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EDITORIAL

Bleeding with the artificial heart: Gastrointestinal hemorrhage in CF-LVAD patients

Grigoriy E Gurvits, Elena Fradkov

Grigoriy E Gurvits, Division of Gastroenterology, New York University School of Medicine/Langone Medical Center, New York, NY 10016, United States

Elena Fradkov, Department of Medicine, New York University School of Medicine/Langone Medical Center, New York, NY 10016, United States

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Correspondence to: Grigoriy E Gurvits, MD, Division of Gastroenterology, New York University School of Medicine/Langone Medical Center, 530 First Avenue, SKI-9N, New York, NY 10016, United States. grigoriy.gurvits@nyumc.org
Telephone: +1-212-2633095

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Abstract

Continuous-flow left ventricular assist devices (CF-LVADs) have significantly improved outcomes for patients with end-stage heart failure when used as a bridge to cardiac transplantation or, more recently, as destination therapy. However, its implantations carries a risk of complications including infection, device malfunction, arrhythmias, right ventricular failure, thromboembolic disease, postoperative and nonsurgical bleeding. A significant number of left ventricular assist devices (LVAD) recipients may experience recurrent gastrointestinal hemorrhage, mainly due to combination of antiplatelet and vitamin K antagonist therapy, activation of fibrinolytic pathway, acquired von Willebrand factor deficiency, and tendency to develop small intestinal angiodysplasias due to increased rotary speed of the pump. Gastrointestinal bleeding in LVAD patients remains a source of increased morbidity including the need for blood transfusions, extended hospital stays, multiple readmissions, and overall mortality. Management of gastrointestinal bleeding in LVAD patients involves multidisciplinary approach in stabilizing the patients, addressing risk factors and performing structured endoluminal evaluation with focus on upper gastrointestinal tract including jejunum to find and eradicate culprit lesion. Medical and procedural intervention is largely successful and universal bleeding cessation occurs in transplanted patients.

Key words: Gastrointestinal bleeding; Left ventricular assist devices; Heart failure; Angioectasia; Endoscopy

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Core tip: Classic descriptors and latest developments in care of left ventricular assist devices (LVAD) patients presenting with gastrointestinal (GI) hemorrhage. Pathophysiology, etiology, clinical presentation, risk factors, location within the GI tract, differential diagnosis, management, complications, and prognosis of LVAD patients with GI hemorrhage. Comprehensive review of aspects of clinical care and future research in this patient population.

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INTRODUCTION

Continuous-flow left ventricular assist devices (CF-LVADs) are the current standard-of care for end stage heart failure, having virtually replaced older pulsatile flow devices due to the smaller size, durability and improved survival outcomes[1,2]. First used experimentally in the late 1960s, LVADs have undergone multiple changes and advances, both structural - becoming smaller and more portable, and functional - changing from pulsatile to continuous flow models. Increased survival and improved quality-of-life in patients with LVADs has lead to significant increase in popularity and utilization among patients and providers over the last decade. The annual rate of device placement in the United States alone has been steadily increasing from 206 LVADs in 2006 to 1451 in 2010, with an overall unchanged rate of heart transplant[3]. The use of the device has also been expanded significantly, as both a bridge-to transplant and a long-term use as destination therapy, especially in patients for whom heart transplant is not an option[1,2]. This increasingly prolonged utilization has led to compounded recognition of additional complications, including gastrointestinal bleeding (GIB)[4].

GIB significantly impacts patient's morbidity and mortality. The annual incidence of gastrointestinal (GI) hemorrhages ranges from 50 to 150 per 100000 of the population of the United States[5]. Upper gastrointestinal tract bleeding (UGIB), from lesions proximal to the ligament of Treitz is responsible for about 20000 deaths annually in the United States[6]. Peptic ulcer disease remains the commonest cause, accounting for nearly 60% of all UGIB[7]. Lower GIB is responsible for 50% as many hospitalizations as upper GIB and carries a mortality rate of 2.4%-3.9%[8]. CF-LVAD recipients are more likely to have a GIB than the general population, and of greater severity, requiring an average of 2-4 units of packed red blood cells per admission[9]. In fact, GIB is the most common adverse event and a frequent cause for early post-transplant readmission in LVAD patients[10]. Historically, 15%-61% of the patients may develop GI hemorrhage after LVAD transplant[11,12]. This bleeding risk is only increasing in significance as LVAD utilization gains acceptance and becomes more widespread with an effective rise in the prevalence. Recent discussions have focused on multidisciplinary approach to anticipation, timely diagnosis, and protocoled management of LVAD patient group[11,13,14]. In this latest review we discuss history of the LVADs, pathophysiology of GIB in LVAD recipients, clinical presentation, risk factors, location within the GI tract, differential diagnosis, management, and prognosis for these patients.

HISTORY

The first ventricular assist device (VAD) was used in 1966 by Michael DeBakey to help wean a woman from the heart-lung bypass machine (of his own design) after cardiac surgery[15]. Early LVADs were large and extracorporeal[16]. Mimicking normal ventricular function, they propelled blood in a pulsatile manner via pneumatic pressure pumps. Technological advances during the last quarter of the 20th century allowed for internalization of devices and a transition to electrical power.

Originally used to assist with myocardial recovery after infarction, LVADS were subsequently approved by the Food and Drug Administration (FDA) as a bridge to cardiac transplant[17]. Despite numerous complications, pulsatile LVADS only gained widespread use after the REMATCH trial in 2001[18]. Shown to prolong life in patients with end-stage heart failure[18], pulsatile LVADs posed a high thrombosis risk with early trials demonstrating a stroke rate as high as 16%[19] and a 50% greater infection rate than continuous-flow devices[8]. Intra-device stasis associated with pulsatility was thought to account for the elevated clotting and infection rates, leading to the development of a continuous flow model (CF-LVAD). Such devices use constant axial-flow through a rotary turbine to pump blood between the left ventricle and the aorta. Several models include Heartmate II (HMII) Left Ventricular Assist System (Thoratec)[20], the MicroMedDeBakey Ventricular Assist Device (MicroMed)[21], the Jarvik 2000 Heart (Jarvik Heart)[22], and the VentrAssist Left Ventricular Assist System (Ventracor)[23].

PATHOPHYSIOLOGY

The risk of bleeding in the setting of CF-LVAD is typically multifactorial - likely due to a combination of exogenous factors (anticoagulation and antiplatelet therapy), endogenous causes (fibrinolysis), intrinsic properties of the machines [the effect of the LVAD on endothelium, platelets, von Willebrand Factor (vWF), and angiogenesis], and the pre-disposing systemic conditions in the patients requiring such devices (hepatic and renal dysfunction).

Biomaterials and device structure

Hematologic responses to biomaterials and their effective interaction with blood components are important factors in augmentation of safe utilization of LVADs over the course of their development and clinical implementation. With significant clinical advances in its mechanical function, LVAD synthetic material

interaction with blood (including effective changes in its immunologic, inflammatory, and hematologic function) has come to spotlight as key concept in decreasing LVAD associated morbidity. To date, LVADs continue to impact expression of a variety of molecules in the coagulation and endothelial systems, including pro-thrombotic and pro-inflammatory intercellular adhesion molecule, E-selectin, and tissue factor[24]. As a result, new devices rely on materials with higher rate of hemocompatability, including titanium, acrylics, and polytetrafluoroethylene and additional optimization of LVAD surface continues to be a focus of both clinical and bench research.

Use of biomaterials and a technological advance away from the pulsatile LVAD systems have effectively decreased the risk of pump thrombosis, however, did not fully eliminate it. Sheer forces of the CF-LVAD change platelet shape and upregulate adhesion factors, which may lead to platelet activation, inducing aggregation and promoting adhesion to the endothelium and the device[25]. CF-LVADs are associated with a 6 per 100 patient-years stroke risk[8], a number comparable to advanced heart failure with atrial fibrillation[26], and a 6% overall thromboembolic event rate[27]. CF-LVAD patients remain on anti-platelet (aspirin or dipyridamole) and anticoagulation (warfarin) medications with a goal international normalized ratio (INR) of 1.5-2.5 immediately post-implantation and for duration of support to decrease the chance of thromboembolic event[28,29].

Early analysis did not demonstrate an increased risk of bleeding with LVAD to be greater than the baseline risk associated with concomitant aspirin and warfarin use[30,31]. However, more recent reviews have shown bleeding rates as high as 40% in HMII patients[32,33]. Additional studies provided further evidence that use of CF-LVAD confers a greater bleeding risk than pulsatile devices. Crow *et al*[34] found a tenfold increase in bleeding episodes per 100 person-years in CF-LVADs compared to pulsatile flow LVADs. A large retrospective review found that the odds of GIB were 3.24 times greater (95%CI: 1.53-6.89) in CF-LVAD recipients[4]. The risk further increases with age: in patients over 65 years old adjusted odds of GIB was 20.5 times greater (95%CI: 2.24-1.88) in continuous flow compared to pulsatile devices[4].

Anticoagulation

Use of antiplatelets and anticoagulants together increases the risk of bleeding above the risk associated with either agent alone and patients with LVAD are maintained on dual therapy for duration of the support. Initial experience with LVAD patients required higher degree of anticoagulation with warfarin but subsequent studies allowed for safe decrease of INR to 1.5-2.5 range without significant compromise in thromboembolic outcome[35]. Low level of auto-anticoagulation exists in advanced heart failure patients, both pre- and post-LVAD implantation[36]. Previous reports suggest a possibility of a specific genetic predisposition to both bleeding and clotting for LVAD patients, the exact mechanism of which is not entirely understood. Warfarin sensitive patients with rare polymorphisms in CYP2C9 (responsible for warfarin metabolism) and vitamin K epoxide reductase complex, subunit 1 (VKORC1) (protein inhibited by warfarin) may have paradoxical increase in both bleeding and thrombosis complications after LVAD placement[37] and additional studies are necessary to elucidate this hypothesis further.

Platelets

While aspirin or dipyridamole impair platelet function, the abnormalities seen in LVAD patients are not solely explained by medication use. Impaired platelet aggregation is partially caused by the interactions with artificial material and turbulent blood flow through the LVAD[35]. Platelets tend to exhibit an increased sensitivity to sheer stress, lysing at velocities far below speeds that would affect erythrocytes[38]. *In vitro*, blood circulated through the centrifugal pump of a VAD caused significant platelet injury[39]. *In vivo*, the MicroMedDeBakey VAD was shown to increase levels of platelet damage markers, platelet-factor 4 (PF4) and beta thromboglobulin β-TG (β-TG)[40], a concept resulting in impaired aggregation and adherence.

A recent study showed that many platelet abnormalities precede LVAD implantation. Patients with New York Heart Association class IV heart failure requiring LVAD placement typically exhibit multisystem dysfunction. Impaired ristocetin-induced platelet aggregation may be present in majority of LVAD recipients and normalizes after heart transplant[41]. Decreased hepatorenal perfusion results in decreased thrombopoetin production and uremia[42,43]. A cohort study of 112 LVAD patients showed that renal dysfunction correlated with increased incidence of GIB. The same study showed that ventricular failure was strongly associated with increased bleeding[13]. This can be explained by the augmented vascular resistance found in heart failure patients that leads to stasis and causes platelets to become hypersensitive and readily degranulate. Such platelets aggregate in a loose manner, are easily broken up by the sheer forces of blood flow and released back into the circulation dysfunctional[44]. Interestingly, there seems to be no difference in platelet aggregometry in patients with LVAD compared to their own blood prior to LVAD implantation, a result with important implications[44]. Indeed, while normalization in perfusion parameters and end organ function may improve after LVAD placement, the device use itself may result in additional burden on platelet function.

Fibrinolysis

Previous studies have indicated an augmented acti-

vation of fibrinolytic systems in older end stage heart failure patients prior to undergoing LVAD implant for destination therapy, likely a result of pre-existing state of inflammation[45]. It has been shown that baseline fibrinogen and d-dimer levels peak at one month and subsequently return to almost normal levels by one year post-implantation[36]. It is possible that early rise in fibrinolysis is related to the initial biomaterial contact with blood protein. Studies have revealed that the majority of bleeding events occur during the first year after LVAD placement, well within this coagulopathic time frame[11]. Further analysis is necessary to better evaluate additional factors in fibrinolytic triggering mechanism of heart failure patients both pre- and post- LVAD implantation.

Acquired von willebrand syndrome

Perhaps one of the more important aspects in understanding propensity to bleed in LVAD recipients is the development of acquired von Willebrand syndrome (avWs). Physiologically, vWF is made in the endothelial cells, released into the bloodstream as a high molecular weight (HMW) multimer that bind factor Ⅷ and plays an important role in hemostasis. It is cleaved by ADAMTS-13, glycosylated and cleared from the bloodstream with a half-life of 12-20 h[46]. Effective hemostasis relies on the proper balance between its production and elimination[47]. However, mechanical forces of LVAD device can easily shear and deform the HMW multimers into smaller, medium and low molecular weight fragments, which impairs thrombosis and interferes with platelet aggregation[41]. In a study of 37 LVAD patients, all subjects showed significant loss of high molecular weight vWFmultimers within 30 d of CF-LVAD placement[48]. vWF multimers and platelet aggregation are significantly reduced in all LVAD recepients but return to normal post-explantation[49]. Importantly, recent analysis with enzyme-linked immunosorbent assay revealed that the LVAD-induced proteolysis is speed dependent[50], resulting in important implications on LVADs setting and bleeding risk and potentially providing an opportunity to regulate hemostasis on individual basis. Finally, normal vWF multimers play an important role in platelet-induced hemostasis as blood flows through angiodysplasias and vWF defragmentation may by itself be pro-angiogenic, an important concept in potential explanation of gastrointestinal hemorrhage in LVAD patients[35,51].

Vascular abnormalities

Finally, most important factor in development of GIB in LVAD patients remains presence of old or development of new gastrointestinal lesion. While existing gastrointestinal pathology may increase likelihood of bleeding, the LVAD placement put patients at risk of angiodysplasia formation in the upper gastrointestinal tract. CF-LVADS create a direct connection between the left ventricle and the aorta bypassing the aortic valve[52]. Similar to the hemodynamic changes in severe aortic stenosis, described by Edward C. Heyde in 1958[53], the continuous flow state of the LVADs create chronic narrow pulse pressure system altering neurovascular physiology, increasing sympathetic tone, intraluminal pressure, and smooth muscle relaxation with resultant distention of submucosal venous plexus and angioectasia formation[54]. Vascular anomalies have also been shown in selected animal studies[55]. Apparent predilection of bleeding angioectasias to the upper gastrointestinal tract may be related to the close proximity of the celiac axis and proximal jejuna branches of the proximal superior mesenteric artery to the LVAD and thus receiving more effective stress compared to distal gastrointestinal vasculature. In addition, chronic low flow state observed in congestive heart failure patients places their capillary systems at the state of equilibrium which is offset immediately by increased cardiac output seen after LVAD implantation[13].

Occult NSAID and other medication use

Many LVAD recipients are elderly and the devices function as destination therapy rather than as a bridge to transplantation. These patients may be concurrently taking over-the counter non-steroidal anti-inflammatory medications (NSAIDs) for other causes, such as arthralgias and arthritis. NSAID use if often not screened for and, as shown in a cross-sectional survey of emergency room departments in the United States, only 58% of the patients are aware of NSAID side effects, with 48% believing that the medications are entirely safe[56]. Polypharmacy is a frequently seen phenomenon in elderly and morbid patients, placing them at risk of drug-drug interaction and additional effects on hemostasis. Common medications used in patients with advanced heart failure and LVAD placement include b-blockers, ACE-inhibitors and calcium channel blockers - all of which may affect platelet function and aggregation[44].

CLINICAL PRESENTATION AND RISK FACTORS

Gastrointestinal hemorrhage in patients with LVAD presents similarly to general population including hematemesis, coffee-grounds emesis, melena, heme positive stools, and rectal bleeding. Patients may complain of worsening fatigue, weakness, lightheadedness, dizziness or show up entirely asymptomatic. Laboratory analysis may reveal worsening anemia, rise in international normalized ratio, decrease in platelet count, and increase in blood urea nitrogen compared to patient's baseline values. The average time to first presentation of GIB is close to 5 mo, but the clinician should be aware of early and late episodes that may occur immediately or long after

LVAD placement. Recurrent GIB is common and may not always follow the initial episode in time or type of presentation. Hemodynamic instability is uncommon but may occur. Male gender and older age may place patients at additional risk of bleeding following LVAD placement. Potential risk factors include history of pre-LVAD GIB, use of concomitant anti-platelet and vitamin K antagonist therapy, increased pump rotary speed, right ventricular dysfunction, and post-LVAD ejection fraction > 30%[9,11,13] - probably a combination of general predisposition, effective potentiation of hemorrhage, and altered neurovascular physiology in LVAD recipients. Additional variables are likely to emerge with time as we gain more insight into disease processes with increased utilization of LVADs in cardiology practice worldwide.

LOCATION IN THE GI TRACT AND DIFFERENTIAL DIAGNOSIS

Various studies have focused on identifying distribution of culprit lesion in the gastrointestinal tract in LVAD recipients. In fact, every part of the bowel may be affected but the majority of the bleeding seems to originate in the stomach, duodenum or jejunum. Over half of the offenders are angiodysplasias of which nearly 90% occur in the upper gastrointestinal tract with 25% of them affecting the jejunum[11]. Other causes may include hemorrhage from Dieulafoy lesions, peptic ulcer disease, bowel ischemia, radiation proctitis, neoplasia, diverticulosis, or hemorrhoids. It is important to realize that while LVAD patients have a propensity for developing angioectasia-associated bleeding from the upper GI tract or have another GI-related etiology of bleeding, the differential diagnosis of anemia in such patients should also embrace the non-gastrointestinal causes including nasal, retroperitoneal, genitourinary, mediastinal, prostate, intracranial bleeding, hemolysis, mineral deficiencies, or anemia related to renal insufficiency or chronic disease[11,12,36]. In fact, several conditions may co-exist and gastrointestinal bleeding may present as an acute drop in the hematocrit level from the baseline abnormal value.

MANAGEMENT

Approach to LVAD patients with gastrointestinal hemorrhage should be multidisciplinary and comprehensive. In the emergency room setting, patient should be immediately assessed and hemodynamically resuscitated. Detailed history and review of systems should be performed and special attention needs to be directed at the medical list including over-the-counter or herbal medications. NSAIDs use should be questioned as it may contribute to the cause of bleeding and is often under-reported by the patient. Physical examination including evaluation of LVAD function by cardiology team and consult to gastroenterology service should be completed in the emergency department. Intensive care admission should be assessed on an individual basis. Urgent bloodwork should include complete blood count, metabolic and hepatic panels, INR, fibrinogen, and a d-dimer assay. Holding of the antiplatelet and anticoagulation therapy with consideration of platelet and/or fresh frozen plasma transfusion should be discussed with cardiology service, weighing in risk of thrombosis. Correction of blood abnormalities, including packed red blood cell transfusion should be performed in patients with significant anemia or signs of hemodynamic compromise. Endoscopic management of the GIB in LVAD patients remains a cornerstone in both identifying the source of hemorrhage and effectively intervening on it to stop the bleeding. Importantly, it should be performed with assistance of cardiac anesthesiologist or a general anesthesiologist trained in LVAD patient management. Known predilection to the upper gastrointestinal tract together with clinical presentation and stool analysis provide gastroenterologist an initial clue to the location of bleeding. Colonoscopy has limited role in evaluation for cause of GIB in patients with LVAD[51] but should be performed for colon cancer screening in age appropriate group according to standard guidelines[57]. With the exception of frank rectal bleeding with hemodynamic stability where a colonoscopy may be the initial approach, upper gastrointestinal endoscopy is always warranted. However, esophagogastroduodenoscopy is non-diagnostic in over two thirds of the patients[11,32]. Patients with melena or hematochezia associated brisk upper gastrointestinal hemorrhage and suspected small intestinal angiodysplastic lesion would require a push enteroscopy to evaluate proximal jejunum. In fact, routine use of push enteroscopy increased diagnostic and therefore therapeutic yield from 29% to 90% in LVAD patients with gastrointestinal hemorrhage and may decrease future GI readmission rates[11,32]. Deep balloon assisted enteroscopy or small bowel video capsule endoscopy may be necessary in the remaining cases. In fact, video capsule (while safe in LVAD patients) may aid in detection of bleeding in up to 40% of the cases, with majority localized in the proximal small bowel[58], although a follow up endoscopic intervention would still be warranted. Easy to perform and well tolerated, capsule endoscopy is not therapeutic, may delay immediate diagnosis, and result in increase in hospital stay[58]. Early use of deep balloon assisted enteroscopy, on the other hand, may decrease transfusion requirements, length and cost of hospitalization, but is cumbersome, riskier, and more invasive that traditional push enteroscopy[59-61]. It may provide additional benefit in cases of suspected hemorrhage in LVAD patients beyond proximal to mid jejunum[11,59]. Endoluminal intervention is directed at the culprit lesion, and given high incidence of

small intestinal angiodysplasias, various hemostatic tools may be employed including argon plasma coagulation alone or in combination with resolution clips/submucosal racemic epinephrine injection[11,62,63]. In fact, utilization of LVAD specific GIB algorithm on institutional basis may provide structured approach to effective management of this patient population and ultimately improve clinical outcome[11].

Discharge planning of LVAD patients with GIB should focus on secondary prevention of future hemorrhages. In fact, up to half of the patients presenting with their first hemorrhage may return to the hospital with recurrent GIB approximately 100 d after initial admission[11] and repeat endoluminal intervention may be warranted. Multidisciplinary approach with risk stratification involving cardiology, hematology, and gastroenterology services may discuss potential alterations in antiplatelet and anticoagulation therapies or adjustment of the LVAD pump speed[13,35]. On a case by case basis, success with octreotide - a somatostatin analogue which acts as a splanchnic vasoconstrictor and may affect angiogenesis - has been reported[64], but its application to control bleeding and prevent future hemorrhages in LVAD patients may be offset by high cost and limited outpatient use[11]. Recent small phase I trial showed octreotide potential effectiveness and general tolerability as early intervention[65] and future large prospective randomized investigations would be necessary to further define its role in LVAD population. There was anecdotal use of antihemophilic factor/vWF complex and hormonal (estrogen, desmopressin) therapy in refractory bleeding[35,66], although unclear if the patient would have benefited from repeated small intestinal evaluation prior to its initiation. Importantly, desmopressin use, while increasing circulating vWF, places the patient at risk of thrombosis[67]. Of interest, blood type A patients may exhibit lowest risk of bleeding[48], an factor that may play a role in risk-stratification of LVAD patients. Possible correlation to GIB may be found in patients with increased nasal hypervascularity after LVAD implantation[68] and may be potentially used to help risk stratify patients. Future research may provide additional insight into management of such patients, perhaps focusing on further definition of various parameters in individual anticoagulation therapy and recognizing patients with highest risk for bleeding at the time of LVAD implant. Primary prevention strategies therefore may come into play with potential pre-LVAD gastrointestinal and hematologic screening.

PROGNOSIS

GIB in LVAD recipients increases overall patient mortality compared to non-bleeders[13] and places additional burden on cost of care[11]. Rebleeding rate may be seen close to 50% in the literature[46,51]. Early jejunal intubation with push enteroscopy significantly increases the yield of detection and correction of angiodysplastic bleeding with four fifths decrease in GIB related hospital readmissions[11] but additional long term studies are required. Recent advances in understanding of pathophysiology, risk factor recognition, etiology, and timely intervention are expected to improve overall prognosis in LVAD GIB patients. Ultimately, cessation of GIB is seen in all patients after cardiac transplantation, likely a reflection of normalization of previously LVAD-induced hematologic and hemodynamic parameters.

CONCLUSION

As CF-LVADS have replaced pulsatile devices, overall outcomes have improved. However, the incidence of gastrointestinal bleeding has increased significantly and hemorrhage remains major concern in patients receiving LVAD placement for end stage congestive heart failure, resulting in rise of hospital admissions, procedure burden, and cost[4,11]. Etiology is multifactorial, a combination of post-operative use of blood thinners, activation of fibrinolytic pathway, acquired vWF deficiency, and device related upper gastrointestinal angiodysplasia formation. Multispecialty approach with individual risk stratification and tailored therapy plays an important role in managing this patient population. Algorithmic endoscopic approach with gastroduodenojejunal evaluation is crucial in hospitalized patients. Future long term randomized studies are necessary and should focus on primary and secondary prevention of GIB in LVAD patients without compromising device function and pump related non-GI complications.

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REVIEW

Role of non-steroidal anti-inflammatory drugs on intestinal permeability and nonalcoholic fatty liver disease

Erika Utzeri, Paolo Usai

Erika Utzeri, Paolo Usai, Department of Medical Sciences, University of Cagliari, 09124 Cagliari, Italy

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Correspondence to: Paolo Usai, Associate Professor, Department of Medical Sciences, University of Cagliari, Via Università, 40, 09124 Cagliari, Italy. usaip@medicina.unica.it
Telephone: +39-70-51096138

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Abstract

The use of non-steroidal anti-inflammatory drugs (NSAIDs) is widespread worldwide thanks to their analgesic, anti-inflammatory and antipyretic effects. However, even more attention is placed upon the recurrence of digestive system complications in the course of their use. Recent data suggests that the complications of the lower gastro-intestinal tract may be as frequent and severe as those of the upper tract. NSAIDs enteropathy is due to enterohepatic recycling of the drugs resulting in a prolonged and repeated exposure of the intestinal mucosa to the compound and its metabolites. Thus leading to so-called topical effects, which, in turn, lead to an impairment of the intestinal barrier. This process determines bacterial translocation and toxic substances of intestinal origin in the portal circulation, leading to an endotoxaemia. This condition could determine a liver inflammatory response and might promote the development of non-alcoholic steatohepatitis, mostly in patients with risk factors such as obesity, metabolic syndrome and a high fat diet, which may induce a small intestinal bacterial overgrowth and dysbiosis. This alteration of gut microbiota may contribute to nonalcoholic fatty liver disease and its related disorders in two ways: firstly causing a malfunction of the tight junctions that play a critical role in the increase of intestinal permeability, and then secondly leading to the development of insulin resistance, body weight gain, lipogenesis, fibrogenesis and hepatic oxidative stress.

Key words: Non-steroidal anti-inflammatory drugs; Intestinal barrier; Intestinal permeability; Non-steroidal anti-inflammatory drugs - enteropathy; Nonalcoholic fatty liver disease; Nonalcoholic steatohepatitis; Microbiota; Metabolic syndrome; Proton pump inhibitors; Endotoxaemia

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Core tip: Among the gastro-intestinal effects, in non-steroidal anti-inflammatory drugs (NSAIDs) users, those of the lower tract seem to be rising. NSAIDs enteropathy is due to the enterohepatic recycling of drugs, resulting in a prolonged and repeated exposure of the intestinal mucosa to the compound and its

metabolites, leading to so called topical effects. The impairment of the intestinal barrier represents the initial damage of NSAIDs enteropathy that leads to the translocation of bacteria and toxic substances of intestinal origin in the portal circulation, promoting an endotoxaemia. This condition, mostly in patients with risk factors for nonalcoholic fatty liver diseas, such as obesity and metabolic syndrome, might lead to liver inflammatory response that could promote the development of nonalcoholic steatohepatitis.

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INTRODUCTION

Nonsteroidal anti-inflammatory drugs (NSAIDs) are among the most consumed drugs in the world thanks to their benefits as analgesic, anti-inflammatory and antipyretic agents[1]. However, these benefits are, in part, overshadowed by the recurrence of digestive system complications which may arise during the course of therapy and can, at times, be severe.

Nevertheless, NSAIDs can cause a variety of functional and structural abnormalities, even in the small and large intestine for patients who make long-term use of the said drugs. About 60%-70% of patients on long-term NSAIDs develop mucosal damage[2-4], including an increase in intestinal permeability, intestinal inflammation, erosions and protein loss, but also more serious complications such as anemia, bleeding, ulcers, perforations, obstruction, diverticulitis, ileal dysfunction and diaphragm-like strictures[5-7] (Table 1). It is estimated that about one third of all complications associated with the use of NSAIDs is represented by severe injuries of the small bowel[8]. Several videoenterocapsule studies have shown that the use of NSAIDs [both nonselective and selective cyclooxygenase-2 (COX-2) inhibitors] may be associated with a high incidence of small-bowel erosion and ulceration (55%-75%)[9-12]; the chronic use of low dose aspirin has also been shown to be associated with the presence of similar small-bowel lesions[13,14]. Recent data has shown that the incidence of complications of the lower gastrointestinal tract, many of these due to the use of NSAIDs and ASA, is on the rise while the incidence of upper gastrointestinal lesions is declining[15].

At the core of this broad spectrum of lesions there is a multifactorial pathogenesis with structural and functional alterations of different components, which make up the intestinal barrier. Additionally, the appearance of factors that interfere with the maintenance and homeostasis of normal bowel functionality can be found.

Table 1 Occurrence of main adverse effects of non-steroidal anti-inflammatory drugs in the lower gastrointestinal tract with non-steroidal anti-inflammatory drug use

Adverse effect	Frequency
Increased gut permeability	44%-70%
Gut inflammation	60%-70%
Blood loss and anemia	30%
Malabsorption	40%-70%
Mucosal ulceration	30%-40%
Protein loss	10%
Mucosal ulceration	30%-40%
Complications requiring hospitalizations	0.3%-0.9%
Diaphragms of the small bowel	< 1%

Table constructed using data from[2,3,5].

Due to the intestinal barrier and its constituent elements alteration, in particular intestinal permeability, luminal substances including toxins, microorganisms and their components can access the portal circulation causing toxemia with pathological effects also in the long term. In this regard, there is strong evidence to confirm the liver as one of the main targets of toxemia resulting from the alteration of intestinal permeability. As a consequence, a process of inflammation, as well as an alteration in the metabolic processes can occur in the liver, contributing to the pathogenesis of Non Alcoholic Fatty Liver Disease and to its various manifestations[16], which, are beyond the scope of this paper.

PATHOGENESIS OF NSAID ENTEROPATHY

Adverse effects mediated by inhibition of COX

The NSAIDs pharmacological target is the inhibition of cyclooxygenase (COX or prostaglandin endoperoxide synthetase) with consequent reduction in the production of prostaglandins (PGE)[17]. The PGE are implicated in a significant number of critical functions in the bowel. The cyclooxygenase consists of two isoforms with distinct functions but both inhibited by NSAIDs: (1) the COX-1, which is constitutively expressed in many tissues, in the gut catalyzes the formation of many cytoprotective PGE involved in the synthesis of mucus, bicarbonate, maintenance of blood flow, the turnover of epithelial cells and the resolution of inflammatory processes; and (2) COX-2 is an inducible form implicated in the resolution of inflammation processes. It is responsible for the production of a variety of PGE that may cause or protect against inflammatory processes[18].

The concept that the inhibition of both isoforms causes enteropathy is strengthened by several studies. Unlike traditional NSAIDs, selective COX-2 NSAIDs results in lower adverse gastrointestinal effects[19], even if this beneficial effect may be lost with long-term use[14].

