derive predictors, and were validation within another claims database.

RESULTS

In the development database, 1004 patients with GI NETs were identified; 251 (25%) had CS and 753 (75%) were controls. In the validation database, 724 patients with GI NETs were identified; 181 (25%) had CS and 543 (75%) were controls. A total of 33 common diagnoses (excluding conditions already known to be associated with CS) in the development database were entered in forward step-wise logistic regression models. In the final, validated logistic regression model, three factors prior to CS diagnosis were found consistently associated with higher risks for CS, including liver disorder [odds ratio (95%CI): 3.38 (2.07-5.51)], enlargement of lymph nodes [2.13 (1.10-4.11)], and abdominal mass [3.79 (1.87-7.69)].

CONCLUSION

GI NET patients with CS were 2-4 times as likely to have preexisting diagnoses (*i.e.*, liver disorder, enlarged lymph nodes, abdominal mass) than non-CS patients.

Key words: Carcinoid syndrome; Gastrointestinal neuroendocrine tumors; Predictive factors; Data mining

Core tip: By assessing patients with gastrointestinal neuroendocrine tumors from two independent United States claim databases, this study found that patients with carcinoid syndrome (CS) were 2-4 times as likely to have a preexisting diagnosis of a liver disorder, enlargement of lymph nodes, or abdominal mass than patients without CS.

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INTRODUCTION

Neuroendocrine tumors (NETs), the most of common of which are carcinoid tumors[1], are characterized by a relatively indolent rate of growth and an ability to secrete a variety of peptide hormones. These tumors can be functional or non-functional and benign or malignant[1]. Even though NETs can develop anywhere in the body, the majority of them occur in the gastrointestinal (GI) tract (67.5%)[2]. Within the GI tract, sites of origin include the stomach, small intestine, appendix, and colorectum[2]. A 6-fold increase in the age-adjusted incidence of diagnosed NETs has been observed from 1.09 new cases per 100000 individuals in 1973 to 6.98 new cases per 100000 individuals in 2012[3,4].

One of the more common functional NET syndromes is carcinoid syndrome (CS), occurring in 8% to 35% of NET patients[5]. CS occurs when functional carcinoid tumors metastasize to the liver or outside the GI tract, and the vasoactive hormones secreted by metastases, such as serotonin, histamine, or tachykinins, are no longer metabolized and inactivated by the liver and reach the general circulation[6,7]. 5-hydroxyindoleacetic acid (5-HIAA), a product of serotonin metabolism of urinary excretion, is used as a first-line test for biochemical detection of suspected CS, in which 5-HIAA excretion is usually elevated[8]. CS includes an array of signs and symptoms. The classical manifestations of episodic flushing and diarrhea affect more than 80% of patients with CS[6]. Other signs include pellagra, wheezing, abdominal pain, telangiectasia and heart disease (usually right sided valvular regurgitation). The clinical presentation with diarrhea and/or abdominal pain dominating often leads to misdiagnosis of CS as irritable bowel disease or small bowel obstruction[9,10] especially in middle-aged perimenopausal females. Delays in the correct diagnosis are common; reports on median time from onset of symptoms to diagnosis range from 2 to 20 years[9].

Other than the typical signs associated with CS, there is limited knowledge on the existence of any other predictors that may be associated with the risk of developing CS. We conducted the current study in patients with GI NETs, the most common type of NETs, to describe factors associated with CS with the intention of assisting physicians with making an earlier diagnosis of CS.

MATERIALS AND METHODS

Data source

We conducted a matched case-control study using data from two large United States healthcare claims databases - IMS PharMetrics Plus and Truven Health Analytics MarketScan. We used IMS PharMetrics Plus as the development database to derive risk factors for CS and MarketScan databases to validate the factors for CS. We used data from 1/1/2009 to 12/31/2014 from both databases.

The PharMetrics Plus database comprises adjudicated

medical and pharmacy claims for approximately 150 million patients enrolled in United States health insurance plans, with an annual capture of 40 million. This database is representative of the United States commercially insured population for individuals under age 65 years. The MarketScan Research Database combines 2 separate databases-the Commercial Claims and Encounters database, and the Medicare Supplemental and Coordination of Benefits database. The commercial database contains the inpatient, outpatient, and outpatient prescription drug experience of about 40 million employees and their dependents, who are covered under a variety of fee-for-service and managed care health plans. The Medicare database contains the healthcare experience of about 3 million retirees with Medicare supplemental insurance paid for by employers. Both databases contain detailed cost, use, and outcomes data for inpatient and outpatient healthcare services. The medical claims are linked to outpatient prescription drug claims and person-level enrollment data through the use of unique enrollee identifiers. Both PharMetrics and MarketScan are Health Insurance Portability and Accountability Act compliant[11,12]. The study was exempt from review by an institutional review board

Study population

The study population consisted of patients who were new diagnosed with GI NETs during the identification (ID) period (1/1/2010-12/31/2014). We identified patients with at least one inpatient or two outpatient claims with International Classification of Diseases, Ninth Revision, Clinical Modification (ICD-9-CM) codes for any NET (209.xx.) Among these, patients who had ICD-9-CM codes for GI NET (209.0, 209.1, 209.2, 209.4, 209.5, and 209.6) as the most frequent NET codes were classified as GI NET. To ensure that patients were newly diagnosed, we excluded patients with GI NETs during the 1-year prior to the first diagnosis date. We excluded patients with pancreatic NETs and Merkel cell carcinoma at any time during the study period.

We matched patients with GI NETs without CS (controls) to patients with CS (cases) based on the diagnosis date (month and year) of the first GI NET diagnosis at a 3-to-1 ratio. For cases, the index date was the first CS diagnosis date. For controls, the index date was assigned to have the same distance from the date of first NET diagnosis as the matched case patients. All patients were required to have at least 1-year continuous enrollment prior to the index date (study baseline).

The algorithm used to identify cases was developed based on a series of analyses performed before undertaking the main study. First, we examined overall prevalence of CS and the distribution of all NET locations (*e.g.*, GI, lung, pancreas) in patients with a CS by identifying cases using a diagnosis (ICD-9-CM code 259.2) and found it did not match the expected distribution. Specifically, we found that 6% of patients with pancreatic neuroendocrine tumor (PNET) had at least two claims with a code for the CS, as did 23% of patients with GI NET. We suspected that the diagnosis code was being applied at an early point in the diagnostic process (*e.g.*, as a "rule out"). We tested the application of more stringent criteria, which required two claims with ICD-9-CM code 259.2 and either a urine 24-h 5-HIAA [Current Procedural Terminology (CPT) code: 83497] or a serum serotonin (CPT code: 84260). In addition, the tests must have been ordered in the period 3 mo before or 3 mo after the CS diagnosis. This stricter algorithm resulted in only 1% of patients with PNET and 11% of GI NET being identified as having CS. As a result, we used the 2 CS diagnosis plus testing algorithm to identify cases with CS for this study.

Patient demographic characteristics included age in years on index date, sex, and geographic region (Midwest, Northeast, South, and West). Clinical characteristics included Charlson Comorbidity Index[13,14], and number of chronic condition indicators [15].

The outcome of interest in this study was the presence of CS.

Statistical analysis

We performed descriptive analyses to assess differences between case/control cohorts and patient demographic and clinical characteristics. χ^2 tests were used for categorical variables and two sample *t*-tests were used for continuous variables.

Data mining is a process of selecting, exploring and modeling large amounts of data in order to discover unknown patterns or relationships that provide a clear and useful result[16]. We used data mining to explore unknown factors associated with CS. Specifically, using the development database (PharMetrics Plus), we assessed the top 50 most frequently observed conditions (based on CS patients) other than symptoms/diagnoses known to be associated with CS during the 1 year prior to the index date. We eliminated conditions or symptoms already known to be associated with CS, those related to screening or health maintenance, other GI cancers, and factors occurring in < 10% of both case and control groups. We began the multivariate analysis by forcing demographics and two general comorbidity measures (Charlson Comorbidity Index and number of chronic conditions) into the model. We used forward-stepwise logistic regression to estimate, for each condition in the final model, the extent to which a patient who went on to be diagnosed with CS would have greater odds of having the condition than a patient who went on to be diagnosed with NET, but not CS. In the forward selection, significant factors ($P < 0.05$) from the list of conditions referred to above were retained. Demographic or comorbidity measures were removed from the final model if they were highly

insignificant ($P \geqslant 0.1$). To validate these predictors, we re-ran the model with completely independent data from another administrative claims database (Truven MarketScan).

RESULTS

Descriptive statistics

Of the 23815 NET patients identified from the IMS PharMetrics Plus, the development database, we excluded 23310 patients who did not meet the inclusion criteria, such as enrollment, age, and definition of CS. Of the 505 patients who met the study selection criteria 251 were GI NET patients with CS (cases) (Figure 1). These 251 cases along with the 753 matched controls (GI patients without CS) were included in the final analytic sample in the development database. Case patients were slightly younger than control patients (mean ± SD: 52.8 ± 10.9 years *vs* 54.4 ± 13.3 years; $P = 0.06$). The majority of patients were in the 55-64 age range for both cohorts (37.5% in CS cohort; 34.1% in non-CS; $P = 0.04$). There were no significant differences in sex, region, and number of chronic conditions between the two cohorts (Table 1).

In the validation database 724 patients with GI NETs were identified, among whom 181 (25%) had CS and 1158 (75%) were controls (Table 1). The mean (SD) age in the CS cohort was 51.8 (9.33) years, compared to 51.7 (10.03) years in the non-CS cohort. There were no significant differences in age, sex, and region (Table 1). With this approach, we identified a total of 33 most common conditions in both CS and non-CS cohorts (Table 2) of the development database for further assessment. In both cohorts, abdominal pain (66.1% of CS cohort; 51.5% of non-CS cohort), hypertension (50.6%; 52.2%), and dyslipidemia (49.4%; 46.1%) were the most prevalent diagnoses (Table 2).

Multivariable analysis

Eight of the 33 common diagnoses tested remained significant in the forward step-wise logistic regression models and were further validated using the validation database (Table 3). In the final, validated logistic regression model, three factors were associated with higher risks for CS, including liver disorder [odds ratio (95%CI): 3.38 (2.07-5.51)], enlargement of lymph nodes [2.13 (1.10-4.11)], and abdominal mass [3.79 (1.87-7.69)] (Table 4).

DISCUSSION

By assessing patients from two independent United States claim databases, this study suggested that in patients with proven GI NETs, patients with CS were 2-4 times as likely to have a preexisting diagnosis of a liver disorder, enlargement of lymph nodes, or abdominal mass than patients without CS.

Among patients with NETs, preexisting enlarged lymph nodes, abdominal mass lesions, and a liver disorder seem to jointly indicate increasing tumor burden, which implies that tumor progression may be associated with a higher risk of developing CS. Although there is no obvious clinical explanation for these findings, we propose the following two considerations: first, patients with CS have a larger systemic tumor bulk at diagnosis than patients who do not manifest CS, implying that increasing tumor burden correlates with increasing tumor products, which in turn led to the syndrome; and second, that patients with CS have more aggressive tumors than those without CS, which leads to more rapid growth of tumor and a larger burden of disease when diagnosed. The first consideration appears less likely since patients with small strategically located tumors (*e.g.*, the ovary or lung) commonly present with CS prior to large bulky liver disease developing. On the other hand, it is well known that patients with CS have a poorer quality of life and outcome than those without CS suggesting that the latter explanation is more likely. To the best of our knowledge, this is only the second study assessing distinctions between CS and non-CS patients. The only other study we could identify in the existing literature is a recent study by Halperin and colleagues[17] that used SEER-Medicare data to assess the clinical factors that may be associated with CS specifically. They found that their CS cohort included more patients with regional and distant metastases, while their non-CS cohort included more patients with local disease; for each distinct site of tumor origin, the percentage of CS increased with tumor progression. In the aforementioned study, CS was also associated with shorter survival compared without patients without CS, further supporting the second explanation for our findings[17]. Of note, our findings extend Halperin's data in that we have now identified three specific predictors of the CS that can be used to direct physicians to specifically consider CS over a more general diagnosis of NET, and to monitor those patients more closely by 5-HIAA or serum serotonin testing In our study, we matched the CS and non-CS cohorts on duration of disease as carefully as possible, knowing that one of the limitations of using claims databases for research is that the precise diagnosis date is not the true date of tumorgenesis. Consequently the duration between the first NET diagnosis and first CS diagnosis was the best proxy for disease duration that we could use. Conditional on the reliability of this proxy, our findings suggest that patients who were eventually diagnosed with CS tended to have additional diagnoses indicating tumor advancement, compared with the non-CS patients who had the same duration of disease.

In general, early diagnosis of cancer can improve quality of life and survival[18]. Patients with CS have a significantly worse quality of life than patients with

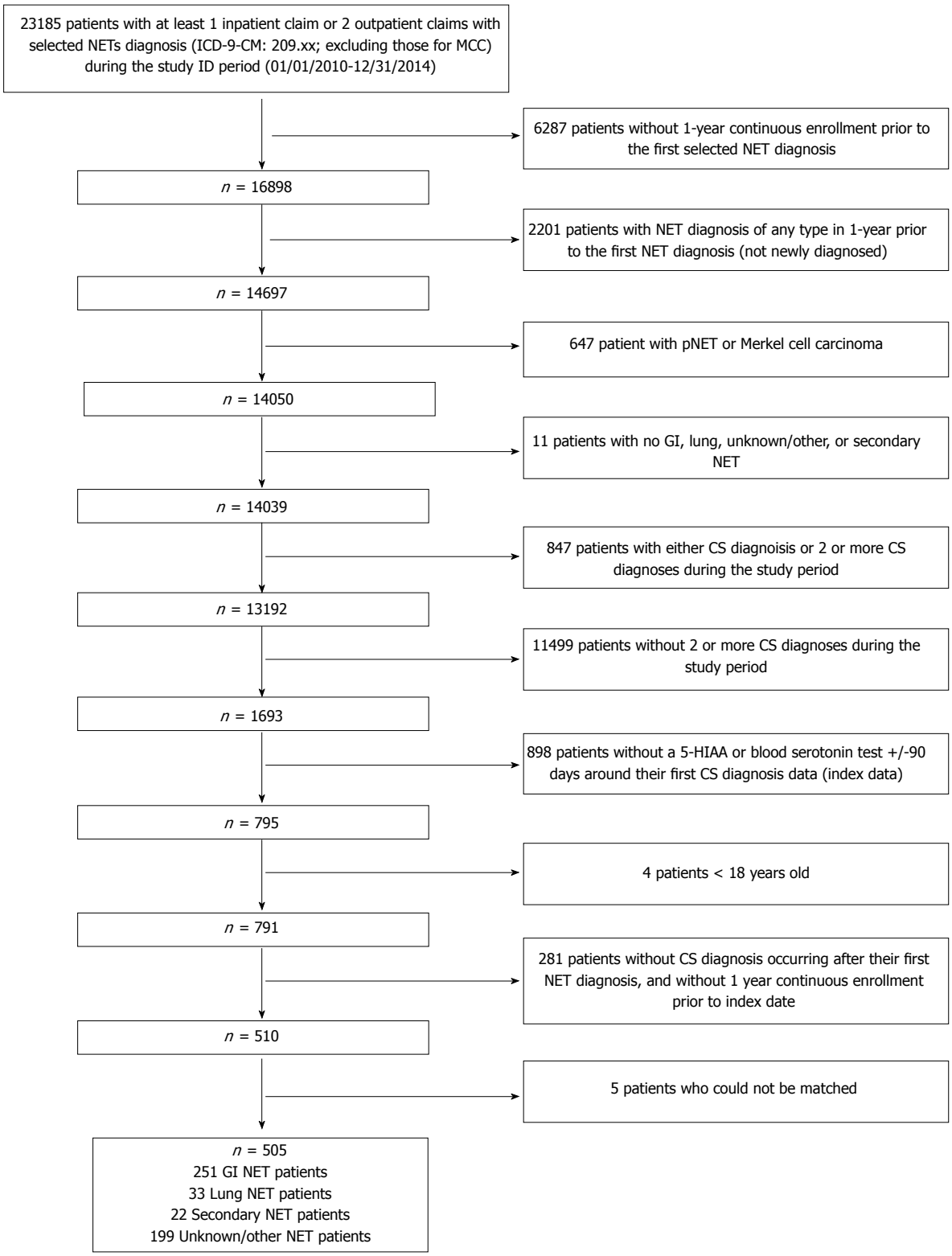

Figure 1 Patient identification. There were 23185 patients with at least one inpatient claim or two outpatient claims with selected NETs diagnosis (ICD-9-CM: 209.xx; excluding those for MCC) during the study ID period (01/01/2010-12/31/2014) in the development database. After excluding patients who were not newly diagnosed and did not have a NET diagnosis of interest; did not have 2 or more CS diagnosis during study period; did not have a 5-HIAA or blood serotonin test +/-90 days around their first CS diagnosis date (index date); were < 18 years old; did not have CS diagnosis occurring after their first NET diagnosis; could not be matched; or were not continuously enrolled in the 1 year pre-index period, there remained 505 NET patients who met the study inclusion criteria. Out of these, 251 were GI NET patients with CS. GI NETs: Gastrointestinal neuroendocrine tumors; CS: Carcinoid syndrome; 5-HIAA: 5-hydroxyindoleacetic acid.

NETs but without CS[19]. Therefore, reducing delays in diagnosis may help improve quality of life in CS. In addition, our findings indicate that the poorer quality of life among patients with CS may also be a composite result of both tumor progression and CS symptoms, as the CS patients also tended to have more advanced

Table 1 Patient demographics *n* (%)

	Development Database				Validation Database			
	CS Cohort	Non-CS Cohort	All	*P* value	CS Cohort	Non-CS Cohort	All	*P* value
n	251	753	1,004		181	543	724	
Age (yr)	52.77 ± 10.88	54.37 ± 13.28	53.97 ± 12.74	0.058	51.76 ± 9.33	51.67 ± 10.03	51.69 ± 9.85	0.913
18-44	52 (20.7)	143 (19.0)	195 (19.4)	0.034	33 (18.2)	100 (18.4)	133 (18.4)	0.637
45-54	80 (31.9)	219 (29.1)	299 (29.8)		64 (35.4)	203 (37.4)	267 (36.9)	
55-64	94 (37.5)	257 (34.1)	351 (35.0)		83 (45.9)	231 (42.5)	314 (43.4)	
65+	25 (10.0)	134 (17.8)	159 (15.8)		1 (0.6)	9 (1.7)	10 (1.4)	
Female	133 (53.0)	407 (54.1)	540 (53.8)	0.770	109 (60.2)	297 (54.7)	406 (56.1)	0.195
Region				0.306				0.667
Midwest	67 (26.7)	219 (29.1)	286 (28.5)		37 (20.4)	118 (21.7)	155 (21.4)	
Northeast	57 (22.7)	200 (26.6)	257 (25.6)		41 (22.7)	100 (18.4)	141 (19.5)	
South	106 (42.2)	269 (35.7)	375 (37.4)		80 (44.2)	254 (46.8)	334 (46.1)	
West	21 (8.4)	65 (8.6)	86 (8.6)		23 (12.7)	71 (13.1)	94 (13.0)	

tumor. Therefore, therapy for CS patients should address both symptom control as well as tumor progression to maintain quality of life.

In addition, we find it rather curious that there were no gender differences in our study. Since flushing is a common symptom of the CS, and since flushing is often confused with the hot flashes of menopause, one might have expected males with CS to be diagnosed earlier than females if the propensity is to misdiagnose CS until the disease is well established (with a known liver disorder, abdominal mass or lymphadenopathy). On the other hand, the delay in identifying CS in NET patients may simply be because flushing is not often sought out by physicians when interviewing patients. Indirectly then, these data suggest that educating physicians about the unusual manifestation of CS may help lead to an earlier diagnosis. The gender equivalence in our study may also be evidence that our algorithm for identifying CS, which required the presence of 5-HIAA or serotonin test around the diagnosis date, was reasonably specific for the condition, so that we minimized the misdiagnosed CS in our CS cohort by requiring this criterion for diagnosis.

Our study had limitations. First, GI NET and CS diagnoses were identified from healthcare claims coded for reimbursement, not research, and misclassification was possible. Errors in coding could bias our analysis. Specifically, patients with CS who have less severe symptoms may never be coded as having the syndrome. Nevertheless, health insurance claims data remain a valuable source of information as they constitute a fairly valid, large sample of patient characteristics and outcomes in a real-world setting. A strength of this study was that it was drawn from two very large underlying databases covering nearly 200 million patients enrolled in United States health insurance plans. The rate of CS among GI-NET patients in our study was higher than in some prior studies[20], and it was lower than at least one other[17]. The criteria we used to identify CS were more restrictive than the study by Halperin *et al*[17]. Current recommendations for diagnosing CS include measuring 5-HIAA[1,21]. We incorporated that recommendation into our identification algorithm, requiring two claims with an ICD-9-CM code for CS and a claim for either a urine 24-hour 5-HIAA or a serum serotonin in the period surrounding that diagnosis, whereas the prior study required two claims for CS, diarrhea, or flushing. Second, because ICD-9-CM codes may be inaccurate, we were not able to identify the specific anatomic location (*e.g.*, portion of large or small bowel) of the GI NET in our study. This could lead to confounding. For instance, small bowl NETs are more commonly associated with CS than large bowel tumors, and previous studies have suggested that hepatic and lymph node metastasis are usually present at time of small bowel NET diagnosis[22]. If our CS cohort systematically includes more small bowel patients than the non-CS cohorts, and the small bowel patients tend to be more advanced at diagnosis, this could explain why we see that CS patients were more likely to have this diagnosis. However, due to the lack of specificity of ICD-9-CM codes, we cannot confirm the anatomic section of the GI tract in which the tumor was identified. Therefore, we could not validate if small bowel NETs may present at a more advanced stage than other NETs based only on the data from a claims database. A study in a data source where the exact location of the tumor could be identified with confidence would be very useful to confirm our findings. Third, our results are reflective of a commercially-insured population, but may not be generalizable to patient populations with other insurance types.

In summary, our study suggests that a liver disorder, enlargement of lymph nodes, or an abdominal mass is associated with higher risk of a future CS diagnosis. There are two aspects with regards how this finding may be applicable to a real world clinical setting. First, CS develops insidiously, and patients who grow accustomed to their syndromic features such as flushing may not think of reporting these symptoms, even when prompted. If physicians are not aware of the presence of CS symptoms in their patients, our findings offer additional signs to prompt them to inquire specifically.

Table 2 Patients with gastrointestinal neuroendocrine tumors: Most frequent diagnoses1 in carcinoid syndrome patients, ordered by prevalence in carcinoid syndrome cohort *n* (%)

Diagnosis description	No. of patients		Difference in Rate (CS-Non-CS)	Relative Risk (CS *vs* Non-CS)
	CS cohort *n* = 251	Non-CS cohort *n* = 753		
Abdominal pain[2]	166 (66.14)	388 (51.53)	14.61%	1.28
Hypertension[2]	127 (50.60)	393 (52.19)	-1.59%	0.97
Dyslipidemia[2]	124 (49.40)	347 (46.08)	3.32%	1.07
Benign neoplasm large bowel	79 (31.47)	241 (32.01)	-0.53%	0.98
Esophageal reflux	74 (29.48)	190 (25.23)	4.25%	1.17
Diverticulosis of colon (without mention of hemorrhage)	68 (27.09)	159 (21.12)	5.98%	1.28
Liver disorder[2]	56 (22.31)	79 (10.49)	11.82%	2.13
Chest pain, unspecified	53 (21.12)	147 (19.52)	1.59%	1.08
Anemia, unspecified	51 (20.32)	141 (18.73)	1.59%	1.09
Pain in limb	41 (16.33)	117 (15.54)	0.80%	1.05
Internal hemorrhoids without mention of complication	41 (16.33)	87 (11.55)	4.78%	1.41
Neoplasm of uncertain behavior of stomach, intestine, and rectum	39 (15.54)	95 (12.62)	2.92%	1.23
Nonspecific (abnormal) findings on radiological and other examination of gastrointestinal tract	38 (15.14)	66 (8.76)	6.37%	1.73
Lumbago	38 (15.14)	79 (10.49)	4.65%	1.44
Enlargement of lymph nodes	36 (14.34)	40 (5.31)	9.03%	2.70
Malignant neoplasm of colon, unspecified site	35 (13.94)	114 (15.14)	-1.20%	0.92
Hypothyroidism, unspecified	34 (13.55)	106 (14.08)	-0.53%	0.96
Abdominal or pelvic swelling, mass, or lump, unspecified site (begin 1994)	34 (13.55)	60 (7.97)	5.58%	1.70
Other and unspecified noninfectious gastroenteritis and colitis	32 (12.75)	73 (9.69)	3.05%	1.32
Type 2 diabetes mellitus[2]	32 (12.75)	158 (20.98)	-8.23%	0.61
Other specified disorders of intestine	29 (11.55)	70 (9.30)	2.26%	1.24
Obesity, unspecified	28 (11.16)	82 (10.89)	0.27%	1.02
Diaphragmatic hernia	28 (11.16)	65 (8.63)	2.52%	1.29
Headache	28 (11.16)	74 (9.83)	1.33%	1.14
Anxiety state, unspecified	27 (10.76)	71 (9.43)	1.33%	1.14
Acute appendicitis, unspecified	26 (10.36)	91 (12.08)	-1.73%	0.86
Reflux esophagitis	26 (10.36)	41 (5.44)	4.91%	1.90
Abdominal or pelvic swelling, mass, or lump, other specified site	26 (10.36)	35 (4.65)	5.71%	2.23
Dyspepsia and other specified disorders of function of stomach	26 (10.36)	26 (3.45)	6.91%	3.00
Urinary tract infection, unspecified	26 (10.36)	96 (12.75)	-2.39%	0.81
Pre-operative cardiovascular examination	25 (9.96)	102 (13.55)	-3.59%	0.74
Tobacco use disorder	24 (9.56)	77 (10.23)	-0.66%	0.94
Constipation, unspecified	24 (9.56)	79 (10.49)	-0.93%	0.91

[1]Claims in 1 year prior to the index date, excluding those for CS, NET, or symptoms/conditions known to be associated with CS; [2]Abdominal Pain (ICD-9-CM 789.0x, 789.6x); Type 2 DM (ICD-9-CM 250.x0, 250.x2); Hypertension (ICD-9-CM 401.xx, 402.xx, 403.xx, 404.xx, 405.xx, 437.2x); Dyslipidemia (ICD-9-CM 272.xx); Liver disorder (ICD-9-CM 573.8x, 573.9x). Variables not shown: Screening (Routine medical examination, Other screening mammogram, Long-term (current) use of other medications, Routine gynecological examination, Vaccination for influenza, Screen for malignant neoplasms of colon, Other specified pre-operative examination; GI cancer (Digestive neoplasm, unspecified); Prevalence < 10% in both CS and non-CS cohort (Screening for malignant neoplasms of prostate, Backache unspecified, Other abnormal blood chemistry, Vitamin D deficiency unspecified, Lumbar or lumbosacral intervertebral disc degeneration, Atrophic gastritis without mention of hemorrhage, Personal history of colonic polyps, Cervicalgia, Iron deficiency anemia unspecified). CS: Carcinoid syndrome; NET: Neuroendocrine tumors.