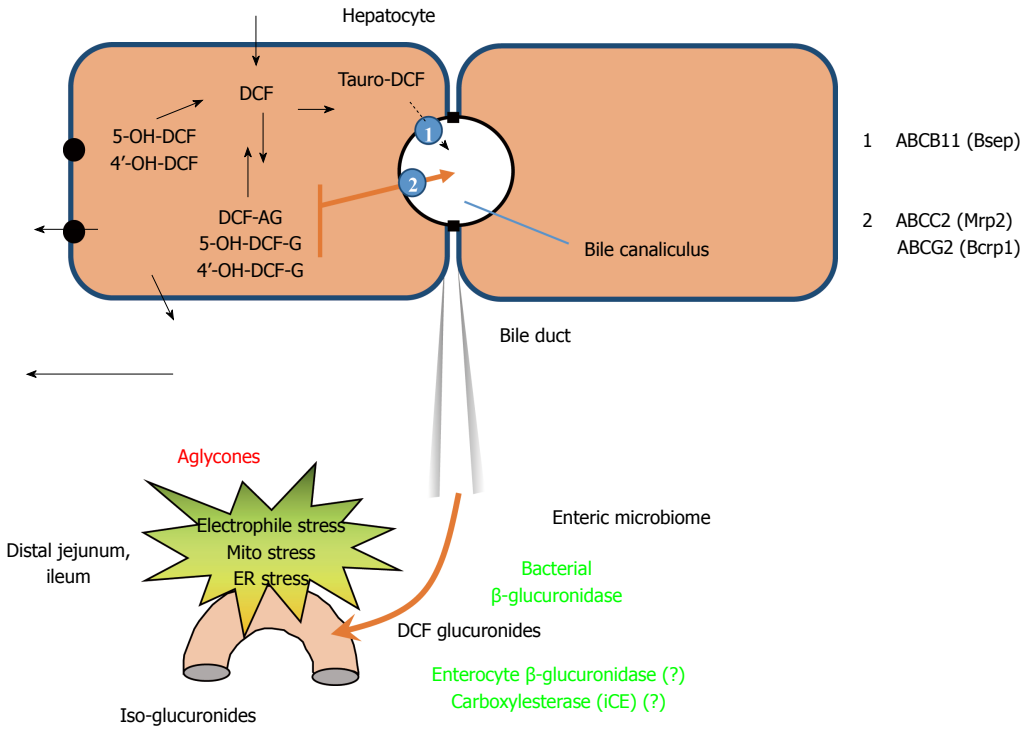

Figure 1 Enterohepatic circulation of non-steroidal anti-inflammatory drugs[21].

Mechanisms of jejunal and ileal mucosal damage

Topical effects: Multi - hit concept: The mechanisms underlying NSAIDs enteropathy are primarily represented by the so-called "topical effects", *i.e.*, those adverse effects coming from the high local concentration of NSAIDs in the intestinal lumen[20] and their enterohepatic circulation. The topical effects are by definition independent from the impact of NSAIDs on COX[21].

From a pharmacokinetic point of view NSAIDs are weak acids (pKa 3-6) which are protonated and then absorbed in the stomach according to their lipophilicity[22,23]. Subsequently, the NSAID-containing carboxylic acids, after their introduction either orally or intraperitoneally, reach the liver *via* the portal system where the drug is glucuronidated: it is conjugated to glucuronic acid[24,25] or taurine[26] or sulfate, and excreted into the bile in large quantities. Specifically, it is exported inside the bile canaliculi against a concentration gradient through the ATP-dependent transporters present on the apical membrane of the hepatocyte, the MRP2 (ABCC2)[27] or Bcrp1 (ABCG2)[28]; the specific carrier of tauro-conjugates is less defined.

At this point the small intestine is exposed to the drug and to its oxidative conjugated metabolites that reach the most distal part where the glucuronide is cleaved by bacterial beta-glucuronidase, forming aglycones, which are free derivatives of NSAIDs or oxidative metabolites[29]. At this point the drug is transferred again into the enterohepatic circulation (Figure 1).

An initial increase in small intestine permeability is a prerequisite of the subsequent development of small intestine inflammation, which is associated with blood and protein loss but is often silent[30].

It would appear that the enterohepatic recycling results in a prolonged and repeated exposure of the intestinal mucosa to the compound[31]. They include the uptake of the drug and its metabolites in the enterocytes where they are metabolized by cytochrome P450 (CYP450) in order to potentially reactivate intermediates with possible bioactivation and the induction of mitochondrial[32-34] and endoplasmic reticulum stress[35,36] (Figure 2). Therefore, the production of reactive metabolites occurs through CYPs of enterocytes, ER stress, oxidative stress and mitochondrial damage[21]. In humans it is mainly CYP2C8/9/19 to be involved in the oxidative biotransformation of many FANS[37]. This step is called the "first hit". After this initial insult of enterocytes, the mucosal epithelium becomes more permeable and the LPS present in the lumen can penetrate deeply into the mucosa and activate the toll-like receptor 4 (TLR4) of macrophages in the lamina propria. This can cause cell damage mediated by the tumor necrosis factor, and subsequently the activation of the innate immune system with the recruitment of inflammatory cells into the injury site. The inflammatory response that follows is the "second hit"[38].

First hit

Mitochondrial damage: most NSAIDs cause a decoupling of oxidative phosphorylation in the mitochondria both *in vivo* and *in vitro*, dissociating

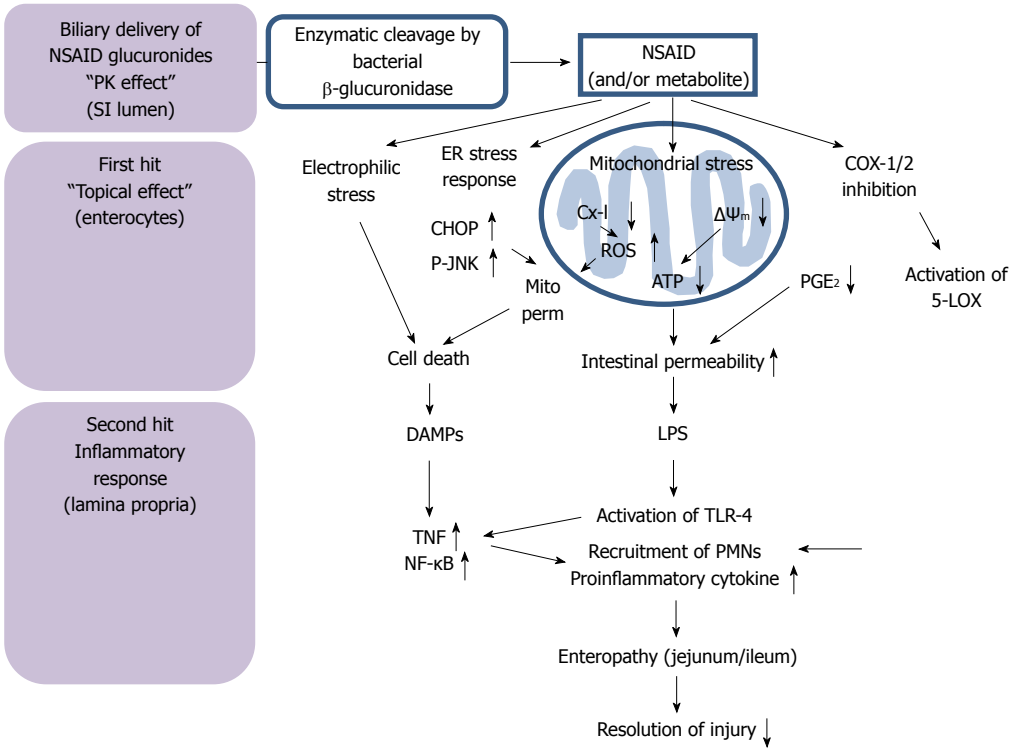

Figure 2 First hit and second hit in non-steroidal anti-inflammatory drugs enteropathy[21].

the breathing from the production of energy and dissipating the inner transmembrane potential of mitochondria[39]. During the absorption of the NSAIDs there is an intracellular accumulation of the drug proportional to its acidity, even at micromolar concentrations: this is able to uncouple oxidative phosphorylation at the mitochondrial level. This event can have two effects on enterocytes. Firstly, an attenuation of ATP production with gradual depletion of cellular ATP; secondly, a collapse of the gradient can determine the opening of the mitochondrial permeability transition pore (mPT) leading to cell death[20]. This ability is due to the structure of NSAIDs. In fact, NSAIDs are weak and lipophilic acids which induce enteropathy through mitochondrial energetic depletion[20]. Some NSAIDs inhibit several complexes of the electron transport chain. Other NSAIDs, such as indomethacin and diclofenac, inhibit the activity of rotenone-sensitive complex I in mitochondria and therefore increase the production of superoxide. This inhibition is reversible with the administration of quercetin, an ubiquinone-mimetic (coenzyme Q)[40].

Interaction with biomembranes: is due to the direct effect of NSAIDs on cell membranes by altering the biophysical properties. One example is the electrostatic interaction between the NSAID and hydrophobic anions and the positive charged nitrogen of phosphatidylcholine, which alters the biophysical properties of the membrane, its fluidity and finally increases the permeability to protons and to bacterial toxins[41].

Detergents properties: NSAIDs are invariably lipophilic weak acids and this makes them the detergents for phospholipid components of the brush border. This causes direct damage to the epithelial surface.

Mitochondrial permeability: NSAIDs can induce mitochondrial permeabilization followed by the release of apoptotic factors from the intermembrane space inside the cytosol. This mechanism is mediated by the opening of the MPT pore, involving both the internal and external membrane, and can be triggered by an increase of Calcium (a mechanism introduced by many NSAIDs), oxidative stress or by the collapse of mitochondrial membrane potential[42,43].

Intestinal permeability: this effect also affects TJ, which are under the control of the actin-myosin ATP-dependent complex. The consequence is an increase in the intestinal permeability[44]. The reduction of mitochondrial ATP production causes a loss of the intestinal barrier function and this can be tested quantitatively by the oral administration of dextran[45].

Oxidative Stress: there is only indirect evidence of its involvement in NSAIDs enteropathy. For example, indomethacin raises the expression of heme oxygenase, an antioxidant enzyme induced by oxidative stress[46]. Another pathway activated by oxidative stress is that of the MAPK (*via* phosphorylation of JNK). These effects can be induced by mitochondrial dysfunction that increase oxidative

stress. This last event may be a side effect triggered by the inflammatory response of the innate immune system cells[32].

ER stress: According to some studies performed on patients taking diclofenac, there is an increase of markers of endoplasmic reticulum stress proteins, like GRP78 and CHOP. CHOP is a transcription factor that induces cell death mediated by mitochondria[47].

Second hit

It consists of the innate immune system and the inflammatory response. The innate response is triggered by bacteria and proinflammatory mediators coming from bacteria that invade the mucous layer over the epithelium. As a result, the signaling pathway TLR-mediated is activated and the neutrophils infiltrate the damaged areas. On the other hand, the adaptive immune system does not seem to play a critical role in NSAIDs enteropathy[21].

TLR and LPS: TLRs recognize specific molecular patterns associated with pathogens, and trigger the inflammatory response. In particular, TLR4 is the LPS receptor and it is expressed in monocytes and macrophages of the lamina propria as an extracellular domain rich in leucinic repetitions and an IL-1R signal intracellular domain[48]. So, the TLR4 activates the NF-kB with consequent production of proinflammatory cytokines including TNF and IL-1 beta[49].

TNF: prostaglandins, and in particular PGE2, inhibit TNF synthesis, while the reduced levels of prostaglandins induced by NSAIDs lead to an increase of its synthesis[50]. TNF is implicated in the apoptosis of enterocytes and in the inflammatory response in the intestine. However, according to some studies, TNF appears to have cytoprotective effects on the intestinal mucosa by inducing the expression of COX2, mediated by EGFR transactivation[51]. Also IL-17A should be mentioned. It is produced by T cells of the lamina propria and regulates the production of proinflammatory cytokines and chemokines[52].

Neutrophils: the NSAID enteropathy is characterized by a massive infiltration of neutrophils in the ulcerated areas, which aggravate the damage through the production of ROS or protease. The biomarkers used for their study is the time-dependent increase in the activity of myeloperoxidase[38].

Bile acid metabolism: the critical role of bile in the pathogenesis of NSAID enteropathy is evident from studies showing that bile duct ligation prevented NSAID-induced intestinal damage in rats[20,53-55]. According to some animal models, the NSAIDs, especially indomethacin, are rapidly excreted *via* the bile and then enter the enterohepatic circulation through the small intestine, resulting in a high concentration of the drugs in the liver and bile. Bile acids are cytotoxic because of their cleansing effect, by binding to phospholipids and directly altering the integrity of the membrane. The phospholipids and cholesterol are considered luminary factors with direct effects that appear to be involved in protective mechanisms on gastrointestinal and liver cells through a cleansing effect on the bile acids. NSAIDs are highly amphiphilic molecules and create stronger links with the phospholipids. Animal and laboratory studies show that NSAIDs reduce the hydrophobic properties of the upper GI barrier, partly determined by the active surface phospholipids. Therefore, NSAIDs secreted in bile interact with its amphipathic components, such as phosphatidylcholine and bile acids; this leads to an alteration of the structure and the stability of these components, and consequently the toxicity of bile in the small intestine is modified[56]. Dial *et al*[57] examined the biliary phosphatidylcholine (PC), which appears to have protective effects on enterocytes, cholangiocytes and erythrocytes against damage induced by bile salts. NSAIDs appear to determine intestinal damage in proportion to their ability to be secreted *via* the bile because of their capacity to chemically bind with the micelles and with the PC, lowering their effects. As previously mentioned, NSAIDs bind the PC and this process takes place in the gastrointestinal tract, where the drug-induced loss of PC, which protects the mucosa, causes mucosal damage. The PC would protect it from both damage induced by bile salts and by NSAIDs[57,58].

ROLE OF GUT MICROBIOTA

The intestinal micro-organisms and their degradation products are necessary for the development of NSAIDs enteropathy, as "germ-free" animals were found to be resistant to indomethacin injuries[14,59]. Therefore, the role of enteric bacteria in determining the NSAID enteropathy is twofold: (1) the toxic insult on the tight junctions determines an increase in intestinal permeability with a subsequent bacterial invasion of the mucous membrane that activates the TLRs essential for the development of NSAID-induced small bowel lesions; and (2) they can metabolically convert NSAIDs glucuronide in aglycones by activation of beta-glucuronidase. Such enzymatic activity would seem greater in the distal part of the small intestine than in the other parts[60]. However, the gus gene, which codes for this enzyme, is not present in all bacterial strains, but only in 50% of the human gut symbiotic bacteria[61].

It has also been shown that treatment with NSAIDs may disrupt the homeostasis of intestinal flora and has been associated with an overgrowth of Gram-negative and anaerobic bacterial species in the small intestine, secreting LPS, which are able to

exacerbate the NSAID-induced intestinal injury[38,62-64]. This mechanism of unbalanced increase of Gram-negative bacteria in NSAID users has not, as yet, been elucidated, however, it seems that the bacteria might easily penetrate into the mucosa when mucosal permeability is enhanced by NSAIDs[65].

Recently more importance has been placed on the co-administration of NSAIDs and the inhibitors of gastric acid secretion, such as proton pump inhibitors and histamine H2 receptor antagonists, in determining a significant alteration of intestinal microbiota composition and exacerbating NSAIDs enteropathy[21,66]. PPI determine hypochloremia causing abnormal growth of bacteria that can colonize the small intestine causing SIBO (Small Intestinal Bacterial Overgrowth) with increased bacterial translocation[67,68]. Wallace *et al*[69] in fact reported that PPIs, in particular omeprazole resulted in significant dysbiosis, with both a substantial increase in Gram-negative bacteria and a significant reduction in the proportion of Actinobacteria (mainly Bifidobacter ssp.) in the jejunum.

INTESTINAL BARRIER AND NAFLD

There is growing evidence that an alterated interaction between gut microbiota and the host at the intestinal mucosa level determine an impairment of gut-liver axis and contribute to a state of low-grade inflammation, endotoxemia, obesity and metabolic liver disorders like nonalcoholic fatty liver disease (NAFLD) and nonalcoholic steatohepatitis (NASH)[70-72]. Through the alteration of the intestinal barrier, as happens in the NSAID enteropathy, a translocation of bacteria and toxic substances of intestinal origin can occur: entering the portal circulation, they can reach the liver and can lead to a large number of pathological alterations such as steatosis, steatohepatitis and liver fibrosis. It can be assumed that this condition promotes the development of NASH in patients with predisposition factors towards the development of NAFLD, such as obesity and metabolic syndrome. Although NAFLD is a multifactorial disease, in recent years more and more importance has been given to the role of microbiota, which seems to be crucial in determining its development, starting from the accumulation of fat in the liver through to the triggering of liver inflammation.

In this regard, several studies have shown that in obese patients there is an alteration of the specific individual bacterial composition, with a reversal of the Bacteroides and Firmicutes ratio, with Bifidobaceri reduction, resulting in an increase of bacteria able to metabolize carbohydrates and assume energy, thus increasing adiposity[73]. These studies also indicate that obese patients do not have a predetermined microbial composition, but it is rather the specific Western diet, which is high in fat, that influences this composition by increasing the Firmicutes. It is also possible to observe a similar prevalence of Firmicutes in patients with NAFLD[74].

Specifically, NSAIDs would act at colic level exposing the patients with NASH to a susceptibility to gut leakiness. In this case, the endotoxemia represents the stimulus required to trigger the necro-inflammatory cascade in hepatocytes, already affected by alteration in lipid homeostasis induced by obesity. According to a study by Farhadi *et al*[75] 2008, the intestinal permeability was measured in patients with steatosis or with NASH and in healthy patients, before and after the administration of aspirin, through the urinary excretion: the lactulose/mannitol (L/M) ratio was evaluated after 5 h, while the sucralose after 24 h. It was observed that the aspirin increases the urinary excretion L/M in the majority of patients, but, especially, it significantly increases the intestinal permeability in patients with NASH. According to this model, patients with NASH would not have a constantly altered intestinal permeability, but this situation could occur due to stress factors such as aspirin, NSAIDs, psychological or physical stress or other. For this reason it would be reasonable that patients with a particular susceptibility to oxidative stress, such as those with metabolic syndrome (obesity, diabetes, NAFLD and insulin resistance) and altered metabolism of fatty acids, avoided agents such as alcohol and NSAIDs that increase intestinal permeability.

LIVER DIRECT EFFECTS OF NSAIDS

The direct effects of NSAIDs in determining liver damage should be emphasized as this is a predisposing factor for the development of nonalcoholic hepatic steatosis (micro or macrovesicular type) and steatohepatitis. In fact, these drugs are implicated in the pathogenesis of the so-called drug-induced liver injury[76], which is diagnosed when the worsening of liver function is given by prescribed medications or not. Steatohepatitis caused by drugs can occur many months after their use and cannot be resolved within 15 d. However, it can be possible that the drugs exacerbate a pre-existing NAFLD[77-80].

The drugs that determine steatosis and NASH interfere firstly with the mitochondrial respiration, beta-oxidation, or both, as shown in one of the first studies performed on Pirprofen[81]. When the hepatic mitochondrial beta oxidation is severely inhibited, the damage of the beta-CoA oxidation increases the levels of non-esterified fatty acids, which are converted into triglycerides determining hepatic steatosis[82]. An increased production of ROS is the result of this process, and, in the most severe cases, this increase leads to liver necrosis[83,84].

THERAPEUTIC APPROACHES

Currently, there are no approved pharmacological strategies that can treat or completely prevent NSAID-mediated enteropathy. Some compounds suitable to

reduce the inflammatory response or to stimulate the effects mediated by prostaglandins were used, but with limited effectiveness or adverse effects[85,86]. Most of the experiments and therapeutic approaches are focused on the inflammatory component that constitutes the second hit, while approaches to protect against the damage of the first hit (mitochondrial stress, endoplasmic reticulum stress, electrophilic stress) or acting on the release of glucuronide and aglicoles, have not yet been fully explored. Among the various approaches, there is the use of NO or H2S -releasing NSAIDs, because, as is well known, nitric oxide and hydrogen sulfide are powerful vasodilatory molecules that protect the mucous membrane and maintain its integrity[87]. Therefore, it could be reasonable to assume that therapeutic strategies that aim to restore the intestinal microbiota firstly with dietary interventions, antibiotics and probiotics could be sound practice.

Since there are multiple mechanisms involved in NSAIDs enteropathy and in the subsequent development of NAFLD, also the therapeutic approach has to aim at applying multiple strategies simultaneously.

CONCLUSION

NAFLD is a rising disease in the Western world due to the increased predisposing diet and lifestyle, and its incidence grows along with that of obesity and metabolic syndrome. Moreover, the simultaneous use of NSAIDs such as analgesics, anti-inflammatory and antipyretic, and the growing prospect of new therapeutic uses also as anti - cancer drugs or in the treatment of Alzheimer's, make them widespread drugs. Despite being beyond the scope of the present work, the many positive effects of NSAIDs cannot be overlooked, primarily their role in malignant transformation. The long-term use of aspirin and other NSAIDs has been shown to reduce the risk of colon cancer and other gastrointestinal organs in addition to cancer of the breast, prostate, lung and skin. NSAIDs restore normal apoptosis and reduce cell proliferation in human adenomatous colorectal polyps. Moreover, NSAIDs, particularly selective COX-2 inhibitors, have been shown to inhibit angiogenesis in cell culture and in rodent models of angiogenesis[88].

The magnitude of serious outcomes from the lower GI tract is not well defined, but recent data suggests that they may be as frequent and severe as upper GI complications. Contrary to what happens in the upper GI tract, treatment and prevention of NSAID enteropathy is difficult, since the pathogenic mechanisms are different and not well understood. Therefore attention should be paid to the administration of NSAIDs or aspirin in patients with particular susceptibility to oxidative stress such as those with metabolic syndrome (obesity, diabetes and insulin resistance) and NAFLD and to the co-administration of antisecretory agents which may exacerbate NSAID-induced intestinal damage. The impairment of the intestinal barrier, represents, the initial damage of NSAIDs enteropathy leading to an endotoxaemia, due to the translocation of bacteria and toxic substances of intestinal origin in the portal circulation. This condition could determine a liver inflammatory response and might promote the development of NASH. Nevertheless, it is necessary to consider the incessant increase of the NSAID enteropathy, and, therefore, research into new therapeutic strategies is needed to prevent or reduce the incidence of this complication and consequent systemic diseases, like NAFLD.

Since the NSAID-induced enteropathy that may accelerate NAFLD/NASH seems at the moment to be an interesting pathogenetic hypothesis, further prospective studies will be necessary in order to definitely confirm such theory.

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REVIEW

Molecular mimicry in *Helicobacter pylori* infections

Magdalena Chmiela, Weronika Gonciarz

Magdalena Chmiela, Weronika Gonciarz, Department of Immunology and Infectious Biology, Institute of Microbiology, Biotechnology and Immunology, Faculty of Biology and Environmental Protection, University of Lodz, 90-237 Lodz, Poland

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Correspondence to: Magdalena Chmiela, MD, PhD, Professor, Department of Immunology and Infectious Biology, Institute of Microbiology, Biotechnology and Immunology, Faculty of Biology and Environmental Protection, University of Lodz, Banacha 12/16, 90-237 Lodz, Poland. chmiela@biol.uni.lodz.pl
Telephone: +48-42-6354186
Fax: +48-42-6655818

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Abstract

Gram-negative bacteria *Helicobacter pylori* (*H. pylori*) colonize gastric mucosa in humans and increase the risk of serious diseases such as gastric and duodenal ulcers, stomach cancers and mucosa associated lymphoid tissue lymphoma. The role of *H. pylori* infection in the pathogenesis of several extragastric diseases has been suggested including immune thrombocytopenic purpura, iron deficiency anemia, vitamin D deficiency, cardiovascular diseases, diabetes mellitus and dermatological disorders. Also neurological diseases and even lung cancer have attracted researchers concern. The relation between *H. pylori* infection and a growth retardation in children has also been suggested. Many mechanisms of molecular mimicry between *H. pylori* and the host have been proposed as a pathogen strategy to manipulate the immune system of the host in order to remain unrecognized and avoid eradication. A lot of effort has been put into the demonstration of homologous sequences between *H. pylori* and host compounds. However, knowledge about how often autoantibodies or autoreactive T lymphocytes induced during *H. pylori* infections cause pathological disorders is insufficient. This review provides data on *H. pylori* antigenic mimicry and possible deleterious effects due to the induction of immune response to the components common to these bacteria and the host.

Key words: *Helicobacter pylori*; Molecular mimicry; Anti-self response; Extragastric effects

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Core tip: Molecular mimicry between *Helicobacter pylori* (*H. pylori*) and the host structures has been suggested as an effective mechanism of antibody production, potentially autoreactive. The chronic character of *H. pylori* infections increases the risk of such production and initiation or maintenance of *H.*

pylori related pathological disorders triggered by the host effector immune mechanisms during infection. The panel of components common to *H. pylori* and the host is still increasing and thus the risk of autoimmune complications is an open problem.

Chmiela M, Gonciarz W. Molecular mimicry in *Helicobacter pylori* infections. *World J Gastroenterol* 2017; 23(22): 3964-3977 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i22/3964.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i22.3964

INTRODUCTION

H. pylori pathogenicity - brief summary

Helicobacter pylori (*H. pylori*) a Gram-negative pathogenic bacterium, which has been described by Warren and Marshall in 1983[1], colonizes the gastric epithelium of humans (on average, 50% of the human population) and induces an excessive inflammatory response with or without symptoms (20% of cases). *H. pylori* infections possibly lead to different disorders such as: gastric and duodenal ulcers and, chronic gastritis, and even malignant diseases, including: mucosa-associated lymphoid tissue (MALT) lymphoma and gastric cancer[2-6]. Polymorphisms of the host genes encoding interleukins (ILs), including IL-1β, tumor necrosis factor (TNF-α) and cyclooxygenase -2 (COX2) have been suggested to increase the risk of infection and its severe consequences[7]. *H. pylori* strains have different genes encoding virulence factors that are important for disease development[8-10], which are either secreted, membrane-associated or translocated into cytosol of the host cells *via* the IV type secretion system, where they can affect the host cell functions[4]. *H. pylori* strains produce different adhesins, such as blood group antigen - binding adhesin (BabA), sialylated blood group - related adhesin (SabA), adherence - associated lipoprotein (AlpA/B) and outer membrane inflammatory protein (OipA), which promote close contact between the bacteria and the gastric epithelium[8-10]. Soluble factors such as urease and vacuolating cytotoxin (VacA) alter gastric cell survival and intercellular adhesion[11-16].

H. pylori CagA (Cytotoxin - associated gene A) is a highly immunogenic protein, which can trigger inflammatory responses in host gastric tissues, and it may influence the cell morphology, polarity, and proliferation; CagA also modulates the activity of immune cells and increases the risk of severe consequences, such as gastric ulcer and cancer[17-25]. Due to bacterial cell lysis, CagA and other *H. pylori* virulence factors can also be delivered to the gastric mucosa in a soluble form and affect the host immune cells infiltrating this milieu[24-27]. Moreover, *H. pylori* continuously produces phospholipid vesicles, which can be distributed by the circulation and function as a secondary extragastric source of CagA and other virulence factors[28-34]. Mucosal recognition of CagA is associated with the stimulation of epithelial cells that produce elevated levels of various cytokines, including IL-1β, IL-6 and IL-8, which is followed by the enhanced infiltration of activated neutrophils and severe mucosal inflammation that increases the risk of gastric cancer[19,35-38].

In addition, flagellin and especially lipopolysaccharide (LPS) were investigated to address their role in *H. pylori* pathogenesis *via* activation of NF-κB and chemokine expression[39]. Previous studies showed that *H. pylori* LPS possesses immunomodulatory properties that diminish the effectiveness of the phagocytosis, cytotoxic activity and the expansion of NK cells and T lymphocytes[40-42].

The interactions of *H. pylori* with host cells result in adherence, induction of inflammatory responses through cytokine/chemokine release, apoptosis or proliferation, which finally result in persistent colonization, severe inflammation, and disruption of the epithelial barrier function[43,44].

This process can enable the translocation of *H. pylori* virulence factors and inflammatory mediators into the circulation and promote or intensify the development of systemic inflammatory response and the possible clinical effects of *H. pylori* infections outside the stomach[45,46].

The role of *H. pylori* in some hematologic conditions has been considered, such as immune thrombocytopenic purpura (ITP), iron deficiency anemia (IDA), and vitamin B12 deficiency. The possible role of *H. pylori* infection in other hematologic diseases, such as non-Hodgkin lymphomas of the stomach, monoclonal gammopathy of undetermined significance, megaloblastic anemia and myelodysplastic syndromes, has been suggested[47]. The elevated risk of childhood leukemia and hemorrhage in patients with coagulation disorders due to *H. pylori* infection has also been considered. The effects of *H. pylori* on other disorders, such as cardiovascular diseases, diabetes mellitus, dermatological disorders, neurological disorders and even lung cancer, have also attracted attention of researchers[48-53]. Data obtained from these studies showed that the immune response induced by *H. pylori* may influence the clinical outcome of these disorders. Many seroepidemiological studies have shown that patients with coronary heart disease (CHD) produce anti-*H. pylori* antibodies[54-57]. A strong immune response triggered by *H. pylori* CagA - positive strains has been suggested to influence the development of atherosclerosis[58]. Many previous studies have stated that chronic infection with *H. pylori* has a significant influence on the immune system. Therefore, the possible mechanisms of *H. pylori* infections in the pathogenesis of the majority of extragastric diseases include chronic local or systemic inflammation and the

initiation of autoimmune responses[59].

CONCEPT OF TRIGGERING AUTOIMMUNE DISORDERS DUE TO MOLECULAR MIMICRY BETWEEN INFECTIOUS AGENTS AND HOST COMPONENTS

Molecular mimicry is a common strategy used by infectious agents to adapt to the host organism and avoid its immune response mechanisms. Molecular mimicry is defined as an antigenic and functional similarity between the second-row microbial structures and host molecules that leads to the production of auto-reactive antibodies, which may contribute to the development of autoimmune disorders. Similarities within and between linear amino acid sequences and spatial structures have been identifiedd[60-62].

Streptococcus pyogenes is one of the most intensely studied bacterial pathogens, that can trigger autoimmune diseases in genetically susceptible individuals. *S. pyogenes* is involved in the development of rheumatic fever and glomerulonephritis due to the induction of antibodies recognizing bacterial M protein and N-acetyl-β-D-glucosamine (GLcNAc) as well as human heart myosin[62,63]. Moreover, infections with Gram-negative bacteria, such as *Klebsiella pneumoniae* and *Campylobacter jejuni*, also stimulate the production of crossreactive antibodies that recognize the human leukocyte antigen (HLA)-B27 or gangliosides[62,64]. Additionally, certain viruses, such as the Epstein-Barr virus and the hepatitis B virus, share similar sequences with proteins in the central nervous system[65,66]. Molecular mimicry combined with the ability of T cells to evade the mechanisms of tolerance has been suggested as a potential mechanism implicated in the pathogenesis of various autoimmune diseases, including multiple sclerosis, diabetes mellitus and spondyloarthropaties[60,66-68].

MOLECULAR MIMICRY BETWEEN *H. PYLORI* AND HOST CELL COMPONENTS

The mechanisms by which *H. pylori* infections lead to various gastric and potentially extragastric disorders are still poorly understood. One concept indicates the role of autoimmune processes. Chronic exposure to *H. pylori* compounds may initiate autoimmune gastritis due to molecular mimicry between *H. pylori* structures and the host tissue. The hypothesis of the induction by *H. pylori* anti-self reactions was proposed after antibodies with reactivity to the gastric antral mucosa were detected in the sera of infected patients[69-71]. Many mechanisms underlying the molecular mimicry between *H. pylori* and the host have been proposed and many efforts have been made to identify homologous sequences between *H. pylori* and host polypeptides, including the P-type adenosine triphosphate (ATP)-ases CopA and CopP that are involved in heavy metal iron transport, 686-bp amino acid ATPase, VacA, and urease beta chain *vs* gastric H+K+-ATPase[72-75], heat shock protein (Hsp) A *vs* GroEs, HspB *vs* 60-kDa host Hsp[76], and hemagglutinin/protease (hap) *vs* carbonic anhydrase[77]. However, whether and how often the autoantibodies induced in response to *H. pylori* infection are involved in various post-infectious pathologies due to the pathogen - induced autoreactive T lymphocytes or antibodies is unclear. The examples of potential autoantigenic host targets for anti-*H. pylori* antibodies are listed in Figure 1.