Table 3 Patients with gastrointestinal neuroendocrine tumors: Selected measures *n* (%)

	Development database				Validation database			
	CS Cohort	Non-CS Cohort	All	*P* value	CS Cohort	Non-CS Cohort	All	*P* value
n	251	753	1004		181	543	724	
Abdominal pain	166 (66.1)	388 (51.5)	554 (55.2)	< 0.001	104 (57.5)	253 (46.6)	357 (49.3)	0.011
Dyslipidemia	124 (49.4)	347 (46.1)	471 (46.9)	0.361	67 (37.0)	209 (38.5)	276 (38.1)	0.724
Diverticulosis of colon	68 (27.1)	159 (21.1)	227 (22.6)	0.050	37 (20.4)	93 (17.1)	130 (18.0)	0.314
Liver disorder	56 (22.3)	79 (10.5)	135 (13.4)	< 0.001	50 (27.6)	44 (8.1)	94 (13.0)	< 0.001
Enlarged lymph nodes	36 (14.3)	40 (5.3)	76 (7.6)	< 0.001	23 (12.7)	25 (4.6)	48 (6.6)	< 0.001
Type 2 diabetes	32 (12.7)	158 (21.0)	190 (18.9)	0.004	30 (16.6)	91 (16.8)	121 (16.7)	0.954
Abdominal mass	26 (10.4)	35 (4.6)	61 (6.1)	0.010	23 (12.7)	16 (2.9)	39 (5.4)	< 0.001
Dyspepsia and other specified disorders of function of stomach	26 (10.4)	26 (3.5)	52 (5.2)	< 0.001	8 (4.4)	35 (6.4)	43 (5.9)	0.318

CS: Carcinoid syndrome

Table 4 Patients with gastrointestinal neuroendocrine tumors: Results of final model

Independent variable[1]	Development database		Validation database	
	OR (95%CI)	*P* value	OR (95%CI)	*P* value
Age group				
18-44 *vs* 65+	2.30 (1.26-4.23)	0.007	4.88 (0.56-42.11)	0.150
45-54 *vs* 65+	1.94 (1.14-3.31)	0.015	3.63 (0.43-30.41)	0.235
55-64 *vs* 65+	2.01 (1.20-3.38)	0.008	3.85 (0.46-31.93)	0.212
Number of chronic conditions	0.94 (0.87-1.01)	0.093	1.13 (1.02-1.24)	0.014
Abdominal pain	1.50 (1.08-2.09)	0.016	1.22 (0.83-1.77)	0.309
Dyslipidemia	1.52 (1.08-2.15)	0.016	0.83 (0.55-1.25)	0.373
Diverticulosis of colon	1.55 (1.08-2.24)	0.019	1.16 (0.72-1.86)	0.537
Liver disorder[2]	2.12 (1.40-3.20)	< 0.001	3.38 (2.07-5.51)	< 0.001
Enlarged lymph nodes	2.33 (1.38-3.92)	0.001	2.13 (1.10-4.11)	0.025
Type 2 diabetes	0.59 (0.38-0.92)	0.021	0.89 (0.54-1.48)	0.653
Abdominal mass	1.89 (1.06-3.37)	0.031	3.79 (1.87-7.69)	< 0.001
Dyspepsia and other specified disorders of function of stomach	2.95 (1.60-5.43)	< 0.001	0.54 (0.23-1.26)	0.154

[1]Variables found consistently significantly associated with risk of CS are bolded; [2]Liver disorder includes other specified disorders of liver (ICD-9-CM 573.8x, 573.9x). CS: Carcinoid syndrome.

Second, patients with a liver disorder, enlarged nodes, or abdominal mass may also not yet have clinically detectable CS symptoms, but may be destined to develop them in the future. In this context, our data suggest that in a patient with NET, even in the absence of typical symptoms, if one of these three findings is noted, consideration should be given to ordering a 5-HIAA or serotonin level. Although validation studies using patients' medical charts are warranted, such a practice could potentially aid physicians in speeding the diagnosis of CS.

ATRICLE HIGHLIGHTS

Research background

Functional neuroendocrine tumors (NETs) have the ability to secrete hormones that may cause carcinoid syndrome (CS), the most common symptoms of which include flushing and diarrhea. Carcinoid syndrome occurs in 8% to 35% of NET patients. Delays in diagnosis of CS are common, ranging from 2 to 20 years. Little is known about predictors that might be related the risk of developing CS.

Research motivation

Patients with CS often face delays in getting the correct diagnosis. Their symptoms, such as diarrhea, are often mistaken for other diseases, such as irritable bowel disease. Finding risk factors that are associated with CS might help physicians with making an earlier diagnosis of CS.

Research objectives

The objective was to identify risk factors that are associated with a future CS diagnosis.

Research methods

The authors conducted the study using data from two large United States health insurance claims databases. These databases contain healthcare cost, use, and outcomes data for covering nearly 200 million insured Americans. We first identified patients newly diagnosed with gastrointestinal NETs between 2010 and 2014 and then compared patients with CS to those without CS. We performed statistical analyses to identify the risk factors associated with CS from one databased and then validated the results using the other database.

Research results

The authors identified 251 patients with CS and 753 without CS in one database, and 386 patients with CS and 1158 patients without CS in the other database. There were no significant differences in age, sex, and region between patients with CS and those without CS. In both databases, we found that CS patients were 2-4 times more likely than non-CS patients to have a diagnosis of liver disorder, enlargement of lymph nodes, or abdominal mass within 1 year prior to their CS diagnosis.

Research conclusions

This study suggests that diagnosis codes of liver disorder, enlargement of lymph nodes, or an abdominal mass are associated with higher risk of a future CS diagnosis. In a patient with NETs, even in the absence of typical symptoms, if one of these three conditions is noted, consideration should be given to ordering a test for CS. Such a practice could potentially aid physicians in speeding the diagnosis of CS.

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ORIGINAL ARTICLE

Retrospective Study

Clinical and pathological characterization of Epstein-Barr virus-associated gastric carcinomas in Portugal

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Abstract

AIM

To determine the prevalence of Epstein-Barr virus (EBV)-associated gastric carcinomas in the North Region of Portugal and to study its clinicopathological characteristics.

METHODS

We have performed a retrospective study including a total of 179 consecutive patients with gastric cancer (GC) submitted to gastrectomy during 2011 at the Portuguese Oncology Institute of Porto. Clinical and pathological data was collected from individual clinical records and inserted on a database with unique codification. Tumour tissues were collected from the institutional tumour bank. EBV was detected by *in situ* hybridization for the detection of EBV-encoded small RNAs (EBERs) and EBV latent proteins (LMP1 and LMP2A) were detected by immunohistochemistry.

RESULTS

The analysis showed that EBV-associated gastric carcinomas (EBVaGC) represents 8.4% (15/179) of all GC cases, with a significant differential distribution among histological types ($P < 0.001$): 100% (3/3) of medullary carcinomas, 100% (1/1) of adenosquamous carcinoma, 8.7% (8/92) of tubular adenocarcinomas, 8.0% (2/25) of mixed carcinomas and 2% (1/51) in poorly cohesive carcinomas. The analysis revealed a higher predominance of EBVaGC in the upper third and middle (cardia, fundus and body) of the stomach ($P = 0.041$), a significant lower number of regional lymph nodes invasion ($P = 0.025$) and a tendency for better prognosis ($P = 0.222$). EBV latent protein expression revealed that all EBVaGC cases were LMP1-negative, nevertheless 6 cases (40%) expressed LPM2A, which reveals that these cases show a distinct EBV-Latency profile (latency II-like).

CONCLUSION

EBVaGC represents 8.4% of all GC in the North Region of Portugal. The EBV-infected patients have specific clinic-pathological features that should be further explored to develop new strategies of management and treatment.

Key words: Gastric cancer; Epstein-Barr virus; Prevalencel; Epstein-Barr virus-associated gastric carcinomas

Core tip: This is the first study to report the prevalence of Epstein-Barr virus (EBV)-associated gastric carcinomas (EBVaGC) in Portugal. The EBVaGCs were found in 8.4% of all gastric cancer cases, being more frequent in upper and middle regions of the stomach and among tubular and medullary carcinomas. Patients with EBV-positive tumours also have a significant lower number of regional lymph nodes with metastasis and a tendency for a better prognosis.

Ribeiro J, Oliveira A, Malta M, Oliveira C, Silva F, Galaghar A, Afonso LP, Neves MC, Medeiros R, Pimentel-Nunes P, Sousa H. Clinical and pathological characterization of Epstein-Barr virus-associated gastric carcinomas in Portugal. *World J Gastroenterol* 2017; 23(40): 7292-7302 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i40/7292.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i40.7292

INTRODUCTION

Gastric cancer (GC) is the fifth most common malignancy worldwide with nearly one million new cases estimated in 2012 (952000 cases) and despite the significant reduction of its incidence, GC still remains the third leading cause of death by cancer[1,2]. Portugal is one of the European countries with the higher GC incidence and mortality rates, where GC represents the fifth most common cancer with about 3000 new cases per year and with 1387 deaths in men and 898 in women[1,2].

Epstein-Barr virus (EBV) is one of the most common viruses and has been associated with several malignancies including Burkitt's Lymphoma, Hodgkin Lymphoma, post-transplant lymphoproliferative disease, a subset of T/NK cell lymphomas and nasopharyngeal carcinoma[3]. Recently, EBV has been associated with the development of GC, with several reports pointing that E BV-associated GC (EBVaGC) might account for nearly 10% of all GC cases[4,5]. The mechanisms of EBVaGC carcinogenesis are not well understood although some authors have suggested that EBV infection can represent a late event after *Helicobacter pylori* (*H. pylori*) infection[6,7].

The presence of EBV in patients with gastric carcinoma was first reported in gastric lymphoepithelioma-like carcinomas (also classified as medullary

carcinomas, gastric carcinomas with lymphoid stroma or uncommon variant), nevertheless it has been detected in different histopathological subtypes of gastric carcinoma[8,9]. The majority of GC are adenocarcinomas and are classified according to the Lauren or World Health Organization (WHO) classification system[10]. These classification systems have a reduced clinical utility and recent studies suggested a new classification based on molecular features of tumours, where EBVaGC arises as a new subtype of gastric cancer[11,12].

Despite the high incidence of both GC and EBV infection prevalence in the Portuguese population, there are no studies reporting the prevalence of EBVaGC in Portugal[2,13]. We aimed to determine the prevalence and characteristics of EBVaGC and analyse the profile of EBV latent proteins expressed in gastric tumours from the North Region of Portugal.

MATERIALS AND METHODS

Study description

We have developed a retrospective study in a cohort of 179 consecutive patients diagnosed with gastric cancer and submitted to surgery at the Portuguese Institute of Oncology of Porto (IPO Porto FG EPE) in 2011. Inclusion criteria: (1) patients with histologically confirmed gastric cancer; (2) submitted to gastrectomy (total or partial); and (3) with representative tumour blocks for adequate evaluation of EBV presence.

All tumour samples were histologically examined by a senior pathologist and classified according to the WHO classification system[10,14]. All clinical and pathological data was collected from individual clinical records and inserted on a database with unique codification. TNM-staging was performed in accordance to the UICC/AJCC system 7th edition[14]. The clinical outcome of the patients was followed from the date of surgery to either the date of death or 1st August 2016. All procedures were approved by the Ethical Committee of IPO Porto (CES IPO 80/2014).

EBV detection

The presence of EBV infection was investigated in all patients using histological sections (3 μm slides) obtained from formalin-fixed paraffin-embedded (FFPE) tissue blocks. EBV was identified using the *in situ* hybridization (ISH) for the detection of EBV-encoded small RNAs (EBERs) in FFPE tissue samples. The ISH was performed using Epstein-Barr virus Probe/Antibody ISH Kit (Leica, Newcastle upon Tyne, United Kingdom) in association with Ultra Vision Large Volume Detection System Anti-Polyvalent, HRP (THERMO SCIENTIFIC, Fremont, United States) according to the manufacturer's instructions. Detection of hybrids is achieved by enzymatic reaction using the *ImmPACT™ DAB* Peroxidase Substrate (VECTOR, Burlingame, CA, United States).

Only gastric tumours with presence of EBV infection in neoplastic cells were considered EBVaGC. For quality control, all EBV-positive cases and 10% of EBV-negative cases were retested and the results were 100% concordant. FFPE samples from a known EBV-positive post-transplant lymphoproliferative disorder (PTLD) tissue were used as positive controls and a sense probe for EBERs were used as the negative controls.

EBV latent proteins analysis

The presence of latent membrane protein 1 (LMP1) and latent membrane protein 2A (LMP2A) were evaluated with specific monoclonal antibodies in EBERs-positive gastric tissues using immunohistochemistry (IHC) (Table 1). IHC was performed using the UltraVision Large Volume Detection System Anti-Polyvalent, HRP kit (THERMO SCIENTIFIC, Fremont, United States) and the signal was detected using the *ImmPACT™ DAB* Peroxidase Substrate (VECTOR, Burlingame, CA, United States). All procedures were realized according to the manufacturer's instructions. PTLD samples with known positivity for LMP1 and LMP2A were used as positive controls. Gastric tumours were considered positive if 10% or more of the neoplastic cells were stained.

Statistical analysis

Statistical analysis was performed using the computer software IBM SPSS statistics for Macintosh, version 20.0 (IBM Corp, Armonk, NY, United States). χ^2 or Fisher Exact-test was used to compare categorical variables, with a significance level of 5%. Overall survival was defined as the time between the date of surgery to the date of last follow-up (censored) or the date of patient death (event). Cases lost to follow-up and those ending in death from any cause other than gastric cancer were regarded as censored data during the analysis of survival rates. The differences in survival times between subgroups were calculated using the log-rank test and the Kaplan-Meier method was used to calculate survival probabilities.

RESULTS

Characterization of the study population

The clinicopathological characteristics of all patients are described in (Table 2). A total of 179 patients (108 males and 71 females) with mean age of 64 ± 12 were included in this study; the majority were submitted to gastrectomy (n = 177), wherein 100 were total resections and 77 partial resections, and the remaining 2 patients were submitted to esophagogastrectomy. Regarding the tumour localisation in stomach: 26 (14.5%) were found in the upper third (cardia or fundus); 46 (25.7%) in the middle region (body); and 107 (59.8%) in lower third (antrum or pylorus).

Tumours were classified according to WHO (2010) classification system and the most common

histological type was tubular adenocarcinoma (n = 92, 51.4%) followed by poorly cohesive carcinoma (n = 51, 28.5%), mixed carcinoma (n = 25, 14%) and less frequent were mucinous adenocarcinoma (n = 5, 2.8%), papillary adenocarcinomas (n = 1, 0.6%), undifferentiated carcinoma (n = 1, 0.6%), adenosquamous carcinoma (n = 1, 0.6%) and medullary carcinomas with lymphoid stroma (n = 3, 1.7%) (Table 2). The correlation of histological type with anatomic localisation (data not shown), revealed that tubular type can be found in any stomach region, being more frequent in the lower third (56.5%) followed by upper and middle regions with 20.8% and 20.7%, respectively; poorly cohesive carcinomas were also more frequently located in the lower third (84.3%) and only 11.8% and 3.9% were in the middle and upper third, respectively; mixed adenocarcinomas were also more prevalent in lower third of the stomach with 72% of cases located in this region, with 16% in the middle and 12% in the upper region; and medullary carcinomas showed a similar distribution throughout the stomach with a percentage of 33.3% in all regions.

Regarding the characteristics of tumours, the most frequent invasion pattern was infiltrative (52.7% *vs* 46.1% expansive); the majority were poorly differentiated (54.5% *vs* 45.5% well or moderate differentiated); and lymphovascular invasion was extremely frequent (57.5% *vs* 42.5%) (Table 2). The tumour staging revealed that the majority of cases (63.8%) were at Stage III and IV and in only 6.7% of all cases presented distant metastasis.

Regarding survival analysis, the median overall survival was of 45 mo (mean 36 ± 20.5; range 1-63). Furthermore, we observed that lymph node metastasis (N), lymphovascular and perineural permeation and infiltrative tumours had a significantly reduced median overall survival (P < 0.001) (Figure 2A, B, C and D).

Characterization of EBVaGC

In our series, out of the 179 patients with GC, fifteen patients (8.4%) were positive for EBV - (Table 2). All EBV positive cases showed EBER positivity staining in > 90 of tumour cells while it was not observed in normal mucosa with or without atrophic gastric, intestinal metaplasia or in stromal cells (endothelial cells and fibroblasts) (Figure 1). In addition, one case showed EBER positivity only in the lymphocytic infiltration but not in tumour cells and therefore it was classified as EBV-negative GC.

Despite no statistical differences, it was observed that EBVaGC is more frequent in males (11.1% *vs* 4.1% in females) and among patients with more than 65 years old (10.1% *vs* 6.7% in younger patients) (Table 2). EBVaGC were not equally distributed among the stomach localisation (P = 0.087), being more often found in upper third and middle of the stomach than in the lower third; and regarding the anatomical site, it was more frequent (P = 0.041) in the body and fundus regions (40.0% and 13.0%, respectively) (Table 2).

Results have revealed statistical significant differences (P < 0.001) when comparing the distribution of EBV among different histological types according to WHO classification: EBV was detected in 8.7% (8/92) of tubular adenocarcinomas, 8.0% (2/25) of mixed carcinomas and only 2.0% (1/51) of poorly cohesive carcinomas. The results have also shown that all medullary (n = 3) and adenosquamous carcinomas (n = 1) were EBVaGC, while no EBV-positive case was identified in papillary adenocarcinomas and undifferentiated carcinomas (Table 2). The analysis of the invasion pattern of the GC, revealed that tumours with expansive patterns have a higher prevalence of EBV when compared with infiltrative patterns (13.0% *vs* 5.7%, respectively), despite not statistically significant (P = 0.237) (Table 2). No significant differences were also observed regarding the tumour differentiation and lymphovascular invasion (P > 0.050).

The results show that EBVaGCs were associated with a low number of regional lymph nodes invasion (P = 0.024) and that all cases had no evidence of distant metastasis, nevertheless there was no evidence of association with tumour stage (P = 0.289). Additionally, comparing the number of lymph nodes with metastasis between EBVaGCs and EBV negative cases, it was observed that EBVaGC cases have a significant lower number (mean difference of 3.7; 95%CI: 1.8-1.5; P < 0.001).

Table 3 resumes the individual characteristics of EBVaGC cases. Briefly, of the 15 EBVaGC cases 12 were males and 3 females with a mean age of 66 years old (range 44-82). Anatomically EBVaGCs were found in the body (n = 6), antrum (n = 4), fundus (n = 2), cardia (n = 2) and pylorus (n = 1). Regarding the histological subtypes, 8 were tubular adenocarcinoma, 3 were medullary carcinoma, 2 mixed carcinoma, 1 poorly cohesive carcinoma and 1 adenosquamous carcinoma. The invasion pattern showed that the majority of cases were expansive (n = 10/15) and regarding the differentiation, cases were equally distributed as poorly and moderately differentiated (n = 7 each). Moreover, there was no EBVaGC with distant metastasis.

The survival analysis revealed that despite not statistically significant, EBVaGC patients have a higher overall survival (41 ± 1.8 mo *vs* 46 ± 5.9 mo, P = 0.222) (Figure 3).

EBV latent proteins expression

Immunohistochemistry results revealed that all of EBVaGC cases were negative for LMP1 while 6 cases (40%) were positive for LMP2A and one case was inconclusive (Table 3). The positive cases for LPM2A expression were randomly distributed among histological subtypes: tubular adenocarcinomas (n = 2),

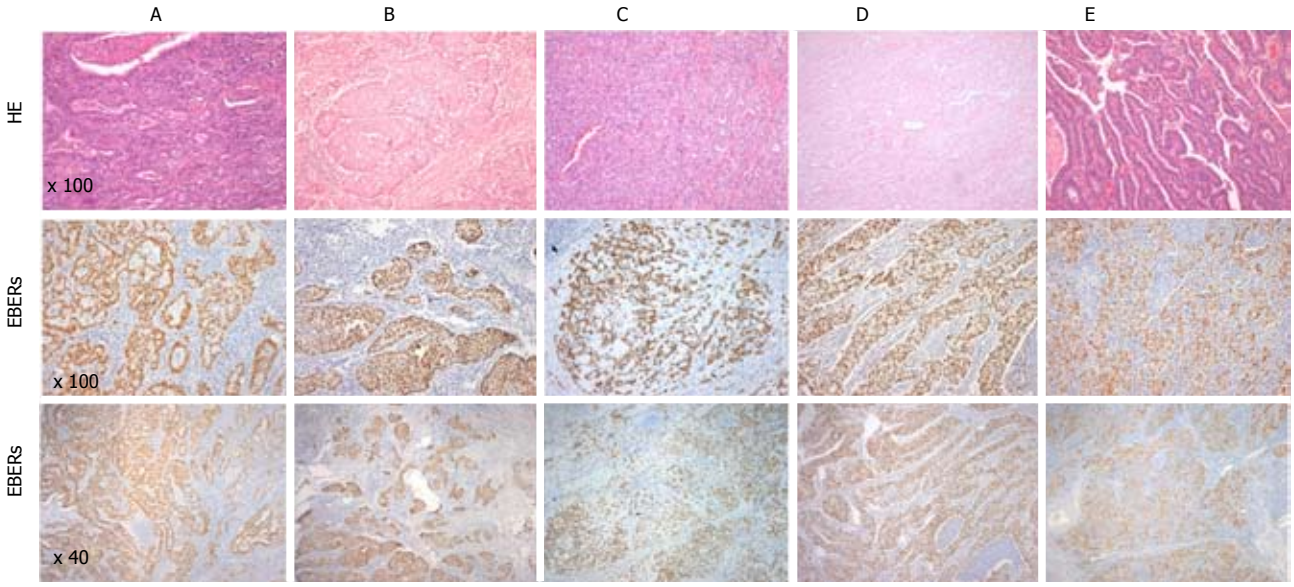

Figure 1 Representative images of EBER-ISH positive results in different histological types of gastric cancer and their respective HE staining. A: Adenocarcinoma tubular; B: Adenocarcinoma tubular; C: Adenocarcinoma Mixed; D: Adenosquamous Carcinoma; E: Medullary caricnoma. HE: Hematoxylin and eosin stain; EBERs: EBV-encoded small RNAs. Figure 1 Representative images of EBER-ISH positive results in different histological types of gastric cancer and their respective HE staining. A: Adenocarcinoma tubular; B: Adenocarcinoma tubular; C: Adenocarcinoma Mixed; D: Adenosquamous Carcinoma; E: Medullary caricnoma. HE: Hematoxylin and eosin stain; EBERs: EBV-encoded small RNAs.

Table 1 Antibodies used in immunohistochemistry

Protein	Primary Antibody	Dilution	Incubation	Retrieval method	Expression in gastric cells
LMP1	NCL-EBV-CS1-4, Leica, Newcastle upon Tyne, United Kingdom	1:100	3h, RT	15 min, microwave in *Vector*® Antigen Unmasking Solution	Cytoplasm
LMP2A	15F9, THERMO SCIENTIFIC, Fremont, United States	1:250	On, 4℃		Cytoplasm and membrane

LMP1: Latent membrane protein 1; LMP2A: Latent membrane protein 2A; RT: Room temperature; On: Overnight.

mixed carcinomas (n = 2), medullary carcinomas (n = 1) and adenosquamous carcinoma (n = 1) (Table 3).

DISCUSSION

Gastric carcinoma is a serious public health problem worldwide with high rates of mortality and Portugal has the highest gastric cancer mortality rates in Western European countries[2,15]. The impact of classification systems in GC clinical decisions has been reduced and more recently two studies suggested a new classification based on molecular features of gastric tumours[11,12]. In this new classification arises four new subtypes of gastric cancer: Epstein-Barr Virus positive tumours (EBVaGC); microsatellite unstable tumours; genomically stable tumours; and tumours with chromosomal instability (CIN)[11,12].

Over the past 30 years, EBV infection has been reported in gastric cancer tumours[16]. Currently, it is accepted that about 10% of all GC represents a specific subset of gastric carcinomas that are associated with EBV carcinogenesis[17]. The gold-standard method for the detection of EBV in tissues is EBER-ISH and the decision to accept one case as EBV-associated should be taken considering the presence of EBERs expression in tumour cells and its absence in the normal surrounding tissue. In our study we found that 8.4% of GC in our population are EBV-associated, based on the EBERs expression in > 90 of tumour cells and its absence in non-malignant or stromal cells. To the best of our knowledge, this is the first study reporting the prevalence of EBVaGC in the North Region of Portugal. The prevalence found in our population is in line with those reported in other studies, which have reported a prevalence ranging 2%-20%[5,8,18]. Similar data was found in other countries from Europe, including Netherlands (7.8%)[18], and Denmark (7.6%)[19] and also from Asiatic countries, such as South Korea (7.8%)[20] and Japan (8%)[21]. Geographic differences have been discussed as a possible reason for the variation of EBVaGC prevalence[8,22] and some studies suggest that EBVaGC prevalence might be inversely correlated with the background incidence of GC[4]. The fact that Portugal has a higher incidence of GC

Table 2 Patients characteristics and distribution of Epstein-Barr virus in gastric cancer patients *n* (%)

characteristics	All cases (*n* = 179)	EBV status		*P* value
		Negative (*n* = 164)	Positive (*n* = 15)	
Gender, *n* = 179				
Male	108 (60.3)	96 (88.9)	12 (11.1)	0.104
Female	71 (51.5)	68 (95.8)	3 (4.2)	
Age, *n* = 179 (64.38 ± 12 yr of age)				
< 65 yr of age	90 (50.3)	84 (93.3)	6 (6,7)	0.433
⩾ 65 yr of age	89 (49.7)	80 (89.9)	9 (10.1)	
Surgical procedure type, *n* = 179				
Total gastrectomy	100 (55.9)	89 (89.0)	11 (11.0)	0.351
Partial gastrectomy	77 (43.0)	73 (94.8)	4 (5.2)	
Esophagogastrectomy	2 (1.1)	2 (100)	-	
Tumour localization, *n* = 179				0.087
Upper third	26 (14.5)	22 (84.6)	4 (15.4)	
Middle	46 (25.7)	40 (87.0)	6 (13.0)	
Lower third	107 (59.8)	102 (95.3)	5 (4.7)	
Anatomical site, *n* = 179				
Cardia	21 (11.7)	19 (95.7)	2 (4.3)	0.041
Fundus	5 (2.8)	3 (60.0)	2 (40.0)	
Body	46 (25.7)	40 (87.0)	6 (13.0)	
Pylorus	13 (7.3)	12 (92.3)	1 (7.7)	
Antrum	94 (52.5)	90 (95.7)	4 (4.3)	
Invasion pattern, *n* = 167				
Infiltrative	88 (52.7)	83 (94.3)	5 (5.7)	0.237
Expansive	77 (46.1)	67 (87.0)	10 (13.0)	
Mixed	2 (1.1)	2 (100)	-	
Differentiation, *n* = 167				
Poor	91 (54.5)	84 (92.3)	7 (7.7)	0.724
Well+Moderate	76 (45.5)	69 (90.8)	7 (9.2)	
Lymphovascular invasion, *n* = 179				
Positive	103 (57.5)	94 (91.3)	9 (8.7)	0.841
Negative	76 (42.5)	70 (92.1)	6 (7.9)	
Histology WHO (2010), *n* = 179				
Papillary adenocarcinoma	1 (0.6)	1 (100)	-	<0.001
Tubular adenocarcinoma	92 (51.4)	84 (91,3)	8 (8.7)	
Mucinous adenocarcinoma	5 (2.8)	5 (100)	-	
Poorly cohesive carcinoma	51 (28.5)	50 (98.0)	1 (2.0)	
Mixed carcinoma	25 (14.0)	23 (92.0)	2 (8.0)	
Medullary carcinoma	3 (1.7)	-	3 (100)	
Undifferentiated carcinoma	1 (0.6)	1 (100)	-	
Adenosquamous carcinoma	1 (0.6)	-	1 (100)	
Primary tumour (T), *n* = 179				
T1a	22 (12.3)	22 (100)	-	0.289
T1b	18 (10.1)	15 (83.3)	3 (16.7)	
T2	25(14.0)	24 (96.0)	1 (4.0)	
T3	69 (38.5)	60 (87.0)	9 (13.0	
T4	7 (4.00)	7 (100)	-	
T4a	37 (20.7)	35 (94.6)	2 (5.4)	
T4b	1 (0.56)	1 (100)	-	
Regional lymph nodes (N), *n* = 179				
N0	73 (40.8)	67 (91.8)	6 (8.2)	0.024
N1	23 (12.9)	21 (91.3)	2 (8.7)	
N2	31 (17.3)	24 (77.4)	7 (22.6)	
N3	8 (4.5)	8 (100)	-	
N3a	23 (12.8)	23 (100)	-	
N3b	21 (11.7)	21 (100)	-	
Distant metastasis (M), *n* = 179				0.18
M0	148 (82.7)	133 (89.9)	15 (10.1)	
M1	12 (6.70)	12 (100)	-	
Mx	19 (10.6)	19 (100)	-	

$P < 0.05$ were considered statistical significant. EBV: Epstein-Barr virus; WHO: World Health Organization.

when compared to other European countries seems to indicate that other factors apart from geography may be influencing the distribution of EBVaGC. Indeed, data from different meta-analyses have failed to show this association[8,22].

We found that EBVaGC was more frequent in males

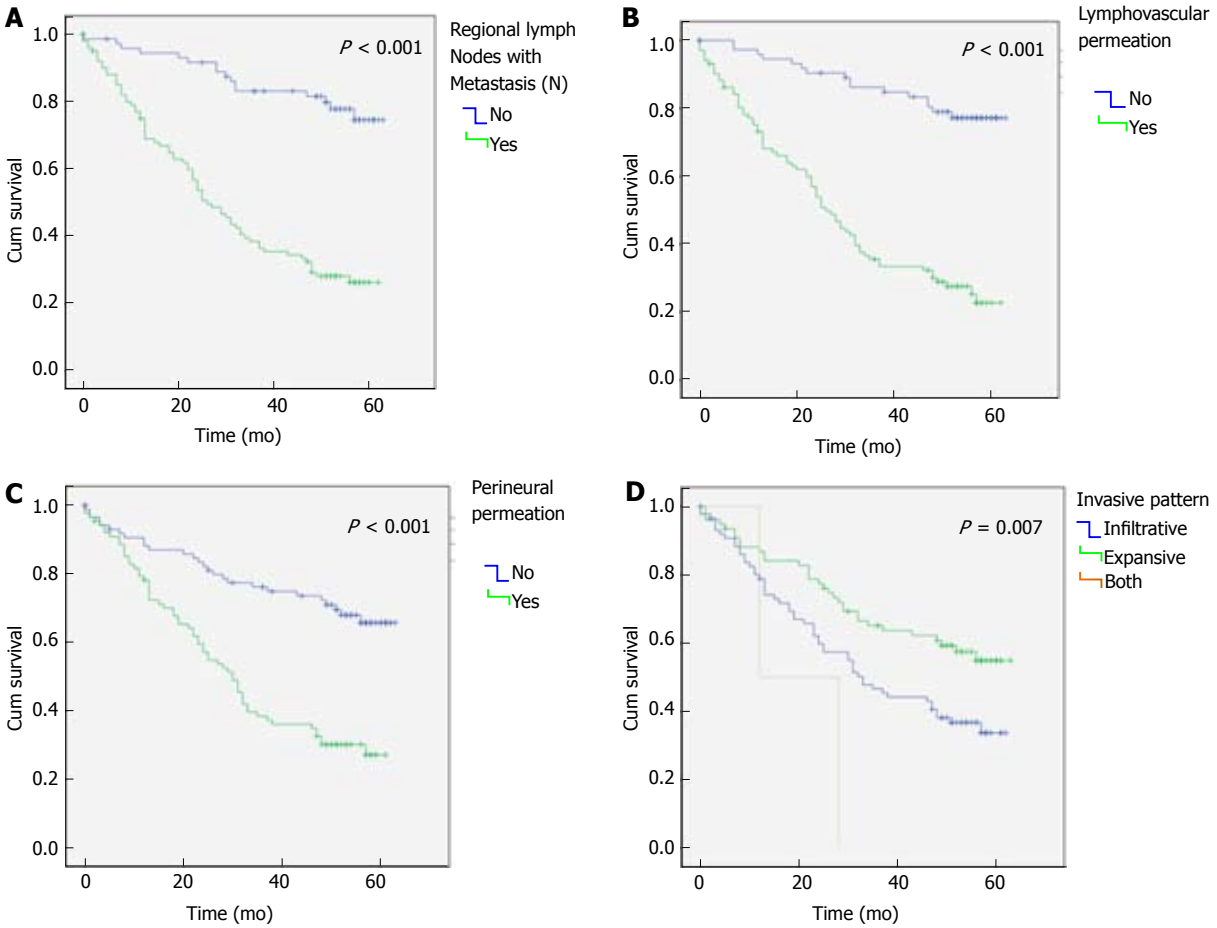

Figure 2 Kaplan-Meier survival curves for overall survival. Comparison of overall survival in gastric cancer patients considering: A: Regional lymph node metastasis; B: Lymphovascular permeation; C: Perineural permeation; and D: Invasive pattern.

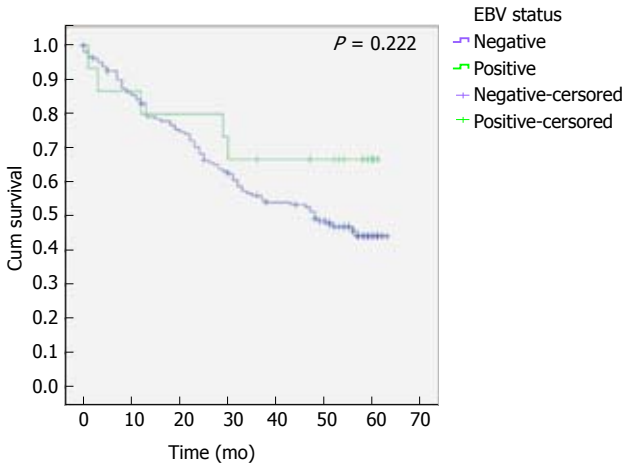

Figure 3 Comparison of overall survival in patients with Epstein-Barr virus-associated gastric carcinomas and Epstein-Barr virus-non associated gastric cancer. Kaplan-Meier curves with log-rank test estimate the overall survival of gastric cancer patients with and without Epstein-Barr virus (EBV)-associated gastric cancer.

than in females, which is in line with several studies and suggests an association with others factors, such as life style or hormonal risks[23-25]. In contrast with other reports, we have found no association between EBV status and patients' age[8,26]. Regarding the tumour location, our study revealed a higher predominance of EBVaGC in upper third and middle of stomach and considering that *H. pylori* preferentially colonises the antral region, these results suggest a possible antagonism of EBV and *H. pylori* in gastric mucosa[27-29]. In fact, Minoura-Etoh *et al*[30] described in an *in vitro* study that reactive products from *H. pylori* seem to induce EBV reactivation from latently in infected gastric epithelial cells, which would avoid EBV transformation of gastric cells in the same areas of *H. pylori* colonization. Despite this association remains unclear, two recent studies have suggested that *H. pylori* may contribute for EBV-associated gastric carcinogenesis by recruiting to gastric tissue the inflammatory cells that are already infected by EBV in the majority of adults[7,31]. This mechanism is not well understood and further studies should be made to establish if the recruitment of EBV-infected lymphoid cells might be the explanation for the infection and subsequent transformation of gastric epithelium. In our series, all EBVaGC cases had EBV only in tumour cells and not in normal tissues, nevertheless, we have

Table 3 Clinicopathological characteristics of Epstein-Barr virus-associated gastric cancers

ID	Gender	Age	Anatomic Site	WHO Classification	Invasion pattern	Differentiation	*n*	TNM Classification	LMP-1	LMP-2A
1	M	75	Body	Tubular Adenocarcinoma	Expansive	Poorly differentiated	6	T4aN2M0	-	-
2	M	80	Antrum	Tubular Adenocarcinoma	Expansive	Moderately differentiated	0	T3N0M0	-	-
3	M	56	Fundus	Tubular Adenocarcinoma	Expansive	Moderately differentiated	2	T2N1M0	-	-
4	M	80	Antrum	Tubular Adenocarcinoma	Expansive	Moderately differentiated	2	T3N0M0	-	-
5	M	64	Body	Tubular Adenocarcinoma	Expansive	Moderately differentiated	0	T3N2M0	-	(+)
6	M	56	Fundus	Tubular Adenocarcinoma	Expansive	Moderately differentiated	0	T3N0M0	-	-
7	M	69	Body	Tubular Adenocarcinoma	Expansive	Moderately differentiated	3	T3N2M0	-	Inc.
8	M	82	Cardia	Tubular Adenocarcinoma	Infiltrative	Moderately differentiated	4	T3N2M0	-	(+)
9	M	55	Body	Mixed Carcinoma	Infiltrative	Poorly differentiated	3	T3N2M0	-	(+)
10	F	66	Antrum	Mixed Carcinoma	NA	Poorly differentiated	6	T1bN2M0	-	(+)
11	F	68	Body	Medullary Carcinoma	Expansive	Poorly differentiated	1	T1bN1M0	-	-
12	M	52	Cardia	Medullary Carcinoma	Expansive	Poorly differentiated	0	T1bN0M0	-	-
13	M	44	Antrum	Medullary Carcinoma	Infiltrative	Poorly differentiated	0	T3N0M0	-	(+)
14	F	65	Pylorus	Poorly cohesive carcinoma	Infiltrative	Poorly differentiated	0	T4aN0M0	-	-
15	M	76	Body	Adenosquamous carcinoma	Expansive	NA	6	T3N2M0	-	(+)

N: Number of regional lymph nodes with metastasis; NA: Not available; (-): Negative result; (+): Positive result; Inc.: Inconclusive result; LMP1: Latent Membrane Protein 1; Latent Membrane Protein.

found one case with EBV only in the lymphocytic infiltration, which we considered negative for EBV, and therefore more studies are required to understand if in such cases tumour cells will be later infected by EBV.

The association between EBV and gastric histological types remains controversial, indeed while one meta-analysis regarding EBV in gastric carcinoma has shown that EBVaGC was associated with diffuse histological type according to Lauren classification[5,8], two other meta-analyses did not find any association between EBV and histology subtypes[22,32]. Several studies demonstrated a strong EBV association with diffuse type while others have reported a similar prevalence between intestinal and diffuse types (classified in our study as poorly cohesive according to the WHO)[9,18,33,34]. Our study revealed that EBVaGC are more frequently associated with specific histologic subtypes. EBVaGC represent about 8% of tubular (intestinal type in the Lauren classification system) and mixed types while it was only found in 2% (n = 1/51) of poorly cohesive carcinomas (diffuse type in the Lauren classification system). These distinct features show that the differences in the GC histological distribution among different populations may impact on the prevalence of EBVaGC subtype, which should gather importance on the detection of EBV in GC. As expected, all medullary carcinomas, also known as gastric carcinoma with lymphoid stroma or lymphoepithelioma-like gastric carcinoma, were EBV positive[35]. In fact, EBV association with gastric cancer was first described in lymphoepithelioma-like gastric subtype and the literature shows that more than 80% of these cases are EBV-positive[5,22,36].

Some studies have revealed that EBVaGC is associated with poor or moderate differentiation of gastric cancer tissues[9,24]. In accordance with these reports, our study showed that 100% of cases were poorly or moderately differentiated and that these differentiation patterns seem to be associated with the histological types. This evidence may be a significant mark of EBVaGC tumours and may provide important information regarding the clinical approach to these tumours.

All these data support the evidence that recent studies have shown by considering EBVaGC as a distinct clinicopathological entity due to its individual features[11,12,18,37]. Interestingly, we found that EBVaGC had a significantly lower number of regional lymph nodes with metastasis and that none have a distant metastasis. Additionally, when analysing the overall survival of EBVaGC compared to all other cases it was observed that EBVaGC seems to have an improved survival, that despite not statistically significant may be explained by the low number of cases in our population. Several studies have reported a better overall survival of EBVaGC, especially in Asiatic populations suggesting that the explanation for this better overall survival could be due to the low

frequency of lymph node involvement and distant metastasis[18,23,24,38]. In Portugal, GC remains at high mortality rates due to the late diagnosis of this disease and these patients are frequently only treated with chemo/radiotherapy. In our study we have included only patients with available tissue sample from surgical procedures and therefore we had not included patients that had received only chemo/radiotherapy, therefore our findings should be applied in patients with recommendation for total or partial gastrectomy as according to the ESMO Clinical Practice Guidelines for Diagnosis, Follow-up[39]. Moreover, others factors may be influencing patients survival and this question should be explored with further large-scale studies.

To elucidate the role of EBV in the pathogenesis of gastric carcinoma several studies have focused on the study of EBV latent proteins expression in gastric tumour cells[40-42]. As expect, in our study LMP1 was not found in any of EBVaGC cases and, in fact, literature also shows that LMP1 is generally absent in EBVaGC except for the data reported in a few studies, which have detected low *albeit* detectable levels of LMP1 mRNA[41-43]. The absence of LMP1 expression in EBV positive cases suggests that LMP1 may not be needed for gastric carcinogenesis or at least not for sustaining the already established malignant tumours. Regarding LMP2A, our results demonstrated that EBV LMP2A is expressed in 40% of EBVaGC cases that is in line with previous studies[41,44]. Recent studies have pointed that LMP2A seems to have an important role contributing to malignant state in epithelial cells by inducing the genome hypermethylation through up-regulation of DNMT1 and phosphorylation of STAT3 and thus contributing for the carcinogenic potential of the cells[45]. A systematic review performed by our group has shown that EBVaGC are usually associated with a distinct latency pattern, characterized by the expression of EBERs, EBNA1, LMP2A and EBV microRNAs. Furthermore, literature also shows that EBV latency I pattern, which is characterized by the expression of EBERs, EBNA 1 and EBV microRNAs[46], has been also frequently found among EBVaGC tumours[47,48]. In line with this, our study demonstrates that the majority of our cases express EBERs with absence of LMP1 and LMP2A, however, 40% of our cases also expressed LMP2A, which does not fit into the standard EBV latency patterns. In fact, literature has described this distinct pattern as latency II-like and has also reported that lytic transcripts such as BARF0 and BARF1 can also be found within EBV-associated gastric neoplasias[44,49]. Our data reinforces that the clarification of which EBV transcripts (including lytic transcripts and EBV microRNAs) are expressed in gastric tumours assumes a great importance to understand the viral carcinogenesis in gastric cancer and may contribute for the identification of new molecular targets for treatment.

In our study, EBVaGC represents 8.4% of our population with gastric cancer and it is more frequent among tubular adenocarcinomas and medullary carcinomas rather than poorly cohesive carcinomas. EBVaGCs were more common in upper and middle regions of the stomach; were correlated with a lower number of regional lymph nodes with metastasis and with no distant metastasis. The results also revealed that EBVaGC patients seem to have an improved survival and better prognosis. These features suggest that EBVaGC is a distinct subtype of gastric cancer and that the mechanisms of carcinogenesis should be further investigated for particular therapeutic targets

COMMENTS

Background

Gastric cancer (GC) is the fifth most prevalent cancer and it remains the third most common cause of cancer-related death in the world. Epstein-Barr virus (EBV) is an oncogenic virus that has been associated with several malignancies, including B-cell lymphomas and nasopharyngeal carcinomas (NPC). Recently, EBV has been also associated with GC development with several studies suggesting that EBV-associated gastric carcinomas (EBVaGC) represent approximately 10% of all GC cases.

Research frontiers

Gastric carcinomas have been widely classified according to the Lauren or World Health Organization classification systems but these systems have a reduced clinical utility. A recent study has suggested a new classification based on molecular features of the tumours, where EBVaGC arises as a new subtype of gastric cancer. Despite the high incidence of both GC and EBV infection prevalence in the Portuguese population, there are no studies reporting the prevalence of EBVaGC in Portugal. The characterization of the EBVaGC in different populations is crucial to the development of new therapies and classification systems.

Innovations and breakthroughs

This is the first study in Portugal to characterize the EBV infection in gastric cancer. It demonstrated that EBV-associated carcinomas represent 8.4% of all gastric cancer cases and that EBVaGCs are a distinct subgroup of GC. Furthermore, it also showed that EBV displays a new latency profile in some of the EBVaGCs.

Applications

The understanding of clinical relevance and carcinogenesis of EBVaGC in different populations worldwide helps the improvement of preventive or management strategies as well as in the identification of more specific therapeutics.

Peer-review

This study showed that 8.4% of GC cases are EBV-associated being more frequent in upper and middle regions of the stomach, among tubular and medullary carcinomas, and having a lower number of regional lymph nodes invasion. Furthermore, this study confirms that EBVaGC may present a new latency-profile which may be useful to study to better characterize this GC subtype.

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ORIGINAL ARTICLE

Retrospective Study

Modified model for end-stage liver disease improves short-term prognosis of hepatitis B virus-related acute-on-chronic liver failure

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Abstract

AIM

To investigate whether the short-term prognosis of hepatitis B virus (HBV)-related acute-on-chronic liver failure (ACLF) could be improved by using a modified model for end-stage liver disease (MELD) including serum lactate.

METHODS

This clinical study was conducted at the First Affiliated Hospital, Fujian Medicine University, China. From 2009 to 2015, 236 patients diagnosed with HBV-related ACLF at our center were recruited for this 3-month follow-up study. Demographic data and serum lactate levels were collected from the patients. The MELD scores with or without serum lactate levels from survival and non-survival groups were recorded and compared.

RESULTS

Two hundred and thirty-six patients with HBV-ACLF were divided into two groups: survival group (S) and

non-survival group (NS). Compared with the NS group, the patients in survival the S group had a significantly lower level of serum lactate (3.11 ± 1.98 *vs* 4.67 ± 2.43, t = 5.43, P < 0.001) and MELD score (23.33 ± 5.42 *vs* 30.37 ± 6.58, t = 9.01, P = 0.023). Furthermore, serum lactate level was positively correlated with MELD score (r = 0.315, P < 0.001). Therefore, a modified MELD including serum lactate was developed by logistic regression analysis (0.314 × lactate + 0.172 × MELD - 5.923). In predicting 3-month mortality using the MELD-LAC model, the patients from the S group had significantly lower baseline scores (-0.930 ± 1.34) when compared with those from the NS group (0.771 ± 1.32, t = 9.735, P < 0.001). The area under the receiver operating characteristic curve (AUROC) was 0.859 calculated by using the MELD-LAC model, which was significantly higher than that calculated by using the lactate level (0.790) or MELD alone (0.818). When the cutoff value was set at -0.4741, the sensitivity, specificity, positive predictive value and negative predictive value for predicting short-term mortality were 91.5%, 80.10%, 94.34% and 74.62%, respectively. When the MELD-LAC scores at baseline level were set at -0.5561 and 0.6879, the corresponding mortality rates within three months were 75% and 90%, respectively.

CONCLUSION

The short-term prognosis of HBV-related ACLF was improved by using a modified MELD including serum lactate from the present 6-year clinical study.

Key words: Hepatitis B virus; Liver failure; Model for end-stage liver disease score; Prognosis; Serum lactate level

Core tip: This is a retrospective study to evaluate the short-term prognosis of hepatitis B virus (HBV)-acute-on-chronic liver failure (ACLF). Two hundred and thirty-six patients with HBV-ACLF were divided into two groups: survival group (S) and non-survival group (NS). In predicting 3-mo mortality using the model for MELD-LAC model, patients from the S group had significantly lower baseline scores compared with those from NS group using model for end-stage liver disease (MELD)-LAC model. AUROC was 0.859 calculated by using the MELD-LAC model, which was significantly higher than those calculated by using the lactate level (0.790) or MELD alone (0.818). The short-term prognosis of HBV-ACLF was improved by using a modified MELD including serum lactate from the present 6-year clinical study.

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INTRODUCTION

Hepatitis B virus (HBV) infection is a severe public health problem all over the world. The prevalence of hepatitis B is particularly high in China. HBV-related acute-on-chronic liver failure (ACLF) is a rapidly progressive disease with a high mortality rate up to 60%-75%[1]. The accurate assessment of the disease severity is critical before clinicians can make decisions on potential treatments like medication or liver transplantation (LT). The model for end-stage liver disease (MELD)[2,3] is a well-accepted model for assessing the feasibility of LT; however, the accuracy of its prediction is still unsatisfying[4]. A more objective and quantitative model with higher repeatability to predict the short-term prognosis of HBV-related ACLF is urgently needed.

Lactate (LAC) is mainly metabolized in the liver and is widely used as an important indicator of organ failure or serious bacterial infection. Hyperlactatemia normally reflects both increased production and impaired clearance in patients with liver dysfunction[5], and a higher level of LAC always indicates a worse prognosis[6]. Although several potential hypoxic and non-hypoxic mechanisms have been implicated on the persistent hyperlactatemia and the high level of LAC, the exact role of these parameters has not been specifically addressed in clinical studies[7,8]. In this study, we investigated the serum LAC level in patients with HBV-related ACLF, and developed a modified MELD including serum lactate (MELD-LAC model) to predict the short-term prognosis (three months) of HBV-related ACLF.

MATERIALS AND METHODS

Patients

Three hundred and ninety-three patients, who were diagnosed with HBV-related ACLF and admitted in our center, were included in the present clinical study between September 2009 and October 2015. The subjects were aged from 18 to 65 years. The diagnosis of ACLF was conducted according to the American Association for the Study of Liver Failure: Update 2011[9]. Three months after diagnosis, these patients were followed by telephone. The patients were divided into two groups: survival group (S group) and non-survival group (NS group), based on whether they were surviving or not three months after admission. The clinical data were collected within the first 24 h after admission, including hepatic, renal, and coagulation functions, as well as the LAC level. The onset time of hepatic encephalopathy (HE) was also noted if it happened. MELD scores were calculated as follows: MELD = 3.8 × Ln(Tbil [mg/dL]) + 11.2 × Ln(INR) + 9.6 × Ln (Cr [mg/dL]) + 6.4 × cause. Since the ACLF was due to HBV infection, the cause was counted as 1 in this study.

On admission, each patient's baseline values of serum LAC level, total bilirubin, creatinine level and

prothrombin time-international normalized ratio were measured. The serum LAC level was measured using a commercial test kit (Johnson & Johnson Inc., United States) with a normal value of 0.9-2.0 mmol/L. Total bilirubin and creatinine levels were tested using an Olympus Chemistry Analyzer (AU2700). All reagents for bilirubin and creatinine tests were purchased from Olympus Corporation (Japan). PT test and INR calculation were performed using a Stago STA Compact coagulation analyzer (Diagnotica Stago, French).

Treatment

After HBV-related ACLF was diagnosed, all the patients were treated with standard protocols, including nutritional support, hepatocyte proliferation, prevention of infection, correction of anomalies in the coagulation system and improvement of cerebral edema. In addition, plasmapheresis was also performed when it was necessary. The survival data were collected after three months.

Statistical analysis

Data analyses were performed using SPSS 17.0 software. The significance of data was tested by the Student's test or the χ^2 test. The correlation between groups was analyzed by the Pearson's product-moment correlation coefficient test. *P*-values less than 0.05 were considered statistically significant. Logistic regression analysis was used to establish the prognosis model. The accuracies of the newly developed prognosis model (MELD-LAC model), LAC and MELD scores in predicting the short-term prognosis of HBV-related ACLF patients were assessed by the area under the receiver operating characteristic (AUROC) curve.

RESULTS

Demographic data

A total of 393 serum samples from patient with HBV-related ACLF were collected, 157 of which were excluded from the present study due to the complications of hepatocellular carcinoma, kidney injury, diabetes or alcoholic liver diseases. According to the follow-up results in the 236 enrolled cases, 130 (110 males and 20 females) were recruited into the S group, and 106 (87 males and 19 females) were recruited into the NS group. The two groups had no statistical difference in gender or age, but had significant differences in TBIL, Cr, INR, LAC levels and MELD scores ($P < 0.05$ for all) (Table 1). There were 68 patients in the S group and 57 patients in the NS group who had received plasmapheresis as an additional enhanced therapy, but it did not provide any positive outcome ($P = 0.896$). No patient in this study had received LT.

Approximately 82.3% (107 of 130) of patients in the S group and 95.2% (101 of 106) of patients in the NS group were found to have elevated LAC level of more than 2 mmol/L. A significant difference was found in the above percentages between the two groups ($\chi^2 = 9.40$, $P = 0.002$). Furthermore, 13.1% of patients (17 of 130) in the S group and 59.4% (63 of 106) in NS group were found to have elevated LAC level of more than 4 mmol/L. A significant difference between the percentages of the S group and the NS group was also found ($\chi^2 = 55.999$, $P < 0.001$). The mean LAC level in the S group (3.11 mmol/L ± 1.98 mmol/L) was significantly lower than that in the NS group (4.67 mmol/L ± 2.43 mmol/L) ($P < 0.001$). No significant difference was shown in the CTP scores between the S and NS groups ($P = 0.373$), but a significant difference was shown in the MELD scores between the two groups ($P = 0.023$).

Using the Spearman analysis method, the correlations between MELD score/serum LAC levels and the mortality in three months were analyzed (Table 2). The results from Pearson's analysis also showed that both serum LAC level and MELD score had a statistically significant correlation with the short-term prognosis of HBV-related ACLF ($r = 0.315$, $P < 0.001$).

Construction of a modified MELD including serum lactate

Based on single factor analysis, both MELD score and LAC level were correlated with the 3-mo mortality of HBV-related ACLF. The forward logistic regression method was used to establish the MELD-LAC model: 0.314 × LAC + 0.172 × MELD score - 5.923 (Table 3). The patients from the S group had significantly lower baseline MELD-LAC scores (-0.930 ± 1.34) compared with those from the NS group (0.771 ± 1.32, $t = 9.735$, $P < 0.001$).

The short-term prognosis of HBV-related ACLF was improved by using the modified MELD including serum lactate from the present 6-year clinical study.

Prediction of the short-term prognosis of HBV-related ACLF by using MELD-LAC model

To predict the 3-month mortality, the LAC model alone had a very similar positive predictive value (PPV), negative predictive value (NPV), sensitivity and specificity to MELD. Interestingly, the new MELD-LAC model by combining these two independent factors had a better predicting score than these two models alone. When the cutoff value of the MELD-LAC model was set at -0.4741, based on the best Yoden index, an AUROC curve of 0.859 (Table 4) was obtained when this equation was applied to evaluate the prognosis, with a sensitivity of 91.5% and a specificity of 80.10%, which were much greater than the other two models. Comparing the AUROCs obtained from the above three models, the prognosis performances were listed as follows: MELD-LAC > MELD > LAC (Figure 1). From our analysis, the patients with an MELD-LAC score of -0.5561, -0.4741 and 0.6879 had the three-month

mortality rate of 75%, 78.86% and 90%, respectively. The PPV and NPV of MELD-LAC were 94.34% and 74.62%, which were significantly improved compared with the scores obtained from the MELD and LAC model alone.

The patients were divided into two groups, when the cutoff value of the MELD-LAC model was set at -0.4741. The first group (MELD-LAC ≥ -0.4741) included 123 patients, and 78.86% were dead after three months. The second group (MELD-LAC < -0.4741) included 113 patients, and only 11.50% died after three months. A statistically significant difference was observed between these two groups (χ^2 = 107.35, P < 0.001). When compared with MELD with a cutoff value of 25, the MELD-LAC model with a cutoff value of -0.4741 apparently achieved better prediction of short-term prognosis.