H+/K+-adenosine triphosphatase as an autoantigen in autoimmune gastritis

Autoimmune gastritis/pernicious anemia is characterized by two phenomena: atrophy in the corpus and fundus of the stomach and autoantibody production against parietal cells (PC) and their secretory component called an intrinsic factor (IF)[78-80]. Anti-PC antibodies, which target H+/K+ ATPase, a gastric proton pump, have been detected in 60%-85% of patients with autoimmune gastritis, whereas antibodies to IF have been detected in 30%-50% of patients with autoimmune gastritis[81-83]. Chronic autoaggression to H+/K+ ATPase may diminish gastric acid secretion, and cause hypergastrinemia and anemia due to iron deficiency[84,85]. Pernicious anemia is also characterized by a vitamin B12 deficiency. Patients suffering from autoimmune gastritis are predisposed to gastric tumors and adenocarcinomas[86]. In patients with type 1 diabetes or autoimmune thyroid disease, the prevalence of autoimmune gastritis is approximately three-fold higher than that of the general population, in which such autoimmune disorder has the frequency of 2%[87]. CD4+ T lymphocytes, which recognize parietal cell H+/K+ ATPase, have been shown to be involved in the development of autoimmune gastritis. H+/K+ ATPase is released from parietal cells during normal cell turnover and is selectively captured and then processed by antigen - presenting cells[88,89]. Another possibility is that *H. pylori* infection may initiate the development of autoimmune gastritis and pernicious anemia through the activation of T lymphocytes that are autoreactive to H+/K+ ATPase due to the antigenic mimicry between gastric H+/K+ ATPase and *H. pylori* at the T cell level[90]. Antibodies to gastric H+/K+ ATPase and their secretory forms are produced by B lymphocytes in cooperation with CD4+ antigen-specific T lymphocytes[91,92]. The deleterious effects of autoantibodies can be a consequence of T cell perforin-dependent cytotoxicity and apoptosis initiated by interaction between the Fas receptor (Fas) an Fas ligand[71]. The role of chronic *H. pylori* infections in the development of atrophic gastritis has

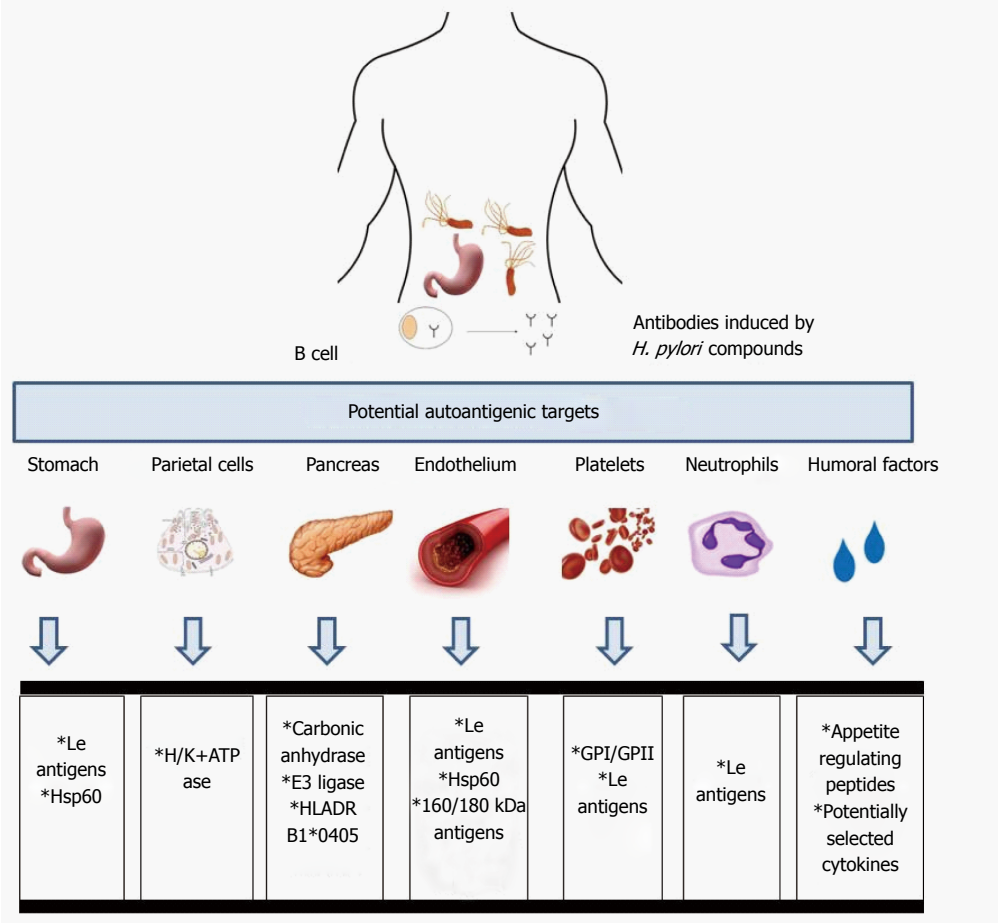

Figure 1 Hypothesis of autoimmune disorders due to molecular mimicry between *Helicobacter pylori* and the host components. Chronic exposure of the host immune system to *Helicobacter pylori* (*H. pylori*) components that have homologous sequences with the host cellular or soluble compounds may initiate the production of autoantibodies. However, how often the autoantibodies arising during *H. pylori* infection are involved in various post-infectious pathologies should be elucidated. The graph shows the examples of host targets for the antibodies induced by *H. pylori* components. GP: Glycoproteins; Hsp: Heat shock protein; H+/K+ ATPase: H+/K+-adenosine triphosphatase; HLA: Human leukocyte antigens; CCRL1: CC chemokine receptor-like 1; Le: Lewis antigens.

been suggested on the basis of the positive correlation between gastric autoantibodies and antibodies specific to *H. pylori* antigens in the majority of patients with pernicious anemia[69,75,93-95]. However, this association has not been confirmed in other studies[96,97].

Anti-Lewis antibodies induced by H. pylori lipopolysaccharide determinants

The presence of antibodies that react with the gastric mucosa in patients infected with *H. pylori* suggested that the autoantibodies induced by this pathogen may play an important role in the *H. pylori* - associated inflammatory response and cause deleterious gastric effects[75,94,98,99]. These antibodies could be stimulated by various Lewis (Le) antigens (Le^x, Le^y and $Le^{x/y}$) that are present in the LPS structure of many *H. pylori* isolates[99,100], and on human cells including polymorphonuclear leucocytes, gastric epithelial cells and endothelial cells. The LPS O-specific chain of the *H. pylori* reference strain NCTC (National Collection of Type Cultures) 11637 was found to possess determinants similar to the human Le^x blood group antigens**,** whereas LPS of the *H. pylori* MO19 strain contains determinants similar to human Le^y[101-103]. Other blood group antigens, including H type 1, Le^a, Le^b, nonfucosylated polylactosoamine (i-antigen), sialyl Le^x, and blood group A but not H type 2 have been detected in various *H. pylori* isolates. Additionally, strains bearing two or three blood group antigens in their LPS have been described[103-106]. The *H. pylori* LPS phase variation, which is defined as the random reversible change in phenotype in a range of blood group determinants, has been described for both reference and clinical strains[107,108]. During *H. pylori* infection different environmental and host factors including - gastric juice acidity may promote the selection of bacteria with the best phenotype in terms of virulence[109,110]. In a rhesus monkey model of *H. pylori* infection, the host Le^y phenotype of the gastric mucosa was shown to select the Le^y - positive phenotype of *H. pylori*, and the Le^x host gastric phenotype was shown to select the Le^x - positive bacteria[109]. Phase variations from Le^x to i-Ag and back to Le^x, from Le^x to Le^x plus Le^y, and from Le^x to Le^y and forming Le^a have been described[104,111-113]. The molecular mechanism of *H. pylori* LPS phase variation

depends on mutations in the genes encoding α3-fucosylotransferases, the activity of these proteins and their preference for carbohydrate residues that determine antigenic specificity[82,108,114-117].

Experiments performed with the use of anti-Le monoclonal antibodies induced by immunization of mice with *H. pylori* showed that these antibodies reacted with both murine and human gastric mucosa, foveolar and glandular epithelial cells and parietal cell canaliculi. The anti-Le^x monoclonal antibodies provoked by *H. pylori* were shown to react with polymorphonuclear leukocytes, gastric mucin and H+/K+ ATPase, which all express Le antigens[118]. However, it is unclear how anti-Le antibodies influence *H. pylori* adhesion and colonization of gastric mucosa in light of the results showing that the attachment of *H. pylori* to the human gastric epithelium is mediated by blood group antigens, including Le^b, Le^x and sialylated-Le^x[8,119-121]. One possible mechanism underlying this effect is the diversification of *H. pylori* in the human host through lipopolysaccharide phase variation due to the heterologous expression of the alpha 1,3-fucosyltransferase gene[107,117]. The role of anti-$Le^{x/y}$ antibodies in the pathogenesis of *H. pylori* - driven deleterious effects is controversial. It has been hypothesized that anti-Le antibodies initiated by *H. pylori*, if bound to the gastric epithelium, can cause complement - dependent cell lysis promoting an excessive inflammatory response[99]. In early studies, these antibodies were not detected at all or only found in a low number of serum samples from individuals infected with a *H. pylori*. Other studies, including our previous work, have revealed that humans may produce anti-Le^x antibodies, particularly those of the IgM class, in the absence of *H. pylori* infection or in the context of *H. pylori* - independent dyspepsia[118,122]. This finding indicates that anti-Le antibodies may be natural antibodies associated with the physiological autoimmunity required for the elimination of self-antigens. However, the incidence of this antibody production increases with age and, can be associated with the history of infections during the life of an individual[123]. The occurrence of anti-Le^x antibodies in the sera of subjects not infected *H. pylori* could be induced by other microorganisms, such as streptococci, *Eikenella corrodens* or *Acinetobacter actinomycetemcomitans* bearing Le^x determinants[124]. However, the possibility that *H. pylori* locally induces anti-$Le^{x/y}$ antibodies, which bind directly to gastric mucosal epitopes, and are absent in the serum, cannot be excluded[88,99]. Interestingly, the frequency of anti-$Le^{x/y}$ antibodies in the sera of patients infected with *H. pylori* and exhibiting gastritis symptoms, as well as in the patients with confirmed ischemic heart disease and *H. pylori* co-infection, was correlated with the increased occurrence of soluble $Le^{x/y}$-anti-$Le^{x/y}$ IgG immune complexes[123-125]. It is possible that the deleterious effects of anti-Le antibodies depend on their ability to bind ligands and form rather small immune complexes, which may be deposited locally in both gastric and endothelial tissues where they can promote the inflammatory response. Perhaps the severity of anti-Le antibody production in *H. pylori* - infected individuals is associated with higher exposure to Le antigens due to inflammation, damage to the gastric epithelium and/or vascular endothelial cells, and the migration and activation of immune cells. Since the expression of $Le^{x/y}$ determinants in *H. pylori* is related to the *cagA* status[126], anti-Le antibodies may increase the inflammatory effects on the gastric mucosa in association with *H. pylori* virulence proteins, such as CagA, VacA and urease. However, Zheng *et al*[127] showed that peptic ulcer disease was not related to *cagA* status, *iceA* (induced by contact with the epithelium) or *vacA* genotypes, but there was an association with increased expression of a combination of Le antigens in *H. pylori*. This finding suggests that gastric disorders related to *H. pylori* infection depend on a specific type of host-pathogen interactions. The bacterial Le determinants may promote the adaptation of bacteria to the host gastric mucosa, which allow them to evade the host immune response and establish a chronic infection, and tissue destruction *via* the induction of anti-Le autoantibodies. The complex strategy of *H. pylori* for survival in the gastric mucosa of the host involves both structural modifications of lipid A in LPS to diminish its endotoxic properties and the expression and variation of Le determinants that mimick host components[125].

Link between H.pylori infection and ITP

In 1988 Gasbarrini *et al*[128], showed that eradication of *H. pylori* resulted in regression of ITP. There are several potential mechanisms that combain *H. pylori* infection with ITP. One is molecular mimicry between *H. pylori* CagA protein and platelet glycoproteins: GPI and GPII[129-131]. In ITP patients infected with CagA positive but not CagA negative *H. pylori* strains a higher number of B lymphocytes producing anti-CagA antibodies that crossreact with the platelet specific peptides have been detected, which was correlated with the elevated levels of such antibodies in the patients sera[129,131]. A complement dependent mechanism of platelet destruction has been suggested[132,133]. Also Lewis antigenic determinants deposited on the surface of platelets may be recognized in ITP patients by anti-Le antibodies. During *H. pylori* infection the production of anti-Le antibodies is enhanced in response to Le antigens present in *H. pylori* LPS[123,130]. The infection can promote platelet aggregation, and the enhancement of expression of phosphatidilserine and p-selectin that may be involved in ITP development[133]. The platelet aggregation is also due to binding the von Willebrand factor by *H. pylori*[134]. Another possibility is that anti-*H. pylori* antibodies that link the platelet GP I protein with

phagocyte FcRIIa receptors may increase the clearance of platelets during phagocytosis. Bacterial LPS if deposited on the surface of platelets may enhance the immune phagocytosis[135]. Th1 lymphocytes activated during infection by *H. pylori* antigens are important for the maintenance of ITP[133]. Concerning the host genetic factors the HLA-DQB1*03 haplotype has been proposed as useful marker for prediction of the platelet response in *H. pylori* infected patients[136].

H. pylotri - related autoimmune hypothesis of cardiovascular disorders

H. pylori infections, especially those with CagA - positive strains, have been suggested to be associated with atherosclerotic vascular disease[58,137]. Patients suffering from CHD were found to be chronically exposed to *H. pylori* at a higher frequency than non-CHD individuals, which was shown by the high frequency and elevated levels of specific anti-*H. pylori* IgG and IgA antibodies[54,138,139] and strong inflammatory response[140], upregulation of biochemical markers, coronary lumen reduction, and elevated levels of low density lipoprotein, C-reactive protein, homocysteine, fibrinogen, plasminogen and inflammatory cytokines and the higher incidence of diabetes in *H. pylori* - infected individuals than in uninfected individuals[141-146]. However, in several studies, no associations between *H. pylori* seropositivity, exposure to CagA and CHD incidence have been found[46,147]. In the search for links between *H. pylori* infection and CHD, it has been suggested that *H. pylori* - induced antibodies with cross-reacting potency towards the host endothelium may play a role in the development and maintenance of atherosclerotic lesions. Autoimmune responses have been shown to participate in the initiation and progression of atherosclerosis[148]. Franceschi *et al*[149], have investigated whether antibodies against CagA cross-reacted with antigens of normal and atherosclerotic arteries, which would provide a possible link to the disorders observed during atherosclerosis. In this study, anti-CagA antibodies interacted with different parts of smooth muscle cells and endothelial cells present in the thin layer sections of atherosclerotic vessels. The antibodies recognized two vascular antigens of 160 and 180 K, which were present in both normal and atherosclerotic artery lysates and a 130 K protein from *H. pylori* lysates[149].

All *H. pylori* isolates produce urease, which can hydrolyze the urea present in the human stomach[150,151]. *H. pylori* urease is composed of a 26.5 kDa UreA subunit (β chain) and a 61.7 UreB subunit (α chain), which are encoded by the *ureA* and *ureB* genes, respectively[152,153]. Although the UreA subunit is the major immunodominant protein the UreB subunit has a higher number of epitopes recognized by anti-urease antibodies[154,155]. The occurrence of anti-urease antibodies was correlated with age and the immunoglobulin class and was linked with the severity of *H. pylori* - related disease symptoms. Superficial gastritis was correlated with a higher production of anti-urease IgA, whereas athrophy of the gastric epithelium was associated with elevated levels of anti-urease IgG immunoglobulins[156]. Recently, a hypothesis linking atherosclerosis and *H. pylori* - induced anti-urease antibodies has been suggested[157]. In the study by Arabski *et al*[158], a significant correlation between the level of antibodies recognizing the 8-mer synthetic peptide corresponding to the UreB minimal flap epitope of *H. pylori* urease and atherosclerosis symptoms was found. This *H. pylori* urease region exhibited similarity to the human CCRL1 (CC chemokine receptor-like 1) protein, which is expressed in heart tissue. Antibodies to *H. pylori* urease initiated during infection might be autoreactive due to the binding of the IKEDV motif in the CCRL1 host receptor. This antigen-antibody interaction may potentially accelerate complement - dependent tissue destruction and the inflammatory response in patients with atherosclerosis lesions and *H. pylori* infections[157,158].

H. pylori synthesizes the two heat shock proteins: HspA (GroES chaperonin or Hsp 10 homologue) and HspB (GroEL chaperonin or Hsp60 homologue)[76,159,160]. Both antigens reportedly induce autoimmune responses[161,162]. The study by Matusiak *et al*[139], supports the idea that chronic exposure to *H. pylori* in patients with CHD may result in an increase in the level of serum lipopolysaccharide - binding protein (LBP) and the production of antibodies against *H. pylori* Hsp B, which crossreact with human Hsp60. Both LBP and anti-Hsp 60 antibodies may facilitate the inflammation in the vascular endothelium. The pathological role of LBP may depend on the phenotype of the vascular endothelium, which exhibits proinflammatory features such as increased expression of pathogen recognition receptors. The involvement of anti-Hsp60 Igs in CHD-related deleterious processes can be explained by the antigenic mimicry and complement - dependent cell damage, which are possibly induced by these antibodies, similarly to the anti-*H. pylori* urease antibodies. Because the expression levels of Hsp proteins, including Hsp60, increases as a result of the inflammatory process in atherosclerotic lesions, it can be assumed that these proteins may be a target for anti-Hsp antibodies initiated by an infectious agent[148]. In regard to HspA, the clinical outcomes of *H. pylori* infection have been shown to be unrelated to HspA antigenicity or amino acid sequence variation[160]. However, age-specific responses to HspA in *H. pylori*-positive subjects have been found[163].

H. pylori infection and autoimmune pancreatitis

The association between *H. pylori* infection and insulin resistance has been suggested[146]. Recently, a significant homology between the human carbonic anhydrase II segment 5-255 and the α-carbonic

anhydrase of *H. pylori* segment 23-239, has been found, with 27% identity and 41% similarity[77]. Anhydrase is a key enzyme for the survival and growth of *H. pylori* in the gastric mucosa. In humans carbonic anhydrase II coordinates the physiological function of the pancreas. Moreover, the homologous regions contain the binding motifs of the HLA DRB1*0405[77]. These observations support the idea that *H. pylori* infection can trigger autoimmune pancreatitis in genetically susceptible individuals. In 2009, Frulloni *et al*[164] showed that in almost all patients with autoimmune pancreatitis there are antibodies against *H. pylori* plasminogen-binding protein (PBP). This PBP protein shows homology with ubiquitin-protein ligase E3 component n-recognition 2, which is an enzyme highly expressed in the acinar cells of the pancreas. This could be another example of *H. pylori* and host molecular mimicry triggering autoimmune pancreatitis.

H. pylori - host antigenic mimicry and growth retardation in children

The relationship between *H. pylori* infections and growth retardation in children is poorly understood. Growth retardation may result from appetite disorders, abnormal metabolism and iron deficiency[165]. Infection with *H. pylori* causes gastrointestinal bleeding, abnormal absorption of iron due to the impaired gastric acid and insulin secretion, and vitamin C uptake. The mechanism driving anemia in children infected with *H. pylori* may be antigenic mimicry. *H. pylori* has an iron-binding protein similar to ferritin, which prevents iron excess. The infection also causes an increase in the concentration of iron-binding lactoferrin in the stomach epithelium[165]. Recent studies indicate the role of the immune system in controlling behaviors related to food intake by producing autoantibodies against peptides and neuropeptides regulating appetite, which may result in a reduction in height and weight[166].

The gastrointestinal microflora, including *H. pylori* may be a source of antigens, which are similar to appetite - regulating peptides. Thus, the bacterial antigens are potentially able to stimulate the immune system of the gastrointestinal tract to produce autoantibodies that are cross-reactive with many of the appetite -regulating peptides and that modify the actions of these peptides. In the sera of pediatric patients with short stature the autoantibodies against 14 key hormones and peptides regulating appetite such as leptin, ghrelin, orexin, and alpha-melanocyte-stimulating hormone (α-MSH), have been detected[166]. These antibodies are also present in healthy subjects, which suggests physiological role for these antibodies in the regulation of hunger and satiety. A number of common sequences between these peptides and proteins of microorganisms have been identified, including antigenic similarity between leptin and the intestinal microflora proteins of *Lactococcus lactis*, *Escherichia coli*, *Lactobacillus bacteriophage* and representatives of *Candida* and *Aspergillus*. The sequence homology of α-MSH and the components of pathogenic *E. coli*, *H. pylori*, *Clostridium tetani*, and *Candida albicans* has also been demonstrated. Regulatory peptides are signaling molecules, and autoantibody blocking of their sequences may alter their biological activity. A recent study highlighted the impact of *H. pylori* on the secretion of ghrelin and leptin[167]. Patients infected with *H. pylori* have been shown to have a significantly lower level of leptin and ghrelin in the plasma. The ghrelin concentration was also lower in the mucous cells of the stomach. After eradication of the infection, the level of ghrelin rose again. However, other authors did not confirm this result[168]. In the studies carried out on a group of Polish children, it was shown that the levels of gastrin in the patients infected with *H. pylori* were significantly higher, whereas the levels of ghrelin and leptin were lower than those of the controls[169]. Growth failure in children due to anemia occurs more often in patients infected with *H. pylori cagA*+ than *cagA*- strains. It has been shown that platelet glycoprotein and the CagA protein are similar and that many patients infected with *H. pylori* with signs of thrombocytopenia have possess anti-platelet antibodies[165]. These results suggest a role for the CagA protein in the development of systemic pathological processes in children infected with *cagA*+ *H. pylori* strains. Further studies are needed to assess the prevalence and the levels of antibodies against the common sequences for CagA protein and peptides regulating appetite in children with short stature. These sequences have been identified by bioinformatic analysis of leptin, ghrelin, visfatin and resistin, which regulate appetite, energy homeostasis, and potentially the immune system[167,170-173]. The release of these proteins is often stimulated by inflammatory processes, growth and gonadal hormones. The results of experiments conducted with serum samples from children with idiopathic short stature and growth hormone deficiency showed that some of the children that were also infected with *H. pylori* and/or exposed to *C. albicans* have antibodies against ghrelin, leptin, orexin A and α-MSH, which may potentially disturb the physiological functions of these molecules[174,175]. This result is potentially due to molecular mimicry between antigens of these microbiota and the mentioned peptides. However, further studies are needed to elucidate this suggested relation.

Newly described gastric potentially autoantigenic proteins as possible targets for antibodies induced by *H. pylori*

Recently, amino acid identity between additional autoantigens derived from the gastric mucosa and gastric adenocarcinoma cells (AGS) and several *H. pylori* proteins has been identified. A proteomics

investigation of anti-gastric autoantibody profiles in the sera of 300 Korean adults infected with *H. pylori*, revealed nearly forty autoantigenic proteins, including nicotinamide adenine dinucleotide phosphate (NADP+) alcohol dehydrogenase, alpha enolase, gastrokine-1, gastric triacylglycerol lipase, Hsp70 kDa protein 1, and peroxiredoxin-2. These proteins were detected in the gastric mucosal tissue[59]. The programmed cell death 6 - interacting protein, serum albumin and T-complex protein 1 subunit gamma were identified in the AGS cells. Several proteins such as albumin, alpha-enolase, annexin A3, cytoplasmic actin 1, Hsp - like 71 kDa protein and leukocyte elastase inhibitor, were detected in AGS cells and gastric mucosal tissue. Furthermore, the alpha-enolase, glutathione S-transferase P, Hsp - like 71 kDa protein, Hsp70 kDa protein 1, mitochondrial Hsp60 kDa, peroxiredoxin-2, 78 kDa glucose-regulated protein precursor, tyrosine-protein phosphatase non-receptor type 11 and tryptophan-aspartic acid repeat-containing protein (WD), showed 60% or even higher amino acid positivity[59]. These newly described gastric proteins may have the ability to control and prevention gastroduodenal disorders linked to *H. pylori* infections, such as chronic gastritis, gastroduodenal ulcers, atrophic gastritis and gastric cancers. However, their role in the pathophysiology of these disorders needs to be examined.

Gastric tissue ulceration initiated by *H. pylori* is related to the elevated production of alarming molecules, including IL-33, which may function as a classic cytokine or transcription factor[176]. IL-33 is suspected to alert the immune system to restore epithelial cell homeostasis. However, IL-33 has also been suspected to play an emerging role in autoimmune diseases[177]. Bioinformatic analysis indicates homology between the amino acid sequences of *H. pylori* CagA and human Hsp60, as well as IL-33. Hypothetically, both host Hsp60 and IL-33 can be targeted by antibodies induced during *H. pylori cagA*+ infections, which may affect gastric inflammatory reactions.

Although the homologous sequences of *H. pylori* and several new host targets have been demonstrated by computer and proteomic analyses, more research is needed to demonstrate the role of these homologous sequences in development of pathological processes due to autoimmune responses initiated by *H. pylori* components.

DISCUSSION

In light of this review, we hypothesize that *H. pylori* possessing antigens that are similar in structure to human cells, tissues and some humoral compounds, which play an important structural and physiological role, through induction of humoral and possible cellular immune responses, may drive tissue destruction and the development a pathological inflammatory response. Chronic exposure of specific memory cells to these *H. pylori* compounds enables their sustained stimulation and transformation into effector lymphocytes, which may be involved in the autoimmune-mediated tissue destruction. Further studies and deeper analyses are necessary to demonstrate the autoimmune potential of specific *H. pylori* antigens.

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REVIEW

Gallbladder cancer epidemiology, pathogenesis and molecular genetics: Recent update

Aarti Sharma, Kiran Lata Sharma, Annapurna Gupta, Alka Yadav, Ashok Kumar

Aarti Sharma, Annapurna Gupta, Alka Yadav, Ashok Kumar, Department of Surgical Gastroenterology, Sanjay Gandhi Post Graduate Institute of Medical Sciences, Lucknow 226014, India

Kiran Lata Sharma, Department of Biochemistry and Medical Genetics, John Buhler Research Centre, University of Manitoba, Winnipeg MB R3E 3P4, Canada

Author contributions: Sharma A, Sharma KL and Kumar A have written the manuscript; Sharma A, Sharma KL and Gupta A has contributed in literature search; Sharma A, Sharma KL, Gupta A and Yadav A have contributed in designing tables as well as referencing; Kumar A is senior Corresponding author; Sharma KL is co-corresponding author; all the authors have approved the final version of the manuscript.

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Correspondence to: Dr. Ashok Kumar, Professor, Department of Surgical Gastroenterology, Sanjay Gandhi Post Graduate Institute of Medical Sciences, Raebareli Road, Lucknow 226014, India. akgupta@sgpgi.ac.in
Telephone: +91-522-2494423
Fax: +91-522-2668017

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Abstract

Gallbladder cancer is a malignancy of biliary tract which is infrequent in developed countries but common in some specific geographical regions of developing countries. Late diagnosis and deprived prognosis are major problems for treatment of gallbladder carcinoma. The dramatic associations of this orphan cancer with various genetic and environmental factors are responsible for its poorly defined pathogenesis. An understanding to the relationship between epidemiology, molecular genetics and pathogenesis of gallbladder cancer can add new insights to its undetermined pathophysiology. Present review article provides a recent update regarding epidemiology, pathogenesis, and molecular genetics of gallbladder cancer. We systematically reviewed published literature on gallbladder cancer from online search engine PubMed (http://www.ncbi.nlm.nih.gov/pubmed). Various keywords used for retrieval of articles were Gallbladder, cancer Epidemiology, molecular genetics and bullion operators like AND, OR, NOT. Cross references were manually searched from various online search engines (http://www.ncbi.nlm.nih.gov/pubmed,https://scholar.google.co.in/, http://www.medline.com/home.jsp). Most of the articles published from 1982 to 2015 in peer reviewed journals have been included in this review.

Key words: Gallbladder cancer; Epidemiology; Molecular genetics; Pathogenesis

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Core tip: The Gallbladder cancer is a fatal malignancy which displays considerable differences in certain ethnicities and geographic regions. Indo-Gangetic plains of India, Mapuche Indians in Chile and South America are most affected regions with this cancer. Because of this cancer is largely unstudied as compare to other cancers Present review provides a comprehensive summery of the studies conducted regarding its Epidemiology, Pathogenesis and molecular genetics. This will be helpful for the researchers to understand the current scenario of research work and how much success we have gained till now. Based on which future research work can be planned in appropriate directions.

Sharma A, Sharma KL, Gupta A, Yadav A, Kumar A. Gallbladder cancer epidemiology, pathogenesis and molecular genetics: Recent update. *World J Gastroenterol* 2017; 23(22): 3978-3998 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i22/3978.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i22.3978

INTRODUCTION

Gallbladder cancer (GBC) is a rare biliary tract malignancy in most western countries, but is much widespread in some other regions of the world. Moreover, this carcinoma is infrequent in developed countries but more common in some developing countries, characterized by its lack of symptoms at initial stage leading to difficulties in treatment.

The extensive variation in geography, ethnicity, and cultural differences in the incidence of gallbladder cancer suggests the role of key genetic and environmental factors associated with the development and progression of the disease[1,2]. The lack of a serosal layer of gallbladder adjacent to the liver thus enabling hepatic invasion and metastatic progression is one of the major cause of its miserable prognosis[3]. The present review provides a recent update of studies regarding epidemiology, pathogenesis and molecular genetics of gallbladder cancer as available in literature.

EPIDIMIOLOGY OF GALLLBLADDER CANCER

Gallbladder cancer shows an unusual geographic distribution worldwide with substantial geographic variation. Data from Mapuche Indians from Valdivia, Chile, South America shows the rate of gallbladder cancer as: 12.3/100000 for males and 27.3/100000 for females[3]. The native people is these countries exceed for gallbladder cancer mortality rates from cervical (8.0/100000), breast (8.7/100000), pancreatic (7.4/100000), and ovarian cancers (7.3/100000)[3]. American Indians in New Mexico, USA, have also very high average annual rate of GBC (8.9/100000)[4], [Surveillance, Epidemiology End-Results Program (SEER) The Four Most Common Cancers for Different Ethnic Populations 2013. Bethesda, MD: National Cancer Institute; 2013].

Although the worldwide occurrence of gallbladder cancer is less than 2/100000 individuals, but this has been recorded with extensive variance[5]. The residents of Indo-Gangetic belt particularly females of northern India (21.5/100000) and south Karachi Pakistan (13.8/100000) have been reported as one of the highest affected regions[4]. Gallbladder cancer is also found in high frequency in Eastern Europe include Poland (14/100000 in Poland), Czech Republic, and Slovakia and Asia whereas south Americans of Indian descent (3.7 to 9.1 per 100000), Israel (5/100000) and Japan (7/100000) have shown intermediate prevalence of gallbladder cancer[4,6]. The residents of Andean-area, North American Indians and Mexican-Americans are specially predisposed of GBC[6]. The majority of the world has decreasing mortality trends in gallbladder cancer but GBC frequency is constantly rising in Shanghai, China which is substantial cause of mortality[7]. Although Gallbladder cancer is more common in females still in some countries like Korea, Iceland and Costa Rica, higher mortality rate has been reported for males as compare to females[8]. The data from National Cancer Institute; SEER Program (http://seer.cancer.gov/) has revealed only little turn down in incidence over the past few decades.