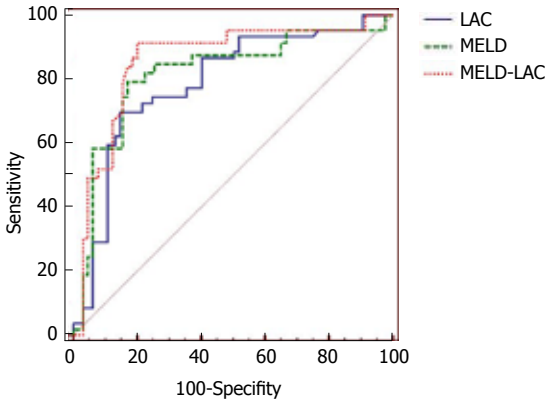

Figure 1 Area under the receiver operating characteristic curves of different models. MELD: Model for end-stage liver disease; LAC: The venous lactate.

DISCUSSION

ACLF is a severe health problem with a high mortality rate[10-12]. In clinical practice, predicting the progression of this disease is the biggest challenge for clinicians. Currently, three scoring systems, namely, King's College Hospital criteria, Child-Turcotte-Pugh (CTP) score and MELD score[4,13] systems, are commonly implemented to predict the prognosis of ACLF patients; CTP score has been used traditionally to assess the prognosis of cirrhosis instead of ACLF; the MELD scoring system has been developed to assess various liver diseases, including assessing the severity of ACLF of all causes to determine the ideal timing of liver transplantation, as well as providing direct information to support medical decision making[14]. The MELD scoring system is widely considered to be better than the CTP score; however, it does have several limitations, for example, the diagnostic sensitivity and specificity of MELD are not high enough.

In the MELD scoring system, several variables associated with poor prognosis of ACLF are not considered, including HE, infections, and hemorrhage. Therefore, modification for MELD is needed to improve its outcome in clinical practice. Addition of Na+ into the MELD (MELD-Na) has been proposed and has been successfully evaluated as a better score in patients with ascites, but only for those patients with sodium levels below normal. However, such a condition is only presented in 30% of patients with decompensated cirrhosis[15].

The elevation of lactate level in ACLF situation has been reported and might be attributed to several suggested mechanisms[16,17]. First, the liver is the organ primarily responsible for LAC clearance, and the LAC clearance may be impaired in the presence of severe liver dysfunction. Second, some ACLF-related complications, *e.g.*, bacterial infection, may also raise the LAC level. Last but not least, hypovolemia-related hypoperfusion is also very likely to induce an elevation of LAC level during the early pre-resuscitative phase. In fact, some previous studies have shown that the acutely injured liver may act as a source of evaluating LAC[18]. Taken together, we suggest that the LAC level could be used as a useful marker for assessing the severity of ACLF. As a simple measure widely available in current hospital system, early measurement of serum LAC can provide important prognostic information in patients with acute variceal hemorrhage requiring ICU admission[19-21]. But to the best of our knowledge, the LAC level had never been considered as a critical biomarker for prognosis prediction of HBV-related ACLF.

Our results showed that LAC could be used as a useful tool to assess the situation of HBV-related ACLF. The evaluated LAC level was found in 82.3% of patients in the S group, but 95.2% of those in the NS group (χ^2 = 9.40, P = 0.002). The mean LAC level in the S group (3.11 mmol/L ± 1.98 mmol/L) was lower than that in the NS group (4.67 mmol/L ± 2.43 mmol/L) (P < 0.001). Further analysis showed that the baseline LAC level was related with patients' prognosis with a rational sensitivity (86.80%). The AUROC for predicting the 3 mo mortality based on the LAC model was relatively lower than that from MELD (0.79 *vs* 0.818), which is consistent with previous studies[21].

In our study, a positive correlation between the LAC and MELD scores was demonstrated, suggesting that a combination of LAC and MELD scores was very likely to increase prediction accuracy for ACLF prognosis. Therefore, we developed a modified MELD including serum lactate level to improve the short-term prognosis prediction of HBV-related ACLF by combining two parameters together (LAC and MELD). By adopting the forward logistic regression method, LAC and MELD scores were subjected to the equation, to yield a new MELD-LAC model.

The new model showed a better (AUROC = 0.859) prediction score for the prognosis of HBV-related ACLF when compared with either LAC (0.790) or MELD (0.818) alone. When the cutoff value was set at -0.4741, the MELD-LAC model had a sensitivity

Table 1 Demographic data, biochemical factors, clinical and surgical characteristics for the S and NS groups

Group	S (n = 130)	NS (n = 106)	t or t'	P value
Age (yr)	43.51 ± 14.13	45.97 ± 10.69	1.480	0.140
Male/Female (n)	110/20	87/19	0.273	0.363
Liver cirrhosis, n (%)	63 (48.46)	68 (64.15)	5.820	0.011
TBil (μmol/L)	344.85 ± 160.82	456.63 ± 180.43	5.040	< 0.001
ALT	92.62 ± 82.80	101.84 ± 83.71	0.847	0.389
AST	92.81 ± 91.84	103.86 ± 79.20	0.978	0.329
GLO	27.72 ± 8.95	27.84 ± 8.85	0.100	0.920
WBC	8.27 ± 4.12	8.21 ± 4.29	-0.117	0.907
PCT	0.53 ± 0.29	0.58 ± 0.54	0.886	0.377
Ammonia	67.29 ± 35.55	69.03 ± 47.57	-0.312	0.755
INR	2.46 ± 0.97	3.11 ± 1.63	3.733	< 0.001
Cr (μmol/L)	79.31 ± 45.25	99.80 ± 88.74	2.293	< 0.001
CHE (U/L)	3956.30 ± 1377.54	3529.00 ± 1509.89	0.170	0.859
Alb (g/L)	31.22 ± 3.98	30.99 ± 3.96	-0.428	0.398
DNA	594275 ± 230160	425886 ± 110810	-0.691	0.491
LAC (mmol/L)	3.11 ± 1.98	4.67 ± 2.43	5.430	< 0.001
< 2.68	76	14	50.685	0
2.68-4	37	29	0.035	0.484
⩾ 4	17	63	55.999	< 0.001
CTP	10.54 ± 1.63	10.74 ± 1.73	0.892	0.373
MELD score	23.33 ± 5.42	30.37 ± 6.58	9.010	0.023
Plasmapheresis	68	57	0.05	0.896

TBil: Total bilirubin; Cr: Creatinine; INR: International normalized ratio for prothrombin time; MELD: Model for end-stage liver disease; LAC: The venous lactate; ALB: Albumin; GLO: Globulin; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; CHE: Cholinesterase; WBC: White blood cell; PCT: Procalcitonin; CTP: Child-Turcotte-Pugh score.

Table 2 Factors correlated with prognosis of hepatitis B virus-related acute-on-chronic liver failure

Factor	Correlation index	P value
MELD	0.548	< 0.001
LAC	0.499	< 0.001
TBiL	0.308	< 0.001
INR	0.289	< 0.001
Cr	0.014	0.828
Alb	0.071	0.273
CHE	0.058	0.373

TBil: Total bilirubin; Cr: Creatinine; INR: International normalized ratio for prothrombin time; MELD: Model for end-stage liver disease; LAC: The venous lactate; ALB: Albumin; CHE: Cholinesterase.

Table 3 The regression coefficients and indices of the model for end-stage liver disease and the venous lactate model

Index	β	SE	Wald	df	P value	Exp(β)
MELD	0.172	0.028	37.198	1	0.000	1.188
LAC	0.314	0.098	10.195	1	0.001	1.368
Constant	-5.923	0.824	51.724	1	0.000	0.003

MELD: Model for end-stage liver disease; LAC: The venous lactate.

of 91.50% and a specificity of 80.10% for predicting the mortality within three months. The patients were further divided into two groups according to the above cutoff value. The mortality was 78.86% in the higher score group (MELD-LAC ⩾ -0.4741) and 7.96% in the lower score group (MELD-LAC < -0.4741), showing a significant difference between these two groups (χ^2 = 107.35, P = 0.000). Under such a cutoff value setting (-0.4741), the MELD-LAC model seemed to provide a much more accurate prediction than normal MELD with a cutoff value of 25 (Table 5). According to our data, the three-month mortality rate of the patients was 75%, and 90% could be precisely predicted by the scores of MELD-LAC (-0.5561 or 0.6879) at baseline. Our results also showed no difference between the S group and NS group in CTP scores (Table 1), suggesting that CTP system is not a good tool in evaluating the outcome of HBV-related ACLF.

There are some limitations of this study. Our study indicated, for the first time, that a modified MELD including serum lactate was a better prediction model for the prognosis of HBV-related ACLF, with higher PPV and NPV. Further research is required to combine this model into the classic model or develop a more accurate prognostic model. Another limitation of this study is that the patient population came merely from a single center in this study and it was a retrospective study; further research and verification need to be done in larger multi-center studies.

Serum LAC test is a simple and mature clinical measurement in modern hospitals, and it reflects both direct liver injury and other organ dysfunction. Dynamic monitoring of LAC was able to provide a new opportunity for clinicians with more accurate disease assessment, prognosis predication and treatment guidance at the early stage of the liver disease. We concluded that the modified MELD including serum lactate is a better prediction model for the prognosis of HBV-related ACLF, with higher PPV and NPV.

Table 4 Prognostic value of the various models

model	AUROC	95%CI	Cut-off value	Sensitivity	Specificity	PPV	NPV
MELD	0.818	0.759-0.877	24.5	87.70 %	63.80%	85.85%	64.62%
LAC	0.79	0.730-0.850	2.68	86.80%	62.10%	86.79%	58.46%
MELD-LAC	0.859	0.807-0.911	-0.4741	91.50%	80.10%	94.34%	74.62%

AUROC: The area under the receiver operating characteristic; PPV: Positive predictive value; NPV: Negative predictive value. LAC: The venous lactate; MELD: Model for end-stage liver disease.

Table 5 Comparison of prediction performance of the model for end-stage liver disease and the modified model

Groups		Non-survival	Survival	χ^2, *P* value
MELD	MELD < 25	16	83	56.98, < 0.001
	MELD ≥ 25	90	47	
MELD-LAC	MELD-LAC < -0.4741	13	100	107.35, < 0.001
	MELD-LAC ⩾ -0.4741	97	26	

MELD: Model for end-stage liver disease; LAC: The venous lactate.

COMMENTS

Background

Hepatitis B virus (HBV)-related acute-on-chronic liver failure (ACLF) is a rapidly progressive disease with a high mortality rate up to 60%-75%. The accurate assessment of disease severity is critically needed, before clinicians make decisions on potential treatments such as medication or liver transplantation (LT). The model for end-stage liver disease (MELD) score model is a well-accepted model for assessing the feasibility of LT; however, the accuracy of prediction is still unsatisfying. A more objective and quantitative model with higher repeatability to predict the short-term prognosis of HBV-related ACLF is urgently needed.

Research frontiers

In the MELD scoring system, several variables associated with poor prognosis of ACLF are not considered, including hepatic encephalopathy (HE), infections, and hemorrhage. Therefore, modification of MELD is needed to improve its outcome in clinical practice. Addition of Na+ into MELD (MELD-Na) has been proposed and has been successfully evaluated as a better score in patients with ascites, but only for those patients with sodium levels below normal. However, such a condition is only presented in 30% of patients with decompensated cirrhosis.

Innovations and breakthroughs

Lactate (LAC) is mainly metabolized in the liver and is widely used as an important indicator in organ failure or serious bacterial infection. Hyperlactatemia normally reflects both increased production and impaired clearance in patients with liver dysfunction, and a higher level of LAC always indicates a worse prognosis. Although several potential hypoxic and non-hypoxic mechanisms have been implicated on the persistent hyperlactatemia and the high level of LAC, the exact role of these parameters has not been specifically addressed in clinical studies. In the present study, they investigated the serum LAC level in patients with HBV-related ACLF, and developed a modified MELD including serum lactate (MELD-LAC model) to predict the short-term prognosis (three months) of HBV-related ACLF.

Applications

This study developed a modified MELD model including serum lactate level to improve the short-term prognosis prediction of HBV-related ACLF by combining the two parameters together (LAC and MELD).

Peer-review

The authors of this paper found that the efficacy of the short-term prognosis of HBV-related ACLF was improved by using a modified MELD including serum lactate from the present 6-year clinical study. The new model showed a better prediction score on the prognosis of HBV-related ACLF, when compared with either LAC or MELD alone.

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ORIGINAL ARTICLE

Observational Study

Chronic opioids in gastroparesis: Relationship with gastrointestinal symptoms, healthcare utilization and employment

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Abstract

AIM

To examine the relationship of chronic scheduled opioid use on symptoms, healthcare utilization and employment in gastroparesis (Gp) patients.

METHODS

Patients referred to our tertiary care academic center from May 2016 to July 2017, with established diagnosis or symptoms suggestive of Gp filled out the Patient Assessment of Upper GI Symptoms, abdominal pain and demographics questionnaires, and underwent gastric emptying and blood tests. They were asked about taking pain medicines and the types, doses, and duration. We used Mann Whitney *U* test, Analysis of Variance, Student's *t* test and χ^2 tests where appropriate for data analyses.

RESULTS

Of 223 patients with delayed gastric emptying, 158 (70.9%) patients were not taking opioids (GpNO), 22 (9.9%) were taking opioids only as needed, while 43 (19.3%) were on chronic (> 1 mo) scheduled opioids (GpCO), of which 18 were taking opioids for

reasons that included gastroparesis and/or stomach pain. Median morphine equivalent use was 60 mg per day. GpCO reported higher severities of many gastrointestinal symptoms compared to GpNO including nausea (mean ± SE of mean of 4.09 ± 0.12 *vs* 3.41 ± 0.12, $P = 0.011$), retching (2.86 ± 0.25 *vs* 1.98 ± 0.14, $P = 0.003$), vomiting (2.93 ± 0.24 *vs* 2.07 ± 0.15, $P = 0.011$), early satiety (4.17 ± 0.19 *vs* 3.57 ± 0.12, $P = 0.004$), post-prandial fullness (4.14 ± 0.18 *vs* 3.63 ± 0.11, $P = 0.022$), loss of appetite (3.64 ± 0.21 *vs* 3.04 ± 0.13, $P = 0.039$), upper abdominal pain (3.86 ± 0.20 *vs* 2.93 ± 0.13, $P = 0.001$), upper abdominal discomfort (3.74 ± 0.19 *vs* 3.09 ± 0.13, $P = 0.031$), heartburn during day (2.55 ± 0.27 *vs* 1.89 ± 0.13, $P = 0.032$), heartburn on lying down (2.76 ± 0.28 *vs* 1.94 ± 0.14, $P = 0.008$), chest discomfort during day (2.42 ± 0.20 *vs* 1.83 ± 0.12, $P = 0.018$), chest discomfort at night (2.40 ± 0.23 *vs* 1.61 ± 0.13, $P = 0.003$), regurgitation/reflux during day (2.77 ± 0.25 *vs* 2.18 ± 0.13, $P = 0.040$) and bitter/acid/sour taste in the mouth (2.79 ± 0.27 *vs* 2.11 ± 0.14, $P = 0.028$). GpCO had a longer duration of nausea per day (median of 7 h *vs* 4 h for GpNO, $P = 0.037$), and a higher number of vomiting episodes per day (median of 3 *vs* 2 for GpNO, $P = 0.002$). Their abdominal pain more frequently woke them up at night (78.1% *vs* 57.3%, $P = 0.031$). They had a lower employment rate (33.3% *vs* 54.2%, $P = 0.016$) and amongst those who were employed less number of working hours per week (median of 23 *vs* 40, $P = 0.005$). They reported higher number of hospitalizations in the last 1 year (mean ± SE of mean of 2.90 ± 0.77 *vs* 1.26 ± 0.23, $P = 0.047$).

CONCLUSION

GpCO had a higher severity of many gastrointestinal symptoms, compared to GpNO. Hospitalization rates were more than 2-fold higher in GpCO than GpNO. GpCO also had lower employment rate and working hours, when compared to GpNO.

Key words: Opioid; Gastroparesis; Symptoms; Hospitalizations; Employment

Core tip: Chronic opioid use can cause gastrointestinal side effects and negatively influence the quality of life. The impact of chronic opioid use on symptoms, healthcare utilization, and employment of gastroparesis patients is not well studied. In our study, gastroparesis patients on chronic scheduled opioids had more severe gastrointestinal symptoms, less work productivity and more frequent hospitalizations compared to gastroparesis patients without opioid use. Whether opioid use is to treat a higher symptom severity from gastroparesis, or the opioid use worsens symptoms requires further study.

Jehangir A, Parkman HP. Chronic opioids in gastroparesis: Relationship with gastrointestinal symptoms, healthcare utilization and employment. *World J Gastroenterol* 2017; 23(40): 7310-7320 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i40/7310.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i40.7310

INTRODUCTION

Opioid use has become a healthcare epidemic in United States with increasing prescription rates in the recent years, and over 3% of the adults are now chronically using opioids[1-3]. In 2013, the overall cost burden from opioids in United States was estimated to be $78.5 billion[4]. A recent systematic review identified mean costs to the payer (commercial or private insurance) of $23000-$25000 per year for the opioid misusers, approximately $15000 more than the non-opioid users[5].

Patients on chronic opioids are more likely to have bowel related issues[2]. The wide array of gastrointestinal symptoms that the patients on chronic opioids may experience, including constipation (38%-63%), gastroesophageal reflux disease (33%), nausea (20%-90%), vomiting (9%-84%), bloating (24%-75%) and delayed gastric emptying are known as opioid induced bowel dysfunction[2,6-13]. The actual incidence of some of these side effects may even be higher as many patients tend to underreport their symptoms, and may self-adjust their regimen to avoid side effects[12]. Approximately 6% of the chronic opioid patients develop worsening chronic or intermittent abdominal pain (AP) even with continuous or increasing doses of opioids, a condition termed narcotic bowel syndrome[8]. The opioid-induced gastrointestinal side effects are often not anticipated while prescribing these medications, and may lead to increase healthcare utilization[9].

Despite the known gastrointestinal side effects of opioids, they are used in some patients with gastroparesis (Gp). Previous studies reported that 30%-46% of the Gp patients regularly use opioids[10,14,15]. In addition to opioids causing constipation, opioids slow gastric emptying and also can cause nausea and vomiting by activating the chemoreceptor trigger zone in the area postrema at the floor of the fourth ventricle[9,16]. Moreover, it has been shown that Gp patients on opioids may have worse outcomes with medical treatment with prokinetics agents and after gastric pacemaker placement[14].

The aim of this study was to examine the relationship of chronic opioid use on symptoms, healthcare utilization and employment in patients referred for Gp. We studied gastroparesis patients; comparing those on chronic scheduled opiates (GpCO) to gastroparesis patients

not taking opiates (GpNO).

MATERIALS AND METHODS

Patients referred to our tertiary care academic center gastroenterology motility clinic at Temple University Hospital (TUH) with established diagnosis of gastroparesis, or patients who presented with symptoms suggestive of gastroparesis from May 2016 to July 2017 (n = 303) were studied. This observational study was reviewed and approved by the TUH Intuitional Review Board. Patients are often referred to our motility clinic for persistent or refractory symptoms of gastroparesis. Subjects were recruited at the end of their regularly scheduled appointments after obtaining informed consent. Inclusion criteria were the following: (1) adults aged 18 to 80 years old; (2) symptoms suggestive of gastroparesis; and (3) delayed gastric emptying on scintigraphy.

On their initial evaluation, patients were asked to fill out a questionnaire about their clinical condition. This questionnaire contained the following: Patient Assessment of Upper Gastrointestinal Symptom (PAGI-SYM), Abdominal Pain Questionnaire, and demographic questionnaire. Patients underwent 4-h gastric emptying scintigraphy (GES)[17] if had not already recently been performed. Blood tests were obtained (see below). A retrospective review of the questionnaires, gastric emptying scintigraphy and blood tests was subsequently performed.

Questionnaires

PAGI-SYM: This validated questionnaire for upper gastrointestinal symptoms of gastroparesis, dyspepsia, and gastroesophageal reflux disease asked patients to rate the severity of 20 common gastrointestinal symptoms over the past two weeks[18]. Patients were asked to rate symptoms over the prior two weeks as none (0), very mild (1), mild (2), moderate (3), severe (4), and very severe (5). They were also asked to rate symptoms of diarrhea and constipation on the same scale. In addition, they were asked about the duration of nausea per day in the past week, number of episodes of vomiting and bowel movements in the past week. We also asked the patients about the frequency of some of their gastrointestinal symptoms over the preceding 3 mo using the Rome IV questionnaires[19].

Abdominal pain questionnaire: This questionnaire was modified from previous studies assessing the presence and severity of abdominal pain in chronic pancreatitis[20,21]. If abdominal pain was present, patients filled out the remaining portion of the questionnaire asking about the duration, location of the most severe pain, relationship of pain to meals and nocturnal awakenings as a result of the pain.

Demographics questionnaire: This questionnaire asked patients to report information such as age, gender, race/ethnicity, height, weight, smoking history, alcohol usage, employment and working hours per week if employed. It also asked about the length of diagnosis with Gp, history of diabetes, and prior abdominal surgeries. Patients were asked about taking pain medicines and the types, doses, frequency, clinical indication and total duration of pain medicine use.

Laboratory analysis

Patients underwent blood drawing including hemoglobin A1c and thyroid stimulating hormone, as hyperglycemia and hypothyroidism are risk factors for delayed gastric emptying[22,23]. We also checked serum trypsinogen level as symptoms of Gp can resemble those of chronic pancreatitis (CP)[24]. Lastly, we ordered random serum cortisol levels as opioid-induced adrenal insufficiency has been reported in the literature[25-27].

Gastric emptying scintigraphy

Gastric emptying scintigraphy was performed using a low-fat, egg white meal with imaging at 0, 1, 2, 4 h after meal ingestion[17]. Patients were instructed to stop medications that could affect gastrointestinal motility (including prokinetics) for 48 h prior to the study and to come to the Nuclear Medicine Section in the morning after fasting overnight, that is, an 8 h fast. The patients on chronic opioids were advised to gradually taper off their opioids as tolerated prior to their scheduled GES to prevent opioid withdrawal. Diabetics have their glucose checked at the beginning of the study, with appropriate treatment measures being taken if low blood sugar (hypoglycemia) or high blood sugar (hyperglycemia > 250 mg/dL) is detected. Gastric emptying scintigraphy is performed using a standard low-fat, Eggbeaters® meal to measure solid emptying. The meal consists of the equivalent of two large eggs radiolabeled with 0.5-1 mCi Tc-99m sulfur colloid served with two pieces of white bread and jelly. Patients are given 120 mL water. Following ingestion of the meal, imaging is performed at 0, 1, 2 and 4 h with the patient standing upright for measuring gastric emptying of Tc-labeled solids. Gastric emptying is analyzed as percent of radioactivity retained in the stomach over time using the geometric center of the decay-corrected anterior and posterior counts for each time point. Gastric retention of Tc-99m > 60% at 2 h. and/or > 10% at 4 h is considered delayed gastric emptying of solids.

Statistical analysis

We used Kolmogorov-Smirnov test to determine the normal distribution of continuous variables; Student's t-test was used for variables with normal distribution, while Mann Whitney U Test was used for variables with skewed distribution and symptoms assessed on ordinal scale. These results are expressed as mean ± SE of mean, or median with interquartile range as

appropriate. χ^2 test was used for categorical data, with results expressed as percentages[28]. A two-tailed *P* value less than 0.05 was considered as statistically significant while comparing chronic opioid using Gp patients to non-opioid users; no adjustment for multiple comparisons was made. Analysis of Variance (ANOVA) was used for comparison of multiple groups, followed by Students; *t*-test with *p* value adjusted with Bonferroni correction for multiple comparisons. Unanswered questions were excluded from the analyses. Gp patients who were using opioids as needed (*n* = 22) were not included in the analyses. Statistical review of the study was performed by an epidemiologist/statistician of our department.

RESULTS

Patients

Of 303 patients referred for symptoms suggestive of gastroparesis in 15 mo, 80 patients had normal gastric emptying tests and were excluded from the analyses. Of the remaining 223 patients, the majority (*n* = 196, 87.9%) had previously been diagnosed with Gp. More than half (52%) of the Gp patients came from outside the catchment area of TUH that we defined as more than 50 miles from our center. The most frequent type of Gp was idiopathic Gp (*n* = 122; 54.7%), followed by diabetic Gp (*n* = 62, 27.8%), post-surgical Gp (*n* = 20; 9.0%), and atypical Gp (*n* = 19; 8.5%) (Table 1). The median age of these patients was 44 years (interquartile range of 32 to 56 years), and 80.7% were females. Amongst 210 patients who reported their race, the most common races were Whites (79.4), African Americans (7.6%) and Hispanics (6.7%). Over two third of patients with Gp (*n* = 158, 70.9%) were not taking opioids, 22 (9.9%) were taking opioids only as needed, while 43 (19.3%) were on chronic scheduled opioids (Table 1).

Gastroparesis patients on chronic scheduled opioids

Amongst the 43 patients on chronic scheduled opioids, 6 (14%) were taking opioids exclusively for Gp and/or stomach pain, while another 12 (27.9%) were taking opioids for reasons that included Gp and/or stomach pain. Other frequent causes of opioid use included back pain (*n* = 27, 62.8%), leg pain (*n* = 10), arthritis (5), fibromyalgia (4), Reflux Sympathetic Dystrophy (3), and neuropathy (2). Nearly one fourth of these patients (*n* =10, 23.3%) were taking more than 1 opioid. The opioids used chronically included oxycodone (18 patients), fentanyl (9), methadone (5), morphine (5), tramadol (5), hydromorphone (4), hydrocodone (3), and oxymorphone (2). Median duration of narcotic use was 2 years (interquartile range of 0.6-6.5 years). Median oral morphine equivalent dose for GpCO was 60 mg/d, with interquartile range of 22.5 mg to 112.5 mg per day.

Patients with diabetic gastroparesis were more likely to be on chronic scheduled opioids (24.2 % *vs* 18.9%), or as needed opioids (14.5% *vs* 4.9%), when compared to the patients with idiopathic gastroparesis who were more likely to be non-opioid users (76.2% *vs* 61.3%), *P* = 0.039. Thirty-three Gp patients on chronic opioids were able to recall the duration of their Gp symptoms as well as chronic opioid use. Out of these, about 1 in every 4 GpCO (27.3%) stated that they started using opioids chronically before their symptoms of Gp started. For another 21.2% of GpCO, the duration of opioid use was the same as the duration of their Gp symptoms. For 51.5% of GpCO, the Gp symptoms started before the use of chronic opioids. Of note however, we had to rely on patients' recall for the duration of their opioids use, and patients reported drug ingestion histories can often be inaccurate[29].

GpCO and GpNO did not have any statistically significant difference in their age, duration of symptoms, gender, body mass index, racial distribution, history of diabetes, and past surgeries on esophagus and stomach (Table 2). GpCO were more likely to be active smokers (31.7% *vs* 13.0%, *P* = 0.004). There was a trend towards higher prior and/or current alcohol use in GpNO compared to GpCO (37.4 % *vs* 23.8% respectively, *P* = 0.100), as well as current alcohol use (20.0% *vs* 7.3% respectively; *P* = 0.057).

Laboratory studies

GpCO were also more likely to have low trypsinogen levels compared to GpNO (23.1% *vs* 4.2% respectively, *P* = 0.004). However, only about half of these patients had their trypsinogen levels drawn (Table 2). Among GpCO, 16.2% had low random serum cortisol levels, *vs* 10.4% among GpNO, *P* = 0.345 (Table 2). We did not confirm the diagnosis of adrenal insufficiency by checking adrenocorticotrophic hormone or by performing stimulation test; this requires further study to determine the actual incidence of adrenal insufficiency in chronic opioid using Gp patients. There was no statistically significant difference in the other laboratory tests, including hemoglobin A1c, thyroid stimulating hormone.

Gastric emptying scintigraphy

On gastric emptying tests, there was no difference between GpCO and GpNO at 2 h (median of 62% *vs* 66%, normal $\leqslant$ 60%) and 4 h (median of 22% *vs* 24%, normal $\leqslant$ 10%) (*P* > 0.05) (Table 2). Amongst GpCO 11 patients (25.6%) had severe delays in gastric emptying (> 35% retention at 4 h), compared to 30 patients (19%) in GpNO (*P* = 0.341).

Opioids did not seem to have a dose-related effect on the delay in gastric emptying, as we did not find any difference in the four quartiles of GpCO based on morphine equivalents per day by comparing the gastric emptying results of these groups using ANOVA (Table 3). We also calculated Pearson correlation coefficient

Table 1 Patients with gastroparesis referred to a tertiary care center between May 2016 and July 2017

Classification	Total	Chronic scheduled opioids	PRN opioids	No opioids
Idiopathic	122	23	6	93
Diabetic	62	15	9	38
Post-surgical[1]	20	2	5	13
Atypical[2]	19	3	2	14
Total	223	43	22	158

[1]Post-surgical gastroparesis consisted of patients with history of duodenal-jejunostomy, esophagectomy, fundoplication, gastric sleeve, gastric banding, gastric bypass, hiatal hernia repair, and vagotomy with pyloroplasty. [2]Atypical gastroparesis consisted of gastroparesis patients with history of Bulimia, Complex Regional Pain Syndrome, Ehlers Danlos Syndrome, Lupus, Parkinson's Disease, Reflux Sympathetic Dystrophy, Scleroderma and Sjogren's Syndrome.

between opioid dose and delay in gastric emptying, and there was no significant correlation between morphine equivalents per day and gastric retention at 2 h ($r < 0.01$, $P = 0.988$) or 4 h ($r = 0.19$, $P = 0.356$).

Of note, 14 patients on chronic scheduled opioids were still taking opioids at the time of their GES, which may have resulted in the delay in their gastric emptying. However, when we compared these patients to GpCO who were able to taper off the opioids prior to the study, there was no difference in gastric retention at 2 h (median of 60% *vs* 70% respectively, $P = 0.461$) and 4 h (16% *vs* 20%, $P = 0.718$).

GI Symptoms

GpCO had higher symptom severities of many GI symptoms including nausea, retching, vomiting, early satiety, post-prandial fullness, loss of appetite, upper abdominal pain, upper abdominal discomfort, heartburn during day, heartburn on lying down, chest discomfort during day, chest discomfort at night, regurgitation/reflux during day, and bitter/acid/sour taste in the mouth compared to GpNO ($P < 0.05$) (Table 4). The severity of constipation was not statistically different between the two groups (2.92 ± 0.30 in GpCO, compared to 2.63 ± 0.14 in GpNO, $P = 0.296$). On PAGI-SYM questionnaire, the total symptom severity score in GpCO was also higher than GpNO (Table 4).

As stated previously the median morphine equivalent use in GpCO was 60 mg per day. When we compared GpCO taking more than 60 mg morphine equivalents per day to GpCO taking 60 mg per day or less, the patients taking more than 60 mg per day reported more severe heartburn during the day (3.30 ± 0.45 *vs* 2.0 ± 0.21, $P = 0.023$). There was also a trend towards more severe heartburn in recumbent position (3.30 ± 0.46 *vs* 2.30 ± 0.38, $P = 0.116$), and bitter, acid or sour taste in the mouth (3.50 ± 0.44 *vs* 2.30 ± 0.36, $P = 0.058$) amongst GpCO taking more than 60 mg morphine equivalents per day (results not shown).

When we compared GpCO with severe delay in gastric emptying (> 35% at 4 h) with GpCO with mild to moderate delay in gastric emptying, there was no difference in symptom severity on PAGI-SYM questionnaire, and we did not notice any differences in the impact of gastroparesis on their employment and healthcare utilization (results not shown).

GpCO had a longer duration of nausea per day in the past week (median of 7 h *vs* 4 h in GpNO, $P = 0.037$), as well as higher number of vomiting episodes per day (median of 3 *vs* 1 in GpNO, $P = 0.002$) but there was no statistically significant difference in the number bowel movements in the past week (Table 5).

AP was frequently present in GpCO and GpNO (84.1% and 85.2% respectively, $P = 0.861$). Epigastrium was the most common location of the most severe AP in GpNO (44.2%); while GpCO most commonly had their most severe AP in periumbilical area (41.7%) followed by epigastric area (38.9%), $P = 0.863$. AP got worse with meal intake in majority of patients (76.5% in GpCO *vs* 80.0% in GpNO, $P = 0.508$). There was no difference in the overall duration of AP (median of 2 years in GpCO *vs* 1.5 years in GpNO, $P = 0.526$). AP more frequently woke up GpCO at night compared to GpNO (78.1% *vs* 57.3%, $P = 0.031$).

Nausea and vomiting were more frequent in GpCO in the past 3 mo (Table 6); 92.5% of GpCO had nausea interfering with their activities at least once a week or more frequently *vs* 76% amongst GpNO ($P = 0.021$).

Employment

The employment rate was lower in GpCO compared to GpNO (33.3% *vs* 54.2%, $P = 0.016$). The average number of working hours per week in GpCO (who were employed) was also lower than GpNO (median of 23 h *vs* 40 h respectively, $P = 0.005$).

It is plausible that a higher severity of abdominal pain in GpCO compared to GpNO was affecting employment. However, when we studied only those patients who had moderate to very severe abdominal pain as one of the symptoms from gastroparesis, GpCO still had a lower rate of employment (35.9% *vs* 54.5%, $P = 0.037$), and lower number of working hours per week (median of 20 h *vs* 40 h, $P = 0.003$), compared to GpNO. In GpCO on more than 60 mg morphine equivalents per day, there was a trend towards less working hours per week compared to GpCO on 60 mg or less morphine equivalents per day (median of 17.5 h *vs* 26 h, $P = 0.071$).

Health care utilization

There were higher number of hospital admissions for GpCO in the past year compared to GpNO (2.90 ± 0.77 *vs* 1.26 ± 0.23, $P = 0.047$) (Table 7). There was a trend towards a higher number of emergency room

Table 2 Gastroparesis patients: Demographics, employment, social history, laboratory tests and gastric emptying test in chronic opioid using gastroparesis patients, and patients with no opioid use

	Chronic scheduled opioids	No opioids	*P* value
Age (median in years with IQR)	49.0 (34.0-56.0)	[4]41.0 (30.0-55.0)	0.091
Age symptoms started (median in years with IQR)	40.0 (24.3-52.0)	[4]33 (22.8-47.3)	0.281
Average duration of symptoms (median in years)	[4]3.0 (1.0-15.5)	[4]4.0 (1.0-10.0)	0.937
Previously established diagnosis of Gp (%)	90.7% (39/43)	86.7% (137/158)	0.482
Female (%)	74.4% (32/43)	81.0% (128/158)	0.341
Body mass Index (median in kg/m^2 with IQR)	[4]24..3 (22.1-29.3)	[4]24.7 (20.3-30.8)	0.983
Race (% White)	83.3% (35/42)	83.9% (125/149)	0.917
Residing outside catchment area (50 miles)	53.5% (23/43)	51.6% (80/155)	0.828
Diabetes (%)	35.7% (15/42)	25.9% (41/158)	0.055
Surgery on stomach/esophagus (%)	28.6% (12/42)	19.1% (28/147)	0.136
Employed (%)	33.3% (14/42)	54.2% (84/155)	0.016
Working hours per week (median with IQR)	23.0 (10.5-35.0)	[4]40 (24.5-40.0)	0.005
Smoking history, current or past (%)	46.3% (19/41)	31.2% (48/154)	0.069
Current smoker (%)	31.7% (13/41)	13.0% (20/154)	0.004
Alcohol history, current or past (%)	23.8% (10/42)	37.4% (58/155)	0.100
Current alcohol use (%)	7.3% (3/41)	20.0% (30/150)	0.057
Random cortisol (% with low cortisol[1])	16.2% (6/37)	10.4% (11/106)	0.345
Hemoglobin A1c (median with IQR)	[4]5.9% (5.3%-7.7%)	[4]5.7% (5.4%-6.4%)	0.377
Thyroid stimulating hormone (% with high TSH[2])	4.9% (2/41)	1.7% (2/116)	0.271
Trypsinogen (% with low trypsinogen[3])	23.1% (6/26)	4.2% (3/72)	0.004
History of chronic pancreatitis	7.0% (3/43)	1.3% (2/158)	0.033
Gastric emptying scintigraphy: Retention at 2 h (median with IQR)	62% (50%-80%)	[4]66% (50%-72%)	0.359
Gastric emptying scintigraphy: Retention at 4 h (median with IQR)	[4]22% (14%-42%)	[4]24% (15%-35%)	0.522

[1]Low cortisol: AM less than 6.2 μg/dL (171 nmol/L), PM less than 2.3 μg/dL (63.4 μg/dL); [2]High TSH: Greater than 4.50 μIU/mL; [3]Low trypsinogen: Less than 19 ng/mL. [4]Results with non-normal distribution. Results expressed as median ± interquartile range, or percentage as appropriate. Gp: Gastroparesis; IQR: Interquartile range; TSH: Thyroid stimulating hormone.

Table 3 Comparison of gastric emptying scintigraphy results at different morphine equivalents per day in gastroparesis patients on chronic opioids using analysis of variance

Gastric emptying scintigraphy	1st quartile (≤ 22.5 mg of morphine equivalents per day)	2nd quartile (> 22.5 mg/d, and ≤ 60 mg/d)	3rd quartile (> 60 mg/d, and ≤ 112.5 mg/d)	4th quartile (> 112.5 mg/d)	*P* value
Retention at 2 h (mean ± SEM)	68% ± 6%	54% ± 8%	84% ± 5%	60% ± 9%	0.157
Retention at 4 h (mean ± SEM)	31% ± 7%	21% ± 8%	37% ± 10%	36% ± 19%	0.678

SEM: Standard error of mean.

visits in the past year in GpCO compared to GpNO (5.13 ± 1.46 *vs* 3.74 ± 0.65) but this did not reach statistical significance ($P = 0.468$).

DISCUSSION

Our study shows that chronic opioid use is present in nearly one fifth of the gastroparesis patients referred for evaluation and treatment of their gastroparesis. Use of chronic opioids in these gastroparesis patients was associated with a higher severity of many gastrointestinal symptoms, especially the symptoms often attributable to gastroparesis. In addition, patients on chronic opioids had decreased work productivity, and more frequent hospitalizations.

The prevalence of chronic scheduled opioid use in our study (19.2%) is less than 30%-46% reported in other studies[10,14,15]. It is plausible that many of our patients on opioids as needed were also taking opioids at least once a day, and including these patients gives a much higher prevalence of opioid use (29.1%). A significantly higher proportion of the Gp patients use opioids compared to the general adult population, which is estimated to be 3%[30].

Our study shows that Gp patients on chronic scheduled opioids have a higher severity of many upper gastrointestinal symptoms especially in those symptoms often attributable to gastroparesis. A higher frequency of upper gastrointestinal symptoms in patients taking opioids for chronic non-cancer pain (CNCP) has previously been reported[2]. Females have been shown to have a 60% greater chance of experiencing nausea and vomiting after receiving opioid therapy, which is noteworthy as majority of patients who suffer from Gp are females[12]. The female preponderance in our study is consistent with the previous literature that shows a higher prevalence of Gp and other functional gastrointestinal disorders in females[31]. The most common types of Gp in our population (idiopathic and diabetic) were also similar

Table 4 Symptom Severity as assessed with Patient Assessment of Upper Gastrointestinal Symptoms questionnaire; comparison between gastroparesis patients on chronic opioids and patients with no opioid use

GI symptom	GpCO (n = 43)	GpNO (n = 158)	P value
Nausea	4.09 ± 0.12	3.41 ± 0.12	0.011
Retching	2.86 ± 0.25	1.98 ± 0.14	0.003
Vomiting	2.93 ± 0.24	2.07 ± 0.15	0.011
Stomach fullness	3.84 ± 0.18	3.59 ± 0.11	0.254
Early satiety	4.17 ± 0.19	3.57 ± 0.12	0.004
Post prandial fullness	4.14 ± 0.18	3.63 ± 0.11	0.022
Loss of appetite	3.64 ± 0.21	3.04 ± 0.13	0.039
Bloating	3.67 ± 0.19	3.36 ± 0.13	0.396
Abdominal distension	2.95 ± 0.25	3.01 ± 0.14	0.753
Upper AP	3.86 ± 0.20	2.93 ± 0.13	0.001
Upper abdominal discomfort	3.74 ± 0.19	3.09 ± 0.13	0.031
Lower AP	2.67 ± 0.27	2.38 ± 0.13	0.315
Lower abdominal discomfort	2.79 ± 0.25	2.38 ± 0.13	0.130
Heartburn during day	2.55 ± 0.27	1.89 ± 0.13	0.032
Heartburn on lying down	2.76 ± 0.28	1.94 ± 0.14	0.008
Chest discomfort during day	2.42 ± 0.20	1.83 ± 0.12	0.018
Chest discomfort at night	2.40 ± 0.23	1.61 ± 0.13	0.003
Regurgitation or reflux during day	2.77 ± 0.25	2.18 ± 0.13	0.040
Regurgitation or reflux on lying down	2.64 ± 0.28	2.21 ± 0.14	0.120
Bitter/acid/sour taste	2.79 ± 0.27	2.11 ± 0.14	0.028
Constipation	2.92 ± 0.30	2.63 ± 0.14	0.296
Diarrhea	1.80 ± 0.30	1.79 ± 0.14	0.891
Total Symptom Severity Score	68.40 ± 2.82	56.63 ± 1.77	0.001

[1]Results with non-normal distribution. Results expressed as median with interquartile range, and percentage as appropriate. AP: Abdominal pain; BMs: Bowel movements; GpCO: Gastroparesis patients on chronic opioids; GpNO: Gastroparesis patients not on opioids.

Table 5 Comparison of gastrointestinal symptoms between gastroparesis patients on chronic opioids and patients with no opioid use trial[10,11,27]

	GpCO	GpNO	P value
Episodes of vomiting in last 1 wk	13.0 (1.0-7.0)	11.0 (0.0-3.0)	0.002
Hours of nausea/day in last 1 wk	17.0 (3.0-18.0)	14 (1.5-12.0)	0.037
Total number of BMs in last 1 wk	14.0 (2.0-7.0)	4.0 (2.0-7.0)	0.714
AP one of the symptoms (%)	84.1% (37/43)	85.2% (132/155)	0.861
Duration of AP (yr)	12.0 (0.5-4.0)	[1]1.5 (0.7-4.5)	0.526
Location of most severe AP	Umbilical 41.7% (15/36)	Epigastric 44.2% (57/129)	0.863
AP wakes up at night (%)	78.1% (7/32)	57.3% (71/124)	0.031
AP worse with meals (%)	76.5% (26/34)	80.0% (100/125)	0.508

[1]Results with non-normal distribution. Results expressed as median with interquartile range, and percentage as appropriate. AP: Abdominal pain; BMs: Bowel movements; GpCO: Gastroparesis patients on chronic opioids; GpNO: Gastroparesis patients not on opioids.

to previous studies on Gp[10,31].

In our study, the higher prevalence of opioid use in patients with diabetic gastroparesis compared to patients with idiopathic gastroparesis is possibly related to other co-morbidities, as both diabetic and idiopathic gastroparesis patients were equally likely to have abdominal pain as one of their symptoms of Gp, and there was no statistically significant difference in the severity of their abdominal pain or discomfort on PAGI-SYM questionnaire (results not shown). GpCO with diabetes more frequently reported using opioids for leg pain and/or neuropathy (33.3%) *vs* GpCO with idiopathic gastroparesis (21.7%), though the difference was not significant (P > 0.05).

The prevalence of abdominal pain in our study groups (> 80%) is towards the higher end of the reported prevalence of abdominal pain in Gp (42%-89%)[15]. Epigastrium was the most common location of the most severe AP, and majority of the patients with AP experienced post-prandial worsening of pain, similar to a previous study performed at our center on a different cohort of Gp patients[32]. Every 3 in 4 GpCO in our study woke up at night from AP, higher than about 1 in 2 of our GpNO patients and significantly higher than the reported 1 in 4 patients on opioids for CNCP reported by Tuteja *et al*[2]. It is possible that the abdominal pain may be the reason these patients were taking opioids. Alternatively, there could be enhanced pain perception in disabling narcotic bowel syndrome.

In this cohort, GpCO did not have increased severity of constipation, which is historically one of

Table 6 Frequency of symptoms in the past 3 mo using Rome IV questionnaire: comparison between gastroparesis patients with chronic scheduled opioid use and patients with no opioid use (percentage of patients with symptoms once a week or more often)

Symptom	GpCO	GpNO	*P* value
Post-prandial fullness interfering with activities	81.6% (31/38)	78.7% (118/150)	0.692
Unable to finish regular sized meal due to fullness	84.6% (33/39)	79.1% (117/148)	0.438
Epigastric pain/burning interfering with activities	75.0% (30/40)	66.9% (103/154)	0.324
Nausea interfering with activities	92.5% (37/40)	76.0% (117/154)	0.021
Vomiting	70.7% (29/41)	50.6% (78/154)	0.022
Bloating or stomach distension	72.5% (29/40)	68.8% (106/154)	0.653
Belching interfering with activities	62.5% (25/40)	51.3% (79/154)	0.206

GpCO: Gastroparesis patients on chronic opioids; GpNO: Gastroparesis patients not on opioids.

the most commonly reported symptoms of opioid induced bowel dysfunction[2]. Fentanyl was the second most commonly used opioid in GpCO, and a randomized cross over trial showed a lower prevalence of constipation in patients taking transdermal fentanyl compared to sustained release oral morphine[7]. Even though our questionnaire did not ask specifically about usage of stool softeners or laxatives, some of these patients had stool softeners (n = 4), stimulant laxatives (2) and osmotic laxative (1) listed on their medication list, and it is plausible that many other patients were on these over-the-counter medications as well, as these medications are often taken prophylactically in patients on chronic opioids[6,33]. Future studies can look into the prevalence of laxative use in opioid using gastroparesis patients. Our finding of higher severity of many gastrointestinal symptoms in GpCO compared to opioid-naïve Gp patients, despite no statistically significant difference in the 2 and 4 h retention in gastric emptying tests between the two groups is not novel, as Karamanolis et al showed that the symptom pattern in Gp is not determined by the severity of delay in gastric emptying[34].

Patients with Gp may have symptoms that mimic clinical manifestations of CP. In fact, Chowdhury *et al*[24] reported that 44% of small duct CP patients may have concomitant Gp. While the prevalence of CP in the general population is only 41.76 per 100000[35], the prevalence of CP in patients with Gp is not known. In our study, GpCO (7%) were more likely to have a history of CP compared to GpNO (1.3%). In addition, nearly one fourth (23.1%) of GpCO who had their trypsinogen levels checked had low levels, compared to < 5% in GpNO, suggesting some of our Gp patients using opioids chronically possibly had severe calcific CP with associated Gp, which might have been causing them abdominal pain. While the sensitivity of serum trypsinogen level in diagnosing CP was only 28%, the specificity was 100% in a study by Pezzilli *et al*[36].

Gp patients have been reported to have the longest length of hospital stay (5 d) amongst the functional gastrointestinal disorders[31]. Recent studies suggest increasing hospitalization due to Gp over the past 20 years[37-39]. A Nationwide Inpatient Sample Study reported 17220 admissions from Gp in 2012, a more than 400% increase compared to 1997[39]. Some authors suggest that the increasing hospitalization due to Gp comes from better recognition of this disorder[40]. Our study suggests a higher number of hospitalizations in GpCO than in Gp patients not taking opiates.

Our finding that GpCO are more likely to be unemployed and work less is consistent with the multi-national questionnaire based study showing that the chronic use of opiates negatively influences the quality of life[41]. We found a higher prevalence of current smoking in GpCO, and a study by Young-Wolff *et al*[42] suggested a higher likelihood of opioid use disorder in current smokers *vs* non-smokers.

Our study has some limitations. Whether opioid use is to manage a higher severity of Gp symptoms, or is responsible for the higher severity of symptoms is unclear as we do not have symptoms of patients prior to starting opioids. Increased opioid use in Gp patients with moderate to severe abdominal pain has previously been reported[15], however nausea, vomiting and retching are frequently reported side effects of opioids and it is plausible that the opioid use itself explains the higher severity of these symptoms amongst GpCO in our study. Secondly, these patients were generally referred from community settings and over half of these patients were referred from outside the catchment area of our tertiary care center, with GpCO and GpNO equally likely to be referred from outside the catchment area. This was a questionnaire based study. Some of the questions were not answered by all the patients, and some patients did not go for the laboratory tests that we requested. These missed questions and laboratory tests were excluded from analyses, however their number were relatively small in most cases, and likely did not to affect the results. Lastly, since this is a questionnaire based study, there is a potential of recall bias as well. This would likely apply both to patients taking and those not taking opioid analgesics.

In conclusion, chronic regular opioid use is present in a significant number (19.3%) of gastroparesis patients. These patients have a higher severity of many gastrointestinal symptoms including those of Gp. They have decreased work productivity compared to non-opioid using Gp patients. Whether opioid use is to

Table 7 Comparison of healthcare utilization between chronic opioid using gastroparesis patients and patients with no opioid use

	GpCO (n = 43)	GpNO (n = 158)	P value
ER visits in last 1 yr from Gp	5.13 ± 1.46	3.74 ± 0.65	0.468
Hospital admissions in last 1 yr from Gp	2.90 ± 0.77	1.26 ± 0.23	0.047

Results expressed as mean ± SE of mean. ER: Emergency room; GpCO: Gastroparesis patients on chronic opioids; GpNO: Gastroparesis patients not on opioids.

treat a higher symptom severity from Gp, or opioid use itself worsens symptoms in patients with Gp requires further study.

ARTICLE HIGHLIGHTS

Research frontiers

Despite the gastrointestinal side effects associated with opioid use, they are used in some patients with gastroparesis. The relationship of opioid use to the gastrointestinal symptoms, healthcare utilization and employment is not known.

Research motivation

As opioid use had become a healthcare epidemic in United States, studies on opioid use in gastroparesis would be useful for clinicians and researchers.

Research objectives

This objective was to study the relationship of chronic scheduled opioid use to gastrointestinal symptoms, healthcare utilization and employment in gastroparesis patients.

Research methods

The authors used Mann Whitney *U* Test, Student's *t*-test, Analysis of Variance, and χ^2 test as appropriate for data analysis.

Research results

This study shows higher severity of many gastrointestinal symptoms, and more frequent hospitalizations in gastroparesis patients on chronic scheduled opioids, compared to gastroparesis patients not using opioids. Chronic opioid using patients also reported their work being effected more frequently by their gastrointestinal symptoms. The prevalence of chronic pancreatitis is also higher in opioid using gastroparesis patients.

Research conclusions

This study confirmed the hypothesis that chronic opioid use in gastroparesis is related with more severe gastrointestinal symptoms, and hospitalizations. Whether opioid use is to manage a higher severity of gastroparesis symptoms, or is responsible for the higher severity of symptoms is not clear as we did not have symptoms of patients prior to starting opioids. In clinical practice, this study implicates that the opioids may need to be used with caution in gastroparesis patients.

Research perspectives

Opioid use is quite prevalent in patients with gastroparesis. Opioid-using gastroparesis patients have more severe gastrointestinal symptoms. These opioid-using patients are more frequently hospitalized, compared to the patients without opioid use. They also more commonly report their employment being affected due to their gastrointestinal symptoms. Patients with gastroparesis may have chronic pancreatitis, possibly contributing to their gastrointestinal symptoms.

Future studies can look into the trends of laxative-use in opioid-using gastroparesis patients. The noticeable prevalence of chronic pancreatitis in gastroparesis patients in this study can be further confirmed in studies with larger sample size. This study found more frequent hospitalizations in gastroparesis patients; future studies to evaluate opioid use during hospitalizations in gastroparesis patients will add useful information to the current literature on gastroparesis.

Future research can look into opioid use in gastroparesis through different perspectives, this could be not only in the tertiary care centers, but also in smaller community settings so that the results more accurately reflect the generalized population. Moreover, bigger databases using diagnosis codes and medication-lists can be used to get a larger sample size.

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ORIGINAL ARTICLE

Observational Study

Medication beliefs predict medication adherence in ambulatory patients with decompensated cirrhosis

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Abstract

AIM

To investigate the impact of medication beliefs, illness perceptions and quality of life on medication adherence in people with decompensated cirrhosis.

METHODS

One hundred adults with decompensated cirrhosis completed a structured questionnaire when they attended for routine outpatient hepatology review. Measures of self-reported medication adherence (Morisky Medication Adherence Scale), beliefs surrounding medications (Beliefs about Medicines Questionnaire), perceptions of illness and medicines (Brief Illness Perception Questionnaire), and quality of life (Chronic Liver Disease Questionnaire) were examined. Clinical data were obtained *via* patient history and review of medical records. Least absolute shrinkage and selection operator and stepwise backwards regression techniques were used to construct the multivariable logistic regression model. Statistical significance was set at alpha = 0.05.

RESULTS

Medication adherence was "High" in 42% of participants, "Medium" in 37%, and "Low" in 21%. Compared to patients with "High" adherence, those with "Medium" or "Low" adherence were more likely to report difficulty affording their medications (P < 0.001), lower perception of treatment helpfulness (P = 0.003) and stronger medication concerns relative to medication necessity beliefs (P = 0.003). People with "Low" adherence also experienced greater symptom burden and poorer quality of life, including more frequent abdominal pain (P = 0.023), shortness of breath (P = 0.030), and emotional disturbances (P = 0.050). Multivariable analysis identified having stronger medication concerns relative to necessity beliefs (Necessity-Concerns Differential $\leqslant$ 5, OR = 3.66, 95%CI: 1.18-11.40) and more frequent shortness of breath (shortness of breath score $\leqslant$ 3, OR = 3.87, 95%CI: 1.22-12.25) as independent predictors of "Low" adherence.

CONCLUSION

The association between "Low" adherence and patients having strong concerns or doubting the necessity or helpfulness of their medications should be explored further given the clinical relevance.

Key words: Medication adherence; Medication beliefs; Illness perceptions; Quality of life; Liver cirrhosis

Core tip: Medication non-adherence is common in people with decompensated cirrhosis but the impact that patients' medication beliefs and illness perceptions have on adherence is under-recognised. Clinician engagment with non-adherent patients should include open discussion of medications and liver disease. Acknowledgement of patient concerns surrounding their medicines, with positive reinforcement of medication necessity in terms of disease management may improve adherence behaviour and patients' quality of life.

Hayward KL, Valery PC, Martin JH, Karmakar A, Patel PJ, Horsfall LU, Tallis CJ, Stuart KA, Wright PL, Smith DD, Irvine KM, Powell EE, Cottrell WN. Medication beliefs predict medication adherence in ambulatory patients with decompensated cirrhosis. *World J Gastroenterol* 2017; 23(40): 7321-7331 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i40/7321.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i40.7321

INTRODUCTION

People with decompensated cirrhosis require intensive inpatient and outpatient management, experience poor quality of life (QoL), and have a median survival of approximately two years. While liver transplantation is a viable treatment for end-stage liver disease, this is not an option for many patients. A complex regimen of medications is usually prescribed to manage complications of portal hypertension and liver insufficiency, however medication mismanagement and non-adherence is relatively common among patients with decompensated cirrhosis.