ETIOLOGICAL FACTORS FOR GBC PATHOGENESIS

The development of gallbladder cancer has been linked to various genetic and environmental factors. Chronic infection of gallbladder or/and environmental exposure to specific chemicals, heavy metals, and even many dietary factors, have been found to be associated with GBC formation. The dramatic association of GBC with female gender and certain geographical regions (mostly developing countries) has been proposed to be influenced by various female hormones, cholesterol cycling and salmonella infections in existing literature[9,10]. Worldwide GBC affects females 2-3 times more commonly than males, but bias varies greatly in different parts of the world mostly in high prevalent regions of GBC[4,6]. To some extent, the female hormone estrogen causes increased cholesterol super saturation in bile and hence involved in gallstone mediated GBC pathogenesis[11]. Although the female gender GBC can be linked with the role of female hormones. However an article published previously has questioned the association of hormone receptor expression to tumor differentiation[12]. So the extent of female hormones contribution in Gallbladder cancer is still not certain and requires more investigation.

Other well-known GBC associated risk factors

Table 1 Etiological factors for gallbladder cancer pathogenesis

Major Independent Etiological factors	Dependent Etiological factors
Age[6]	Tobacco consumption[15]
Sex[6], BMI[16]	Mustard oil[17] Argemone oil (AO) and butter yellow (BY)[18]
Family history[7,19]	Early age at first pregnancy[20]
Cholelithiasis[6,22-24]	Use of Oral contraceptives[15,25,26]
Chronic cholecystitis, porcelain gallbladder[27,28]	Red Chili pepper[29,30]
Chronic infection by *Salmonella* species, *S. paratyphi* or *S. typhican*[6,10,31-34]	Occupational exposure, Benzene[17,35]
Helocobacter pylori[36,37]	Secondary bile acids[13,38-40]
High parity[20,21,24,26]	Xanthogranulomatous cholecystitis[41]
Anomalous pancreatobiliary duct junction[42,43]	Heavy metals[44,45]
Porcelain gallbladder[46]	Genetic factors[48]
Gallbladder polyp[47]	
Obesity[49]	Free radical oxidation products[50]

such as porcelain gallbladder, Mirizzi's syndrome and bile reflux has also been playing a major role as a predisposing factors of this disease[9]. Family history of gallstones, tobacco consumption, chemical exposure, residence in Gangetic belt and high concentrations of secondary bile acids, excessive intake of fried foods (reused oil), increases the risk for GBC[13]. Present data suggest that gallstones are a major risk factor for GBC but their role as a cause for gallbladder cancer is still not certain. A review article by Shrikhande *et al*[14] has also supported the fact that the populations reporting high incidence of gallbladder cancer with associated gallstones, prophylactic cholecystectomy should be done only after correlating with the epidemiological profile of the place. Convincing evidence also exists for the presence of gallstones as strongly associated factor for gallbladder cancer etiology[7]. Most of the etiological factors are summarized in Table 1[6,7,10,13,15-50].

Familial and linkage studies

Swedish family-cancer database and Utah cancer registry has reported the first ever data for familial clustering of GBC[51]. This study has provided the first data on familial clustering of gallbladder cancer based on medically confirmed records, in which it was estimated that 26% of gallbladder cancers are familial. The significant risk in 3rd degree relatives and the disease manifestation in several high risk pedigrees as reported in previous studies gives a strong indication for genetic susceptibility to GBC[51]. The high risk heritable factors are likely to contribute to a large extent to this cancer further modulated by environmental factors. The nationwide Swedish Family-Cancer Data base from the Swedish Cancer Registry (10.2 million individuals from the year 1961-1998), has reported maternal transmission favoring over paternal in familial gallbladder cancers[52]. Furthermore, the clustering of gallbladder cancer within families is suggestive of a critical role of genetics in its development[19]. Carcinoma gallbladder was detected in two siblings from Brazil as reported by Trajber *et al*[53]. Role of allele specific mutations in pathogenesis of carcinoma gallbladder has also been reported[54]. Another report by Pandey *et al*[55] has shown higher frequency of carcinoma gallbladder in patients with A+ and AB+ blood groups to which the reason is still unknown.

GENETIC AND MOLECULAR ALTERATION REPORTED IN GALLBLADDER CARCINOMA

The present existing information regarding genetic and molecular alterations in GBC is still very much limited. Like other neoplasms, GBC is a multifactorial disorder involving multiple genetic alterations[56-58]. Abnormality in tumor suppressor genes, oncogenes, and DNA repair genes, presence of microsatellite instability (MSI) and epigenetic alterations mainly caused by aberrant promoter methylation of gene areas are some of the various well known factors reported till now. The serious of genetic alteration leading to gallbladder cancer formation is still not established clearly. Some of the molecular alterations reported so far are enumerated in Tables 2-4.

GENETIC ALTERATIONS IN GBC

KRAS

KRAS act as initial key player in numerous signal transduction mechanisms and associated pathways. Many pathogenic mutations have been reported in *KRAS oncogene* in Gallbladder cancer tissue[58-63]. K*RAS* gene mutations identified in GBC mostly affects codons 12, 13 and 61. In north India *KRAS* codon 13 mutation is more common (about one third) than codon 12 and 61[64]. However many other studies have not detected any mutations in this gene[65,66]. Any activating point mutations in *KRAS* oncogene can give rise to abnormal growth signals which is one of the hallmarks of cancer. The previous reports have co-related a condition called anomalous arrangement of the pancreatico-biliary duct with presence of gallbladder cancer as patients harboring this condition have a higher frequency of *KRAS* gene mutation as compare

Table 2 Mutations detected in gallbladder cancer by low throughput methods

Studied gene	Type of study	Methods used	Studied population	Ref.
KRAS	Mutation at codon-12 (8%)	PCR-RFLP	India	[64]
	Mutation at codon-12 (29%-30%)	PCR-RFLP	Chile	[76,77]
	Mutation at codon-12 (0%-59%)	PCR-RFLP, Direct sequencing	Japan	[60,78,79]
	Mutation at codon-12 (50%-80%)	ELMA, SAB, PCR-SSCP, Direct sequencing	Japan	[63,80]
INK4A (p16)	Mutation, deletion	PCR-RFLP, direct sequencing, IHC	Japan, Chile	[54,79,81,82]
D310 mtDNA	Mutation (Displacement loop)	PCR-based assay, direct sequencing	Chile	[83]
TP53	Mutation, overexpression, LOH	PCR-RFLP, direct sequencing, IHC	Greece, Japan, Chile	[84-86]

Table 3 Mutations studies in gallbladder cancer by high throughput methods

Platform	Number of samples	Study population	Research planned	Key findings	Ref.
Sequenom Mass ARRAY technology	49 FFPE	India	390 mutations in 30 genes	*PIK3CA* (4%), *KRAS* (2%), *CTNNB1* (4%), *TP53* (18%)	[95]
Mass spectroscopy-based	57 FFPE	MD Anderson Centre	159 mutations in 33 genes	14 hotspot mutations in 9 cases including (*KRAS, NRAS, PIK3CA, IDH1, ALK, MET*) 26 mutations in 15cases	[94]
Next-generation sequencing (NGS)	15 FFPE		NGS of 182 cancer-related genes	(*P53, STK11, RICTOR,TSC2, FGF3-TACC* fusion, *FGF10* amplification) Preponderance of mutations involving the PI3 kinase pathway	[94]
Whole Exome and transcriptome Sequencing	29 Fresh Frozen	Japan	64 non silent mutations signatures	*EGFR, ERBB3, PTEN, ARID2, MLL2, MLL3, APOBEC, TERT* *APOBEC*-associated mutation signature were observed in GBC	[96]
Exome sequencing and targeted gene sequencing	57 Fresh Frozen	China	Whole exome sequencing	*TP53* (47.1%), *KRAS* (7.8%) and *ERBB3* (11.8%) ERBB pathway genes mostly mutated	[93]

FFPE: Fresh frozen paraffin embedded.

Table 4 Summary of global gene expression studies in gallbladder cancer

Biological sample used	Platform/studies key findings	Ref.
17 gallbladder tissue specimens (6 advanced GBC , 6 early GBC cancers and 5 normal control	Oligonucleotide Microarray platform Unregulated genes: 2270 Downregulated genes: 2412	[97]
5-Normal biliary epithelial scrapings, 11- surgically resected biliary carcinomas, 9-biliary cancer cell lines	Oligonucleotide Microarray platform Unregulated genes : 282 genes Downregulated genes: 513	[98]
37 biliary tract carcinomas (15 bile duct, 11 gallbladder, 11 of ampulla of Vater)	cDNA array platform 118 genes were identified with a prognostic value	[99]
12 advanced gallbladder carcinoma tissue 3 samples of normal control gallbladder epithelium	Oligonucleotide Array platform Upregulated: (TOPO II-alpha, cyclin B2, CDC28, ubiquitin-conjugating enzyme E2C), and one metabolism-related: (gamma-glutamyl hydrolase)	[100] [101]
34 biliary tract cancers including 13 intrahepatic (IHC), 12extrahepatic (EHC), 9 (GBC)	Oligonucleotide Array platform 1281 genes with deregulated expression pattern	

to normal condition[65,67,68]. However mutation of *KRAS* gene has never been detected in GBC having adenoma carcinoma sequence of development[69] (Table 2).

TP53

TP53 is a well-known tumor suppressor gene and has various mechanisms of anticancer function and plays significant role in maintenance of genome integrity, apoptosis, genomic stability, and inhibition of angiogenesis etc. Loss of *TP53* function allows deregulated survival of genetically impaired abnormal cells which can lead to neoplastic conversion of later on[70]. *TP53* mutations are relatively more common in later stages of the disease[63,66,71-73]. Most of the *TP53* mutations associated with GBC are missense mutations that produce a non-functional protein with an increased half-life. The existing literature has reported mutations of the *TP53* gene in between approximately 27% to 70% of gallbladder carcinomas[74]. Many codons of the *TP53* codons are affected by pathogenic

mutations of this gene. Functional molecular studies have discovered that mutations in exons 5 and 8 of *TP53* gene causes deregulation of this gene[75]. Details are shown in various existing literature is shown in Table 2[54,60,63,64,76-86].

C-ERB-B2

The oncogene *c-erb-B2* is a homologue for epidermal growth receptor, encoding a protein with tyrosine kinase activity. The immunohistochemical expression of c-erb-B2 has been found positive between 10%-46% of gallbladder cases. However its expression has been found to be absent in dysplasia or adenomas as shown by previous reports[87,88]. Animal model studies in transgenic mice have shown that *erbB2* overexpression in the basal layer of the biliary tract epithelium led to the development of GBC in all (100%) of mice. Moreover, the expression of HER2/neu was positively observed in 28% of GBCs which was directly correlated with advanced stage of cancer[89]. Therefore, it can be hypothesized that some oncogene is associated with in Gallbladder cancer progression. In a study from India, C-erbB2 was frequently expressed in well differentiated and stage Ⅱ to stage Ⅳ in about 9.4% of GBC cases[90]. A recent report showed HER2/neu overexpression occurred in 14% of the advanced gallbladder cancer cases, and this subgroup was expected to be benefited from HER2/neu pathway inhibitors[91]. Therapeutic targeting of *EGFR/HER2* pathways boosts the anti-proliferative effect of gemcitabine in biliary tract and gallbladder carcinomas as shown by a previous study[92]. Based on facts it can be concluded that *C-ERB-B2* expression can become a marker for a poor prognosis.

HIGH THROUGHPUT MUTATION STUDIES IN GBC

High throughput research has made large scale repetition of experiments feasible as it automates the experiments thus it has now become possible to study how all 21000 genes potentially contribute to cell function or disease. But in case of gallbladder cancer there are very limited high throughput studies. One of the pioneer studies published in nature genetics using high throughput approach by Chinese population has found recurrent mutations in ErbB pathway[93]. Javle *et al*[94] has found 26 missense mutations with more common *TP53* and *PIK3CA* mutations in GBC tumor using NGS technology. Mutation profiling of gallbladder cancer tissue in Indian population has found *PIK3CA* and *KRAS* mutations as most common among this ethnicity[95]. The variability in the results is an indicator of intra-tumoral heterogeneity of cancer, which describes the observation of different tumor cells showing distinct morphological and molecular profiles including variable gene expression but ultimately leading to a common phenotype. The high throughput mutation studies in GBC are presented in Table 3[93-96].

GENE EXPRESSION STUDIES IN GBC

In order to identify potential biomarkers for GBC progression, many studies have been performed to find out the differential gene expression profiles between normal and tumor cells. Existing data various greatly, despite of same grade and stage of the included study subjects. Table 4[97-101] and Table 5[54,66,75,84,86,90,102-180] are summarizing global and single gene expression studies reported in GBC respectively.

LOSS OF HETEROZYGOSITY AND MICROSATALLIE INSTABILITY

Loss of heterozygosity (LOH) is a common genetic alteration in cancer genome. The events like heterozygous deletion of one of the two alleles, or duplication of a maternal or paternal chromosome or chromosomal region and concurrent loss of the other allele gives rise to LOH. The studies focused to detect loss of heterozygosity (LOH) in GBCs have shown frequent heterozygous allelic loss which spans in 18 different chromosomal regions[57]. Cytogenetic locations involved in frequent loss of heterozygosity *i.e.,* 3p, 8p, 9p, and 22q regions have also been identified in GBC from different populations; which have also been reported in several other cancers like Retinoblastoma, melanoma, Squamous cell carcinoma of larynx[181-183]. In particular, gallbladder tumor shows numerous site of allelic loss in the short arm of chromosome 3, which harbors several known or putative tumor suppressor genes[109,181]. High degree of microsatellite instability (MSI) in 10% of GBC cases was observed as reported in research article published by Goldin *et al*[184]. A different pattern of allelic loss has also been detected in Japanese population. In this report the allelotype analysis of gallbladder carcinoma revealed an interesting associated with anomalous junction of pancreatico-biliary duct[68]. Table 6[54,57,66,68,109,112,185-193] enlists various studies conducted in GBC regarding LOH and MSI.

METHYLATION AND GALLBLADDER CANCER

Understanding of DNA methylation patterns of gallbladder tumors can prove to be important biomarkers to refine the diagnosis and prognostic information which ultimately helps in appropriate therapeutic selection. Hypermethylation in gene promoter regions is a common epigenetic mechanism for the inactivation of tumor suppressor genes. One of the important research article published previously has found an important link between methylation and survival. In this study methylation of genes *p73, MGMT,* and *DCL1* was significantly associated with

Table 5 Summary of single gene expression studied reported in gallbladder cancer

Studied single genes	Expression pattern	Studied population	Ref.
TP53	Expression (20%-70%)	India, Slovenia, Greece, Taiwan, Japan, Chile	[75,84-86,102-106]
p16	Overexpression	South Korea	[107]
FHIT	Expression loss (45%-75%)	Japan, Chile	[108,109]
ERBB2	Overexpression (25%-64%)	India, Japan, China, South Korea	[66,103,110,111]
	Expressed in 9.4%cases of well differentiated and stage Ⅱ to stage Ⅳ tumors	India	[90]
RB	20% cases allelic loss 4%-14%- loss of expression	Japan	[54,112]
CDKN1A	Reduced expression 49% cases	Japan	[113]
Cyclin D1, Cyclin E	Overexpression (41%-49%)	Japan	[114,115]
COX2	Over-expressed	Slovenia, Japan, Chile	[104,116,117]
BCL2	Over-expressed	Japan	[118]
CKIT	Expression 45%	Japan	[119]
SOX-4	Overexpression	China	[120]
Chemokine (C-X-C motif) ligand 12	Increased expression	South Korea	[121]
CXCR4, CXCR7	Increased expression	China	[122]
hedgehog pathway components (Shh, Ptch1 and Gli1)	*Shh*: 81.7% of cases expressed *Ptch1*: 75.3% of cases *Gli1*: 70.0% of cases	China	[123]
CD56, CD99	Altered expression	South Korea	[124]
CD97, CD55	*CD97*: 69.6% of cases expressed *CD55*: 65.2% of cases	China	[125]
HMGA2 and CD9	*HMGA2* positive expression *CD9* negative expression	China	[126]
cholecystokinin type-A	44.1% of cases expressed	India	[127]
vascular endothelial growth factor-A	53.6% of cases expressed	China	[128]
VEGF-C, VEGF-D	*VEGF-C*: 64.0% of cases *VEGF-D*: 62.0% of cases	China	[129]
Tumor endothelial marker 8 protein	Increased expression	India	[130]
L1 cell adhesion molecule	Increased expression	South Korea	[131]
Tissue factor pathway inhibitor-2	Down-regulated	China	[132]
HIF-1α	Increased expression	China	[133]
VHL	Reduces expression		
ERCC1(excision repair cross-complementing 1)	High expression in best differentiated tumors	Chile	[134]
NF-E2-related factor 2 (Nrf2)	Increased expression	China	[135]
CD34 , CA15-3	Highly expressed in stroma and in epithelium	Italy	[136]
ADAM-17	Overexpression	China	[137]
Cdx2	Aberrant expression	Japan	[138]
TLR4	Expressed in glandular and luminal epithelium	China	[139]
MiRNA	Loss of Dicer and Drosha expression	China	[140]
Inducible Nitric Oxide Synthase iNOS	Expressed	China	[141]
Prostate stem cell antigen (PSCA)	Down-regulated	Japan, China	[142]
OCT-4	Down-regulated	China	[143]
hTERT/Telomerase	Expressed in 56.66% cases	India	[144]
Aquaporins (AQPs)	Positive expression	Japan	[145]
Ornithine decarboxylase (ODC) and glutamate decarboxylase 65 (GAD65)	Overexpression	China	[146]
Alpha-methylacyl coenzyme A (racemase) AMACR	Overexpression	Taiwan	[147]
Sonic Hh (Shh)	Elevated expression	Japan	[148]
TGF-β induced miR-182	Overexpression	China	[149]
SLP-2	Overexpression	China	[150]
TMPRSS4	Higher expression	China	[151]
zinc finger X-chromosomal protein	Suppressed	China	[152]
multidrug resistance-associated protein 2 (MRP2)	Overexpression	South Korea	[153]
HuR	Overexpression	Taiwan	[154]
miR-155	Overexpression	Japan	[155]
LAPTM4B-35	Overexpressed(76%)	China	[156]
p27, P21	*p21* (75% cases) and *p27* (25% cases)	Jordan	[157]
Thymidylate synthase (TS)	Low expression	Japan	[158]
CD146	Elevated expression	China	[159]
AEG-1	Highly expressed (63.4%)	China	[160]
CCKAR	Expression increased (76.6%)	India	[127]
Nemo-like kinase (NLK)	Overexpression of *NLK*	China	[161]
C-erbB2	Overexpression (9.4%)	India	[90]

Phospho-mTOR expression	Positive expression (64.1%)	Chile	[162]
human telomerase reverse transcriptase (hTERT)	Expression increased	India	[163]
Phosphoglycerate kinase 1 (PGK1)	Decreased expression (54.7%)	China	[164]
Notch 1 and Notch 3	Positive expression	China	[165]
CCK-A	Decreased expression	India	[166]
3-phosphoinositide-dependent protein kinase 1 (PDK1)	Positively expressed	China	[167]
Zinc finger X-chromosomal protein (ZFX)	Overexpression	China	[151]
miR-138	Over expression	China	[168]
HSP gp96	Expression (90.7%)	China	[169]
Long non-coding RNA-LET	Overexpression	China	[170]
Survivin	higher expression (2.9- fold)	India	[171]
Long non-coding RNA CCAT1	Overexpressed	China	[172]
TEM8	Expression increased	India	[130]
Fhit,Mlh1, P53	Reduced expression of Fhit and Mlh1 protein and Overexpression of P53	Japan	[108]
NDRG2, CD24	NDRG2 down-regulation, CD24 up-regulation	China	[173]
IL-6	Overexpressed	China	[174]
SLP-2	Overexpression	China	[150]
BCL6, p19(ARF)	*BCL6* overexpression , p19 (ARF) Low Expression	Taiwan	[175]
VEGF-A	High expression of *VEGF-A*	Chile	[176]
MALAT1	Upregulation of MALAT1	China	[177]
miR-182	Upregulation of miR-182	China	[149]
miR-155	High expression level of miR-155	Japan	[155]
p53, S100A4, p27, p16, RB, Smad4, FHIT, E-cadherin and PML	*p53* and *S100A4* overexpressed, Loss of p27, p16, RB, Smad4, *FHIT*, E-cadherin and *PML* expression	South Korea	[178]
PEG10, TSG101	*PEG10* and *TSG101* overexpressed	China	[179]
CK7, CK20	*CK7* (69.05%), *CK20* (28.57%) expressed	Greece	[180]

Table 6 Loss of heterozygosity and microsatellite instability studies reported in gallbladder cancer

Studied reported in respective population	LOH/MSI	Ref.
Chilean	LOH reported in : 3p, 6q, 7q, 8p, 9p, 9q, 11q, 12q, 17p, 18q, 19p, 22q, and Xq	[57]
Japan	LOH reported in : 2p, 4p, 4q, 8q,9q, 10p,14p,14q,16p, 19p, 21p and Xp [Maximum deletion-2p24, 14q22 and 21q22]	[68]
Chilean, Japan	p53, 9p.8p, DCC, KRAS, p16, 16q24, 3p,9q, 22q and p161NK4	[54,66,109,112,185]
Greece	BAT-26	[186]
Chile, Japan	MSI reported (20%-33%)	[187,188]
India	E-cadherin (CDH1) 2p, 2q, 6q, 7q,17p	[189]
India	Fragile histidine triad (FHIT) MSI-H 17.5% LOH :27.5%	[190]
Japan	High incidences of LOH at 1p36 (19/36:53%), 9p21 (12/32:38%), 13q14 (20/36: 56%), 16q24 (31/54: 61%), and 17p13 (15/36: 42%)	[191]
Chile	FHIT gene locus (3p14.2)	[109]
India	LOH at 8 loci, that is 3p12, 3p14.2, 5q21, 9p21, 9q, 13q, 17p13, and 18q for tumor suppressor genes (DUTT1, FHIT, APC, p16, FCMD, RB1, p53, and DCC genes)	[192]
India	genomic instability at 2p, 2q, 6q, 7q, and 17p loci	[189]
Chile	DUTT1 (3p12), FHIT (3p14.2), BLU, RASSF1A, SEMA3B and hMLH1 (3p21.3)	[193]

LOH: Loss of heterozygosity; MSI: Microsatellite instability.

survival of gallbladder cancer patients[194,195]. The study was conducted in a series of 109 advanced gallbladder cancer cases. However genes like *CDH13* and *FHIT* did not show any significant tendency with respect to gallbladder cancer patient's survival[194,195]. Multivariate analysis found *MGMT* gene to be an independent prognostic factor for survival found, representing the important role of epigenetic process in gallbladder carcinogenesis[195]. The recent report showed that promoter methylation of specific genes like *CDH1, CDKN2A-p16*, *REPRIMO* (tumor suppressor gene family) and *UCHL1* (also known as PGP9.5) have important role in gallbladder carcinogenesis[196]. Other studies conducted on GBC have shown variable methylation pattern of a number of genes Table 7[81,82,193-208].

In addition, with the help of advanced technologies like high resolution allele stratification (allelotyping analysis) investigated very high frequencies of 3p (100%), 8p (100%), 9q (88%), 22q (92%) sites in gallbladder cancer that lead to positional identification of tumor suppressor genes associated with GBC malignancies and pathogenesis[57,58,109,209]. Moreover, some well-known tumor suppressor genes that

Table 7 Aberrant promoter methylation gene studies summary in gallbladder cancer

Gene	Full name	Function	Meth Freq	Population	Ref.
CDH1	Cadherin 1, type 1, E-cadherin (epithelial)	Tissue invasion (cell-cell adhesion)	11%-65%	Japan, Chile	[194-200]
FHIT	Fragile histidine triad gene	Regulation of DNA Replication, and apoptosis	30%-57%	Chile	[81,193-195,199]
APC	Adenomatous polyposis coli	Tumor suppressor gene (Cell migration, adhesion and apoptosis)	26%-35%	Chile, United States	[81,194,195,198,199]
hMLH1	Human homologs of MutL gene of bacteria	Mismatch repair	0%-14%	Chile, United States	[81,193-195,199]
p16	Cyclin-dependent kinase inhibitor 2A	Cell cycle regulation	15%-60%	Chile, United States, Germany	[81,82,195,197-199,201,202]
p15	Cyclin-dependent kinase inhibitor 2B	Cell cycle regulation	22%-44%	Chile	[81,198]
DAPK1	Death-associated protein kinase 1	Serine-threonine kinase	8%-61%	Japan, Chile	[81,197,198]
DLC1	Deleted in liver cancer 1	GTPase-activating protein	39%	Chile	[81]
RASSF1	RAS association domain family protein 1A	Signal transduction	0%-36%	Japan, Chile South Korea	[81,193,197,198,203]
MGMT	O-6-methylguanine-DNAmethyltransferase	Methyltransferase	13%-30%	Chile, United States	[81,195]
CDH13	CDH13 Cadherin 13, H-cadherin(heart)	Tissue invasion (cell-cell adhesion)	44%-70%	Chile	[81,198]
TIMP3	Metallopeptidase inhibitor 3	Degradation of extracellular matrix	0%-39%	Chile	[81,198]
GSTP1	Glutathione S-transferase pi 1	Conjugation of hydrophobic and electrophilic compounds	13%	Chile	[198]
RARβ2	Retinoic acid receptor, beta	Encodes retinoic acid receptor beta	4%-44%	Chile, United States	[81,198]
REPRIMO	TP53 dependent G2 arrest mediator candidate	Cell cycle regulation (p53 mediator)	62%	Chile	[204]
SHP1	Protein tyrosine phosphatase, non-receptor type 6	Regulate cell growth, differentiation, mitotic cycle	80%	Chile	[198]
3-OST-2	Heparan sulfate (glucosamine) 3-O-sulfotransferase 2	Osulfotransferase	72%	Chile	[198]
RUNX3	Runt-related transcription factor 3	TGF-beta signal pathway	22%-32%	Chile	[197,198]
RIZ1	PR domain containing 2, with ZNF domain	Histone/protein methyltransferase	26%	Chile	[198]
HPP1	Transmembrane protein with EGF-like and two follistatin-like domains 2	TGF-beta signal pathway	20%		[198]
P73	Tumor protein p73	Induction of apoptosis and cell cycle regulation	14%-28%	Chile, United States	[81,198]
SOCS-1	Suppressor of cytokine signaling 1	JAK-STAT pathway	12%	Chile	[198]
DCR2	Tumor necrosis factor receptor superfamily, member 10d	TNF-receptor superfamily	6%	Chile	[198]
SEMA3B	Sema domain, immunoglobulin domain (Ig), short basic domain, secreted,(semaphorin) 3B	Induction of apoptosis	92%	Chile	[193]
DUTT1	Human homolog of Drosophila Roundabout (ROBO1)	Cell migration and metastasis	22%	Chile	[193]
BLU	Zinc finger, MYND-type containing 10	Cell cycle regulation	26%	Chile	[193]
p14	Ribonuclease P/MRP 14 kDa subunit	Cell cycle regulation	40%	Germany	[201]
MASPIN	Mammary serine protease inhibitor	Tumor suppressor gene	70%	India	[205]
THBS1	Thrombospondin 1	Platelet aggregation, angiogenesis, and tumorigenesis	52%		
HLTF	Helicase-like transcription factor	Regulate transcription	16%		
MYC	V-Myc Avian Myelocytomatosis Viral Oncogene Homolog transcription factor	Cell cycle progression, apoptosis and cellular transformation	80%	Brazil	[206]
APC	Adenomatous polyposis coli	Tumor suppressor gene	71%-95%	Chile	[207]
CDKN2A	Cyclin-dependent kinase inhibitor 2A	Cell cycle			
ESR1	Estrogen receptor 1	Transcription factor			
PGP9.5	Protein gene product 9.5	Neural and/or nerve sheath differentiation			
SSBP2	Single-stranded DNA-binding protein 2	Microsatellite instability			

PGP9.5	Protein gene product 9.5	Neural and/or nerve sheath differentiation	27.2%	South Korea	[208]
MLH1, CDKN2A	MutL homolog 1	Mismatch repair	5%	Chile	[194]
	Cyclin-dependent kinase inhibitor 2A	Cell cycle	35%		
FHIT	Fragile histidine triad protein	Purine metabolism	21%		
APC	Adenomatous polyposis coli	Tumor suppressor genes	25%		
CDH1	Cadherin-1	Cell cycle	66%		

are present in chromosomes like 3p, 5q, 8p,13q and 18q can also influence the gallbladder cancer formation[57,58,109,209].

Candidate genes for gallbladder cancer susceptibility

The merely successful mechanism for identifying low or moderate penetrance cancer genes, is the analysis of genes involved in candidate loci. Therefore, these genes are also termed as candidate genes. The candidate gene analysis is done via case-control study, in which allele frequencies in cancer patients and healthy controls are compared and obtained results are analyzed statistically. Candidate modifier genes are selected on the basis of biological plausibility. Most studies are based on genes that encode proteins, thought to be involved in carcinogenesis, such as those involved in apoptosis, cell-cycle control, DNA repair, xenobiotic metabolism, hormonal and inflammatory pathway or other risk factors. Moreover, known genes account for a small proportion of the heritability of gallbladder cancer, and it is likely that many genes with modest effects are yet to be found.

A study by Wang *et al*[210] from china suggested about CCK-induced impaired gallbladder emptying in patients having gallstones. Most of the candidate genes identified so far are related to the classical rate limiting enzymes and proteins of lipid metabolism, steroidogenesis, lipid transport, bile acid synthesis, bile canalicular transport, gallbladder contractility, cell cycle, DNA repair and Inflammatory pathway[211-233]. Till now there are very limited studies in GBC which are independently replicated which includes *OGG1*$_{rs1052133}$, *TP53*$_{rs1042522}$, *GSTM1* null polymorphism and *CYP1A1*$_{rs1048943}$ polymorphism[48]. No definitive conclusions can be drawn due to limited number of studies. Hence there is a great need to explore genes related to GBC susceptibility. Table 8[30,214-273] shows an overview of candidate gene studies reported in GBC.

The only one genome-wide association study conducted in gallbladder cancer identified a SNP (rs7504990) in *DCC* gene which was associated with six times gallbladder cancer risk in the Japanese population. It has also been reported that reduced expression of *DCC* gene (deleted in colorectal cancer, 18q21.3) was designated to be associated with the greater aggressiveness of the disease which include increased proliferation, poorly differentiated histology, and metastasis through loss of adhesiveness[234]. However genome wide association study (GWAS) identified SNPs was replicated in Indian population and the study found no individual association of *DCC*$_{rs7504990}$ but haplotype analysis of *DCC* gene found the cumulative effect of G$_{rs2229080}$-A$_{rs4078288}$-C$_{rs7504990}$ A$_{rs714}$ haplotypes in Gallbladder Cancer predisposition[235].