Medication adherence is defined by the World Health Organisation (WHO) as "the extent to which a person's behaviour - taking medication, following a diet, and/or executing lifestyle changes - corresponds with agreed recommendations from a health care provider"[1]. Similar to other chronic diseases where approximately 50% of patients are thought to be non-adherent, up to 70% of patients with cirrhosis identify

as having "Low" or "Medium" levels of medication adherence[2].

Non-adherence with medications has been associated with increased mortality in diabetes, coronary heart disease and heart failure[3,4]. It has been estimated that between 22% and 37% of 30-d readmissions among patients with decompensated cirrhosis may be potentially preventable with improved management of pharmacotherapy[5,6]. For example, non-adherence with lactulose, a non-absorbable disaccharide syrup used in the treatment of hepatic encephalopathy (HE), is reported to be as high as 69%[7], and has been associated with approximately 36% of potentially preventable 30-d readmissions[6]. Intentional non-adherence due to adverse effects (diarrhoea, flatulence and abdominal pain)[7] or misunderstanding of the indication[6] carries a 3-fold risk of HE recurrence[8]; a substantial, potentially preventable burden on patients, carers and the healthcare system[9]. The one-year survival probability following an episode of overt HE is 42%[10], and persisting cognitive impairment or covert HE[11] may in turn lead to unintentional non-adherence with other medications.

Non-adherence and mismanagement of diuretic therapy, which is prescribed in the management of abdominal ascites, peripheral oedema or pleural complications, contributes to 55% of potentially preventable 30-d readmissions in people with decompensated cirrhosis[6]. While prevalence has not been reported in cirrhosis, 30%-66% of patients prescribed loop diuretics in the management of heart failure are non-adherent with therapy[12,13]. Incorrect use of diuretics in cirrhosis can lead to severe electrolyte disturbances and renal impairment, and may contribute to a requirement for recurrent large volume paracentesis. Bacterial infections in people with decompensated cirrhosis are common and often precipitate further deterioration. Spontaneous bacterial peritonitis (SBP) carries a mortality rate of 31.5% at one month and 66.2% at one year[14], with a one-year recurrence rate of 61% without prophylaxis[15]. Prophylaxis with antibiotic therapy considerably reduces SBP recurrence, development of hepatorenal syndrome, and improves probability of one year survival[15], however adherence with long-term antibiotics is known to be low in other patient groups[16,17].

The WHO has identified non-adherence as an international priority in the prevention of patient harm and optimisation of limited health resources[1]. However, medication adherence is a complex health behaviour that is the result of numerous interacting dynamic variables including health literacy, self-efficacy, psychological perceptions of medicines and disease, quality of life, and other internal and external barriers[1]. Previous studies in liver disease patients have identified that side effects and changes to routine are the most commonly cited reasons for non-adherence[7,18]. Illness perceptions have been shown to influence patients' confidence to self-manage alcoholic liver disease and limit self-efficacy[19], while self-perceived disease stigma can affect medical-care seeking behaviours and impact on QoL[20]. However there is limited information available about how perceptions and beliefs surrounding medications and liver disease affect adherence behaviours in people with decompensated cirrhosis. Identifying potentially-modifiable factors that influence adherence behaviour is important, as strategies to support adherence can be tailored to individual patients' needs.

The aim of this study was to: (1) investigate medication non-adherence in a cohort of ambulatory patients with decompensated cirrhosis; and (2) to identify the effect of patients' medication beliefs, illness perceptions, quality of life and clinical and demographic factors on medication non-adherence.

MATERIALS AND METHODS

A convenience sample of adult patients with cirrhosis who had experienced a decompensating event (abdominal ascites, spontaneous bacterial peritonitis, hepatic hydrothorax, encephalopathy or variceal bleeding) within the preceding two years were invited to participate when they attended routine outpatient follow-up at the Princess Alexandra Hospital in Brisbane, Australia, between February and October 2016. Patients were excluded if they were less than 18 years of age, unable to provide informed consent, undergoing transplant workup, or receiving intensive management by the palliative care team.

A structured questionnaire was used to obtain measures of medication adherence, quality of life, medication beliefs and illness perceptions. Questionnaires were completed independently by the patient or with the assistance of a carer, family member, or study coordinator, according to patient preference. Clinical data was collected from patients and/or their medical records, including standard biochemical and serological assays and liver imaging to confirm the diagnosis of cirrhosis and decompensation history. Socio-demographic items included patient-reported individual-based measures of education level and employment status, and residential area-based measures (Index for Relative Socioeconomic Disadvantage[21] and the Accessibility/Remoteness Index of Australia[22] for classification of remoteness of residence).

Medication adherence

Self-reported medication adherence was examined using the 8-Question Morisky Medication Adherence Scale (MMAS-8) which contains seven questions with yes/no alternatives, and one question which features a 5-point Likert scale. The scores from completed

questionnaires are categorised into "High" (score = 8), "Medium" (score 6 to < 8) and "Low" (score < 6) adherence groups[23-25].

Medication beliefs

The Beliefs about Medications Questionnaires (BMQ-General and BMQ-Specific) were used to elicit patients' beliefs about medications. Participants responded to eighteen statements across four domains using a 5-point Likert scale, from "strongly disagree" (score = 1) to "strongly agree" (score = 5). The BMQ-General contains 4 items that examine beliefs about Overuse of medicines by doctors, and 4 items about medication Harms. The BMQ-Specific contains 5 items in the Necessity domain and 5 items in the Concerns domain, which elicit patients' respective necessity and concern beliefs about the medicines prescribed for their liver disease. Scores derived from the Concerns domain can be subtracted from the Necessity domain to give a Necessity-Concerns Differential (N-C differential)[26].

Illness perceptions

The Brief Illness Perceptions Questionnaire (Brief-IPQ) was used to examine the strength of patients' perceptions about liver cirrhosis. The Brief-IPQ contains eight items, including identity (severity of symptoms) and consequences of disease on daily life, personal control and treatment control over disease, timeline for disease duration, self-perceived coherence or understanding of the disease, in addition to concerns and emotional representation that are caused by the disease. Patients self-measure each item on a scale from 0 to 10, where one end of the scale represents a benign perception and the other represents a threatening perception of illness[27].

Quality of life

The Chronic Liver Disease Questionnaire (CLDQ) was chosen to measure health related quality of life as it comprises specific domains relevant to the study population[28]. The full CLDQ contains 29 items across six domains; however responses to the twenty-ninth item have been excluded from analysis due to inapplicability to the majority of study participants. The twenty-ninth item pertains to worry about the availability of a liver if the patient requires a liver transplant; however patients being actively assessed for liver transplant were excluded from the study.

The shortened CLDQ used for the present study therefore contained eight questions related to Emotional Function, five questions within the domains of Systemic Symptoms and Fatigue, three questions within the domains of Abdominal Symptoms and daily Activity, and four items related to Worry. Individual item scores range from 1 to 7 and domain scores are calculated by averaging item scores within each domain. Higher scores represent better perceived health-related quality of life.

Data analysis: Statistical review of the study was performed by a biomedical statistician. Data was analysed using IBM® SPSS© Version 20.0. Where a question was missed within a validated tool, but at least 85% of the tool had otherwise been correctly completed, the missing value was imputed based on the mean or median response to that question, according to data skew. Imputation was required for a single value for two participants: one Brief IPQ-timeline (imputed median score 10) and one CLDQ-anxiety (imputed mean value 4).

Continuous and normally-distributed variables are presented as mean ± SD. Differences between groups were analysed by one-way ANOVA. Non-normally distributed data are presented as median (range) and have been analysed using the Kruskal-Wallis *H* test. Categorical data are presented as proportional percent and analysed using Pearson's chi-squared (χ^2), Fisher's Exact test or Linear-by-Linear Associated test for trend as denoted. Statistical significance was set at alpha = 0.05.

The relationship between "Low" medication adherence and patients' clinical, demographic and self-reported medication beliefs, illness perceptions and QoL were determined by calculating the odds ratio (OR) and 95%CI. Continuous variables were systematically assessed to identify optimal cut-points. Least absolute shrinkage and selection operator and stepwise backwards regression techniques were used to construct the final multivariable logistic regression model. Both methodologies identified the same significant factors for inclusion in the final model. The Hosmer-Lemeshow test was used to assess goodness-of-fit. Interactions between individual variables were not found to be statistically significant.

RESULTS

Demographic and clinical

One-hundred and thirty-four eligible patients were invited to participate in the study (Figure 1). Fourteen patients declined. Five patients indicated that they did not take medicines for their liver disease and thus did not complete essential components of the questionnaires. Of these five patients, three were unaware that their current pharmacotherapy included treatment for hepatic decompensation events, and two had ceased their therapy without medical advice. An additional fifteen patients incompletely filled out the questionnaires for other reasons.

Of the 100 patients who completed the questionnaire tools, the majority (65.0%) were male and the mean age was 58.4 ± 10.2 years. Primary disease aetiology was alcoholic liver disease (ALD) in 49 patients, hepatitis C (HCV) in 35, non-alcoholic fatty liver disease in 9, primary sclerosing cholangitis in two, hepatitis B in one, autoimmune hepatitis in one, drug-induced liver injury in one, alpha-1-antitrypsin

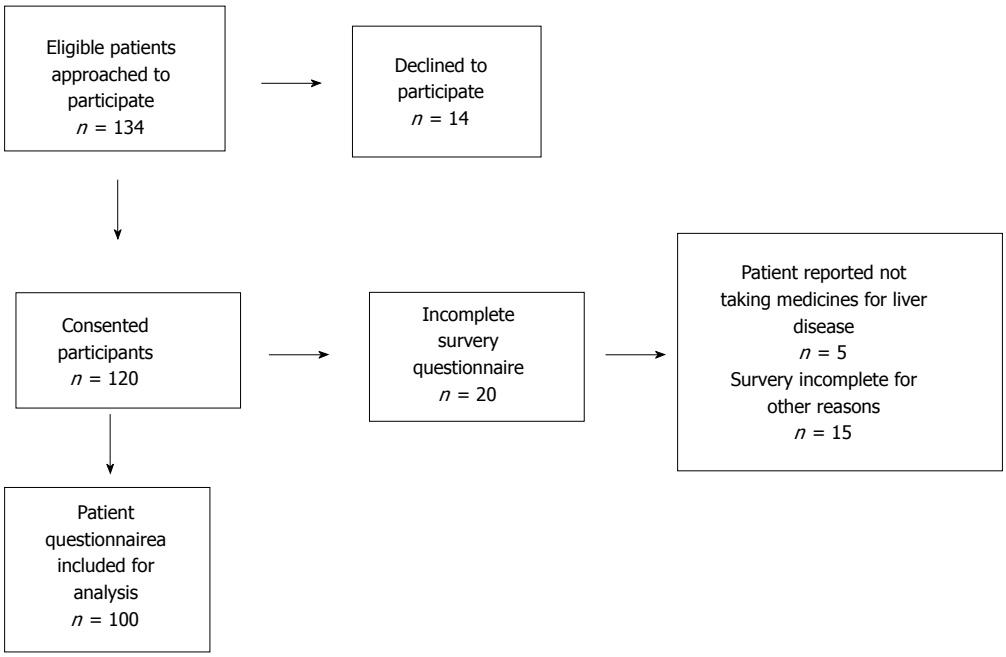

Figure 1 Participant recruitment flow diagram.

deficiency in one and unknown in one. Of the 51 patients who were not considered to have ALD, alcohol was a documented cofactor in 21 patients (41.2%).

Forty-two participants (42%) were categorised as having "High" medication adherence, 37% with "Medium" adherence, and 21% with "Low" adherence. Demographic and clinical characteristics of patients, and their association with medication adherence, are presented in Table 1. Male gender (P = 0.015) and inability to afford medications (P < 0.001) were associated with lower levels of medication adherence. Self-reported ability to afford medicines could not be predicted by employment status, relative sociodemographic disadvantage, or other sociodemographic factors (P > 0.05).

Medication beliefs

Compared to "Low" adherence, there was a non-significant increase in the strength of Necessity beliefs in "High" and "Medium" adherence groups, and a non-significant increase in the strength of Concerns, Harms and Overuse beliefs from "High" to "Low" adherence groups. Compared to patients with "Medium" and "High" medication adherence, patients with "Low" medication adherence reported a lower mean Necessity-Concerns Differential (P = 0.003; Table 2). Three patients had a negative Necessity-Concerns Differential and eight patients had a differential of zero, indicating that their Concerns about their liver disease medicines outweighed or were equal to the perceived Necessity of therapy respectively.

Patients were more likely to have a lower Necessity-Concerns Differential if they were male (6.6 ± 4.3 *vs* 8.5 ± 5.2, P = 0.046), reported inability to afford medications (4.8 ± 4.6 *vs* 7.8 ± 4.5, P = 0.011) or had fewer comorbidities (Pearson's r = 0.263, P = 0.008). Medication beliefs measured using the BMQ scales were not related to age, disease severity, education, sociodemographic status or other clinical and demographic variables.

Illness perceptions

Overall, participants had generally high levels of concern about their liver disease, felt they did not have much personal control over it, and perceived that it would persist for a long duration of time (Table 3). Patients with "Low" medication adherence reported lower perception of how much treatment could help their liver disease (treatment control, P = 0.003) and a lower self-perceived understanding of their liver disease (coherence, P = 0.014).

Patients with HCV reported experiencing more severe symptoms (identity score 6.5 ± 2.4 *vs* 4.9 ± 3.1, P = 0.009), greater impact of disease on daily life (consequences score 6.9 ± 2.4 *vs* 5.5 ± 3.3, P = 0.019) and perceived that their disease would persist for a shorter duration of time (timeline median score 8 [range 0-10] *vs* 10 [range 2-10], P = 0.010) compared to patients who did not have HCV. Female patients reported greater emotional impact of disease (emotional representation score 5.8 ± 3.3 *vs* 4.3 ± 3.4, P = 0.041) while patients with higher levels of education (completed high school, formal trade qualification, university degree *etc.*) perceived greater impact of disease on daily life (consequences score 6.7 ± 2.8 *vs* 5.3 ± 3.2, P = 0.024) compared to patients educated up to middle school. There was a negative correlation between the total Brief-IPQ and CLDQ scores (r = -0.707, P < 0.001), indicating that stronger threatening perceptions of illness are associated with

Table 1 Demographic and clinical factors in patients with high, medium and low medication adherence *n* (%)

Demographic and clinical variables		All patients	Medication adherence ranking			*P* value
			High (*n* = 42)	Medium (*n* = 37)	Low (*n* = 21)	
Age		58.4 ± 10.2	57.9 ± 10.9	58.2 ± 9.0	57.8 ± 10.8	0.787
Male gender		65 (65.0)	21 (50.0)	30 (81.1)	14 (66.7)	0.015
Primary aetiology	ALD	49 (48.0)	19 (45.2)	19 (51.4)	11 (52.4)	0.842
	HCV	35 (33.0)	14 (33.3)	13 (35.1)	8 (38.1)	0.963
	Other	16 (16.0)	9 (21.5)	5 (13.5)	2 (9.5)	0.383
[1]Child-Turcotte Pugh class	A	24 (24.0)	6 (14.3)	12 (32.4)	6 (28.6)	0.684
	B	59 (59.0)	29 (69.0)	21 (56.8)	9 (42.8)	
	C	17 (17.0)	7 (16.7)	4 (10.8)	6 (28.6)	
MELD score		14.4 ± 5.2	14.6 ± 4.6	14.2 ± 5.1	14.2 ± 6.7	0.936
Ascites at review (incl. suppressed by medication)		80 (80.0)	37 (88.1)	28 (75.7)	15 (71.4)	0.187
Encephalopathy at review (incl. suppressed by medication)		36 (36.0)	12 (28.6)	13 (35.1)	11 (52.4)	0.184
Hepatocellular carcinoma		8 (8.0)	3 (7.1)	3 (8.1)	2 (9.5)	1.00
Number of self-reported medicines		7.1 ± 3.5	7.2 ± 3.7	7.1 ± 3.6	6.9 ± 3.1	0.923
Number of comorbidities		5.5 ± 2.8	5.4 ± 2.8	5.8 ± 3.0	5.2 ± 2.5	0.703
[2]Unable to afford medicines		19 (20.2)	1 (2.5)	12 (36.4)	6 (28.6)	< 0.001
[3]Education	Nil, Primary, Middle school	39 (42.4)	14 (34.1)	13 (43.3)	12 (57.1)	0.215
	High school, Trade, University	53 (57.6)	27 (65.9)	17 (56.7)	9 (42.9)	
[4]Employment status	Employed	18 (19.1)	9 (22.0)	6 (18.2)	3 (14.3)	0.842
	Government welfare	72 (76.6)	30 (73.2)	25 (75.8)	18 (85.7)	0.602
ARIA	Highly accessible	89 (89.0)	36 (85.7)	34 (91.9)	19 (90.5)	0.713
	Accessible–remote	11 (11.0)	6 (14.3)	3 (8.1)	2 (9.5)	
IRSD	Most disadvantaged	32 (32.0)	16 (38.1)	7 (18.9)	9 (42.9)	0.093
	Low–moderate disadvantage	68 (68.0)	26 (68.9)	30 (81.1)	12 (57.1)	

Normally distributed data presented as mean ± SD and analysed using one-way ANOVA. Categorical data presented as proportional percent (%) of column (adherence ranking) and analysed using Pearson's χ^2 or Fisher's Exact test unless otherwise noted. [1]Analysed using the Linear-by-Linear Exact Association test for trend; [2]Excluding 6 patients for whom data was not available (*n* = 40 High adherers, *n* = 33 Medium adherers and *n* = 21 Low adherers); [3]Excluding 8 patients for whom data was not available (*n* = 41 High adherers, *n* = 30 Medium adherers and *n* = 21 Low adherers); [4]Excluding 5 patients for whom data was not available (*n* = 41 High adherers, n = 33 Medium adherers and *n* = 21 Low adherers). *P*-values represent "Employed" *vs* "Unemployed" (data not shown), and "Government Welfare" *vs* "Not receiving Government Welfare" (data not shown) respectively. Two patients reported concurrent part-time employment and Government Welfare support and are represented twice. "Employed" includes full-time, part-time, casual and self-employment. "Government Welfare" includes disability support, aged pension, carer's pension, total permanent disability, Newstart allowance. Six patients reported no active income (living off savings or financially supported by family). ALD: Alcoholic liver disease; HCV: Hepatitis C virus ARIA: Accessibility/Remoteness Index of Australia; IRSD: Index for relative socioeconomic disadvantage.

Table 2 Responses to the beliefs about medications questionnaires in patients with high, medium and low medication adherence

Medication beliefs domains	All patients (*n* = 100)	Medication adherence ranking			*P* value
		High (*n* = 42)	Medium (*n* = 37)	Low (*n* = 21)	
Necessity	19.3 ± 3.8	19.6 ± 3.1	19.9 ± 4.1	17.6 ± 4.0	0.064
Concerns	12.0 ± 3.6	11.2 ± 3.3	12.2 ± 3.3	13.3 ± 4.2	0.074
Necessity-Concerns Differential	7.3 ± 4.7	8.4 ± 4.8	7.6 ± 4.2	4.3 ± 4.2	0.003
Harms	8.3 ± 2.5	7.9 ± 2.8	8.3 ± 2.0	8.8 ± 2.7	0.405
Overuse	10.3 ± 3.1	9.5 ± 3.0	10.7 ± 3.0	11.3 ± 2.9	0.053

Data presented as mean ± SD and analysed using one-way ANOVA. Higher scores indicate stronger medication beliefs within a given domain. Harms and Overuse scores range from 5 to 20. Necessity and Concerns scores range from 5 to 25. The Necessity-Concerns Differential (score range 0 to 20) is calculated by subtracting individual patients' Concern scores from their Necessity scores.

lower quality of life.

Quality of life

The greatest overall health-related QoL impacts reported by patients were in the domains of Fatigue and Worry. Impact of QoL on medication adherence is presented in Table 4. "Low" medication adherence was associated with lower QoL in terms of shortness of breath that impacted on daily activity (*P* = 0.030), greater emotional disturbances (*P* = 0.050), particularly irritability (*P* = 0.017) and mood swings (*P* = 0.031), and greater frequency of abdominal and bodily pain (*P* = 0.023 and *P* = 0.037, respectively). Patients with moderate or large ascites at the time of review reported greater impact of abdominal bloating (CLDQ-abdominal bloating score 3.47 ± 2.04 *vs* 4.69 ± 1.88, *P* = 0.014) and those with a history of HE reported more frequent irritability (CLDQ-irritability score 4.31 ± 1.57 *vs* 5.04 ± 1.72, *P* = 0.029), but these did not translate into an effect on medication

Table 3 Illness perceptions in patients with high, medium and low medication adherence

Brief illness perception questionnaire items	All patients (n = 100)	Medication adherence ranking			P value
		High (n = 42)	Medium (n = 37)	Low (n = 21)	
Consequences How much does your liver disease affect your life? 0 = No affect; 10 = Severely affects my life	6.0 ± 3.1	6.1 ± 3.0	6.0 ± 3.1	5.7 ± 3.2	0.846
Timeline How long do you think your liver disease will continue? 0 = Very short time; 10 = Forever	10.0 (0-10)	10 (3-10)	10 (0-10)	10 (3-10)	0.962
Personal Control How much control do you feel you have over your liver disease? 0 = Absolutely no control; 10 = Extreme amount of control	4.8 ± 3.0	4.6 ± 3.2	4.8 ± 2.9	5.0 ± 2.8	0.893
Treatment Control How much do you think your treatment can help your liver disease? 0 = Not at all; 10 = Extremely helpful	8.0 (0-10)	8 (2-10)	10 (4-10)	7 (0-10)	0.003
Identity How much do you experience symptoms from your liver disease? 0 = No symptoms; 10 = Many severe symptoms	5.5 ± 3.0	5.6 ± 3.0	5.5 ± 3.0	5.2 ± 2.9	0.905
Concern How concerned are you about your liver disease? 0 = Not at all; 10 = Extremely concerned	8.0 (0-10)	8 (0-10)	9 (0-10)	8 (0-10)	0.416
Coherence How well do you feel you understand your liver disease? 0 = Don't understand at all; 10 = Understand very clearly	8.0 (0-10)	8 (3-10)	8 (0-10)	7 (2-9)	0.014
Emotional Representation How much does your liver disease affect you emotionally? 0 = Not at all; 10 = Extremely affected emotionally	4.9 ± 3.4	4.7 ± 3.2	5.1 ± 3.9	4.7 ± 3.1	0.874

Normally distributed data presented as mean ± SD and analysed using one-way ANOVA. Non-normally distributed data presented as median group score and (range) and analysed using the Kruskal-Wallis H Test.

adherence.

Patients who reported feeling hassled about sticking to their treatment plan (n = 10) and those that stated they did not take their medicines the preceding day (n = 9) reported lower overall QoL (average CLDQ score 2.96 ± 1.08 *vs* 4.30 ± 1.17, P = 0.001 and 2.97 ± 0.78 *vs* 4.28 ± 1.20, P = 0.002 respectively), especially within the domains of Activity, Emotion and Fatigue. Feeling hassled about sticking to the treatment plan was particularly associated with increased irritability (CLDQ-irritability score 3.50 ± 2.01 *vs* 4.81 ± 1.60, P = 0.019) and mood swings (CLDQ-mood swings score 3.33 ± 1.32 *vs* 4.81 ± 1.66, P = 0.003 respectively).

Factors associated with low medication adherence

Bivariate analysis indicated that patients with Necessity-Concerns Differential ⩽ 5, Brief Illness Perception Questionnaire "treatment control" score ⩽ 8 and "coherence" score ⩽ 8, or CLDQ score ⩽ 3 in the items of bodily pain, abdominal pain, shortness of breath or irritability, had higher odds of reporting "Low" medication adherence (Table 5). However, when included in the regression model having a Necessity-Concerns Differential ⩽ 5 (OR = 3.66, 95%CI: 1.18-11.40), Brief IPQ-coherence score ⩽ 8 (OR = 8.15, 95%CI: 0.98-67.78) or a CLDQ-shortness of breath score ⩽ 3 (OR = 3.87, 95%CI: 1.22-12.25) were the only independent predictors of "Low" medication adherence.

DISCUSSION

In our study, self-reported medication non-adherence in ambulatory patients with decompensated cirrhosis was prevalent, with over one-fifth of patients categorised with "Low" adherence and more than one-third categorised with "Medium" adherence. We have identified that lower levels of medication adherence in this group are associated with stronger patient Concerns about their medication relative to their belief in its Necessity, lower self-perceived understanding of liver disease, and lower QoL.

The relationship between medication beliefs and medication adherence behaviour has been explored in numerous chronic diseases, including asthma, cardiovascular disease and mental health disorders[29]. Perceptions of illness have also been shown to influence medication adherence in asthma, diabetes, hypertension and heart failure[30], though the impact of the different illness perception items on adherence appears to differ between diseases. In the present study, people with decompensated cirrhosis were more likely to have "Low" medication adherence if they had a lower Necessity-Concerns Differential, poorer self-perceived understanding of their hepatic disease (coherence), and had lower perceptions of the benefits of treatment for their hepatic disease (treatment control). It can therefore be inferred that decompensated cirrhosis patients who have a

Table 4 Health-related quality of life in patients with high, medium and low medication adherence

Quality of life domains	All patients (n = 100)	Medication adherence ranking			P value
		High (n = 42)	Medium (n = 37)	Low (n = 21)	
Abdominal symptoms	4.7 ± 1.5	4.6 ± 1.4	5.1 ± 1.4	4.2 ± 1.6	0.063
Abdominal bloating	4.5 ± 2.0	4.3 ± 2.0	4.9 ± 1.8	3.9 ± 2.1	0.109
Abdominal pain	4.9 ± 1.8	4.8 ± 1.7	5.5 ± 1.5	4.1 ± 2.2	0.023
Abdominal discomfort	4.4 ±1.8	4.7 ± 1.8	4.9 ± 1.7	4.6 ± 1.9	0.715
Activity	4.5 ± 1.5	4.6 ± 1.6	4.5 ± 1.3	4.1 ± 1.5	0.377
Not been able to eat as much as you would like	5.1 ± 1.9	5.0 ± 2.0	5.2 ± 1.6	5.0 ± 2.0	0.814
Trouble lifting or carrying heavy objects	3.4 ± 2.1	3.8 ± 2.3	3.3 ± 1.9	2.5 ± 1.9	0.067
Bothered by a limitation of your diet	5.0 ± 1.9	5.0 ± 1.9	5.0 ± 2.0	4.7 ± 2.1	0.824
Emotion	4.3 ± 1.5	4.7 ± 1.3	4.3 ± 1.7	3.7 ± 1.3	0.050
Anxiety	4.4 ± 1.9	4.8 ± 1.6	4.3 ± 2.1	3.8 ± 1.8	0.126
Unhappiness	4.5 ± 1.9	4.8 ± 1.9	4.6 ± 2.1	3.8 ± 1.3	0.125
Irritability	4.7 ± 1.7	5.0 ± 1.6	4.9 ± 1.8	3.8 ± 1.4	0.017
Difficulty sleeping at night	3.6 ± 2.2	4.0 ± 2.1	3.4 ± 2.3	3.1 ± 2.2	0.259
Mood swings	4.7 ± 1.9	5.1 ± 1.7	4.7 ± 2.1	3.8 ± 1.7	0.031
Unable to fall asleep at night	3.9 ± 2.3	4.1 ± 2.3	3.7 ± 2.3	3.7 ± 2.3	0.687
Felt depressed	4.6 ± 1.9	5.1 ± 1.8	4.5 ± 2.1	3.9 ± 1.6	0.058
Problems concentrating	4.3 ± 1.9	4.5 ± 2.0	4.5 ± 1.9	3.7 ± 1.9	0.232
Fatigue	3.2 ± 1.5	3.5 ± 1.6	3.3 ± 1.5	2.9 ± 1.3	0.340
Tired or fatigued	3.0 ± 1.6	3.0 ± 1.6	3.1 ± 1.7	2.7 ± 1.4	0.622
Sleepy during the day	3.2 ± 1.8	3.3 ± 1.9	3.5 ± 2.0	2.7 ± 1.2	0.255
Bothered by having decreased strength	3.6 ± 2.0	3.9 ± 2.0	3.4 ± 2.0	3.1 ± 2.0	0.281
Decreased level of energy	3.0 ± 1.8	3.2 ± 1.7	2.9 ± 1.8	2.6 ± 1.6	0.361
Drowsiness	3.9 ± 1.7	4.1 ± 1.8	3.8 ± 1.7	3.6 ± 1.6	0.466
Systemic symptoms	4.3 ± 1.3	4.3 ± 1.2	4.4 ± 1.4	4.0 ± 1.5	0.519
Bodily pain	4.2 ± 2.0	4.2 ± 1.8	4.7 ± 2.2	3.3 ± 2.0	0.037
Shortness of breath	4.5 ± 1.9	5.0 ± 1.8	4.4 ± 1.9	3.7 ± 1.8	0.030
Muscle cramps	4.2 ± 1.9	4.2 ± 2.0	4.1 ± 1.7	4.2 ± 2.2	0.957
Dry mouth	3.9 ± 2.0	4.0 ± 2.0	3.9 ± 2.0	3.7 ± 1.9	0.833
Itching	4.5 ± 2.1	4.2 ± 2.2	4.6 ± 2.0	5.0 ± 2.1	0.392
Worry	3.9 ± 1.8	4.1 ± 1.8	3.8 ± 2.0	3.6 ± 1.6	0.491
Worry about impact of liver disease has on family/friends	3.9 ± 2.1	4.0 ± 2.0	3.9 ± 2.2	3.7 ± 2.2	0.876
Worried that symptoms will develop into major problems	3.7 ± 2.1	4.0 ± 2.1	3.6 ± 2.2	3.1 ± 2.0	0.357
Worry about condition getting worse	3.9 ± 2.0	4.1 ± 2.0	3.7 ± 2.1	3.6 ± 2.0	0.569
Worry about never feeling any better	4.2 ± 2.2	4.5 ± 2.2	4.2 ± 2.3	3.8 ± 1.8	0.436

Normally distributed data presented as mean ± SD and analysed using one-way ANOVA. Item scores range from 1 to 7 where a lower score indicates lower quality of life (*i.e.*, more frequent symptoms). Domain scores (range from 1 to 7) are calculated averages of items within the domain.

weaker belief in the necessity or helpfulness of their medications, and those who do not understand the consequences of cirrhosis, are less likely to perceive a need to take their medications and may therefore exhibit non-adherent behaviour.

Interestingly, perceptions of symptom frequency/severity (identity), concerns and consequences of disease on daily life were not associated with adherence behaviour. This was further confirmed using the CLDQ which identified that adherence was not influenced by the domains of Activity, Fatigue, Worry, Systemic or Abdominal Symptoms. This may be explained by changes to patients' priorities in the terminal stages of illness which may affect their decisions for self-care. For example, people with palliative diseases such as cancer and end-stage heart failure may exhibit non-adherent behaviour in an attempt to maintain control, reduce adverse events, or in response to social or financial circumstances[31-33]. Similar issues may affect people with decompensated cirrhosis, as this is a palliative condition for patients who are ineligible for transplantation. Most patients in the present study were aware of the incurable nature of their disease as evidenced by responses to the timeline item. The presence of hepatocellular carcinoma, considered to be an "imminently terminal" occurrence in people with decompensated cirrhosis who are ineligible for liver transplant, was not associated with adherence, which indicates that perceptions of palliation alone may not strongly influence adherence behaviour in this group.

Notably, people with HCV perceived that their liver disease would last for a shorter duration of time compared to people with other aetiologies of cirrhosis, possibly related to availability of new HCV direct-acting antiviral therapies. Unfortunately, this may be the result of false hope in this group as it is possible that many patients with HCV cirrhosis will have persisting complications despite a HCV "cure". People with HCV also perceived greater impact of symptom frequency/severity (identity) and consequences of disease on their daily lives compared to other aetiologies, but this was not associated with adherence behaviour. Where improved control over debilitating symptoms may be an incentive for better adherence for some

Table 5 Crude and multivariable predictors of low medication adherence in patients with decompensated cirrhosis

		Crude		[1]Multivariable		*P* value
		OR	95%CI	OR	95%CI	
Age ⩾ 60 yr		1.20	0.46-3.16	1.79	0.56-5.70	0.325
Gender, male		1.10	0.40-3.04	0.74	0.21-2.58	0.639
Unable to afford medicines		1.85	0.60-5.66	0.88	0.25-3.44	0.857
N-C Differential	⩽ 5	4.79	1.74-13.25	3.66	1.18-11.40	0.025
Overuse	⩾ 13	1.69	0.59-4.86	0.85	0.23-3.15	0.813
Brief IPQ- treatment control	⩽ 8	3.63	1.21-10.88	3.23	0.92-11.39	0.068
Brief IPQ- coherence	⩽ 8	13.62	1.74-106.62	8.15	0.98-67.78	0.052
CLDQ-bodily pain (3)	QoL score ⩽ 3	2.72	1.02-7.27	1.69	0.54-5.35	0.369
CLDQ-abdominal pain (5)	QoL score ⩽ 3	4.19	1.45-12.09	1.73	0.50-6.05	0.389
CLDQ-shortness of breath (6)	QoL score ⩽ 3	3.93	1.44-10.71	3.87	1.22-12.25	0.022
CLDQ-irritability (15)	QoL score ⩽ 3	3.12	1.08-9.04	1.70	0.47-6.11	0.416
CLDQ-mood swings (19)	QoL score ⩽ 3	2.09	0.75-5.82	0.93	0.24-3.58	0.917

[1]Odds ratio adjusted for N-C Differential ⩽ 5, Brief IPQ-treatment control score ⩽ 8, Brief IPQ-coherence score ⩽ 8 and CLDQ-shortness of breath score ⩽ 3 (indicative of experiencing the symptom "all the time", "most of the time" or "a good bit of the time") in the multivariable logistic regression model.

patients, others could perceive side effects of therapy to outweigh benefits. This may be evidenced by the increased likelihood for "Low" adherence in patients who had stronger Concerns (*i.e.,* side effects) relative to Necessity (*i.e.,* benefits) beliefs, and those with less understanding about cirrhosis (coherence) and the role of medicines in disease and symptom management (treatment control). Alternatively, studies in other chronic diseases with a heavy symptom burden like decompensated cirrhosis, such as asthma and heart failure, have suggested that many patients with good adherence report a more benign perception of illness identity, concerns and consequences[34,35]. This may be attributed to improved disease control or fewer episodes of exacerbation because of good adherence with treatment, and may partially explain the heterogeneity of responses to these Brief-IPQ items among people with decompensated cirrhosis.

A previous investigation by Polis *et al*[18] in cirrhosis patients has identified that medication adherence, defined as "never missing medications", was associated with having less abdominal symptoms and increased emotional well-being[18]. Similarly, we have identified that decompensated patients with "Low" medication adherence reported more frequent emotional disturbances, greater abdominal and bodily pain, and activity-limiting shortness of breath. Rather than a cause, these symptoms are postulated to be an effect of non-adherence. Conversely, irritability and mood swings were associated with patients reporting they felt "hassled about sticking to their treatment plan" and failure to take medications the day preceding the survey. Irritability and mood swings are thus postulated to impact on adherence in this group. While emotional disturbances are often associated with hepatic encephalopathy, irritability and mood swings were not necessarily more common in people with a history of HE, and these symptoms were not found to relate to non-adherence in patients prescribed lactulose. While the effect of adverse drug events experienced by individual patients on medication adherence was not specifically explored, the BMQ items designed to capture negative beliefs potentially related to adverse effects were not strongly related to nonadherence. Interestingly, disease severity measured using the Child-Turcotte Pugh classification was not related to QoL at either the domain or item level. This is contrary to other studies in cirrhosis patients[36,37], however by the nature of this study we aimed to recruit patients with a diverse decompensation history, which may explain the heterogeneity of responses to the CLDQ.

Strengths and limitations

The single study site, the General Hepatology Clinic at the Princess Alexandra Hospital, is one of the largest hepatology centres in Australia. The Clinic delivers ambulatory care to a substantial proportion of south-east Queensland hepatology patients, in addition to regional, remote and interstate patients who travel to access specialist services. Despite the broad representation of people with chronic liver diseases at our site, an element of selection bias may exist in the present study due to recruitment constraints. If two or more eligible patients were scheduled for hepatology review simultaneously (parallel hepatology clinics), patients who were taking more medicines for decompensation events were selectively approached. Thus, the patients included may represent the more severe end of the disease spectrum. Furthermore, participants could only be recruited if they were scheduled to attend clinic on a day the principal researcher (KH) was present and as such a convenience sample of patients was recruited.

As far as we are aware, this is the first study to use the BMQ and Brief-IPQ to measure beliefs and perceptions about medications and illness in people with decompensated cirrhosis. While these tools have not been previously validated in this cohort, our findings are comparable with studies in other chronic diseases. Non-equal questioning methodology (due

to patients' preference, need for carer/family member involvement, researcher assistance and feasibility) may have introduced an element of bias, however this was difficult to avoid given the study group of interest often requires assistance completing complex tasks and reflects a "real-world" clinic setting. To minimise potential bias, the study coordinator read survey questions aloud verbatim and did not actively seek additional information beyond what patients volunteered.

In conclusion, a large proportion of ambulatory patients with decompensated cirrhosis are non-adherent with prescribed medications. The association between "Low" medication adherence and patients having strong concerns or doubting the necessity of their medications should be explored further given the potential clinical relevance. Interventions that promote positive reinforcement of the value and necessity of medications in addition to education about disease and medication management tailored to individual patient needs may improve adherence.

COMMENTS

Background

Non-adherence and medication-mismanagement in people with decompensated cirrhosis have been associated with substantial potentially-preventable harm. The current study describes the relationship between patients' self-reported adherence and medication beliefs, illness perceptions and quality of life. The study was conducted in a cohort of patients receiving ambulatory care at a large tertiary government hospital.

Research frontiers

Existing data on medication-nonadherence behaviour in people with decompensated cirrhosis is largely qualitative. The present study intended to quantitatively measure these variables using validated tools, to correlate potentially-modifiable beliefs, perceptions and quality of life with medication adherence.

Innovations and breakthroughs

In this study, the authors have identified that patients with lower levels of medication adherence were more likely to have lower perception of treatment helpfulness, higher concerns relative to necessity beliefs surrounding their medicines, and poorer quality of life.

Applications

The authors findings have important implications for evolving clinical practice, as medication-management and disease education interventions can be developed to target these potentially-modifiable patient variables.

Terminology

Levels of adherence (High, Medium and Low) were assessed using a validated adherence tool, the Morisky Medication Adherence Scale-8 (MMAS-8). Patients' beliefs and perceptions towards their medicines were elicited using previously validated questionnaires (Beliefs about Medicines Questionnaire and Brief Illness Perception Questionnaire), which have been correlated with adherence behaviour. Quality of Life was measured using the Chronic Liver Disease Questionnaire.

Peer-review

This is a very interesting article dealing with the adherence to therapy by decompensated cirrhotic patients.

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CASE REPORT

Case of familial hyperlipoproteinemia type III hypertriglyceridemia induced acute pancreatitis: Role for outpatient apheresis maintenance therapy

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Abstract

Hypertriglyceridemic pancreatitis (HTGP) accounts for up to 10% of acute pancreatitis presentations in non-pregnant individuals and is the third most common cause of acute pancreatitis after alcohol and gallstones. There are a number of retrospective studies and case reports that have suggested a role for apheresis and insulin infusion in the acute inpatient setting. We report a case of HTGP in a male with hyperlipoproteinemia type Ⅲ who was treated successfully with insulin and apheresis on the initial inpatient presentation followed by bi-monthly outpatient maintenance apheresis sessions for the prevention of recurrent HTGP. We also reviewed the literature for the different inpatient and outpatient management modalities of HTGP. Given that there are no guidelines or randomized clinical trials that evaluate the outpatient management of HTGP, this case report may provide insight into a possible role for outpatient apheresis maintenance therapy.

Key words: Apheresis; Pancreatitis; Plasmapheresis; Outpatient; Hypertriglyceridemia

Core tip: There are a number of retrospective studies that have suggested a role for apheresis and insulin infusion in the acute management of hypertriglyceridemic pancreatitis (HTGP) but the post-discharge course and outpatient management of HTGP remain unclear. We report a case of HTGP in a male with hyperlipoproteinemia type Ⅲ who was treated successfully with insulin and apheresis on the initial inpatient presentation followed by bi-monthly outpatient maintenance apheresis sessions for the prevention of recurrent HTGP. We also reviewed the literature for the different inpatient and outpatient management modalities of HTGP.

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INTRODUCTION

Hypertriglyceridemic pancreatitis (HTGP) accounts for up to 10% of acute pancreatitis presentations in non-pregnant individuals and is the third most common cause of acute pancreatitis after alcohol and gallstones[1,2]. Both genetic and secondary causes of lipoprotein metabolism have been implicated in HTGP. A serum triglyceride (TG) level of 10 g/L or greater is associated with acute pancreatitis. The risk of HTGP is approximately 5% with TG levels above 10 g/L and 10% to 20% with TG > 20 g/L[3]. The 2013 American College of Gastroenterology (ACG) guidelines in managing acute pancreatitis recommend obtaining a triglyceride level on all patients with acute pancreatitis without a known history of alcoholism or gallstones. While the ACG guidelines extensively explore the management of pancreatitis in general, there is a lack of data on the specific management of HTGP[4]. A number of retrospective studies and case reports in the past decade have suggested a role for insulin infusion with or without apheresis as an approach to rapidly lowering TG levels in an attempt to treat HTGP[1,5-18]. There are no randomized clinical trials evaluating the benefit of insulin infusion or apheresis in managing HTGP. Furthermore, there is a lack of characterization of post-discharge course and outpatient management of HTGP[19,20]. To our knowledge, there is only one case report of two patients with HTGP in 1996 who were managed with monthly plasmapheresis as maintenance therapy to prevent HTGP recurrence[21]. We report a case of HTGP in a male with hyperlipoproteinemia type Ⅲ who was treated successfully with insulin and apheresis followed by bi-monthly maintenance apheresis sessions post-discharge as prevention of recurrent HTGP. This case report may provide insight into a possible role for outpatient apheresis maintenance therapy.

CASE REPORT

A 40-year-old Caucasian male with a past medical history significant for hyperlipoproteinemia type Ⅲ (on atorvastatin 80 mg daily, fenofibrate 200 mg daily and omega-3 polyunsaturated fatty acids), coronary artery disease status post 3-vessel coronary artery bypass graft, peripheral vascular disease, hypertension, diabetes mellitus (DM) type 2, and one reported episode of acute pancreatitis in the past presented with epigastric pain, nausea and decreased oral intake over 3 d. Physical exam was remarkable for tachycardia to 100 beats/min, localized epigastric tenderness, and xanthomas with striae palmaris. Labs were remarkable for mildly elevated lipase of 334 U/L (reference range 114-286 U/L) and TG levels of 45.3 g/L (reference range 0-149). Abdominal CT scan showed moderate fat stranding around the pancreas and stable pseudocyst. He was started on insulin drip at a rate of 1 unit/h. TG levels trended down after 3 d of insulin infusion to 9.57 g/L. The patient, however, continued to have severe abdominal pain and inability to tolerate oral intake. After 3 d of inpatient stay, patient received his first session of apheresis. After 24 h of apheresis, patient improved symptomatically and was able to tolerate oral intake. Triglyceride levels trended down to 4.61 g/L after 24 h of apheresis and were 6.75 g/L on the day of discharge.

Given that the patient had a complicated cardiac history and that this was his second HTGP presentation in one year, a decision was made to have the patient undergo maintenance apheresis sessions bi-monthly as an outpatient to prevent recurrent pancreatitis and cardiac complications. Patient was discharged on home cardiac medications which consisted of hydrochlorothiazide 12.5 mg daily, Plavix 75 mg daily, aspirin 325 mg daily, metoprolol tartrate 50 mg BID, atorvastatin 80 mg daily, fenofibrate 200 mg and omega-3 polyunsaturated fatty acids. Twelve mo post-discharge course was remarkable for a total of three admissions due to recurrent pancreatitis with one admission attributed to poor adherence to apheresis, fat-free diet and lipid lowering medications. His TG levels mostly remained otherwise successfully below 15 g/L. Repeat abdominal CT scans over 12 mo demonstrated resolution of previous pseudocyst and absence of local complications (Figure 1).

DISCUSSION

Patients with HTGP present with symptoms typical of

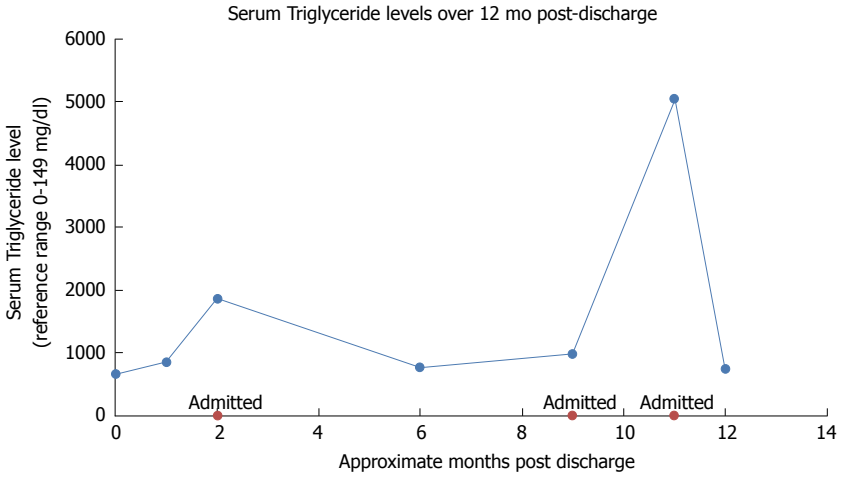

Figure 1 Serum triglyceride levels over 12 mo post-discharge. Hospital admissions are highlighted in red.

acute pancreatitis[1]. Specific features of HTGP that can help identify the etiology include xanthomas on extensor surfaces of arms and legs, lipemia retinalis and hepatosplenomegaly[22]. Lactescent serum is found in 45% of patients with mean Hypertriglyceridemia (HTG) level of 4537[2]. A serum TG level of 10 g/L or greater is associated with acute pancreatitis. The risk of HTGP is approximately 5% with TG levels above 10 g/L and 10% to 20% with TG > 20 g/L[23]. HTG by itself is not toxic to the pancreas, however, the breakdown of TG into free fatty acids (FFA) by pancreatic lipase causes lipotoxicity during acute pancreatitis, leading to a systemic inflammatory response[22]. Primary HTG that includes Frederick's phenotype Ⅰ-Ⅴ is associated with HTGP. Our patient was diagnosed with hyperlipoproteinemia type Ⅲ, an autosomal recessive trait, characterized by presence of Apo E2/E2[24]. Apo E ligand clears chylomicrons and Very low-density lipoprotein (VLDL) remnants from the circulation. Thus, this disorder leads to accumulation of VLDL and chylomicrons leading to HTG. Secondary HTG is caused by DM, alcoholism, hypothyroidism, pregnancy, and certain medications such as thiazides, beta blockers, corticosteroids, isotretinoin, immunosuppressants and antipsychotics[1].

A number of retrospective studies and case reports in the past decade suggested a role for insulin infusion with or without apheresis as an approach to rapidly lower TG levels in an attempt to treat HTGP[1,5-18]. There are no randomized clinical trials evaluating the benefit of insulin infusion, heparin or apheresis in managing HTGP to date. However, lowering of TG levels to below 5 g/L has been shown to advance clinical improvement in HTGP[25]. Apheresis is used to lower triglyceride level > 10 g/L and improve signs of severe inflammation such as hypocalcemia and lactic acidosis. One study reported (average TG of 14.06 g/L) a 41% decrease in TGs after one session of plasma exchange[18]. To our knowledge, there is only one case report of two patients with severe HTGP in 1996 who were managed with monthly plasmapheresis maintenance therapy[21]. Their 32-38 mo course was remarkable for only one episode of acute pancreatitis in one patient, suggesting a beneficial role for maintenance plasmapharesis[21].

With regard to anticoagulation during apheresis, studies have shown that citrate as opposed to heparin is associated with decreased mortality[18]. Intravenous insulin has been shown to be more effective than subcutaneous insulin in managing HTGP[26]. Insulin increases lipoprotein lipase which in turn accelerates chylomicron and VLDL metabolism to glycerol and FFA. It also inhibits lipase in adipocytes. Heparin is controversial in efficacy when used alone. It is thought to stimulate the release of endothelial lipoprotein lipase into the circulation, but the mechanism of lowering TG remains unclear[27].

Lipid lowering agents are indicated in the management of HTGP[25]. However, this is further complicated by the association between statin therapy and the development of acute pancreatitis. A series of case-control studies in Taiwan demonstrated that individuals using a statin therapy for the first time are more likely to develop an episode acute pancreatitis when compared to individuals who are not on statin therapy. These studies included Simvastatin (OR = 1.3, 95%CI: 1.02-1.73), Atrovastatin (OR = 1.67, 95%CI: 1.18-2.38), and Rosuvastatin (OR = 3.21, 95%CI: 1.70-6.06)[28-31]. Table 1 provides a summary of HTGP management strategies.

In summary, we presented a case of HTGP in a male with hyperlipoproteinemia type Ⅲ who was treated successfully with insulin and apheresis followed by outpatient bi-monthly maintenance apheresis sessions with a 12 mo post-discharge course remarkable for total of three admissions due to recurrent pancreatitis but with TG levels mostly remaining successfully below 15 g/L and repeat abdominal imaging demonstrating resolution of previous pseudocyst and absence of local

Table 1 Summary of hypertriglyceridemic pancreatitis management strategies

Management strategy	Description	Indication	Outcomes	Case report/Ref.
Diet restriction	Absolute restriction of fat intake	HTG, Primary prevention	Effective when combined with lipid lowering agents[15]	Tsuang *et al*[15], 2009
Lipid lowering agents	Fibrates (gemfibrozil 600 mg twice daily), niacin, N-3 fatty acids, statins	First line in HTG Adjuvant therapy in HTGP	Triglyceride level lowered about 60% by fibrates, about 50% by niacin, about 45% by omega-3 fatty acids[15]	Tsuang *et al*[15], 2009
Apheresis	Therapeutic Plasma Exchange which is removal of plasma and replacement with colloid solution (albumin, plasma). Citrate is used as an anticoagulant. Goal is TGH < 500	HTGP without contraindication to Apheresis such as inability to obtain central access or hemodynamic instability	Appears to be effective based on multiple case reports and case series. about 41% decrease in HTG levels. Apheresis within 48 h associated with better outcomes[16]	Furuya *et al*[16], 2002
Insulin	Intravenous regular insulin drip (0.1 to 0.3 units/kg/h). Goal is TGH < 500. Used alone or in combination with apheresis and/or heparin	Apheresis unavailable unable to tolerate apheresis hyperglycemia > 500	Intravenous insulin is more effective than subcutaneous[17] Effective in lowering triglyceride levels	Berger *et al*[17], 2001
Heparin	Combined with insulin. Subcutaneous heparin 500 units BID in 2 case reports	Controversial in HTGP	Controversial. Associated with increased mortality when compared to citrate (both combined with apheresis)[18].	Gubensek *et al*[18], 2014
Periodic apheresis	Described in 2 patients as monthly apheresis in 1996	Recurrence prevention especially in noncompliant patients	Reported success in one case report (2 patients in 1996)[21].	Piolot *et al*[21], 1996

HTGP: Hypertriglyceridemic pancreatitis; HTG: Hypertriglyceridemia; BID: Bis in die; TGH: Triacylglycerol hadrolase.

complications. This may suggest a beneficial role for outpatient apheresis as maintenance therapy in HTGP patients.

COMMENTS

Case characteristics

A 40-year-old Caucasian male with a past medical history significant for hyperlipoproteinemia type Ⅲ (on atorvastatin 80 mg daily, fenofibrate 200 mg daily and omega-3 polyunsaturated fatty acids), coronary artery disease status post 3-vessel coronary artery bypass graft, peripheral vascular disease, hypertension, diabetes mellitus type 2, and one reported episode of acute pancreatitis in the past presented with epigastric pain, nausea and decreased oral intake over 3 d.

Clinical diagnosis

Physical exam was remarkable for tachycardia to 100 beats/min, localized epigastric tenderness, and xanthomas with striae palmaris.

Differential diagnosis

Acute pancreatitis, gastritis, peptic ulcer disease, gastroenteritis, gastroesophageal reflux disease.

Laboratory diagnosis

Labs were remarkable for mildly elevated lipase of 334 U/L (reference range 114-286 U/L) and triglyceride levels of 45.3 g/L (reference range 0-149 g/L).

Imaging diagnosis

Abdominal CT scan showed moderate fat stranding around the pancreas and stable pseudocyst.

Treatment

Insulin, apheresis, atorvastatin 80 mg daily, fenofibrate 200 mg and omega-3 polyunsaturated fatty acids.

Related reports

There are a number of case reports in the literature that have demonstrated a benefit from apheresis and insulin therapy in the inpatient management of hypertriglyceridemic pancreatitis (HTGP). There is only one case report that have suggested a role for outpatient apheresis as a maintenance therapy.

Term explanation

HTGP is acute pancreatitis caused by high levels of serum triglycerides. Apheresis is the process of removal and separation of blood components and replacement with colloid solution (albumin, plasma).

Experiences and lessons

Multiple studies have demonstrated a role for apheresis in the acute management of HTGP. This case report suggests a role for outpatient apheresis in preventing recurrent attacks of HTGP.

Peer-review

This is a nice case report on a timely topic: hypertriglyceridemia-induced acute pancreatitis and role for outpatient apheresis maintenance therapy. This manuscript is generally of interest. The authors provided the complete review of this issue. The manuscript provides the updated evidence to the readers. It can be accepted for publication.

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CASE REPORT

Rescue case of low birth weight infant with acute hepatic failure

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Abstract

We report a case involving a rescued low birth weight infant (LBWI) with acute liver failure. Case: The patient was 1594 g and $32^{3/7}$ gestational wk at birth. At the age of 11 d, she developed acute liver failure due to gestational alloimmune liver disease. Exchange transfusion and high-dose gamma globulin therapy were initiated, and body weight increased with enteral nutrition. Exchange transfusion was performed a total of 33 times prior to living donor liver transplantation (LDLT). Her liver dysfunction could not be treated by medications alone. At 55 d old and a body weight of 2946 g, she underwent LDLT using an S2 monosegment graft from her mother. Three years have passed with no reports of intellectual disability or liver dysfunction. LBWIs with acute liver failure may be rescued by LDLT

after body weight has increased to over 2500 g.