Molecular pathogenesis of GBC

Gallbladder carcinoma develops through a serious of events before converting in to invasive malignancy. Any exposure to carcinogens may convert normal gallbladder epithelium to condition called metaplasia which subsequently forms dysplasia to carcinoma *in situ* (CIS), and finally proceeding to invasive carcinoma in about 15 years[274,275]. The multistage pathogenesis of gallbladder carcinoma begins with gallstones giving rise to a condition called chronic cholecystitis, which increases to risk to gallbladder cancer formation. More than 90% of patients with gallbladder carcinoma show dysplasia and CIS[274,275]. There is an unusual asymmetric thickening of the gallbladder wall with infiltration to surrounding structures in gallbladder cancer. Maximum cases reported in carcinomas of gallbladder are adenocarcinomas (80%-95%). Adenocarconomas can further be of papillary, tubular, mucinous, or signet cell type. Some other types which are present in very low frequency include: squamous cell carcinoma (16%), undifferentiated or anaplastic carcinoma (2%-7%), and adeno-squamous carcinoma (1%-4%)[276]. Most of GBCs (60%) are found in the fundus, near about 30% in the body, and 10% in the neck region.

Tumor markers in GBC

Till date there is no reliable tumor marker developed which can be employed in diagnosis of gallbladder cancer. The only two markers *i.e.,* carcino-embryonic antigen (CEA) and carbohydrate antigen 19-9 are most often elevated in advanced stages with a low specificity. So most often they are not used in stand-alone diagnosis of GBC[277]. However, there are other tumor markers like CA125, CA199, CEA (carcino-embryonic antigen), cancer antigens (CA) and CA242, which are for diagnosis of different other types of cancer (*e.g.*, gastric, liver, pancreatic), have also been researched in diagnosis of gallbladder cancer but the obtained results are highly inconsistent[278-280]. In addition some previous reports have shown CA 242, RCAS1 (receptor binding cancer antigen expressed on SiSo cells) CA15-3, Mac-2BP (macrophage

Table 8 Candidate gene studies (low susceptibility genes) in gallbladder cancer

Pathway involved	Gene	Polymorphism	Population	Ref.
DNA repair pathway genes	*XPC*	(rs2228000) Ala499Val	China	[236]
		(rs2228001) Lys939Gln	China	
	ERCC2	(rs1799793) Asp312Asn	North Indian	[232]
		(rs13181) Lys751Gln	North Indian	
	MSH2	(rs2303426) IVS1+9G>C		
		(rs2303425) -118T>C		
	OGG1	(rs2072668) 748-15C>G		
	TP53	(rs1042522) Pro72Arg	Chilean, Hungary, Japanese	[237-239]
	XRCC1	(rs1799782) Arg194Trp	North Indian Shanghai, China	[222,231]
		(rs25487) Arg399Gln		
	APEX1	(rs3136820) Asp148Glu	Shanghai, China	[222]
	RAD23B	(rs1805335) IVS5-15A>G		[223]
		(rs1805329) EX7+65C>T		
	FEN1	FEN1-69G>A and haplotypes	China	[240]
Hormonal pathway genes	*CCKAR*	(rs1800857) IVS1-5T>C	North Indian	[227]
	CCK and CCKAR	(rs2071011G>C, rs915889C/T,	Shanghai, China,	[241]
		rs3822222C/T, rs1800855T/A		
	ESR1	(rs2234693) IVS1-397T>C	Shanghai, China, North India	[241-243]
		(rs3841686) IVS5-34->T		
		(rs2228480) Ex8+229G>A		
		(rs1801132) Ex4-122G>C		
		(rs9340799) IVS1-351A>G		
	ESR2	(rs1256049) Val328Val	Shanghai, China	
	PGR	Ins/Del	North India	
	AR	(CAG)n	Shanghai, China	[244]
		(rs4633) His62His	Shanghai, China	[224]
	COMT	(rs4818) Leu136Leu		
	CYP1A1	(rs2606345) IVS1+606G>T		
	CYP1B1	(rs10012)Arg48Gly		
		(rs1065778) IVS4-76A>G	Shanghai, China	[224]
	CYP19A1	(rs700518) Val80Val		
		(rs2304463) IVS7-106T>G		
		(rs700519) Arg264Cys		
		(rs1065779) IVS9-53G>T		
		(rs4646) Ex11+410G>T		
	HSD3B2	(rs1819698) Ex4-133C>T	Shanghai, China	[224]
		(rs1361530) Ex4-88C>G		
	HSD17B3	(rs2066479) Gly289Arg		
	HSD17B1	(rs2830) Ex1-486G>A		
	SHBG	(rs6259) Ex8+6G>A		
	SRD5A2	(rs523349) Ex1-17G>C		
	RXR-a	(rs1536475) IVS6+70A>G	Shanghai, China	[245]
		(rs1805343) IVS1-27A>G		
	RXR-b	(rs2744537) G392T		
		(rs2076310) C51T		
	INS	(rs689) A-6T	Shanghai, China	[245]
	PPARD	(rs2016520) Ex4+15C>T	Shanghai, China	
	PPARG	(rs3856806) His477His	Shanghai, China	
Inflammatory pathway genes	*CR1*	(rs2274567) His1208Arg	North Indian	[230]
		(rs12144461) Intron 27, HindIII		
	IL1RN	86-bp VNTR	North Indian	[220]
		(rs689466) -1195G>A		[233]
	PTGS2	(rs20417) -765G>C		
		(rs5275) +8473T>C	North Indian Shanghai, China	[233,246]
	IL1B	(rs16944) -1060T>C	Shanghai, China north Indian	[220,247]
	IL10	rs1800871)- 7334T>C	Shanghai	[247]
		(rs1800872) -6653A>C	Shanghai	
	IL-8	(rs10805066) IL8 -13985C>G	China	[248]
	EGF	(rs4444903) +61A>G	North Indian	[221]
	TGFb1	(rs1800469)-509C>T	Shanghai, north Indian	[219,221,247]
	TNF-α	(rs1800629) -308G>A		
	IL6	(rs1800795) 236C>G)		
	IL8	(rs10805066) -13985C>G	China	[248]

	MMP-2	(rs2285053) -735 C>T (rs9340799) -1306 C>T	North Indian	[249]
	MMP-7	(rs11568818) -181 A>G (rs2250889) P574R		
	MMP9	(rs 17576) R279Q (rs 17577) R668Q		
	TIMP2	(rs8179090) -418 G>C		
Metabolic pathway genes	*MTHFR*	(rs1801133) Ala222Val	Indian	[228]
	APOB	(rs17240441) 35_43del9	Indian	[217]
	NAT2	(rs1799929) NAT2*5A (rs1799930) NAT2*6B rs1799931, NAT2*7A	Indian	[216]
	GSTT1 *GSTP1*	Null polymorphism (rs1695) Ile105Val	Indian	[215]
	CYP17	(rs743572) Ex1+27T>C	Shanghai Indian (265)	[250,251]
	GSTM1	Null polymorphism	Indian, Chilean Hungary Japanese	[215,237,238]
	CYP1A1	(rs4646903) CYP1A1*2A (rs1048943) Ile462Val (*2C)	Indian, Chilean, Hungary Japanese China, Chilean, Hungary Japanese	[218,237,239] [224,237-239]
	Cyp1a1 cyp1b1	CYP1A1-MspI, CYP1A1-Ile462Val, and CYP1B1-Val432Leu	India	[252]
		(rs5930) EX10+55G>A	Shanghai	[253]
	LDLR	(rs6413504) IVS17_42A>G (rs14158) EX18+88G>A	Shanghai	
	LPL	(rs263) IVS5-540C>T		
	ALOX5	(rs2029253) IVS3+100G>A		
	ApoB	rs693) Thr2515Thr	Indian Chilean	[30,217]
	ABCG8	(rs11887534) Asp19His	North Indian Shanghai China	[229,254]
	CETP	(rs708272) TaqIB (rs1800775) -629C>A	Chilean Shanghai China Shanghai China	[30,254] [254]
	LRPAP1	(rs11267919)752_177_752_176 I 37	North Indian Shanghai China	[214,254]
	CYP7A1	(rs3808607) -204 A>C	North Indian	[255]
	CYP7A1	(rs3824260) -469 T>C	North Indian	
	CYP17	(rs743572)A/G	North Indian	[250,251]
	ApoB	(rs676210) Pro2739Leu (rs673548) IVS23-79T>C rs520354) IVS6+360C > T (rs1367117) Thr98Ile (rs440446) IVS1+69C>G	Shanghai	[253]
	CYP2C19	(rs4244285) CYP2C19*2, (rs4986893) CYP2C19*3	Japanese	[256]
	ADRB3	(rs4994)A/G	North Indian	[257]
Apoptosis pathway	*CASP8*	(rs3834129) -652 6N ins/del (rs1045485) Asp302His (rs3769818 A) IVS12-19 G>A	North Indian	[258]
Nuclear Receptors	*Lxr-alpha, Beta*	LXR-α (rs7120118) and LXR-β (rs35463555 and rs2695121)	North Indian	[259]
Cancer Stem cell gene	*CD44*	CD44 (rs13347) C>T, CD44 (rs353639)A>C, CD44 (rs187116) G>A, CD44 (rs187115) T>C	North Indian	[260]
	NANOG, ALCAM, EpCAM, SOX-2, OCT-4, NANOG	NANOG (rs11055786)T>C, ALCAM (rs1157)G>A EpCAM (rs1126497)T>C, SOX-2(rs11915160)A>C OCT-4 (rs3130932)T>G, NANOG (rs11055786)T>C	North Indian	[261]
Prostate stem cell antigen	*PSCA*	(rs2294008) T/C and rs2978974)	India, Japan	[262,263]
miRNA	*hsa-miR-146a*	(rs2910164) G>C	North Indian	[264]
	hsa-mir-196a2	(rs11614913) C>T		
	hsa-mir-499	(rs3746444)T>C		
	miR-27,miR-570,miR-181	miR-27a (rs895819)A>G, miR-570(rs4143815)G>C, miR-181a(rs12537)C>T	North Indian population	[265]
GWAS-associated genes	*DCC*	(rs7504990)C>T	Japan	[234]
		(rs2229080) C>G (rs4078288) A>G (rs7504990) C>T (rs714) A>G	North Indian	[235]

Wnt signaling pathway	*SFRP4, DKK2, DKK3, APC, AXIN-2, B-CATENIN, GLI-1*	SFRP4 (rs1802073) G>T, DKK2 (rs17037102) C>T DKK3 (rs3206824) C>T, APC (rs4595552)A/T APC (rs11954856) G>T, AXIN-2 (rs4791171)C>T β-CATENIN (rs4135385) A>G, GLI-1(rs222826) C>G	North Indian	[266]
Other genes	*KRAS*	codon 25 Gln25His	Eastern India	[267]
	ACE I/D	(rs4646994) 289 bp del	North Indian	[268]
	DNMT3B	(rs1569686) -579 G>T	North Indian	[269]
	TLR2	-196-174del	North Indian	[270]
	TLR4	(rs4986791) Thr399Ile	North Indian	
	Adrenergic receptors (ADRA)	ADRA2A C-1291G, ADRβ3 T190C or Trp64Arg, and ADRβ1 C1165G or Arg389Gly	North Indian	[271]
	Death Receptors and their ligands (DR4)	DR4 (rs20575, rs20576 and rs6557634), FAS (rs2234767) FASL (rs763110)	North Indian	
	PlCE1	(rs2274223) A>G and. (rs7922612) T>C	North Indian	[272]
	Vitamin D receptor (VDR)	FokI C>T	China	[273]

galactose-specific lectin-2 binding protein), Fragments of cytokeratin-19 (CYFRA 21-1) are frequently present in blood of cancer patients and shown to be associated with GBC with variable sensitivity and specificity[277,281,282].

CONCLUSION

Various lines of evidence suggest role for various environmental risk factors in Gallbladder carcinoma. Despite of many articles regarding genetic predisposition of gallbladder cancer there is no established genetic marker. Also, very limited Genome wide association studies (GWAS) have been conducted in gallbladder cancer till now.

The evidence-based model of gallbladder carcinogenesis and its dissemination by Barreto *et al*[283] serves as a basic platform for elucidation of molecular mechanisms involved in cancer development which based on recent data can be improved by discovery of other signature mutations using high throughput studies. Technological advancement can be helpful more understanding of pathogenic mechanisms underlying neoplastic conversion of gallbladder cancer muscosa. The tumor markers available for diagnosis GBC has also not of very high specificity and not discovered until advanced stage of the disease leading to complexity of the treatment. Exome sequencing of gallbladder cancer tissue has found ERBB pathway as most dysregulated pathway in this disease. Although the studies have been published in highly distinguished journals but they need to be validated before clinical implication. Moreover, limited studies with small sample size are not robust enough to conclude anything. Regardless of improvement in technologies in research field there is no accountable betterment in the prognosis of GBC patients. The future therefore should be engaged towards good quality research focused on early diagnosis and refinement of prognostic information to ultimately improve the management strategies of gallbladder cancer. Present review provides a comprehensive summery of the studies conducted regarding its Epidemiology, Pathogenesis and molecular genetics under a single umbrella. This will be helpful for the researchers to understand the current scenario of research work and how much success we have gained till now. Based on that future research work can be planned in appropriate directions.

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ORIGINAL ARTICLE

Basic Study

Serelaxin increases the antifibrotic action of rosiglitazone in a model of hepatic fibrosis

Robert G Bennett, Ronda L Simpson, Frederick G Hamel

Robert G Bennett, Ronda L Simpson, Frederick G Hamel, Research Service, Nebraska-Western Iowa Health Care System, Omaha, NE 68198, United States

Robert G Bennett, Ronda L Simpson, Frederick G Hamel, Department of Internal Medicine, University of Nebraska Medical Center, Omaha, NE 68198, United States

Robert G Bennett, Department of Biochemistry and Molecular Biology, University of Nebraska Medical Center, Omaha, NE 68198, United States

Frederick G Hamel, Department of Pharmacology and Experimental Neuroscience, University of Nebraska Medical Center, Omaha, NE 68198, United States

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Correspondence to: Robert G Bennett, PhD, Professor, Department of Internal Medicine, University of Nebraska Medical Center, Omaha, NE 68198, United States. rgbennet@unmc.edu
Telephone: +1-402-3468800
Fax: +1-402-4490604

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Abstract

AIM

To determine the effect of combined serelaxin and rosiglitazone treatment on established hepatic fibrosis.

METHODS

Hepatic fibrosis was induced in mice by carbon tetrachloride administration for 6 wk, or vehicle alone (nonfibrotic mice). For the final 2 wk, mice were treated with rosiglitazone, serelaxin, or both rosiglitazone and serelaxin. Serum liver enzymes and relaxin levels were determined by standard methods. The degree of liver collagen content was determined by histology and immunohistochemistry. Expression of type I collagen was determined by quantitative PCR. Activation of hepatic stellate cells was assessed by alpha-smooth

muscle actin (SMA) levels. Liver peroxisome proliferator activated receptor-gamma coactivator 1 alpha (PGC1α) was determined by Western blotting.

RESULTS

Treatment of mice with CCl_4 resulted in hepatic fibrosis as evidenced by increased liver enzyme levels (ALT and AST), and increased liver collagen and SMA. Monotherapy with either serelaxin or rosiglitazone for 2 wk was generally without effect. In contrast, the combination of serelaxin and rosiglitazone resulted in significantly improved ALT levels ($P < 0.05$). Total liver collagen content as determined by Sirius red staining revealed that only combination treatment was effective in reducing total liver collagen ($P < 0.05$). These results were supported by immunohistochemistry for type I collagen, in which only combination treatment reduced fibrillar collagen levels ($P < 0.05$). The level of hepatic stellate cell activation was modestly, but significantly, reduced by serelaxin treatment alone, but combination treatment resulted in significantly lower SMA levels. Finally, while hepatic fibrosis reduced liver PGC1α levels, the combination of serelaxin and rosiglitazone resulted in restoration of PGC1α protein levels.

CONCLUSION

The combination of serelaxin and rosiglitazone treatment for 2 wk was effective in significantly reducing established hepatic fibrosis, providing a potential new treatment strategy.

Key words: Relaxin; Peroxisome proliferator-activated receptors; Liver cirrhosis; Liver diseases; Fibrosis

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Core tip: Hepatic fibrosis is a chronic condition that can lead to cirrhosis, but treatment options are limited and ineffective. Agonists of peroxisome proliferator-activated receptor gamma (PPARγ), such as rosiglitazone have shown limited efficacy. The hormone relaxin has antifibrotic effects, and increases the activity of PPARγ, leading to the hypothesis that combination treatment may be more effective. Mice with established hepatic fibrosis were treated with relaxin and rosiglitazone alone or in combination. Combination treatment reduced liver fibrosis, and increased the level of a PPARγ coactivator. These results suggest that relaxin and PPARγ co-therapy could be a more effective treatment for hepatic fibrosis.

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INTRODUCTION

Relaxin is a polypeptide hormone of the insulin/relaxin superfamily[1]. One important action of relaxin is the widespread remodeling of extracellular matrix, which involves altered secretion and degradation of matrix components[1,2]. The case for a role for relaxin as a general protective agent against fibrosis was dramatically strengthened by observations made using the relaxin-null mouse. These mice spontaneously developed age-related pulmonary, cardiac, dermal, and renal fibrosis[2-4]. This has led to the use of relaxin in the treatment of experimentally-induced pulmonary and renal fibrosis in rodents, which could be reversed by systemic relaxin treatment[5,6].

Relaxin also has effects in the liver. Relaxin treatment of rats caused acute changes in the hepatic microcirculation[7], and morphological changes were detected in nonparenchymal sinusoidal cells[8]. In addition, the relaxin-null mouse developed increased liver weight[9]. Work by our laboratory and others showed that relaxin had antifibrotic effects on activated hepatic stellate cells (HSC), which are the major collagen-producing cells in liver injury. Relaxin treatment of activated HSC had numerous effects, including decreased total collagen deposition, collagen synthesis, and collagen-I secretion, and decreased smooth muscle actin expression, but had no effect on HSC proliferation or apoptosis[10,11]. Relaxin promoted a matrix degrading phenotype in HSCs by increasing matrix metalloproteinase expression and activity, and inhibiting secretion of the tissue inhibitors of metalloproteinases[10,11]. The effects of relaxin were mediated by activation of the relaxin family peptide 1 (RXFP1) receptor, which is expressed predominantly in the HSC in liver[12,13]. Finally, using *in vivo* models of experimental hepatic fibrosis, relaxin prevented hepatic collagen content[10,14], and was effective in treating established hepatic fibrosis[13,15]. Therefore, there is considerable evidence to support a functional role for relaxin effects in the liver.

A second critical regulatory element in HSC activation is the PPARγ pathway. PPARγ is a transcription factor activated by the antidiabetic thiazolidinedione (TZD) drugs, such as rosiglitazone and pioglitazone, and some prostaglandins[16]. Expression of PPARγ is detectable in quiescent HSC, but is lacking in activated HSC and myofibroblasts[17]. Restoration of PPARγ expression, either by treatment of activated HSC with PPARγ ligands or by forced expression of PPARγ, induced a reversion of the HSC to a state that closely resembled the quiescent phenotype, as shown by decreased proliferation, reduced SMA, collagen and TIMP expression, increased MMP-13 expression, and restoration of lipid-storage[18]. Importantly, treatment of experimentally-induced fibrosis with PPARγ ligands

prevented hepatic fibrosis in some *in vivo* models[19-21]. However, recent studies have suggested that TZD treatment may be ineffective for established fibrosis in rodents, casting some doubt on the utility of using TZDs alone for this purpose[22-24].

As discussed above, PPARγ has numerous anti-fibrotic effects, and relaxin reduced many of the same markers reported for PPARγ agonists in HSC in culture and *in vivo.* We reported that relaxin activates PPARγ transcriptional activity in cells expressing RFXP1 in a manner that did not require the addition of exogenous PPARγ ligands[25]. More recently, we identified the mechanism for this stimulation[26]. Relaxin increased the expression of a coactivator protein in activated HSC, known as PPARγ coactivator 1α (PGC1α) through cAMP and p38-MAPK dependent pathways, and that these pathways were intact in the human hepatic stellate cell line LX2. Therefore, relaxin treatment may enhance the response to TZDs in hepatic fibrosis. To test this hypothesis, we compared the effectiveness of the recombinant form of relaxin (serelaxin), rosiglitazone, or their combination, in the treatment of established models of hepatic fibrosis.

MATERIALS AND METHODS

Mouse model of hepatic fibrosis and treatment

Fibrosis was induced in male C57BL/6 mice (20-24 g, Charles River Laboratories, Wilmington, MA) as described[15]. Briefly, mice received twice-weekly intraperitoneal injections of CCl_4 (diluted 1:7 in sunflower oil) at 1 mL/kg body weight, for a total of 6 wk to induce hepatic fibrosis. Control (nonfibrotic) mice received oil alone. For the final 2 wk of treatment, mice were randomly assigned to receive implantation of subcutaneous osmotic pumps (model 1002, Durect, Cupertino CA) to deliver serelaxin (generously provided by Dennis Stewart, Novartis) at 150 μg/g per day, or vehicle (citrate buffer). Rosiglitazone (4 mg/kg per day Enzo Life Sciences, Farmingdale, CA) or vehicle (5% DMSO in phosphate buffered saline) was also administered daily by oral gavage for the final 2 wk. Each group contained 5 mice. Mice were sacrificed 72 h after the final CCl_4 injection, and liver and blood were collected. Mice were maintained at 22 ℃ under 12-h light/dark cycles, and had free access to food and water throughout the study. All procedures were conducted in accordance with The Guide for the Care and Use of Laboratory Animals[27], and were approved by the VA Nebraska Western-Iowa Institutional Animal Care and Use Committee.

Histology and immunohistochemistry

Liver tissue was fixed in 4% buffered formalin, embedded in paraffin, and sections were mounted onto slides. Sections were dewaxed and then stained with picrosirius red to visualize total collagen, as described[28]. For immunohistochemistry, tissues were subject to antigen unmasking by heating in citrate buffer (Vector Labs, Burlingame CA), then probed overnight at 4 ℃ with antibodies directed against type Ⅰ collagen (ab21286, Abcam, Cambridge, MA) at 1:250 dilution, or α-smooth muscle actin (SMA, clone 1A4, Sigma Chemical, St. Louis, MO) at 1:400 dilution. Positive staining was detected using the DAB Envision System (Dako, Carpenteria, CA). Images were captured and analyzed using ImageJ software as described previously[15].

Gene expression analysis

Total liver RNA was extracted using the Purelink kit (Thermo Fisher, Carlsbad, CA), with on-column DNase treatment as per the manufacturer's instructions. RNA integrity and lack of contaminating genomic DNA was confirmed by visualization on agarose gels, and RNA concentration was determined using the Ribogreen assay (Thermo Fisher, Carlsbad, CA). A total of 2 μg of RNA was converted to cDNA using the TaqMan High Capacity Reverse Transcription kit (Thermo Fisher, Carlsbad, CA) in a final volume of 20 μL. Quantitative PCR was conducted using TaqMan hydrolysis probe assays, using 2 μL of cDNA (diluted 1:15), 10 μL Taqman universal PCR master mix, 1.0 μL Taqman primer/probe mix in a final volume of 20 μL per reaction. The mouse gene expression assays used included procollagen type Iα2 (*Col1a2*; Mm00483888_m1), αSMA (*Acta2*, Mm01546133_m1), and TATA-box binding protein (*Tbp*; Mm01277045_m1). All expression levels were normalized to that of *Tbp* in the same sample, and the data expressed as the expression level relative to nonfibrotic controls, using the $\Delta\Delta C_T$ method.

Serum measurements

Serum levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were determined by standard clinical chemistry assays. Human relaxin levels were determined using the Quantikine kit (R&D Systems, Minneapolis MN), which does not detect mouse relaxin or other insulin- and relaxin-related peptides. Mouse adiponectin levels were measured by immunoassay (Alpco, Salem NH).

Western blotting

Lysates were prepared from liver tissue and protein levels were determined by the bicinchoninic acid assay (Thermo Fisher, Carlsbad, CA). A total of 50 μg protein was applied to 10% SDS-PAGE gel, then transferred to PVDF membranes. The membranes were probed overnight at 4 ℃ with antibodies directed against PGC1α (#101707, Cayman Chemical, Ann Arbor, MI, 1:500) or GAPDH (MAB374, Millipore, Temecula, CA, 1:2000). After washing, membranes were probed with fluorescently-labeled secondary antibodies (Li-Cor, Lincoln, NE), and immunoreactive proteins detected using an Odyssey fluorescent

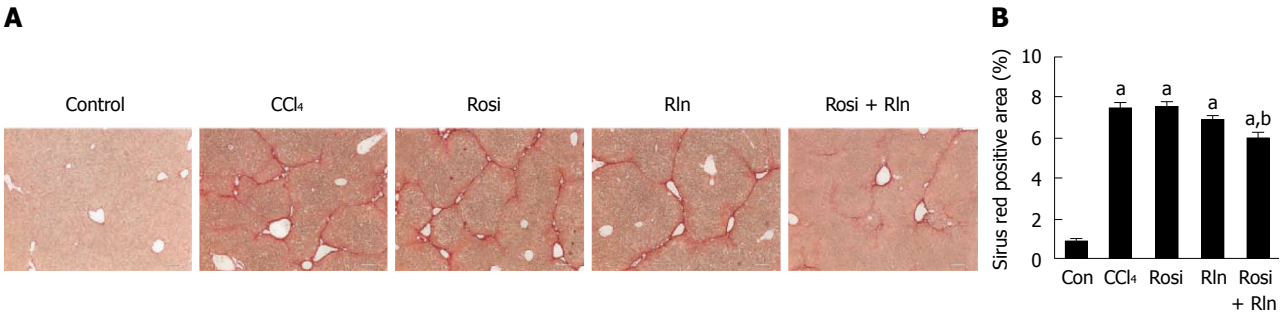

Figure 1 Total liver collagen content. A: Sirius red staining of liver tissue from control (Con), fibrotic (CCl_4), rosiglitazone (Rosi), serelaxin (Rln) or combination-treated (Rosi + Rln) mice. Bar: 100 μm. B: Sirius red staining quantified. Data are expressed as mean ± SE, and analyzed by ANOVA (n = 5). [a]P < 0.001 *vs* Con; [b]P < 0.05 *vs* CCl_4, Rosi, or Rln.

Table 1 Serum measurements in control and fibrotic mice

	Control	CCl_4 only	Rosi	Rln	Rosi + Rln
Body weight (g)	26.7 ± 0.9	25.6 ± 0.8	24.8 ± 1.0	24.9 ± 1.0	24.7 ± 0.9
Liver weight (g)	1.7 ± 0.1	1.7 ± 0.1	1.5 ± 0.1	1.6 ± 0.1	1.4 ± 0.1
Liver (% body wt)	6.3 ± 0.1	6.7 ± 0.3	6.1 ± 0.3	6.4 ± 0.4	5.8 ± 0.2
ALT	91.8 ± 5.7	2656 ± 538[a]	3892 ± 676[a]	1755 ± 610[a,b]	3227 ± 313[a]
AST	368 ± 33	1621 ± 282[a]	2496 ± 339[a]	1278 ± 277[a,b]	1863 ± 165[a]
Human relaxin (ng/mL)	ND	ND	ND	28.6 ± 7.2	20.5 ± 7.4
Adiponectin (ug/mL)	35.0 ± 3.4	32.6 ± 3.9	86.0 ± 12.2[c]	32.4 ± 2.2	147.5 ± 18.7[c]

[a]P < 0.05 *vs* control; [b]P < 0.05 *vs* rosiglitazone (Rosi); [c]P< 0.05 *vs* control, CCl_4 only or relaxin (Rln). ND: Not detected.

scanner (Li-Cor, Lincoln, NE).

Statistical analysis

Statistical analysis was performed using Prism5 software (GraphPad, La Jolla, CA). Differences between groups were analyzed using one-way analysis of variance (ANOVA) with the Newman-Keuls post-test. Data are expressed as mean ± SE of means.

RESULTS

Serum levels of serelaxin were analyzed by a specific assay that does not detect mouse relaxin. Serelaxin was successfully delivered, as evidenced by detectable human relaxin in treated mice, but not control mice (Table 1). As expected, rosiglitazone treatment caused an increase in serum adiponectin levels, confirming successful treatment and bioactivity of rosiglitazone. Fibrotic mice (CCl_4 group) had significantly elevated levels of ALT and AST. None of the treatments resulted in a significant change in ALT or AST levels compared with CCl_4 treatment alone. A significant difference was detected between Rosi and Rln treatments alone, due to opposite but statistically insignificant differences caused by each treatment individually. There was no significant difference in body or liver weight under any treatment condition (Table 1).

The level of total collagen deposition determined by Sirius red staining was markedly increased with CCl_4 treatment, confirming development of hepatic fibrosis (Figure 1). As demonstrated previously[14], 2 wk treatment with relaxin alone did not reduce Sirius red staining. Similarly, rosiglitazone alone had no significant effect on the total collagen deposition. In contrast, the combination of relaxin and rosiglitazone significantly reduced the degree of Sirius red staining (Figure 1). To more precisely assess the relative levels of fibrillar collagen, immunohistochemistry for type Ⅰ collagen was performed (Figure 2). Consistent with the Sirius red staining, only the combination of relaxin and rosiglitazone reduced the overall level of type Ⅰ collagen.

The primary cell type responsible for the deposition of collagen in fibrosis are the activated hepatic stellate cells (HSC). Using immunohistochemistry for the activated HSC marker α-smooth muscle actin (αSMA), robust induction of HSC activation was induced by CCl_4 treatment (Figure 3). Treatment with rosiglitazone was without effect, while relaxin caused a modest but significant, decrease in αSMA staining. The combination of relaxin and rosiglitazone induced a significant reduction in the level of HSC activation as exemplified by the reduction in αSMA level. These effects were confirmed at the transcriptional level, as similar effects were observed on the gene expression level of type Ⅰ collagen as determined by qPCR.

Relaxin was previously shown to increase the levels of PGC1α in cultured hepatic stellate cells[26]. To determine the effect of serelaxin and rosiglitazone treatment on PGC1α protein levels in hepatic fibrosis *in vivo*, Western blotting was performed on liver lysates. The level of PGC1α was decreased after CCl_4 treatment

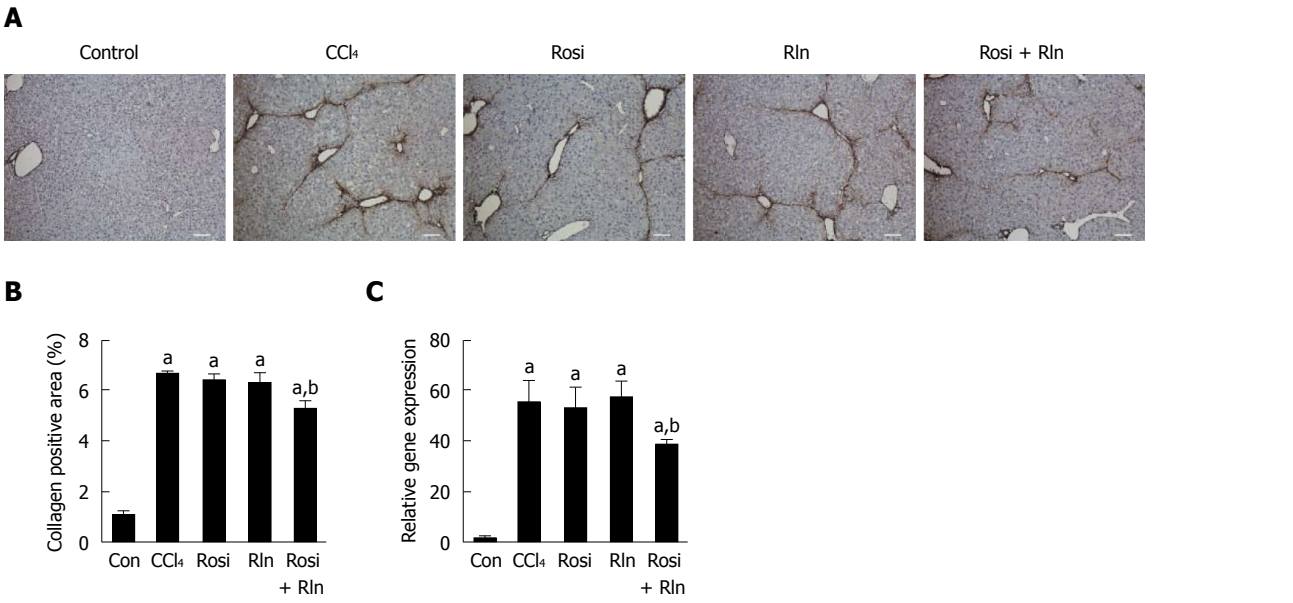

Figure 2 Liver type Ⅰ collagen content. A: Immunohistochemical staining of liver tissue from control (Con), fibrotic (CCl_4), rosiglitazone (Rosi), serelaxin (Rln) or combination-treated (Rosi + Rln) mice. Bar: 100 μm; B: Type Ⅰ collagen staining quantified; C: Type Ⅰ collagen gene expression determined by qPCR. Data are expressed as mean ± SE, and analyzed by ANOVA (n = 5). $^aP < 0.05$ *vs* Con; $^bP < 0.05$ *vs* CCl_4, Rosi, or Rln.