Key words: Liver transplantation; Acute liver failure; Low birth weight infant; Transplantable body weight; Monosegment graft

Core tip: We report a case involving a rescued low birth weight infant (LBWI) with acute liver failure. The patient was 1594 g at birth. At the age of 11 d, she developed acute liver failure due to gestational alloimmune liver disease. Medications were initiated, and body weight increased with enteral nutrition. Her liver dysfunction could not be treated by medications alone. At 55 d old with a body weight of 2946 g, she underwent living-donor liver transplantation (LDLT) using an S2 monosegment graft. Conclusion: LBWIs with acute liver failure may be rescued by LDLT after body weight has increased to over 2500 g.

Okada N, Sanada Y, Urahashi T, Ihara Y, Yamada N, Hirata Y, Katano T, Ushijima K, Otomo S, Fujita S, Mizuta K. Rescue case of low birth weight infant with acute hepatic failure. *World J Gastroenterol* 2017; 23(40): 7337-7342 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i40/7337.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i40.7337

INTRODUCTION

Neonatal acute hepatic failure is a rare but serious disease[1]. Reports have indicated that the cause of neonatal acute hepatic failure is most frequently gestational alloimmune liver disease (GALD), but in rare cases, it can be metabolic disorder, viral infection, or mitochondrial disorder, among other possibilities[2]. The initial treatment for neonatal acute liver failure is apheresis and medication while the cause of hepatic failure is determined. If hepatic recovery has not been achieved with medication alone, liver transplantation is indicated[1,3]. However, the living donor liver transplantation (LDLT) procedure for neonatal recipients is challenging due to the size mismatch between the liver graft and the body of the recipient. Moreover, management during the perioperative period is also challenging[4]. LDLT is particularly difficult for low birth weight infants (LBWIs) for the reasons discussed above. In such cases, LDLT can result in recovery if the body weight of the infant can be increased by nutritional management[5]. However, few reports have discussed LDLT for neonatal recipients with low body weights[6]. Herein, we report a case involving a rescued LBWI with acute liver failure and discuss the limitation of body weight as an indicator for neonatal LDLT.

CASE REPORT

The patient was born at $32^{3/7}$ gestational wk because of fetal distress. Her birth weight was 1594 g (Figures 1 and 2). Immediately after birth, hypoglycemia and hypotension appeared. A daily administration of hydrocortisone and continuous administration of dopamine hydrochloride were started at the age of 1 day. At the age of 11 d, coagulation dysfunction and an elevated serum ferritin level due to acute liver failure were observed (ferritin 2865 ng/mL, total bilirubin 12.5 mg/dL, PT-INR 4.57). The patient's older sister was highly suspected of GALD by clinical course and pathological findings and underwent LDLT at 13 d of age. GALD was highly suspected in this case after excluding the possibility of metabolic disorder or infectious disease. The patient did not undergo magnetic resonance imaging or salivary grand biopsy for GALD diagnosis. We explained the high recurrence rate of GALD in siblings to her parents; however, the parents gave birth to a baby in another hospital without informing us. Starting at 11 d of age, exchange transfusion and medication therapy using high-dose gamma globulin and deferoxamine were initiated. The patient's body weight had been gradually increased with enteral nutrition using commercially available nutrients, administered 8 times per day *via* a gastric tube. She then recovered from hypotension, and the continuous administration of dopamine hydrochloride was finished. Exchange transfusion was performed a total of 33 times prior to LDLT. The patient was transported to our hospital at 44 d of age; at that time, her body weight was 2525 g. We speculated that her target body weight for LDLT would be over 2500 g based on the estimated graft volume. Computed tomography scan revealed a markedly atrophied liver (resected liver was 78 g). Laboratory data showed repeated coagulopathy and hyperbilirubinemia with daily exchange transfusion and a pediatric end-stage liver disease (PELD) score of 15.8 (T-bil 5.09 mg/dL, PT-INR 1.91, Albumin 3.4 g/dL). A liver biopsy revealed a marked loss of hepatocytes. The remaining hepatocytes were multinucleated, and there was widespread fibrosis around Glisson's sheath and in the parenchymal area (F3-4) (Figure 2F and G). Thus, her liver dysfunction could not be successfully treated *via* medications alone, and she was judged to be indicated for liver transplantation at that time. We tried to perform LDLT at 45 d of age, but before LDLT, she went into shock following hemothorax due to failure of catheter insertion with injury of the right subcla*via*n artery. After the recovery period, she underwent LDLT at 55 d of age using an S2 monosegment graft from her mother (107 g, graft-recipient weight ratio (GRWR) 3.6%; Figure 2 A). The body weight of the patient at LDLT was 2946 g. At the time of LDLT, the PELD score of the patient was 21.9 (T-bil 13.74 mg/dL, PT-INR

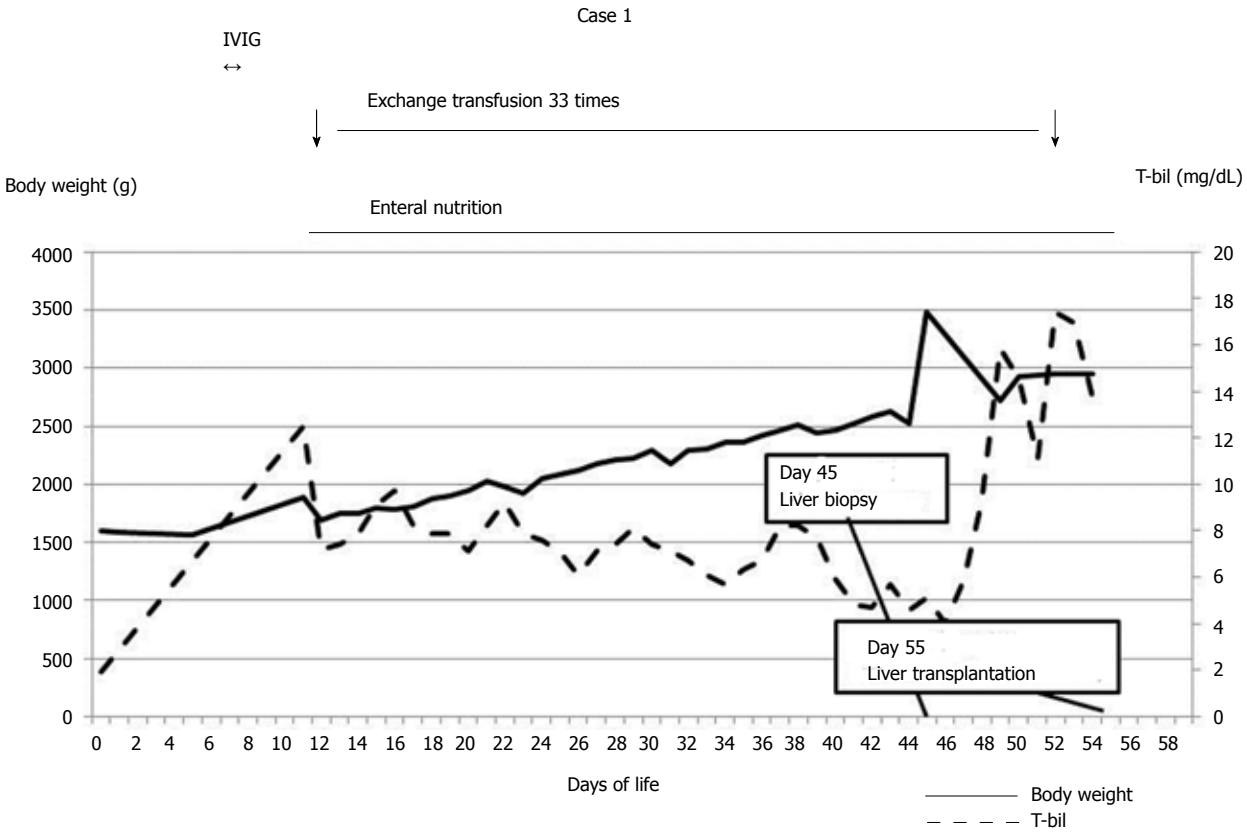

Figure 1 The preoperative treatment and changes in body weight and total bilirubin. The patient was able to gain weight due to the use of internal medication and enteral nutrition.

2.12, Albumin 3.7 g/dL). The operation duration was 13 h and 37 min and bleeding was 700 mL [238 mL/recipient body weight (kg)]. A transverse incision was created, and the liver was resected with temporary bypass of the portal vein. The resected liver was 78 g (Figure 2E). The recipient's right hepatic artery (2.0 mm) was anastomosed to the graft's left hepatic artery (2.5 mm) *via* a dorsal position of the portal vein anastomosis using a microsurgical technique. The graft-to-recipient distance ratio (GRDR) was 2.4 (58/24). Biliary reconstruction was performed using a Roux-en-Y hepaticojejunostomy. The abdominal wound could not be closed because respiratory failure occurred due to abdominal compartment syndrome (Figure2B and C). Intraoperative water balance was +1645 mL [558 mL/recipient body weight (kg)]. After LDLT, continuous hemodiafiltration (CHDF) had been performed for systemic edema, removing water as long as blood pressure and portal vein flow remained steady. Respiratory failure due to abdominal compartment syndrome and lung edema gradually improved. Thus, on postoperative day (POD) 5, the abdominal skin of the patient was closed without closing the abdominal fascia (Figure 2D). Tacrolimus and methylprednisolone were used as the standard postoperative immunosuppression therapy regimen. Acute rejection occurred on POD 17, so steroid pulse therapy was initiated. After steroid pulse therapy, liver enzyme, PT-INR and T-bil were nearly normalized. Respiratory failure due to large-for-size graft syndrome had been prolonged; however, it gradually improved, and she was extubated on POD 81. Cytomegalovirus infection and catheter infection occurred several times and were treated using antivirus or antibiotic drugs. The patient showed difficulty eating sufficient meals and required habilitation to eating. She was discharged on POD 225. Vessel complications did not occur. Three years have passed since LDLT, with no reports of intellectual disability or liver dysfunction.

DISCUSSION

Liver transplantation for a small recipient

We rescued a case involving an LBWI with acute liver failure; however, few reports to date describe such a case (Table 1). To our knowledge, the lightest LDLT recipient had a body weight of 2.4 kg at the time of LDLT[7], and the youngest LDLT recipient in Japan was 9 d of age[8-10]. For deceased donor liver transplantation, the smallest reported recipient was 2 kg, and the youngest was 7 d of age[11]. No consensus has been reached regarding the safe lower limit of body weight for small recipients. A recent UNOS database analysis of infants weighting less than 5 kg revealed one-year patient and graft survival rates of 77.7% and 66.1%, respectively[12]. A recent Japanese

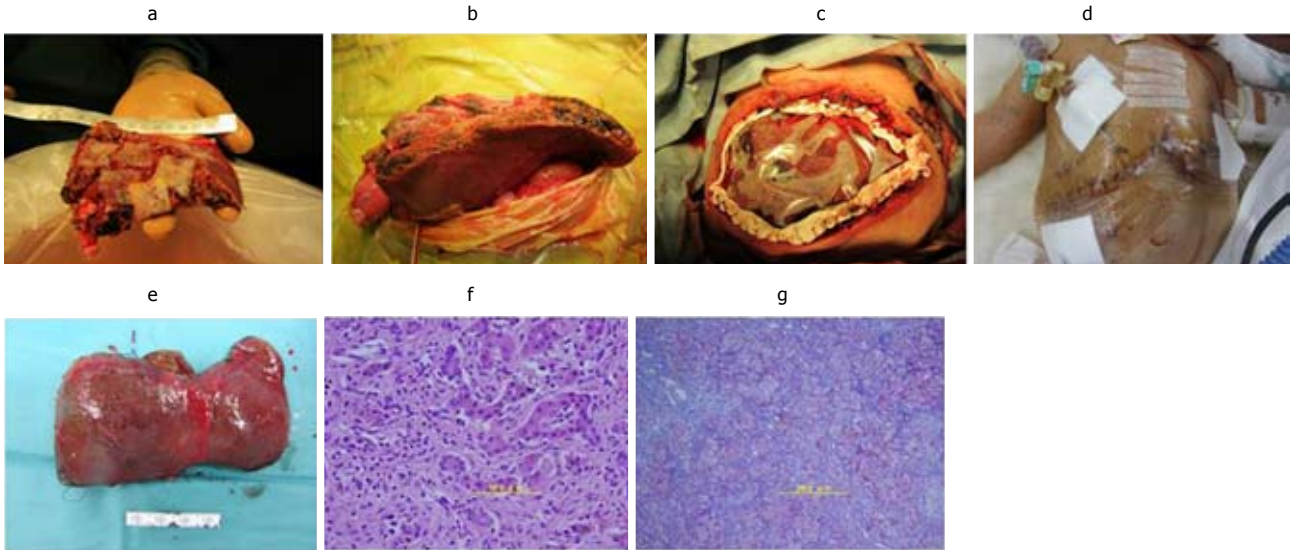

Figure 2 Images. A: The S2 monosegment graft (107 g) on the back table; B: An image obtained after reperfusion, the graft was too large to close the abdominal fascia; C: The abdominal fascia could not be closed at the time of living donor liver transplantation (LDLT), excess water was removed by continuous hemodiafiltration after LDLT; D: Secondary skin closure was performed on postoperative day 5; E: The resected liver was 78 g; F: Hematoxylin and eosin staining revealed a marked lack of hepatocytes and the presence of multinucleated hepatocytes; G: Azan staining revealed widespread fibrosis around Glisson's sheath and the parenchymal area (F3-4).

Table 1 Problems and management for low birth weight infant with acute liver failure

	Problems	Management
Pre-LT	Low body weight	Enteral nutrition targeting to over 2500 g
	Liver failure	Apheresis (exchange transfusion, plasmapheresis)
	Donor	Informed consent
LT	Large-for-size graft syndrome	Monosegment graft
	Hepatic artery reconstruction	Brunch patch, dorsal approach
	Abdominal compartment syndrome	Skin closure
		Open management→secondary skin closure
Post-LT	Fluid overload	Aggressive water removal using CHDF
	Respiratory failure	

CHDF: Continuous hemodiafiltration.

study reported an improved survival rate of 90.1% in an infant recipient within 3 mo[7]. Recipients who were within 3 mo of age also showed a higher rate of biliary complications[7]. However, our case did not have biliary complications. The problems we experienced in the described case were related to the following: (1) large-for-size graft syndrome[4]; (2) the transplantable body weight of the recipient; and (3) vessel reconstruction difficulties[13]. Challenges in managing transplantation for LBWIs with acute liver failure are nearly always due to the low body weight and fragility of the recipient.

The lower limit of the recipient's body weight for living donor liver transplantation

In liver transplantation, the transplantable body weight of a neonatal recipient is limited by graft size. If the GRWR is greater than 4.0%, the risk of abdominal compartment syndrome and insufficient blood supply in relation to a large-for-size graft are increased; the recipient abdomen cannot be closed, or massive hepatocyte necrosis may occur in the transplanted graft[8,10,14,15]. In pediatric LDLT, particularly in cases for which the recipient's body weight is less than 6 kg, monosegment grafts or hyper-reduced left lateral grafts have been transplanted[4]. A total of 268 pediatric patients underwent LDLT 275 times between May 2001 and December 2015 at Jichi Medical University Hospital. These transplantation procedures involved 196 left lateral segment grafts, with a median graft weight of 230 g (range, 138-382 g). Monosegment grafts were transplanted in 13 cases (including 9, 3, and 1 cases involving S2, reduced S2, and S3 monosegment grafts, respectively); in these cases, the median graft weight was 124 g (range, 93-180 g). A hyper-reduced left lateral segment graft was transplanted in 1 case, and the graft volume was 172 g. The smallest graft that we have transplanted was 93 g, and the median GRWR was 3.6% (2.4%-4.2%). A previous study reported hyper-reduced left lateral segment graft weights of 72-189 g,

with most graft weights being 100-150 g[7]. The lower limit of the smallest graft volume from a living donor is approximately 100 g[10]. Given this graft weight, transplantable recipient body weights of 2500 g or more will result in a GRWR of 4.0% or less. Based on this estimation, 2500 g is a reasonable body weight for such recipients.

The specific peritransplant management of living donor liver transplantation for low birth weight infant

In our experience, the body weights of the described patient were increased by enteral nutrition and became greater than 2500 g at 44 d of age. Another case of increased body weight by enteral nutrition was reported, and the body weight increased to greater than 2500 g at 41 d of age[5]. Despite the existence of acute liver failure, the body weights of the patients increased to over 2500 g *via* enteral nutrition while exchange transfusions were performed. Thus, in cases of acute liver failure, body weight may be increased with enteral nutrition if appropriate medication therapy that includes apheresis, such as exchange transfusion or plasmapheresis, is performed.

At times, it is impossible to close the abdominal fascia of the recipient during LDLT due to abdominal compartment syndrome. In this case, the abdominal fascia could not be closed during LDLT; instead, the case required secondary skin closure. In cases involving pediatric LDLT patients, apheresis and dialysis are reportedly effective[16]. Aggressive water removal using CHDF was required in this case. Thus, even if LDLT for an LBWI with acute liver failure is successfully performed after an increase in body weight, circulation and respiratory management are challenging due to volume overload. Under these circumstances, aggressive water removal using CHDF and secondary abdominal closure approaches are useful and important tools for successful postoperative management.

Hepatic arterial reconstruction during living donor liver transplantation

Vessel reconstruction in cases similar to the described case is difficult due to the high GRDR between the hepatic vein and the portal vein bifurcation and to diameter mismatch for the hepatic artery between the graft and the recipient[13]. Hepatic artery reconstruction by the dorsal approach of portal vein anastomosis has been reported to help reduce the risk of hepatic artery complications among small recipients if the GRDR is over 2.4[13]. At times, the hepatic arteries of such recipients are thin. The hepatic artery of this case was not thin; however, the GRDR was 2.4, and the hepatic artery was anastomosed by a dorsal approach. In our experience of 13 cases of LDLT using monosegment grafts, the thinnest artery among the recipients was 1.2 mm, with 3 cases involving hepatic arteries of less than 1.5 mm. The graft-to-recipient diameter ratio was 0.67: 1.67. In addition, the dorsal position was selected for hepatic artery anastomotic approaches in 7 cases (53.8%), and the branch patch technique was selected in 5 cases (38.5%). Hepatic artery complications after LDLT occurred in 3 cases, although interventional radiology was sufficient for achieving recovery in all 3 cases.

We rescued a case of LBWI with acute liver failure by performing LDLT. In cases involving LBWIs with acute liver failure, infants may be rescued by LDLT after their body weights have been increased to over 2500 g *via* repeated exchange transfusions and enteral nutrition.

COMMENTS

Case characteristics

The patient was 1594 g, $32^{3/7}$ gestational wk at birth and immediately after birth, hypoglycemia and hypotension appeared.

Clinical diagnosis

At the age of 11 d, coagulation dysfunction and an elevated serum ferritin level due to acute liver failure were observed.

Differential diagnosis

The case was highly suspected of gestational alloimmune liver disease and differential diagnosis was metabolic disorder, infectious disease and Neimann-Pick disease type C.

Laboratory diagnosis

Laboratory data showed repeated coagulopathy and hyperbilirubinemia with daily exchange transfusion and a pediatric end-stage liver disease score of 15.8.

Imaging diagnosis

Computed tomography scan revealed a markedly atrophied liver.

Pathological diagnosis

A liver biopsy revealed a marked loss of hepatocytes and the remaining hepatocytes were multinucleated, and there was widespread fibrosis around Glisson's sheath and in the parenchymal area (F3-4).

Treatment

The case underwent living donor liver transplantation at 55 d of age using an S2 monosegment graft from her mother (107 g).

Related reports

Kasahara M *et al* and Mizuta K *et al* reported the living donor liver transplantation for small recipient (*Exp Clin Transplant* 2014; 12 Suppl 1:1-4, *Am J Transplant* 2010; 10: 2547-2552).

Term explanation

Low birth weight infant (LBWI) is defined as a birth weight of a infant of 2499 g or less regarding of gestational age.

Experiences and lessons

In cases involving LBWI with acute liver failure, infants may be rescued by living donor liver transplantation after their body weights have been increased to over 2500 g.

Peer-review

The authors presented an interesting case with LBWIs (1594g, 323/7 wk) with acute liver failure.

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LETTERS TO THE EDITOR

S-Adenosyl-L-methionine towards hepatitis C virus expression: Need to consider *S*-Adenosyl-L-methionine's chemistry, physiology and pharmacokinetics

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Abstract

S-Adenosyl-L-methionine (SAM) is a cofactor serving as a methyl donor in numerous enzymatic reactions. It has been reported that SAM has the potential to modify antioxidant-enzymes, glutathione-biosynthesis and methionine adenosyltransferases-1/2 in hepatitis C virus -expressing cells at millimolar concentrations. The efficacy of SAM at micromolar concentrations and the underlying mechanisms remain to be demonstrated.

Key words: *S*-Adenosyl-L-methionine; Bioavailability; Concentration; Liver

Core tip: *S*-Adenosyl-L-methionine (SAM) serves as a cofactor for enzymes that transfer its methyl group to nucleophilic functionalities of various biomolecules including DNA and RNA. Exogenous SAM has been shown to be a useful pharmacological agent in liver-associated diseases. SAM is a labile species, undergoes spontaneous decomposition in biological samples, and its oral bioavailability is only about 2%. Lozano-Sepulveda and colleagues observed that SAM modulates antioxidant enzymes, restores glutathione synthesis, and switches MAT1/MAT2 turnover in hepatitis C virus (HCV) expressing cells. The authors suggested that this may be a likely mechanism by which HCV expression is diminished by SAM. This SAM concentration range was chosen on the basis of cell viability experiments and is up to 1000 times higher than physiological intracellular. Other groups have used SAM in the concentration range 0 - 1000 nmol/L. The efficacy of SAM, its pharmacological effects towards

HCV and possibly adverse effects beyond cell viability need to be elaborated in further studies using SAM concentrations much lower than 1 mmol/L.

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TO THE EDITOR

S-Adenosyl-L-methionine (SAM) is the common cofactor of methylating enzymes, the methyl transferases. These enzymes catalyze the transfer of the methyl group of SAM to various nucleophilic functionalities of low-molecular-mass and high-molecular-mass biomolecules. Catechol amines, DNA, RNA, and proteins are well-investigated substrates of methyl transferases. SAM deficiency is associated with many different pathogenic conditions including liver diseases, depression and inherited methylation disorders. SAM supplementation in such diseases is a therapeutic means[1-5]. Lozano-Sepulveda and colleagues recently reported in the *World Journal of Gastroenterology* that SAM decreased hepatitis C virus (HCV) -RNA levels by 50% to 70% and induced a synergistic antiviral effect with standard IFN treatment[6]. The authors found that SAM modulated several antioxidant enzymes (*e.g.,* superoxide dismutase-1 and -2, thioredoxin), restored glutathione (GSH) synthesis, and switched methionine adenosyltransferase (MAT) turnover in HCV-expressing cells. The study by Lozano-Sepulveda and colleagues adds to the pleiotropic effects of SAM. However, this study by Lozano-Sepulveda and colleagues suffers from a major limitation, namely the use of very high SAM concentrations (range, 1 - 5 mmol/L)[6]. The choice of this SAM concentration range appears arbitrary. Another study limitation is that no SAM concentration/dose-response experiments have been performed.

SAM is a physiological substance and is widely distributed in extracellular and intracellular compartments of the human body[7-11]. The concentration of SAM in plasma of healthy subjects is of the order of 150 nmol/L, seemingly independent of the concentration of total homocysteine[7]. The intracellular SAM concentration in human lymphocytes has been reported to be about 5 nmol/10^6 cells; in mouse liver the SAM content was determined to be 0.5 nmol/mg protein[7]. The latter values are close to those reported by others using different analytical methods[12]. In freshly isolated human erythrocytes the concentration of SAM is of the order of 4 μmol/L[13]. This value agrees with more recently reported median SAM concentrations in erythrocytes of diabetic (3.8 μmol/L) and non-diabetic (3.5 μmol/L) male and female subjects[14].

The pharmacokinetics of SAM has been frequently investigated in animals as well as in healthy and diseased humans[15-17]. The oral bioavailability of SAM is of the order of 1% - 4%. Ingestion of 1000 mg SAM as tosylate disulfate salt resulted in maximum plasma SAM concentrations of about 2.5 μmol/L in men and women[3]. Intravenous injection of 1000 mg SAM resulted in maximum plasma SAM concentrations of about 211 μmol/L[15]. Another study found that oral administration of 10 mg SAM/kg body weight did not result in significant increases in systemic SAM concentration[16]. Thus, the SAM concentration range used in the Lozano-Sepulveda's study[6] is almost 1000-fold higher than physiological and pharmacologically used SAM concentrations (0-1000 nmol/L), and even 5 - 25 times higher than plasma SAM concentrations from intravenously injected SAM.

Use of very high SAM concentrations in *in vitro* experiments, even if not toxic[6], may lead to entirely different or contradictory results than the use of physiological and pharmacological SAM concentrations[18]. Oral administration of radiolabeled SAM (*i.e.,* [*methyl*-^{14}C]SAM) in mice resulted in radioactivity accumulation in the liver due to authentic [*methyl*-^{14}C]SAM and [*methyl*-^{14}C]phosphatidylcholine. The latter was found to be about 8 (after 60 min) and 25 (after 240 min) times higher concentrated than [*methyl*-^{14}C]SAM[16]. In aqueous solution, SAM is unstable and decomposes spontaneously to its components including *S*-methylthioadenosine, adenosine, adenine, and homoserine lactone[19]. Above pH 6, SAM is chemically very labile. Its inherent reactivity towards nucleophilic functionalities of biomolecules such as DNA and proteins is about 1000 times higher than that of methylated folates[19]. These observations suggest that SAM does not only function as an universal cofactor in methyltransferases-catalyzed reactions, but also undergoes both spontaneous methylation reactions with various biomolecules and decomposition to species such as *S*-methylthioadenosine and homoserine lactone[19]. Possibly, SAM decomposes to additional substances with not yet known biological activities, albeit not necessarily acutely cell toxic. The decrease in total glutathione concentration in the HCV-expressing cells upon incubation with SAM at 1 mmol/L for 1 and 2 h seen by Lozano-Sepulveda *et al*[6] may be an indication of a (spontaneous) reaction of SAM with reduced glutathione (GSH) to form *S*-methyl-glutathione which cannot be detected by the Ellman's method. At least in rat kidney proximal tubules, *S*-methyl-glutathione is rapidly degraded by gamma-glutamyl-transpeptidase[20]. Measurement of oxidized glutathione, *i.e.,* glutathione disulfide (GSSG), is a much more suitable and direct approach to assess oxidative stress. Yet, no GSSG data

were reported in the paper[6]. It is worth mentioning that SAM (at 4 mmol/L) can also inhibit thioredoxin-mediated protein disulfide reductase activity[20]. This and further reports[22] are supportive of the chemical lability of SAM that makes it a spontaneous unselective methylating agent. Spontaneous decomposition of SAM considerably contributes to *S*-adenosyl-homocysteine which is a potent inhibitor of methyltransferases including protein arginine methyltransferases[23].

Lozano-Sepulveda and colleagues reported in their article interesting results and proposed possible mechanisms for the explanation of the effects exerted by SAM in HCV-expressing cells seen in their study[6]. Yet, the SAM concentrations used in the study are difficult to be reached within cells even by intravenous injection of SAM salts. The high chemical reactivity of the *S*-methyl group of SAM towards biomolecules and its spontaneous decomposition is likely to bear potential adverse effects. The efficacy and the safety of SAM, especially its pharmacological effects towards HCV, need to be elaborated in further studies taken into consideration the pharmacokinetics of SAM. Use of SAM at mmol/L-concentrations may raise unrealizable expectations.

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