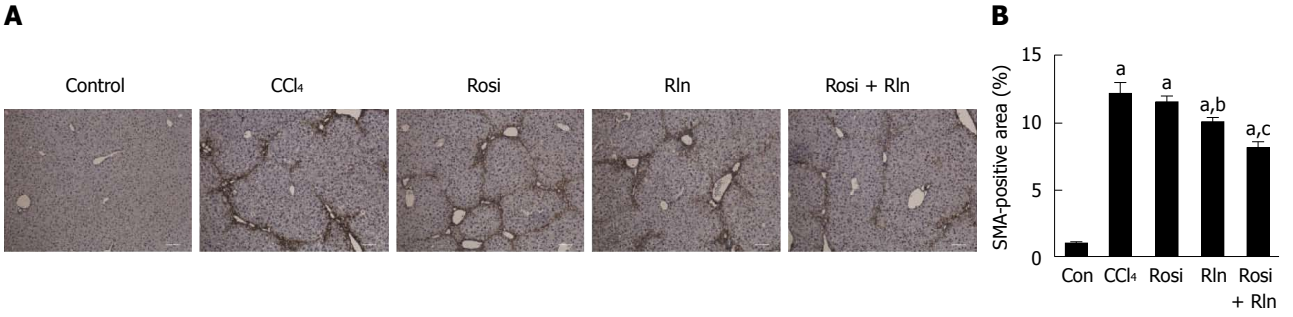

Figure 3 Liver smooth muscle actin content. A: Immunohistochemistry of liver tissue for SMA content from control (Con), fibrotic (CCl_4), rosiglitazone (Rosi), serelaxin (Rln) or combination-treated (Rosi+Rln) mice. Bar: 100 μm; B: SMA staining quantified. Data are expressed as mean ± SE, and analyzed by ANOVA (n = 5). $^aP < 0.001$ *vs* Con; $^bP < 0.05$ *vs* CCl_4; $^cP < 0.05$ *vs* CCl_4, Rosi, or Rln.

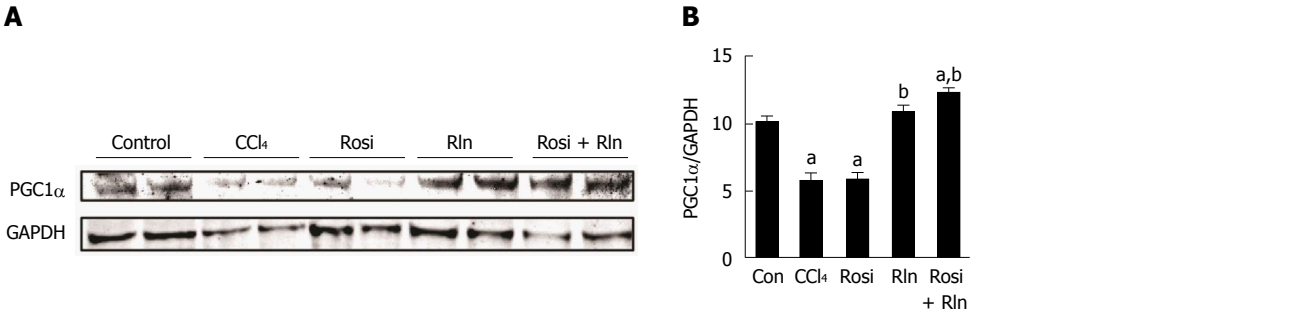

Figure 4 Western blotting of PGC1α content in livers from control (Con), fibrotic (CCl_4), rosiglitazone (Rosi), serelaxin (Rln) or combination-treated (Rosi + Rln) mice. A: Liver tissue extracts were analyzed by Western blotting for PGC1α. The levels of GAPDH are shown as a loading control; B: The levels of PGC1α relative to GAPDH were determined by densitometry. Data are shown as mean Data are expressed as mean ± SE, and analyzed by ANOVA (n = 5). $^aP < 0.05$ *vs* Con; $^bP < 0.01$ *vs* CCl_4 or Rosi.

(Figure 4). While single treatment with rosiglitazone was without effect, serelaxin alone or in combination with rosiglitazone restored PGC1α levels.

DISCUSSION

While hepatic fibrosis is a major health concern worldwide, the options for treatment are limited. The effectiveness of TZDs in the treatment of human liver disease remains to be studied. Early studies of the antidiabetic PPARγ agonists of the thiazolidinedione class, including rosiglitazone and pioglitazone, reduced the activation of HSCs[29-31], and had preventive effects in rat models of hepatic fibrosis[20,21,32,33]. However, in more clinically relevant studies exploring the effectiveness of TZDs in the treatment of established hepatic fibrosis in rats, pioglitazone was only effective when introduced very early in the course of the disease[23]. Furthermore, pioglitazone was ineffective in reducing the fibrotic phenotype of mouse HSC, and did not prevent CCl_4-induced hepatic fibrosis in mice[22]. These findings dampened enthusiasm for the utility of TZD treatment of hepatic fibrosis.

The reason for the failure of mice to respond to thiazolidinediones is unknown, but may be related to the lack of PPARγ expression in activated mouse HSC[23]. If this is the case, then strategies to increase PPARγ signaling might restore responsivity to TZDs. We previously identified PPARγ as a downstream target of relaxin signaling through its receptor, RXFP1[25]. Furthermore, we demonstrated that relaxin activated PPARγ through a ligand-dependent mechanism mediated by increased expression of the PPARγ coactivator PGC1α[26]. The co-treatment of cells with relaxin and the PPARγ agonist rosiglitazone resulted in greater PPARγ transcriptional activity than either relaxin or rosiglitazone alone, suggesting that relaxin was acting to enhance the activity of PPARγ[25]. The purpose of the present study was to test this relationship using an *in vivo* model of established hepatic fibrosis. In earlier studies, we found that short-term relaxin treatment of hepatic fibrosis (2 wk) was insufficient to significantly reduce collagen deposition, and that 4 wk of treatment was required for significant results[14,15]. We therefore chose to 2 wk of serelaxin treatment for this study. While serelaxin alone had no effect on total collagen or type I collagen, it did significantly reduce αSMA content and therefore, HSC activation. This suggests that the effects of serelaxin on HSC activation precede the degradation of excess collagen. We also confirmed, using rosiglitazone, the lack of effectiveness of TZD treatment alone on mouse hepatic fibrosis, reported previously using pioglitazone[23]. However, consistent with our earlier cell culture studies[25], the combination of relaxin and rosiglitazone significantly decreased collagen content and HSC activation, and reduced AST levels. The effects occurred with only 2 wk of treatment, and in the face of continued delivery of toxin (CCl_4), suggesting that the combination treatment accelerated the rate of the antifibrotic effect.

Our previous findings showed that relaxin enhanced PPARγ signaling through increased expression of PGC1α[26]. In the present study, CCl_4 treatment reduced the level of PGC1α, as shown previously in models of liver injury[34,35]. Treatment with serelaxin, or the combination of serelaxin and rosiglitazone, restored PGC1α levels. This finding supports the previous findings suggesting that relaxin acts to enhance PPARγ activity through increased expression of PGC1α. However, since relaxin treatment alone for 2 wk failed to reduce collagen levels, induction of PGC1α alone is not sufficient for resolution of hepatic fibrosis, and the presence of PPARγ agonists is necessary for maximum effectiveness.

Taken together, these data suggest that the combination of serelaxin and rosiglitazone may be a more effective treatment for hepatic fibrosis than either agent alone. This raises the possibility of new approaches to the treatment of hepatic fibrosis that can exploit the combined effects of both RXFP1 and PPARγ activation. Further studies are needed to determine if combination therapy can be effective in alternative models of hepatic fibrosis, or extended to extrahepatic fibrotic conditions.

COMMENTS

Background

Hepatic fibrosis is characterized by excess collagen deposition in response to a variety of causes of liver injury. There are currently no effective treatments for hepatic fibrosis and cirrhosis. The hormone relaxin has antifibrotic effects in a number of tissues, and was recently found to increase the activity of peroxisome proliferator-activated receptor γ (PPARγ).

Research frontiers

Relaxin is quickly emerging as an antifibrotic agent, and in preclinical studies has shown efficacy in the treatment of a variety of fibrosis models. The use of PPARγ agonists have also been explored for antifibrotic effects, but have had limited success in models of hepatic fibrosis.

Innovations and breakthroughs

This is the first study to explore the effect of combined relaxin and PPARγ agonist treatment of established hepatic fibrosis. The results suggested that the combination treatment was more effective that either treatment alone.

Applications

The findings provide evidence that combined use of relaxin and PPARγ agonists may represent a potential new approach for the treatment of hepatic fibrosis.

Terminology

Relaxin is a polypeptide hormone with important roles in pregnancy, cardiovascular function, and extracellular matrix regulation. PPARγ is a nuclear transcription factor that regulates the expression of target genes.

Peer-review

The authors have provided evidence that combined treatment with relaxin and rosiglitazone was effective in a model of hepatic fibrosis.

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ORIGINAL ARTICLE

Basic Study

Bcl-2 degradation is an additional pro-apoptotic effect of polo-like kinase inhibition in cholangiocarcinoma cells

Svenja Sydor, Sami Jafoui, Lena Wingerter, Sandra Swoboda, Joachim C Mertens, Guido Gerken, Ali Canbay, Andreas Paul, Christian D Fingas

Svenja Sydor, Sami Jafoui, Lena Wingerter, Guido Gerken, Ali Canbay, Department of Gastroenterology and Hepatology, University Hospital Essen, University Duisburg-Essen, 45122 Essen, Germany

Sandra Swoboda, Andreas Paul, Christian D Fingas, Department for General, Visceral and Transplantation Surgery, University Hospital Essen, University Duisburg-Essen, 45122 Essen, Germany

Joachim C Mertens, Division of Gastroenterology and Hepatology, University Hospital Zurich, 8091 Zurich, Switzerland

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Institutional animal care and use statement: For this study we did not perform animal experiments or use experimental material derived from animals. Provision of an institutional animal care and use committee statement is not applicable in this case.

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Correspondence to: Christian D Fingas, MD, PhD, Associate Professor, Department for General, Visceral and Transplantation Surgery, University Hospital Essen, University Duisburg-Essen, Hufelandstraße 55, 45122 Essen, Germany. christian.fingas@uk-essen.de
Telephone: +49-201-723 83676
Fax: +49-201-7231131

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Abstract

AIM

To examine the influence on apoptotic mechanisms following inhibition of polo-like kinases as therapeutically

approach for cholangiocellular cancer treatment.

METHODS

As most cholangiocarcinomas are chemotherapy-resistant due to mechanisms preventing tumor cell death, we investigated the effect of Cisplatin on cholangiocellular carcinoma (CCA) cell lines KMCH-1 and Mz-Ch-1. Polo-like kinases (PLK) are important regulators of the cell cycle and their inhibition is discussed as a potential therapy while PLK inhibition can regulate apoptotic mediators. Here, cells were treated with PLK inhibitor BI6727 (Volasertib), Cisplatin, and in combination of both compounds. Cell viability was assessed by MTT; apoptosis was measured by DAPI staining and caspase-3/-7 assay. Western blot and qRT-PCR were used to measure expression levels of apoptosis-related molecules Bax and Bcl-2.

RESULTS

The cell viability in the CCA cell lines KMCH-1 and Mz-Ch-1 was reduced in all treatment conditions compared to vehicle-treated cells. Co-treatment with BI6727 and cisplatin could even enhance the cytotoxic effect of cisplatin single treatment. Thus, co-treatment of cisplatin with BI6727 could slightly enhance the cytotoxic effect of the cisplatin in both cell lines whereas there was evidence of increased apoptosis induction solely in Mz-Ch-1 as compared to KMCH-1. Moreover, PLK inhibition decreases protein levels of Bcl-2; an effect that can be reversed by the proteasomal degradation inhibitor MG-132. In contrast, protein levels of Bax were not found to be altered by PLK inhibition. These findings indicate that cytotoxic effects of Cisplatin in Mz-Ch-1 cells can be enhanced by co-treatment with BI6727.

CONCLUSION

In conclusion, BI6727 treatment can sensitize CCA cells to cisplatin-induced apoptosis with proteasomal Bcl-2 degradation as an additional pro-apoptotic effect.

Key words: Tumor necrosis factor-related apoptosis-inducing ligand; Myeloid cell leukemia-1; Hedgehog pathway; Cisplatin; Chemotherapy resistance

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Core tip: This manuscript addresses the timely and topical roles of cell cycle/apoptosis modulating enzymes for the tumor biology of human cholangiocarcinoma. These data suggest that polo-like kinases -Inhibition by BI6727 (volasertib) sensitizes some cholangiocarcinoma cell lines to cisplatin-induced apoptosis. Our findings include an enhanced cytotoxic effect of cisplatin by co-treatment with BI6727 (volasertib) and results in decreased protein expression levels of the anti-apoptotic molecule Bcl-2, which appears to be mediated *via* proteasomal degradation. Taken together, these data reveal another pro-apoptotic mechanism of polo-like kinase inhibition emphasizing the potential therapeutic benefit of polo-like kinase inhibitors for the treatment of cholangiocarcinoma.

Sydor S, Jafoui S, Wingerter L, Swoboda S, Mertens JC, Gerken G, Canbay A, Paul A, Fingas CD. Bcl-2 degradation is an additional pro-apoptotic effect of polo-like kinase inhibition in cholangiocarcinoma cells. *World J Gastroenterol* 2017; 23(22): 4007-4015 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i22/4007.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i22.4007

INTRODUCTION

Cholangiocellular carcinoma (CCA) represents the most common primary liver cancer with biliary differentiation and its incidence is increasing constantly in Western countries[1-5]. Therapeutic options are limited for CCA as tumors can be multifocal in advanced stages being surgically non-accessible. Additionally, CCA is often resistant to conventional chemotherapy and, therefore, associated with poor prognosis[6]. Development and progression of CCA are in part mediated by mechanisms that prevent tumor cell death[3,7]. For example, these cancer cells paradoxically express tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) as well as its cognate receptors[8-10] but are quite resistant to TRAIL-induced apoptosis[10-13]. The underlying mechanisms are complex and seem to be mediated by effective survival signals that prevent TRAIL-induced apoptosis (*e.g.*, upregulation of anti-apoptotic proteins of the Bcl-2 family)[8,14,15].

Polo-like kinases (PLK) represent a highly conserved family of several members of serine/threonine kinases regulating cell cycle division and are often overexpressed in tumor tissue of many different tumors including CCA[16]. PLK 1/2 expression is known to be associated with a poor prognosis and short overall survival rates in CCA[17]. PLK2 has been shown to be upregulated by Hedgehog (Hh) signaling - another important survival mechanism in CCA[8,18].

Thus, PLK2 appears to be an important mediator of Hh survival signaling as its expression is reduced when Hh signaling is inhibited[8]. In this context, PLK inhibition is discussed as a new potential therapeutical approach for the treatment of different cancers and has been described to decrease myeloid cell leukemia-1 (Mcl-1) - an anti-apoptotic member of the Bcl-2 protein family that has been identified as an important survival factor in CCA[15,19-21]. Members of the Bcl-2 family include anti- as well as pro-apoptotic proteins. The anti-apoptotic members Bcl-2 and Mcl-1 can prevent apoptosis induction by inhibition of mitochondrial cytochrome C release while the pro-apoptotic protein Bax can induce apoptosis by stimulating cytochrome C release[22-24]. We have recently shown that PLK2 inhibition can decrease Mcl-1 levels by proteasomal degradation inducing apoptosis

in CCA cells, which finally results in tumor suppression *in vivo*[8].

Beside gemcitabine, cisplatin is a conventional chemotherapeutic drug used for CCA treatment that can induce cell death in fast replicating cells by inhibition of DNA replication causing DNA damage and apoptosis[22,25]. However, treatment with cisplatin often results in chemoresistance and therapy failure[25]. Many different underlying mechanisms have been described in this context such as defective DNA binding, premature degradation, and increased activation of specific transporter proteins[25].

In this study, we aimed to investigate the impact of PLK inhibition in cisplatin-treated CCA cell lines and its effect on several Bcl-2 family members (beside Mcl-1) that might be involved in the mechanism of apoptosis resistance in CCA.

MATERIALS AND METHODS

Cell culture

Human CCA cell lines KMCH-1 and Mz-Ch-1 were cultured in Dulbecco's modified eagle medium/high-glucose medium (Invitrogen, Carlsbad, CA, United States) containing 10% fetal bovine serum, 1000 U/mL Penicillin, 0.1 mg/mL streptomycin and 2 mmol/L L-glutamine (PAA, Pasching, Austria) at 5% CO_2 and 37 ℃. The selective PLK-inhibitor BI6727/Volasertib[26] (Selleckchem, Houston, TX, United States) and the proteasome inhibitor MG-132 (Merck, Rockland, MA, United States) were dissolved in DMSO (Sigma-Aldrich, St. Louis, MO, United States), Cisplatin (Merck, Rockland, MA, United States) was dissolved in PBS, stock solutions of substances or vehicle as control were subsequently diluted in cell culture medium for experiments.

MTT cell viability assay

For assessing cell viability 5 × 10^4cells/well were plated in 96-multiwell plates. Twenty-four h after plating, cells were incubated with 200 nmol/L BI6727 or 1 mmol/L cisplatin for 24 h as described previously[8]. Cell viability was measured by MTT assay. The cells incubated with vehicle (DMSO) for 24 h were considered 100% viable.

Quantitation of apoptosis

Apoptosis in CCA cells was quantified by assessing characteristic nuclear changes of apoptosis after staining with 4′, 6-diamino-2-phenylindole dihydrochloride (DAPI; Sigma-Aldrich, St. Louis, MO, United States) using fluorescence microscopy as described previously[27]. Caspase-3/-7 activity was assessed by Caspas-3/-7 assay (Promega, Madison, WI, United States) according to manufacturer's recommendations.

RNA isolation and qRT-PCR

Cells were seeded at a density of approx. 1 × 10^6 cells/cm^2 for *in vitro* experiments. At the end of the stimulation period total RNA extraction and purification was performed using RNeasy Mini Kit (Qiagen, Hilden, Germany) following manufacturer instructions. Reverse transcription was performed with the QuantiTect RT kit (Qiagen; Hilden, Germany) using 1 μg of total RNA. Quantitative realtime PCR (qRT-PCR) for specific mRNA sequences was performed on a CFX96 Touch Real-Time PCR Detection System (Bio-Rad, Hercules, CA, United States) using QuantiTect SYBR Green Kit (Qiagen, Hilden, Germany) in a final volume of 15 μL including 2 μL of cDNA. Oligonucleotide sequences for Bax primers were used as follows: Bax forward: 5′-TCTGACGGCAACTTCAACTG-3′; Bax reverse: 5′-GGAGGAAGTCCAATGTCCAG-3′. Melting curves were collected to ascertain specificity of PCR products. Changes in mRNA expression were calculated by the ΔΔ-ct method and are presented as foldchanges in relation to expression of a reference gene (hypoxanthine-guanine phosphoribosyltransferase, HPRT) in vehicle-treated cells.

Protein isolation and western blot

Cells were seeded at a density of approx. 1 × 10^6 cells/cm^2 for *in vitro* experiments, at the end of the stimulation period protein lysates were prepared using lysis buffer (50 mmol/L Tris-HCl; 150 nmol/L NaCl; 0.1% NP-40; 1% desoxycholic acid) containing complete mini EDTA-free protease inhibitor cocktail and phosphostop (Roche, Mannheim, Germany). In all, 30 μg of total protein were separated using SDS-PAGE, immunoblotting was performed using standard procedures with the following primary antibodies (incubation: overnight at 4 C): Actin (1/1000; #5125; Cell Signaling, Cambridge, United Kingdom), Bax (1/1000; #2772; Cell Signaling) and Bcl-2 (1/1000; #2876; Cell Signaling), cleaved PARP (1/1000; #5625, Cell Signaling). After incubation with the appropriate horseradish peroxidase-conjugated secondary antibody, bound antibodies were visualized using chemiluminescence reagent ECL-Prime reagent (GE Healthcare, Chalfont St. Giles, United Kingdom) according to the supplier's protocol. Blotting images and densitometric quantification of protein bands was generated using Fusion detection system (PeqLab Biotechnology, Erlangen, Germany).

Statistical analysis

Statistical significance for in vitro experiments was determined by one-way ANOVA (with Tukey's post-hoc test for individual experimental conditions) and by a two-tailed unpaired Student t test performed with Prism 5 (GraphPad Software, Inc.; San Diego, CA, United States). Data are presented as mean ± SEM. Differences were considered significant at $P < 0.05$.

RESULTS

Cytotoxic effects of cisplatin are enhanced by co-treatment with PLK inhibitor BI6727

In previous studies, we demonstrated that the PLK

inhibitor BI6727 had a pro-apoptotic effect in CCA cell lines and could exacerbate TRAIL-induced cell death[8]. Cisplatin is one of the conventional cytostatic drugs that are used for the chemotherapy of CCA. Here, we aimed to further investigate the effect of the PLK inhibitor BI6727 on cell death in CCA cell lines in the presence or absence of cisplatin. We measured viability in the CCA cell lines KMCH-1 and Mz-ChA-1 treated with cisplatin and BI6727 for 24 h *via* a MTT viability assay. Cell viability was reduced in all treatment conditions compared to vehicle-treated KMCH-1 (Figure 1A) and Mz-Ch-1 cells (Figure 1D). Co-treatment with BI6727 and cisplatin could even enhance the cytotoxic effect of cisplatin single treatment. By means of the MTT viability assay, we could demonstrate that cell viability was reduced under different treatment conditions in these two cell lines; however, with this assay the type of cell death cannot be determined. To assess apoptosis induction caused by the different treatments, we performed DAPI staining with quantitation of apoptotic nuclei by fluorescence microscopy as well as fluorescent analysis of caspase-3/-7 activity. In KMCH-1 cells treated with BI6727, cisplatin and the combination of BI6727 and cisplatin, some apoptotic nuclei were found (Figure 1B). Moreover, caspase-3/-7 activity was slightly induced in BI6727-treated KMCH-1 cells (Figure 1C). In KMCH-1 cells treated with the cytotoxic drug cisplatin, caspase-3/-7 activity was reduced compared to vehicle-treated cells whereas co-treatment with BI6727 and cisplatin induced caspase -3/-7 activitiy as compared to cisplatin only-treated cells. In KMCH-1 cells, viability was reduced by treatment with BI6727 or cisplatin and the combination of BI6727 with cisplatin could even exacerbate this effect (which does not appear to be mediated by apoptosis induction). In Mz-Ch-1 cells, we could observe a similar effect as co-treatment of BI6727 and cisplatin could slightly enhance the cytotoxic effect of both single agents (Figure 1D). Quantification of apoptotic nuclei demonstrated that BI6727 or cisplatin treatment increased the number of apoptotic nuclei. The number of apoptotic nuclei was increased in BI6727-treated cells as compared to cisplatin-treated cells while the combination of these substances enhanced the apoptotic effect of cisplatin single treatment (Figure 1E). Caspase-3/-7 activity was induced in BI6727-treated as compared to vehicle-treated cells whereas cisplatin treatment did not enhance caspase activity (Figure 1F). Under co-treatment conditions with BI6727 and cisplatin, caspase-3/-7 activity could be stronger induced as compared to cisplatin single treatment. In order to confirm apoptosis induction we also checked cleavage of the chromatin-associated enzyme PARP [poly (ADP-ribose) polymerase 1]. PARP is involved in DNA repair and replication but PARP cleavage has been described to be an early event during apoptosis[28] while cleavage may diminish DNA-repair and replication processes[29]. In KMCH-1 cells PARP cleavage was not detected (data not shown) while in Mz-Ch-1 cells, protein levels of cleaved PARP were slightly induced after BI6727 single treatment and cisplatin/BI6727 combination treatment (Figure 2A and B).

Thus, co-treatment of cisplatin with the PLK-inhibitor BI6727 could (slightly) enhance the cytotoxic effect of the cytostatic drug cisplatin in both cell lines whereas there was evidence of increased apoptosis induction solely in Mz-Ch-1 cells as compared to KMCH-1 cells.

PLK Inhibition by BI6727 alters Bcl-2 but not Bax expression

As shown previously, pro-apoptotic effects of BI6727 treatment are in part mediated by Mcl-1 down regulation[8]. As other important regulators of apoptosis, we here aimed to investigate the effect of BI6727 on the anti-apoptotic protein Bcl-2 as well as the pro-apoptotic Bcl-2 family member Bax. We determined *Bax* mRNA expression levels in KMCH-1 and Mz-Ch-1 cells by qRT-PCR treated with BI6727, cisplatin or both substances. In KMCH-1 cells, BI6727 treatment induced *Bax* expression and this effect was also present in cells co-treated with BI6727 and cisplatin. Interestingly, cisplatin as a single agent did not induce *Bax* expression (Figure 3A). In contrast, Bax protein levels, determined *via* western blot analysis and quantified by densitometric measurement, were not changed in KMCH-1 cells treated with BI6727 and/or cisplatin (Figure 3B and C). In Mz-Ch-1 cells, *Bax* expression was significantly induced by cisplatin treatment whereas BI6727 treatment had no effect on *Bax.* Following combination treatment of BI6727 with cisplatin, *Bax* was not further induced (Figure 3D). Western blot analyses did not show alterations regarding the Bax expression levels in Mz-Ch-1 cells after treatment with one of the components or after combination treatment (Figure 3E and F).

In Mz-Ch-1 cells, protein levels of Bcl-2 were reduced after treatment with cisplatin single treatment, cisplatin/BI6727 combination treatment, and especially BI6727 single treatment (Figure 3G and H). To assess whether the Bcl-2 decrease is a result of proteasomal degradation, cells were co-treated with the potent proteasome inhibitor MG-132. Indeed, inhibition of proteasomal degradation by co-treatment of BI6727 with MG-132 restored Bcl-2 to normal levels similar to the vehicle control.

Thus, PLK inhibition reduces Bcl-2 protein levels in a posttranslational manner by proteasomal degradation resulting in enhanced apoptosis (Figure 3G and H).

DISCUSSION

This study reveals an additional pro-apoptotic effect of polo-like kinase inhibition in CCA cell lines. The

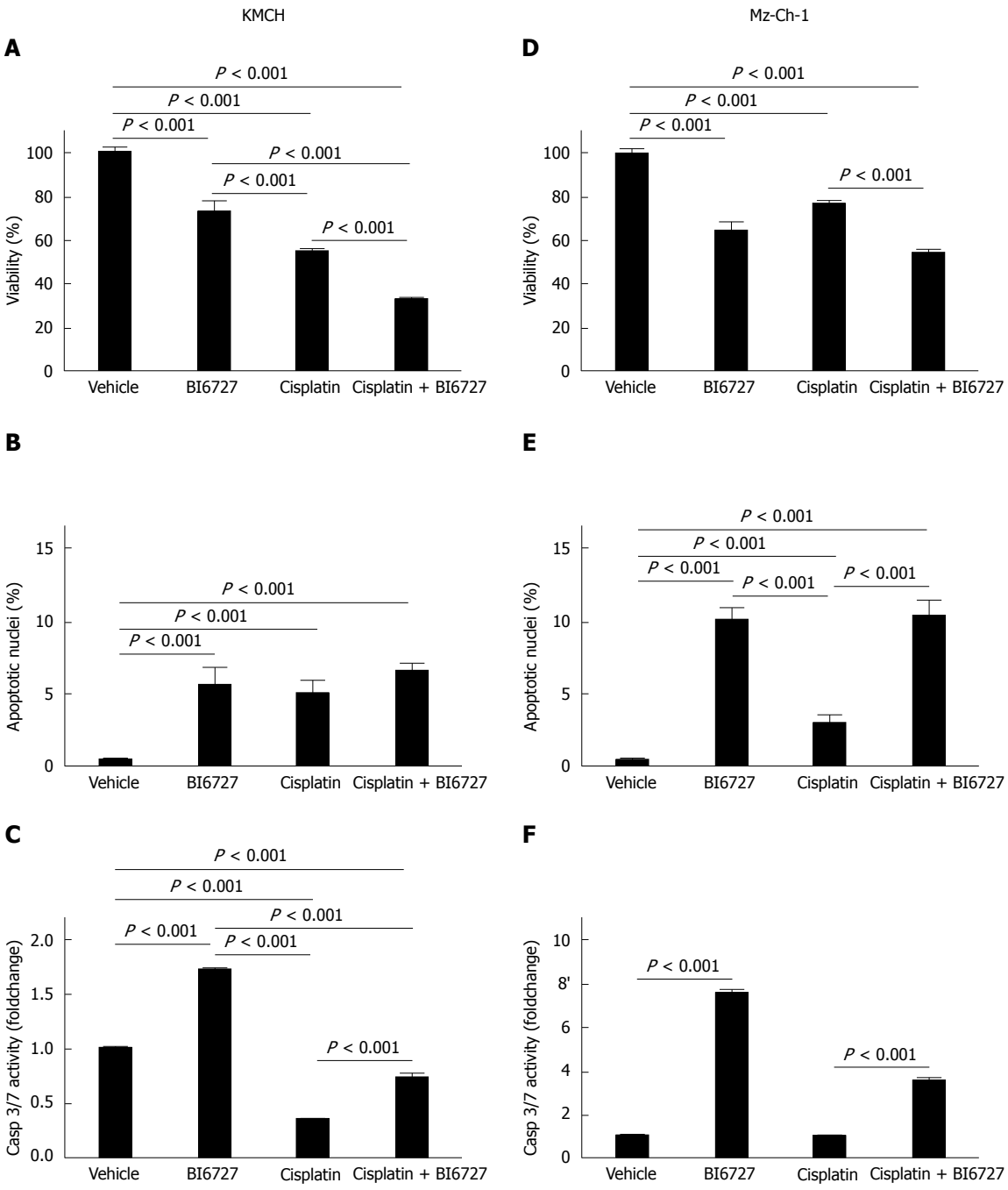

Figure 1 PLK-inhibitor BI6727 reduces cell viability and acts pro apoptotic in cholangiocellular carcinoma cell lines. Cell viability of CCA cell lines KMCH-1 (A) and Mz-Ch-1 (D) treated with the PLK-inhibitor BI6727 (200 nmol/L for 24 h), the cytostatic drug cisplatin (1 mmol/L for 24 h) or both components was assessed by MTT assay (A, D) shown as % of viable cells (viability) compared to vehicle-treated cells (mean ± SEM, *n* = 3). Apoptotic nuclei were determined in DAPI stained KMCH (B) and Mz-Ch-1 (E) cells after treatment using fluorescence microscopy. The number of apoptotic nuclei was normalized to the total number of nuclei (mean ± SEM, *n* = 4). Apoptosis induction in KMCH-1 (C) and Mz-Ch-1 (F) cells was measured by fluorescent Caspase-3/-7 activity assay shown as foldchange of vehicle-treated cells (mean ± SEM, *n* = 3). PLK: Polo-like kinase; CCA: Cholangiocarcinoma; MTT: 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromid; DAPI: 4', 6-diamino-2-phenylindole dihydrochloride.

findings indicate that the cytotoxic effect of cisplatin can be enhanced by co-treatment with the PLK inhibitor BI6727 and that PLK inhibition (beside Mcl-1) decreases Bcl-2 *via* its proteasomal degradation[8].

Cholangiocellular carcinoma represents a deadly disease with rising prevalence in Western countries and its pathogenesis is understood insufficiently. Effective treatment options are still rare due to missing understanding of pathogenic mechanisms. TRAIL has been discussed as an effective agent to target tumor cell growth and to support existing therapy options in different types of cancers[30-32]. However CCA tumor cells already express TRAIL and its cognate receptor *in vivo* but are resistant to TRAIL[14]. Many other pathways and factors regulating cell growth, migration and invasion have become potential targets in cancer therapy, such as the Wnt, Notch or Hh pathway (which has already been associated to PLK)[8,33-35]. Various

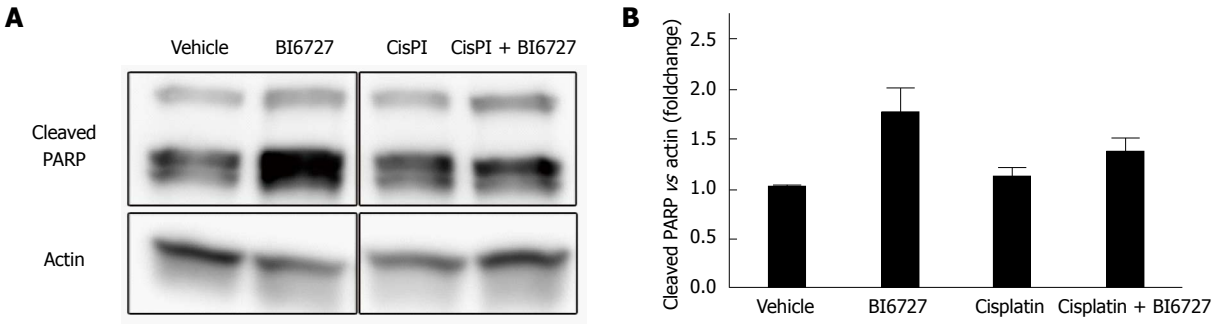

Figure 2 PLK-inhibitor BI6727 induces apoptotis in Mz-Ch-1 cells. Cleavage of PARP in Mz-Ch-1 treated with the PLK-inhibitor BI6727 (200 nmol/L for 24 h), the cytostatic drug cisplatin (1 mmol/L for 24 h) or both components was assessed by Western blot analysis (A) and densitometric quantification followed by normalization to loading control actin and shown as foldchange compared to vehicle-treated cells (B, mean ± SEM, *n* = 2). PLK: Polo-like kinase; PARP: Poly(ADP-ribose) polymerase 1.

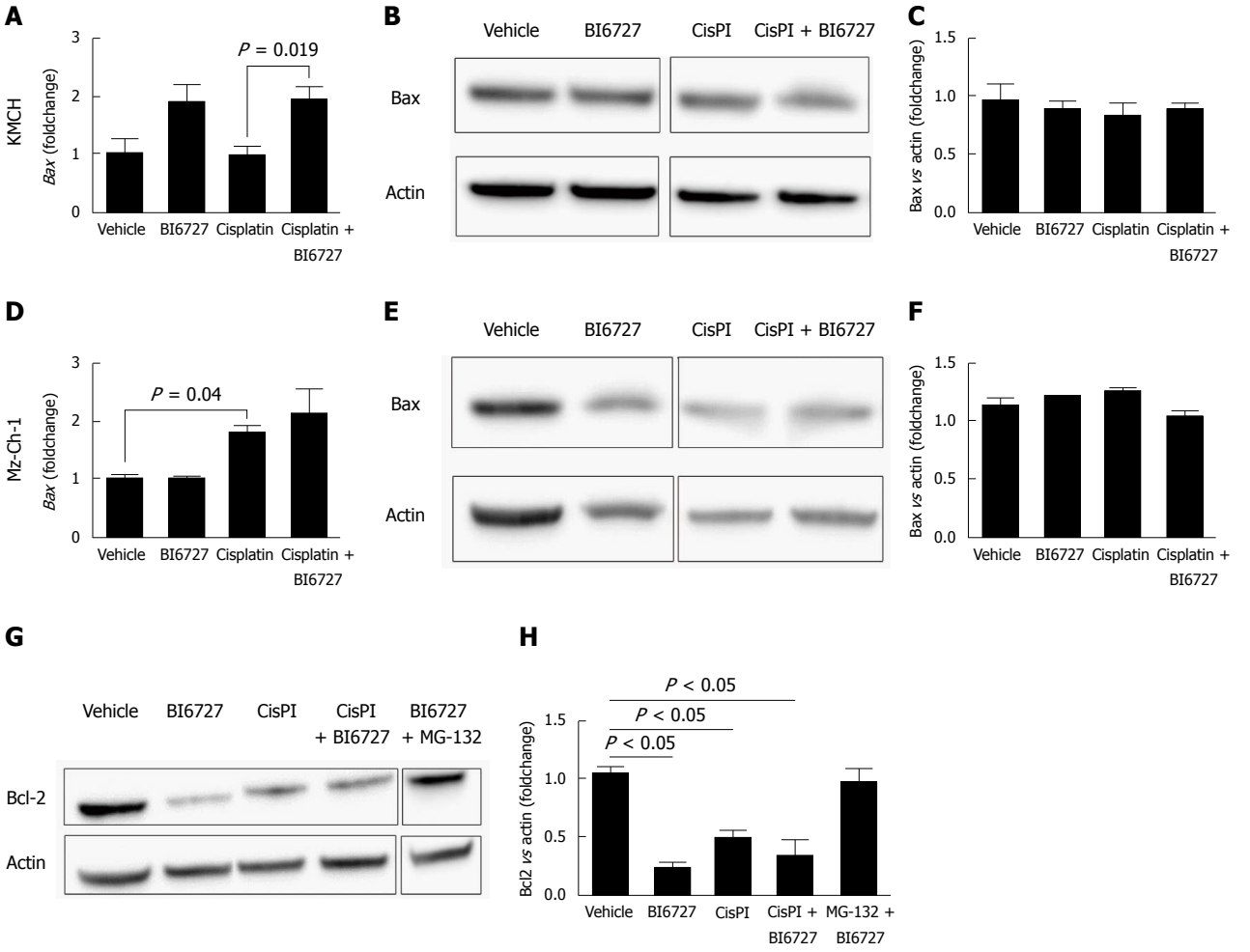

Figure 3 Impact of treatment with BI6727 and cisplatin on Bax and Bcl-2 expression in cholangiocellular carcinoma cell lines. Bax expression levels were determined in KMCH-1 (A-C) and Mz-Ch-1 (D-F) cells after treatment with BI6727 (200 nmol/L for 24 h), cisplatin (1 mmol/L for 24 h) or the combination of both components. Bax mRNA levels were measured by qRT-PCR (A, D; mean ± SEM, *n* = 3), shown as foldchange compared to vehicle-treated cells. Representative Western blot images of Bax and the loading control actin are shown in panels B and E. Bcl-2 protein levels were determined using Western blot analysis in treated Mz-Ch-1 cells. Proteasomal degradation was inhibited by co-treatment with MG-132 (1 μmol/L for 24 h) (G, H). Protein levels were quantified by densitometry, normalized to the loading control and shown as foldchange compared to vehicle-treated cells (C, F, H, mean ± SEM, *n* = 3); *P*-values were determined using student *t*-test. CCA: Cholangiocarcinoma; Bcl-2; B-cell lymphoma 2.

specific inhibitors are tested in different studies for their usability as potent anti-cancer drugs or additives to existing therapies[8,36]. The potent PLK inhibitor BI6727 (volasertib) has been identified as a promising candidate for cancer therapy as PLK are upregulated in many different cancers[16,17,37-39]. PLK depletion in different stromal cancer cells using small interfering RNA technique resulted in cell cycle arrest and increased apoptosis[38]. Using KMCH-1 and Mz-Ch-1 CCA cancer cells, we here could demonstrate increased

overall cell death and enhanced apoptosis induction following treatment with the PLK-inhibitor BI6727. In combination with the conventional chemotherapeutic drug cisplatin, BI6727 could even (slightly) enhance cell death in KMCH-1 and Mz-Ch-1 cells whereas the pro-apoptotic effect was more potent in Mz-Ch-1 as compared to KMCH-1 cells. It is well known that PLK inhibition has an impact on regulation of proteins belonging to the Bcl-2 family as PLK inhibition decreases protein levels of Mcl-1 in esophageal squamous cell carcinomas and osteosarcomas as well as in KMCH-1 cells[8,38,40]. Here, we focused on the effect of BI6727 on the pro-apoptotic protein Bax and the anti-apoptotic molecule Bcl-2. *Bax* mRNA levels were slightly induced in KMCH-1 and Mz-Ch-1 cells treated with BI6727 and cisplatin, whereas Bax protein levels were not found to be changed in both cell lines. Moreover, Bcl-2 levels were decreased in Mz-Ch-1 cells treated with BI6727 (and cisplatin). This effect was not enhanced after combination of both components. In a previous study we could demonstrate that BI6727 treatment reduced Mcl-1 levels but not Bcl-2 levels after 8 h of incubation[8]. In the present study we observed a significant reduction of Bcl-2 protein levels due to a longer incubation period with BI6727 of 24 h.

Thus, the pro-apoptotic effect of BI6727 treatment appears to be mediated by proteasomal degradation not only of Mcl-1 but also of the anti-apoptotic protein Bcl-2 (without affecting Bax protein levels). Overexpression of Bcl-2 is common in many types of human cancer and has been correlated with decreased susceptibility to chemotherapeutical drugs[22]. However, in CCA, Mcl-1 plays a more pivotal role as tumor cell survival factor than Bcl-2[19,41].

Treatment of CCA cells with the PLK inhibitor BI6727 beside Mcl-1[8] decreases Bcl-2 protein levels thereby reducing cell viability and enhancing apoptosis. In combination with the chemotherapeutical drug cisplatin, BI6727 treatment could even enhance the cytotoxic effect of cisplatin single treatment.

In conclusion, BI6727 treatment sensitizes some CCA cell lines to cisplatin-induced apoptosis with proteasomal Bcl-2 degradation as an additional pro-apoptotic effect.

COMMENTS

Background

Most cholangiocarcinoma are chemotherapy-resistant and these tumors show a poor therapeutic prognosis. Development and progression of cholangiocarcinoma are in part mediated by complex mechanisms that prevent tumor cell death by stimulation of death receptors such as tumor necrosis factor-related apoptosis-inducing ligand (TRAIL). Polo-like kinases are important regulators of the cell cycle and their inhibition is discussed as a potential therapeutic approach for cancer treatment as polo-like kinase inhibition can decrease protein levels of anti-apoptotic mediators such as myeloid cell leukemia-1 (Mcl-1). The authors here aimed to study the chemotherapeutic effect of the conventionally used drug cisplatin in combination with polo-like kinase inhibition in cholangiocarcinoma cell lines in order to investigate mechanisms of drug resistance and potential benefits of polo-like kinase inhibition in cancer and especially in cholangiocarcinoma therapy.

Research frontiers

The manuscript addresses the very timely and topical roles of cell cycle/apoptosis modulating enzymes for the tumor biology of human cancer and especially in cholangiocarcinoma. In particular, the data suggest that polo-like kinase inhibition can sensitize some cholangiocarcinoma cell lines to cisplatin-induced apoptosis and therefore can address a new mechanism for enhancements of cancer therapies.

Innovations and breakthroughs

It was already known that polo-like kinase inhibition could decrease expression levels of the anti-apoptotic molecule Mcl-1 in cholangiocarcinoma cells. Data of this manuscript reveal another pro-apoptotic mechanism of polo-like kinase inhibition emphasizing the potential therapeutic benefit of polo-like kinase inhibitors for the treatment of cholangiocarcinoma. Polo-like kinase inhibition by BI6727 (volasertib) could enhance cytotoxic effect of cisplatin in cholangiocarcinoma cell lines by reducing expression of the anti-apoptotic molecule Bcl-2 that seems to be mediated *via* proteasomal degradation.

Applications

These data reveal another pro-apoptotic mechanism of polo-like kinase inhibition emphasizing the potential therapeutic benefit of polo-like kinase inhibitors for the treatment of cholangiocarcinoma.

Terminology

Polo-like kinases: Polo-like kinases are important cell cycle regulating enzymes with a conserved N-terminal kinase domain and a C-terminal polo box domain. Polo-like kinases are involved in formation of the spindle apparatus in mitosis and may activate cdk/cycline complexes of the cell cycle. In different tumors PLK1 has been described to be up regulated. Due to pro-proliferative effects these tumors are associated with enhanced tumor growth and worse outcome and therefore PLK-inhibition is tested as potential cancer treatment. TRAIL: Tumor necrosis factor related apoptosis-inducing ligand belongs to the TNF/TNFR superfamily that can induce apoptosis in target cells *via* binding to special death receptors. Important target cells represent tumor cells while "normal" non-tumorous cells are less susceptible to TRAIL-induced cell death and mainly remain less harmed compared to tumor cells after treatment with TRAIL. Due to these findings in different *in vitro* and *in vivo* experiments TRAIL has been discussed as a potential cancer treatment agent. Interestingly in cholangiocellular carcinoma TRAIL seems to contribute to therapy resistance of tumors.

Peer-review

The manuscript is interesting, but needs more improvements. For example, the confirmation of apoptosis at the molecular level by the analysis of PARP cleave, or caspase-3 using western blot analysis. Also, the analysis of Bax expression at the protein level.

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ORIGINAL ARTICLE

Basic Study

Effect of *CXCR3/HO-1* genes modified bone marrow mesenchymal stem cells on small bowel transplant rejection

Ming-Li Yin, Hong-Li Song, Yang Yang, Wei-Ping Zheng, Tao Liu, Zhong-Yang Shen

Ming-Li Yin, Tianjin First Central Hospital Clinic Institute, Tianjin Medical University, Tianjin 300070, China

Hong-Li Song, Department of Organ Transplantation, Tianjin First Central Hospital, Tianjin Clinical Research Center for Organ Transplantation and Tianjin Key Laboratory of Organ Transplantation, Tianjin 300192, China

Yang Yang, Wei-Ping Zheng, Zhong-Yang Shen, Department of Organ Transplantation, Tianjin First Central Hospital, Tianjin 300192, China

Tao Liu, Tianjin First Central Hospital and Key Laboratory of Emergency and Care Medicine of Ministry of Health, Tianjin 300192, China

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Correspondence to: Zhong-Yang Shen, MD, PhD, Professor, Department of Organ Transplantation, Tianjin First Central Hospital, No. 24 Fukang Road, Nankai District, Tianjin 300192, China. shenzhy@tmu.edu.cn
Telephone: +86-22-2362698
Fax: +86-22-2362698

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Abstract

AIM

To investigate whether bone marrow mesenchymal stem cells (BMMSCs) modified with the *HO-1* and *CXCR3* genes can augment the inhibitory effect of BMMSCs on small bowel transplant rejection.

METHODS

Lewis rat BMMSCs were cultured *in vitro*. Third-passage BMMSCs were transduced with the *CXCR3/HO-1* genes or the *HO-1* gene alone. The rats were divided into six groups and rats in the experimental group were pretreated with BMMSCs 7 d prior to small

bowel transplant. Six time points (instant, 1 d, 3 d, 7 d, 10 d, and 14 d) (n = 6) were chosen for each group. Hematoxylin-eosin staining was used to observe pathologic rejection, while immunohistochemistry and Western blot were used to detect protein expression. Flow cytometry was used to detect T lymphocytes and enzyme linked immunosorbent assay was used to detect cytokines.

RESULTS

The median survival time of BMMSCs from the *CXCR3/HO-1* modified group (53 d) was significantly longer than that of the *HO-1* modified BMMSCs group (39 d), the BMMSCs group (26 d), and the NS group (control group) (16 d) (P < 0.05). Compared with BMMSCs from the *HO-1* modified BMMSCs, BMMSCs, and NS groups, rejection of the small bowel in the *CXCR3/HO-1* modified group was significantly reduced, while the weight of transplant recipients was also significantly decreased (P < 0.05). Furthermore, IL-2, IL-6, IL-17, IFN-γ, and TNF-α levels were significantly decreased and the levels of IL-10 and TGF-β were significantly increased (P < 0.05).

CONCLUSION

BMMSCs modified with the *CXCR3* and *HO-1* genes can abrogate the rejection of transplanted small bowel more effectively and significantly increase the survival time of rats that receive a small bowel transplant.

Key words: Bone marrow mesenchymal stem cells; CXCR3; HO-1; Small bowel transplantation; Rejection

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Core tip: In this paper, transplant recipient rats were pretreated with bone marrow mesenchymal stem cells (BMMSCs) in advance, and these rats were in a compromised immune state. *CXCR3/HO-1* gene modified BMMSCs were injected into recipient rats after small bowel transplantation. The survival time of these rats was significantly prolonged; the number of cells that underwent apoptosis was significantly lower in the *CXCR3/HO-1* modified BMMSCs group compared with rats from the *HO-1* gene modified BMMSCs group, the native BMMSCs group, and the NS group. Furthermore, the percentage of regulatory T cells was significantly increased. Proinflammatory cytokines (IL-2, IL-6, IL-17, IFN-γ, and TNF-α) were significantly reduced, while anti-inflammatory cytokines (IL-10 and TGF-β) were significantly increased. Our data suggest that BMMSCs modified with the *CXCR3* and *HO-1* gene can reduce rejection of the small intestine more effectively than *HO-1* modified BMMSCs and native BMMSCs.

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INTRODUCTION

In recent years, with improvements in surgical transplantation, immunosuppressive regimens, prevention of infection, and other key technological advances, small bowel transplantation has become an effective treatment for intestinal failure[1-4]. Currently, small bowel transplantation poses more clinical problems compared with transplantation of other organs such as the liver and kidney[5]. Specific problems include high rejection rates and the lack of effective preventive and treatment methods for such rejection. Clinical studies demonstrate that 30%-40% of recipients experience chronic rejection 5 years after transplantation, which requires removal of the transplanted small bowel, restoration of parenteral nutrition, or re-transplantation[6]. Therefore, the prevention and treatment of rejection after small bowel transplantation are a significant clinical problem that remains to be solved.

Bone marrow mesenchymal stem cells (BMMSCs) have the ability to proliferate *in vitro*, differentiate into cells with massive potential for immune regulation, and promote tissue repair in injured tissue[7,8]. They can secrete soluble cytokines through paracrine mechanisms, regulate inflammation and the immune response[9,10], and participate in complex regulation of immune cell function[11], mainly by inhibiting the most influential immune cells such as NK cells, T cells, and B cells[12]. A number of studies have shown that the immunomodulatory capacity of BMMSCs is capable of curing diseases, suggesting that they might be a novel approach for the treatment of disorders of the immune system[13,14]. BMMSCs are ideal candidates for cell transplantation and for the treatment of autoimmune diseases and prevention of rejection after solid organ transplantation[15]. However, a possible limitation to these cells is their ability to survive and to specifically target lesions[16]. Indeed, if BMMSCs are transplanted directly into a lesion, most tend to die within a few hours[17]. Therefore, the ability of BMMSCs to survive, proliferate, and migrate is an important problem that must be solved, if they can ever be used clinically. By modifying BMMSCs and interfering with or enhancing the expression of a certain gene may be an effective way to improve the survival rate of these cells in stem cell transplantation. In this study, the chemokine receptor (*CXCR3*) gene for chemotaxis[18] and the heme oxygenase-1 (*HO-1*) gene, which can enhance stem cell activity and prolong stem cell function[19], were transduced into BMMSCs to assess whether such modified BMMSCs could prevent or reduce rejection of transplanted small bowel in a rat model.

MATERIALS AND METHODS

Main reagents

DMEM-F12 (Gibco, Grand Island, CA, United States), RPMI-1640 (Gibco), fetal bovine serum (FBS) (Biowest, Loire Valley, France), gel preparation kit (SDS-PAGE) (Boster, Suzhou, China), reverse transcription kit (Thermo Scientific, Massachusetts, MN, United States), SYBR Green kit (TaKaRa, Japan), antibodies against CD34 (FITC) (Santa Cruz Biotechnology, Santa Cruz, CA, United States), CD29 (PE), CD45 (PE), CD90 (FITC), RT1A (PE), RT1B (FITC) (Biolegend, San Diego, CA, USA), CD4 (FITC), CD25 (PE), FoxP3 (PE-Cyanine5) (eBioscience Inc, San Diego, CA, United States), Heme Oxygenase 1 (HO-1) (Abcam, Cambridge, United Kingdom), and CXCR3 (Affinity Bioreagent, United States), YAC-1 (Chinese Academy of Medical Sciences, Beijing, China), AD-Hmox1 (Shanghai Genechem Co. Ltd, Shanghai, China), AD-fusion gene (*CXCR3/HO-1*) (Shanghai Genechem Co. Ltd), and interleukin (IL)-2, IL-6, IL-10, IL-17, IL-23, tumor necrosis factor (TNF)-β, interferon (IFN)-γ, transforming growth factor (TGF)-β, and diamine oxidase (DAO) enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Minneapolis, MN, United States) were employed in this study, as was HRP polymer (Jinmai Gene, Tianjin, China).

BMMSCs preparation and in vitro isolation

We surgically removed femurs and tibias from 2-3-wk-old Lewis rats that were sacrificed by cervical dislocation. The weight of the rats ranged from 40-60 g. After the bones were immersed in 75% alcohol for 5-10 min, the medulla canals of the bones were rinsed with DMEM-F12 and 10% FBS (complete medium) in order to generate single cell suspensions. Cells were incubated at 37 ℃ in an atmosphere containing 50 mL/L CO_2. Approximately 10 d later, cells filled the bottom of the culture bottles, and these were the first cell culture passage. The cells were then subpassaged at a 1:1 ratio, amplified, purified, and expanded to third-passage cells. Finally, the third-passage BMMSCs were induced to differentiate as steatoblasts and osteoblasts after the phenotype test[20].

Method for preparing BMMSCs modified with the CXCR3/HO-1 genes or the HO-1 gene

We prepared six 75-mm culture bottles for culturing 5×10^6 third-passage BMMSCs with 20 mL complete medium for 24 h. Then we discarded the medium. Two of the bottles received 5 mL of complete medium, two received 5 mL of *HO-1* modified cells in complete medium, and the last two received 5 mL of *CXCR3/HO-1* modified cells with complete medium, ensuring that all steps were done in dark conditions. The ratio of BMMSCs to genetically modified cells was 1:10. After incubation for 6 h, we discarded the medium and then added 20 mL of complete medium to culture for a further 48 h. Observation of expressed fluorescent protein was performed by fluorescence microscopy in the dark. Two types of BMMSCs were used to extract RNA and protein.

Establishment of the animal model

Experimental animals: The donors for small bowel transplantation were healthy male Brown Norway (BN) rats aged 6-8 wk and weighing 180-200 g. The recipients for small bowel transplantation were healthy male Lewis rats aged 6-8 wk and weighing 180-200 g. All animals were provided by the Experimental Animal Center of the Academy of Military Medical Sciences and standard rat food was provided *ad libitum*. The experimental animals were kept at 23 ℃ with 50% humidity and a 12 h light/dark cycle for 2 wk, with free access to water and food, and regular replacement of cage and clean bedding before the experiments. All experiments on animals followed the experimental animal ethical regulations, and were approved by the Ethics Committee of Tianjin First Central Hospital.

Donor surgery: After the BN rats were weighed and appraised, we performed abdominal anesthesia with 5% chloral hydrate (0.5 mL/100 g) and fully exposed the abdominal cavity by making an incision from the xiphoid to the pubic symphysis. We isolated a section of the superior mesenteric artery and portal vein for vascular anastomosis, and ligated the surrounding arteriovenous vein. Then, we separated a section of the small bowel from the Treitz Ligament to the cecum. We preserved the tissue in lactated Ringer's solution at 4 ℃ after low-pressure enema with 5 mL of normal saline (NS) and then performed vascular perfusion with heparinized lactated Ringer's solution (25 U/mL) at 4 ℃.

Recipient surgery: Previously detailed steps for Lewis recipient rats were similar to those for donors. We isolated the inferior vena cava and abdominal aorta 2 cm under the left renal artery for vascular anastomosis, then occluded the proximal and distal branches of the abdominal aorta and inferior vena cava. Recipient abdominal aorta and inferior vena cava stomas, and the superior mesenteric artery and portal vein of the transplanted small bowel were subjected to arterial-arterial and venous-venous anastomosis. We then opened the blood vessels and straightened the intestine, before generating fistula on both sides of the abdominal wall. Using warm NS, we repeatedly washed the abdominal cavity, before finally closing the abdomen. According to different groups included in the experimental design, we injected the penis dorsal vein with the corresponding liquid.

Postsurgical management: Lewis rats were placed under 60 W heated lamps to awaken the animals, before they were all marked and fed. They were then

isolated for 1-2 d. After the health of the rats had improved, they were placed in their respective groups. We observed the health of the rats and tended the stomas daily.

Experimental groups: The rats were divided into six groups. These was a "no small bowel transplantation" (NSBT) group and an IsoT group where transplantation occurred between the Lewis rats. According to different postoperative injections in the BN and Lewis rats, we divided another four groups into a normal saline group (NS), a BMMSCs (BM) group, an Adv-HO-1/BMMSCs (HB) group, and an Adv-(CXCR3+HO-1)/BMMSCs (HCB) group. Six time points (instant, 1 d, 3 d, 7 d, 10 d, and 14 d) (n = 6) were chosen for each group. Lewis rats in the IsoT and NS groups were injected with 1 mL of sterile NS *via* the dorsal penile vein, 7 d before surgery. Rats in the B, HB, and HCB groups were injected with a single-cell suspension that included 5 × 10^6 BMMSCs and 1 mL of sterile NS *via* the dorsal penile vein, 7 d before the operation. After transplantation, the IsoT group and NS group recipient rats were injected intravenously with 1 mL of sterile NS *via* the dorsal penile vein, while the BM group recipient rats were injected with a single-cell suspension including 5 × 10^6 BMMSCs and 1 mL of sterile NS. The HB group recipient rats were injected with a single-cell suspension including 5 × 10^6 Adv-HO-1 /BMMSCs and 1 mL of sterile NS, and the HCB group recipient rats were injected with a single-cell suspension that included 5 × 10^6 Adv-(CXCR3+HO-1)/BMMSCs and 1 mL of sterile NS. In addition, we prepared five more rats for each group and used them for survival analysis.

Small bowel transplantation and consequent effects: The survival of the rats in each group was observed, including the weight of the rats, changes in the mucosa, and secretions from the fistulas. The survival time and cause of death were recorded, and the survival rate was calculated.

Pathology of transplanted intestine

The abdominal cavity was opened under anesthesia to observe the appearance of the transplanted intestine, including color, lumen diameter, and presence or absence of inflammatory masses and surrounding tissue adhesions. We then resected 2 cm of the intestine 12 cm distant from the stoma. After the resected intestine was cleaned and excised, it was soaked in 40 g/L of formaldehyde solution for detection of aforementioned immunological markers. We used paraffin to embed the sections, then made tissue sections for hematoxylin-eosin staining (HE staining), before observing the sections by optical microscopy. A pathologist who was blinded to the procedures examined the tissue sections under a light microscope. Six high power fields (× 200) were randomly selected from each slice to observe intestinal mucosal injury. Finally, we established the index of intestinal injury by referring to the literature[21].

Fluorescence immunohistochemistry

Fluorescent double staining: The tissue was embedded in paraffin and 5 μm continuous sections were made. The sections were dewaxed and endogenous peroxidase was blocked by addition of 3% H_2O_2. After hydration, antigen repair and closure, we incubated the mixed primary antibodies (HO-1 at 1:250, CXCR3 at 1:400) with the sections and kept them in a 4 ℃ wet box overnight. On the next day, we rewarmed the sections and incubated them with the corresponding secondary antibody at 37 ℃ for 1 h. We then photographed the sections under a microscope. A negative control was run by using PBS instead of primary antibody.

Terminal deoxynucleotidyl transferase dUTP nick-end labeling: We strictly followed the kit instructions after baking at 70 ℃. We used glycerol for sealing and selected multiple fields under a fluorescence microscope for analysis.

Western blot detection of cellular proteins: We collected protein samples from supernatant after adding the cells to 500 μL of RIPA lysis buffer for 30 min and centrifuging the contents in a 1.5 mL centrifuge tube (13000 × g for 5 min). The expressed proteins were detected by Western blot. After calculating the amount of sample and measuring the protein concentration, we performed electrophoresis and transfer, added primary antibodies against HO-1 (1:800) CXCR3 (1:800), and GAPDH (1:5000), and incubated them in a 4 ℃ wet box overnight. The secondary antibody was incubated at room temperature, and the color was then developed. The relative expression of HO-1 and CXCR3 was calculated by quantitative analysis.

In addition, we also prepared small intestinal tissue that was soaked in RIPA lysis buffer reagent (100 mg per 1 mL lysis buffer), macerated and centrifuged in 1.5 mL tubes (13000 × g for 5 min). After calculating the amount of sample and measuring the protein concentration, we performed electrophoresis and transfer, treated the samples with primary antibodies against HO-1 (1:400), CXCR3 (1:800), and GAPDH (1:5000), and incubated them at 4 ℃ overnight. The secondary antibody was incubated at room temperature, and the color was then developed. The relative expression of HO-1 and CXCR3 was calculated by quantitative analysis.

Quantitative PCR for measurement for mRNA expression

Trizol (1 mL) was added to each of the culture bottles and mixed for 10 min. After we measured the concentration, degree, and purity of RNA, cDNA was

Table 1 Primer sequences for quantitative PCR

Target gene	Primer sequence
HO-1	Forward: 5'-CTGGCTCTTTTCTTGG -3'
	Reverse: 5'-ATGGTCAGAACATGGAC-3'
CXCR3	Forward: 5'-TCATGGCCTACTGCTATGC-3'
	Reverse: 5'-CGACTTGGCCACGTCTAC-3'
β-actin	Forward: 5'-GCGTGACATTAAAGAGAAGCTG-3'
	Reverse: 5'-AGAAGCATTTGCGGTGCAC-3'

synthesized by reverse transcription and PCR was performed. With β-actin as a reference, the expression of *HO-1* and *CXCR3* mRNA was calculated by relative quantitative analysis. We used the Ct value as the statistical parameter; the relative ratio of the target gene and the β-actin gene was used as the evaluation standard, then relative quantification of the target gene was performed. The primer sequences are shown in Table 1.

Identification of BMMSCs and T lymphocytes by flow cytometry

Flow cytometry of BMMSCs: Third-passage BMMSCs were digested and prepared as single cell suspensions in 100 μL of PBS away from light. The number of cells was 5 × 10^5. We then added 0.625 μL of CD29 (PE), 2.5 μL of CD34 (FITC), 0.625 μL of CD45 (PE), 0.25 μL of CD90 (FITC), 0.625 μL of RT1A (PE), and 0.25 μL of RT1B (FITC), respectively. The blank group did not include antibody. All samples were incubated at 4 ℃, washed and centrifuged (300 × *g* for 5 min), and resuspended in 200 μL of PBS. Flow cytometry was used to analyze the data.

Flow cytometry of T lymphocytes: We used 5% chloral hydrate while performing abdominal anesthesia. Rat spleens were harvested and macerated into a single cell suspension using a 200-μm nylon mesh. The cells were washed in RPMI 1640 medium and added to the separated lymphocytes at a 1:1 ratio. After centrifugation at 500 × *g* for 20 min, we collected buffy coat cells. Lymphocytes were precipitated after washing and centrifugation. The lymphocyte concentration was adjusted to 2 × 10^6/100 μL and then divided into a positive group and a control group. The control group did not have antibody. The positive group contained regulatory T cells (Tregs) to which we added 0.5 μL of CD4 antibody (FITC) and 0.625 μL of CD25 antibody (PE). After incubation at 4 ℃, the cells were washed and centrifuged at 300 × *g* for 5 min, and perforated at 4 ℃ overnight, before being ruptured and resuspended in 100 μL PBS. Then, we added 5 μL of FoxP3 antibody (PE-Cyanine 5) and incubated the cells at 4 ℃. The cells were then washed, centrifuged, and resuspended in 100 μL of PBS. $CD4^+CD25^+FoxP3^+$ T cells were then detected.

Detection of NK cell activity: The target cells (YAC-1) were subcultured 24 h prior to the experiment, and cell viability was confirmed when live cells accounted for > 95% of the total population. Whole blood was taken from the inferior vena cava of the rats, and the lymphocyte separation fluid was added to obtain a cell ratio of 1:1. The buffy coat was collected after centrifugation. Then, we washed, centrifuged, and precipitated the cells to obtain effector cells (NK cells). Effector cells were resuspended. The ratio of effector cells to target cells was 100:1. The lactate dehydrogenase method[22] was used to detect NK cell activity.

ELISA

Blood from normal and postoperative Lewis rats was collected *via* the inferior vena cava and stored at -80 ℃. The ELISA kit was used to detect the cytokines IL-2, IL-6, IL-10, IL-17, IL-23, IFN-γ, TNF-α, and TGF-β, and DAO. We strictly followed the kit instructions.

Statistical analysis

SPSS statistical software, version 17.0 was used for statistical analyses. The measurement data, expressed as mean ± SD, were analyzed using single factor analysis of variance. Count data, expressed as percentages (%), were analyzed using the χ^2 test. *P*-values < 0.05 were considered statistically significant.

RESULTS

Characteristics of HO-1 modified BMMSCs and CXCR3/HO-1 modified BMMSCs

Identification of BMMSCs: Primary BMMSCs (Figure 1A) were cultured *ex vivo* and third-passage BMMSCs (Figure 1B) were typically spindle-like in appearance. BMMSCs were induced to differentiate into adipocytes (Figure 1C, with lipid droplets in the cytoplasm) and osteoblasts (Figure 1D, with calcium deposits in the cytoplasm) using different media. Flow cytometry analysis showed that the positive rates of CD29, CD90, and RT1A in the third-passage Lewis rat BMMSCs exceeded 98%; the negative rates of CD34, CD45, and RT1B also exceeded 95% (Figure 1E-G).

Characteristics of *HO-1* modified BMMSCs: After infection with recombinant adenovirus expressing the *HO-1* gene for 48 h, the BMMSCs were observed under a fluorescence microscope. The positive expression rate of green fluorescent protein (GFP) exceeded 85% (Figure 2A and B). The expression of HO-1 in *HO-1* modified BMMSCs detected by Western blot was significantly higher than that of native BMMSCs ($P < 0.05$; Figure 2C and D). RT-PCR results showed that the expression of *HO-1* mRNA in *HO-1* modified BMMSCs was up-regulated, by a factor of 7.89 times compared with native BMMSCs ($F = 39.49$, $P < 0.05$; Figure 2E). These results showed that the *HO-1* gene

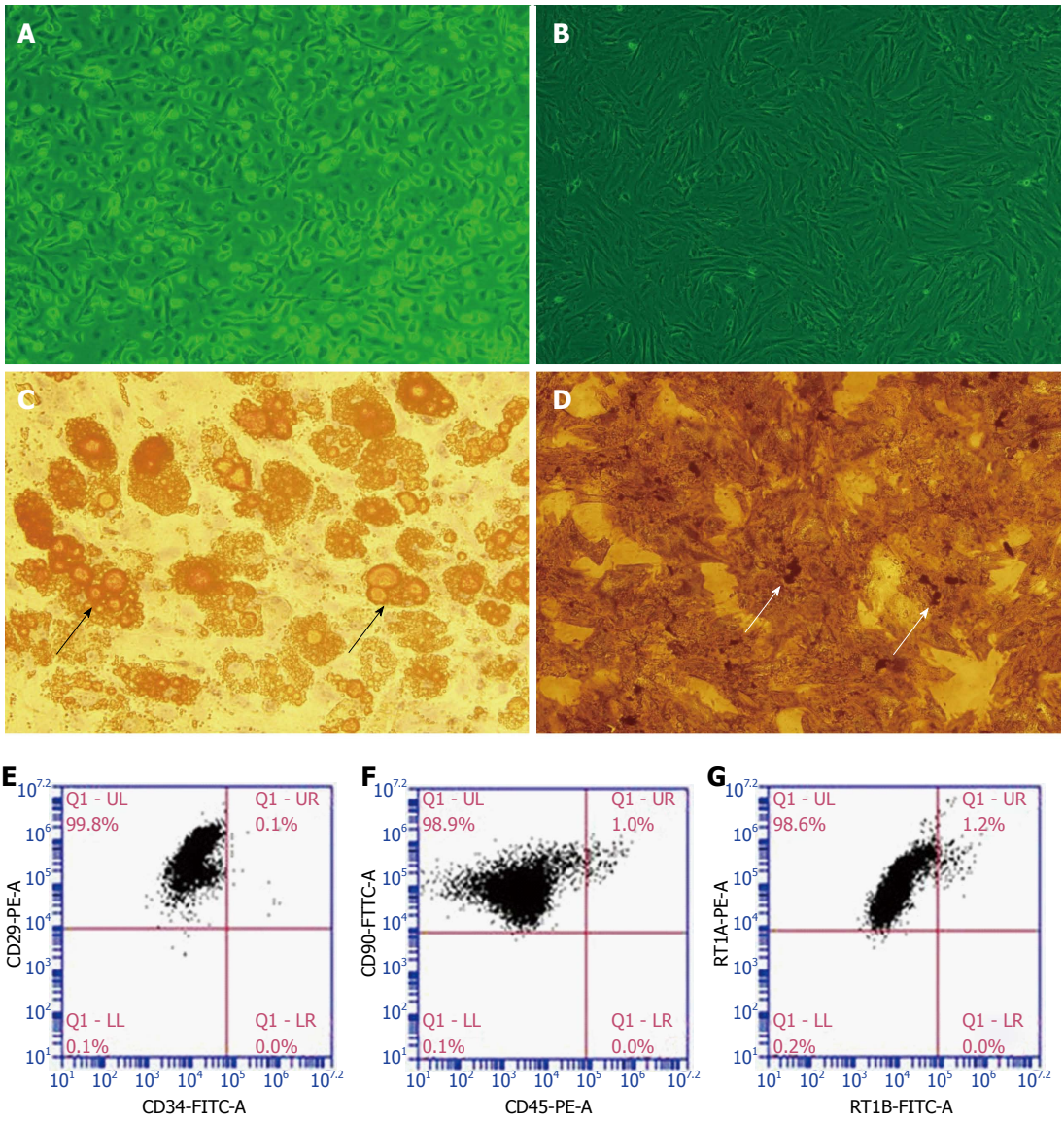


Figure 1 Identification of bone marrow-derived cells as bone marrow mesenchymal stem cells. Morphology of first-passage (A) and third-passage (B) BMMSCs (× 100). The differentiation capability of BMMSCs into adipogenic or osteogenic cells was evaluated by oil red O (× 200) (C) or Von Kossa staining (D) (× 200). The arrows indicate the red lipid droplets (C) or the black calcium deposition (D); The proportion of CD29-positive and CD34-negative cells was 99.8% (E); The proportion of CD90-positive and CD45-negative cells was approximately 98.9% (F); The proportion of RT1A-positive and RT1B-negative cells was 98.6% (G). BMMSCs: Bone marrow mesenchymal stem cells.

was successfully overexpressed in BMMSCs.

Increased expression of CXCR3 and HO-1 in BMMSCs after transduction with *CXCR3/HO-1* genes: After infection with the GFP-labeled *CXCR3/HO-1* gene for 48 h, the BMMSCs were observed under a fluorescence microscope. The positive expression rate of GFP exceeded 85% (Figure 3A and B). The expression of CXCR3 protein in *CXCR3/HO-1* modified BMMSCs detected by Western blot was significantly higher than that of native BMMSCs (Figure 3C and D). RT-PCR results showed that the expression of *CXCR3* mRNA in *CXCR3/HO-1* modified BMMSCs was up-regulated, by a factor of 6.69 times higher than that of native BMMSCs (F = 33.28, P < 0.05; Figure 3E). The expression of HO-1 protein in *CXCR3/HO-1* modified BMMSCs detected by Western blot was significantly higher than that of native BMMSCs (Figure 3F and G). RT-PCR results showed that the expression of *HO-1* mRNA in *CXCR3/HO-1* modified BMMSCs was up-regulated, by a factor of 7.71 times compared with native BMMSCs (F = 35.81, P < 0.05; Figure 3H). These results showed that the combination of *CXCR3/HO-1* genes was successfully transduced into BMMSCs.

Establishment of a rejection model for heterotopic small bowel transplantation

Pathological rejection of transplanted small bowel: Pathological results showed that there was no rejection in the NSBT group, while the IsoT group showed obvious rejection, and the injuries caused by ischemia-reperfusion fully recovered 7 d after

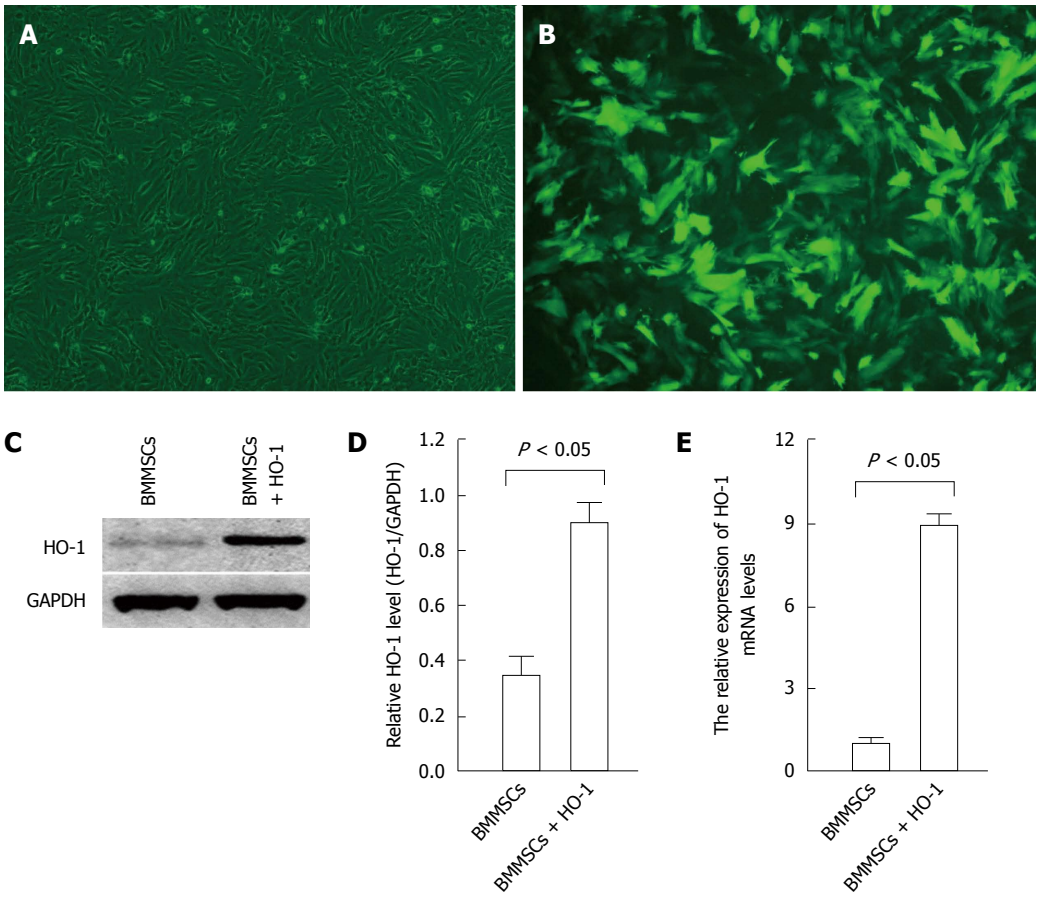


Figure 2 Expression of fluorescence, protein, and mRNA of HO-1 in HO-1 modified BMMSCs. A: Bright field microscopy of HO-1 modified BMMSCs; B: Fluorescent microscopy of HO-1 modified BMMSCs (The positive expression rate of GFP exceeded 85 %); C: Expression of HO-1 protein in HO-1 modified BMMSCs and native BMMSCs; D: The expression of HO-1 protein in HO-1 modified BMMSCs (0.903 ± 0.072) was significantly higher than that in native BMMSCs (0.348 ± 0.065; $P < 0.05$); E: The relative expression of HO-1 mRNA in HO-1 modified BMMSCs (8.89 ± 0.40) was 7.89 times higher than that in native BMMSCs (1.00 ± 0.24; $P < 0.05$). BMMSCs: Bone marrow mesenchymal stem cells; HO-1: Heme oxygenase-1.

transplantation. The rejection was similar between the NS group, BM group, HB group, and HCB group at instant and 1 d after transplantation; at the other time points, the rejection in the NS group was the most profound, and the injury was significantly higher than that of the BM group, HB group, and HCB group at the same time point (Figure 4). At the same time, HCB group had the least rejection, and the damage was significantly lower than that in the NS group, BM group, and HB group (Figure 4). At day 3, the BM group and HB group had only lymphocyte infiltration; at day 7, the BM group and HB group experienced mild rejection; at day 14, the BM group showed moderate rejection, while the HB group did not achieve moderate rejection (Figure 4).

General condition of the rats after small bowel transplantation: All of the rats recovered normally after transplantation. Rats in the NSBT group were normal in health. The mucosal color and secretion from the intestinal stoma were normal in the rats of the IsoT group. Rats in the NS group showed mild rejection 3 d after transplantation, with mucosal congestion and edema, and increased volume of thin secretions. The rejection was moderate 7 d after transplantation, with mucosal congestion turning dark, and the secretion becoming thick and in large volumes. The rejection was severe 14 d after transplantation, with severe mucosal necrosis and decreased volume of secretion. Rats in the BM group and the HB group showed mild rejection at day 7 and moderate rejection at day 14 after transplantation, and the rats in the HB group experienced less severe rejection compared with those in the BM group. Rats in the HCB group exhibited mild rejection at day 10 after transplantation, and showed no significant aggravation at day 14 after transplantation.

The average body weight (BW) of rats in the NSBT group increased by 3.82 g/d. The BW of rats in the IsoT group decreased slightly after transplantation, recovered to the preoperative BW at day 7 after transplantation, and later increased by 3.32 g/d. The average BW of rats at day 3 in the HCB group was higher than those in the NS group, BM group, and HB group ($P < 0.05$). There was no significant difference between the BM group and HB group ($P > 0.05$) but both groups had higher BW than the NS group ($P < 0.05$). Any two groups of the NS group, BM group, HB

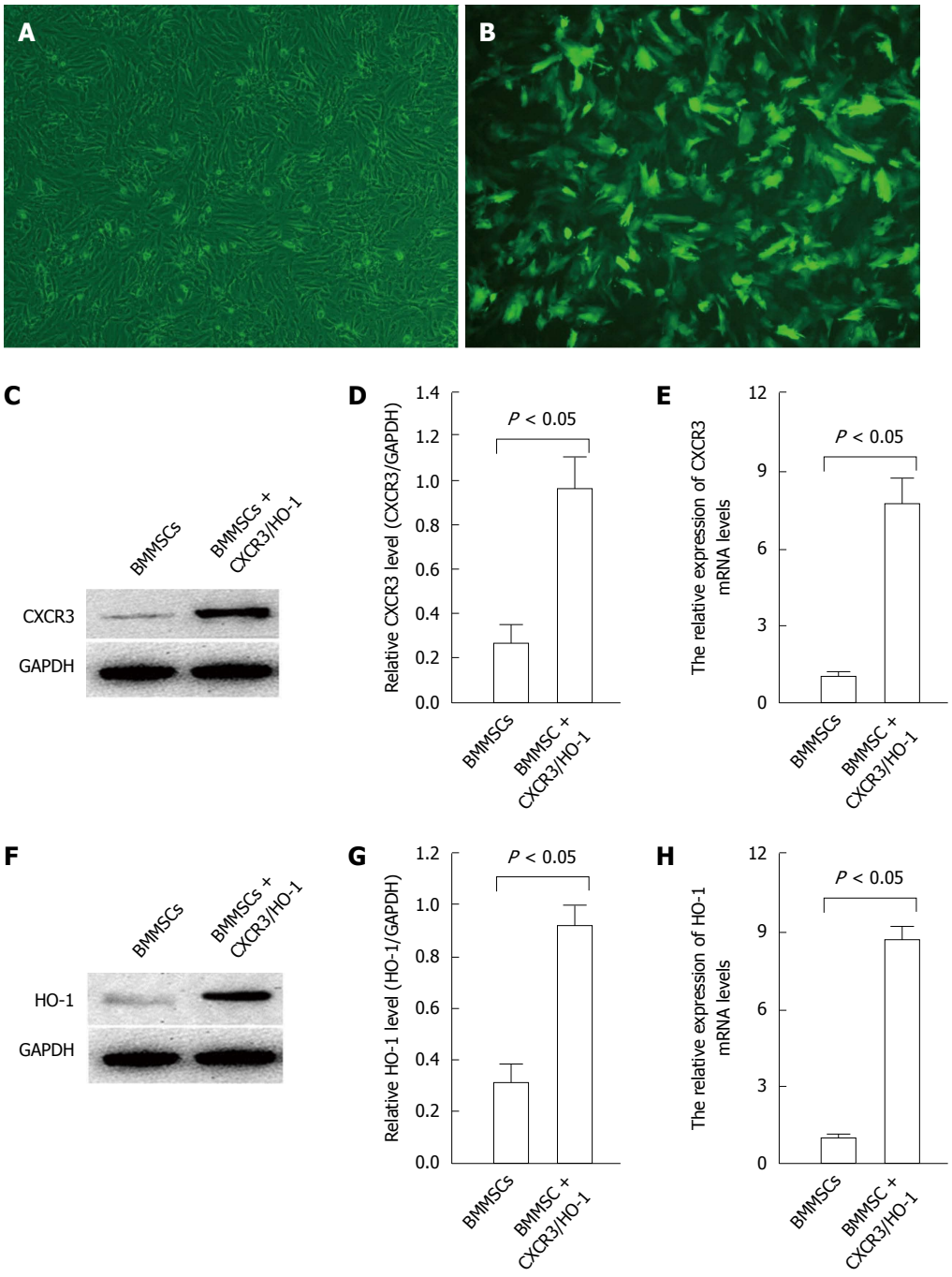


Figure 3 Expression of fluorescence, protein and mRNA of CXCR3 and HO-1 in CXCR3/HO-1 modified BMMSCs. A: Bright field microscopy of CXCR3/HO-1 modified BMMSCs; B: Fluorescent microscopy of CXCR3/HO-1 modified BMMSCs (the positive expression rate of GFP exceeded 85%); C: Expression of CXCR3 protein in CXCR3/HO-1 modified BMMSCs and native BMMSCs; D: The expression of CXCR3 protein in CXCR3/HO-1 modified BMMSCs (0.96 ± 0.14) was significantly higher than that in native BMMSCs (0.25 ± 0.09, $P < 0.05$); E: The relative expression of CXCR3 mRNA in CXCR3/HO-1 modified BMMSCs (7.69 ± 1.06) was 6.69 times higher than that in native BMMSCs (1.00 ± 0.20, $P < 0.05$); F: Expression of HO-1 protein in CXCR3/HO-1 modified BMMSCs and native BMMSCs; G: The expression of HO-1 protein in CXCR3/HO-1 modified BMMSCs (0.91 ± 0.081) was significantly higher than that in native BMMSCs (0.305 ± 0.071, $P < 0.05$); H: The relative expression of HO-1 mRNA in CXCR3/HO-1 modified BMMSCs (8.71 ± 0.51) was 7.71 times higher than that in native BMMSCs (1.00 ± 0.19, $P < 0.05$). BMMSCs: Bone marrow mesenchymal stem cells; HO-1: Heme oxygenase-1.

group, and HCB group showed a significant difference in the average BW of rats at 7 d, 10 d, and 14 d after transplantation, respectively ($P < 0.05$), among which BW in the HCB group was the highest, and BW in the NS group was the lowest (Figure 5). These results suggested that *CXCR3/HO-1* modified BMMSCs significantly improved the survival quality of rats after transplantation than *HO-1* modified BMMSCs and native BMMSCs.

Improved post-transplant survival after treatment by *CXCR3/HO-1* modified BMMSCs: The normal recipient (Lewis) rats in the NSBT group and IsoT group survived for more than 200 d after small

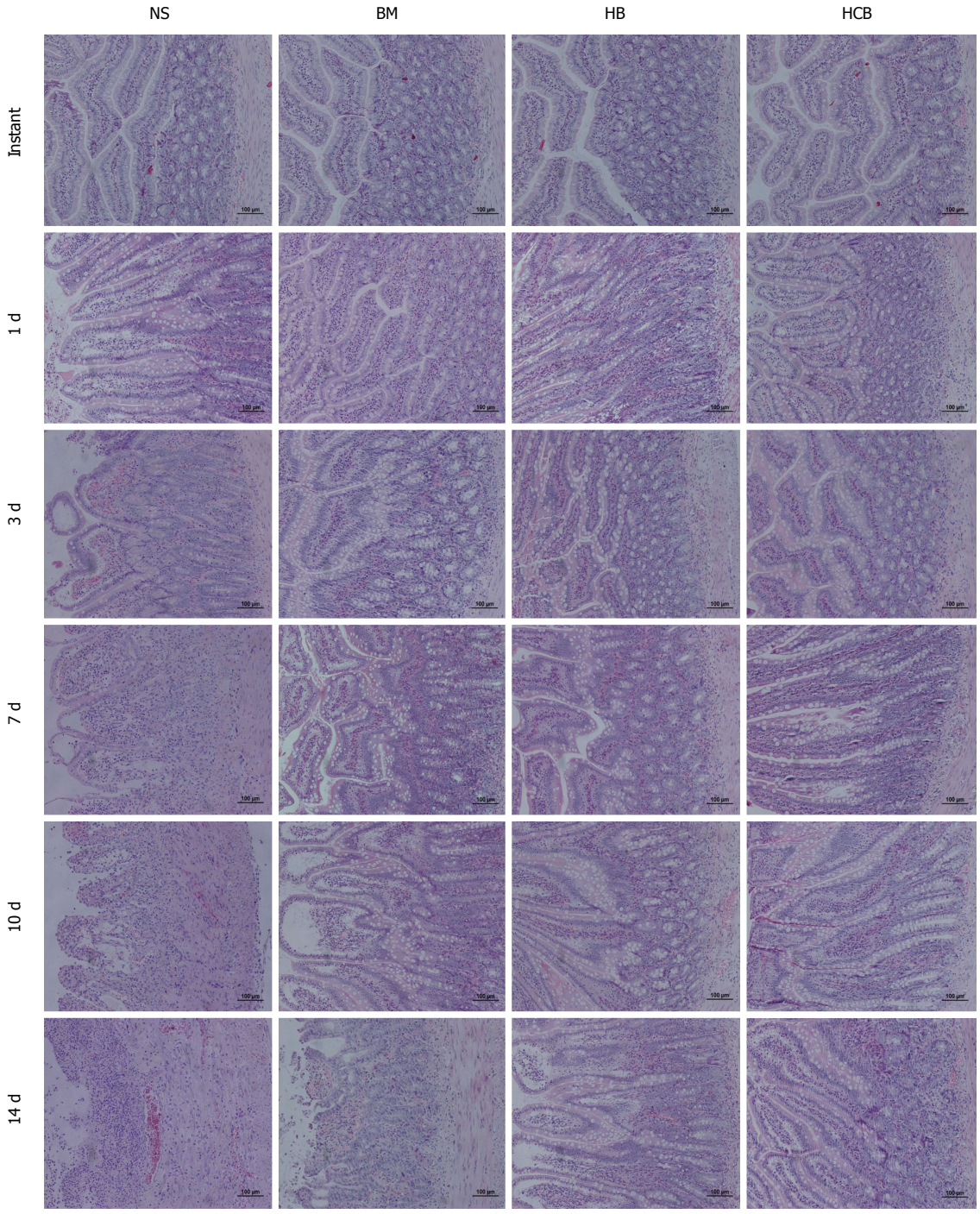


Figure 4 Pathology of transplanted small bowel (HE staining, × 100). All four groups showed normal histological results immediately after transplant. The NS group showed mild rejection at day 1 after transplantation: shorter and bifurcated intestinal villi, mild submucosal edema, cryptic epithelial cells with mild damage (cytoplasmic basophilic increase, nuclear enlargement, and color deepening) and increased apoptosis, more than six apoptotic bodies per 10 crypt cells, and mild mononuclear cells which are the main inflammatory cells found during inflammatory infiltration of the lamina propria; in contrast, the BM group, HB group, and HCB group showed only mild edema. The pathological changes at 3 d after transplantation in the NS group were more severe than those at 1 day, where the submucosal edema was aggravated, and there was increased inflammatory infiltration of the lamina propria; the BM group and HB group showed mild inflammatory infiltration of the lamina propria, and the HCB group was similar to the normal intestine. The NS group showed moderate rejection at day 7 after transplantation: a reduced ratio of villous height to crypt, partial necrosis of glandular epithelial cells, aggravated edema and inflammation, diffuse crypt damage and increased apoptosis, and mild arteritis and congestion of lamina propria and submucosa; the BM group and HB group showed mild rejection: the BM group had shorter intestinal villi, mild submucosal edema, inflammatory cell infiltration, mild crypt epithelial injury, and increased apoptosis; the HB group had mild inflammatory cell infiltration, mild crypt injury, with other signs not being obvious; the HCB group were indeterminate for rejection: mild cryptic epithelial damage and the number of apoptotic bodies increased, with mild, local inflammatory cell infiltration of the lamina propria. The NS group showed severe rejection at day 10 after transplantation: intestinal villus changes were further aggravated, with serious shedding of the intestinal mucosal epithelial cells, while crypt epithelial injury was very serious; inflammatory cell infiltration involved the muscle layer, resulting in severe arteritis; the pathological changes in the BM group, HB group, and HCB group at day 10 were more severe than those at day 7 after transplantation. The structures of the intestinal mucosa layer were completely destroyed and the intestinal wall became thinner with necrosis in the NS group 14 d after transplantation; the BM group showed moderate rejection; the HB group did not achieve moderate rejection but rejection was more severe than mild; the HCB group showed mild rejection.

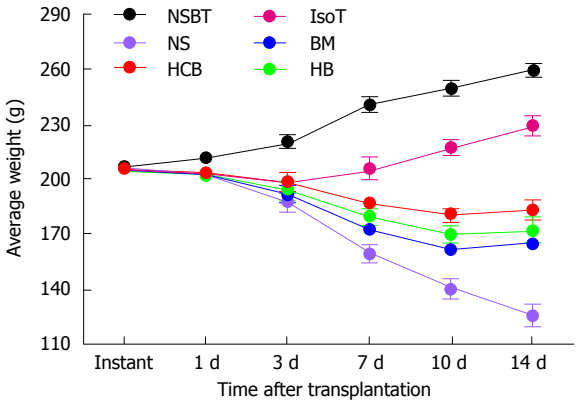


Figure 5 Average body weight of rats after small bowel transplantation. The average body weight (BW) of rats in the NSBT group increased by 3.82 g/d. BW of rats in the IsoT group decreased slightly after transplantation, recovered to preoperative BW at day 7 (206.08 ± 3.87 *vs* 205.45 ± 2.10), and later increased by 3.32 g/d. BW of rats at day 1 showed no significant difference between the NS group, BM group, HB group, and HCB group (203.78 ± 3.01 g *vs* 202.87 ± 2.33 g *vs* 202.16 ± 2.14 g *vs* 203.76 ± 2.65 g, $P > 0.05$). Any two groups of the NS group, BM group, HB group, and HCB group showed significant differences in the average BW of rats at 3 d, 7 d, 10 d, and 14 d after transplantation, respectively (3 d: 188.34 ± 2.13 g *vs* 192.00 ± 3.39 g *vs* 194.21 ± 2.99 g *vs* 198.65 ± 2.02 g; 7 d: 160.02 ± 4.87 g *vs* 172.67 ± 2.17 g *vs* 179.35 ± 2.37 g *vs* 187.21 ± 3.28 g; 10 d: 140.25 ± 4.45 g *vs* 161.83 ± 3.01 g *vs* 170.05 ± 2.72 g *vs* 180.32 ± 3.85 g; 14 d: 125.74 ± 6.63 g *vs* 165.32 ± 2.89 g *vs* 172.23 ± 6.92 g *vs* 183.65 ± 5.44 g; $P < 0.05$). BW in the HCB group was the highest, followed by the HB group and BM group, and BW in the NS group was the lowest. The average BW of rats at day 3 showed no significant difference between the BM group and the HB group ($P > 0.05$).

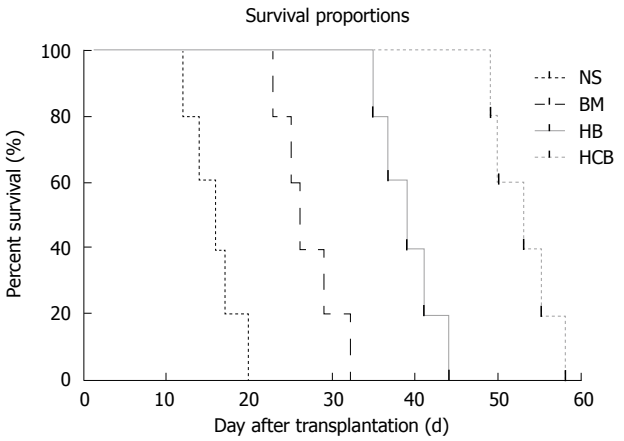


Figure 6 Survival rates of rats after small bowel transplantation. The survival time of rats in the HCB group was longer than those in the NS group, BM group and HB group (median survival time: 53 d *vs* 16 d *vs* 26 d *vs* 39 d; $P < 0.05$). The survival time of rats in the HB group was longer than that in the BM group ($P < 0.05$). The survival time of rats in the NS group was shorter than those in the BM group, HB group, and HCB group ($P < 0.05$). The χ^2 and P values for comparison of survival rates in different groups are the same ($\chi^2 = 9.701$ and $P < 0.01$).

bowel transplantation. The survival time of rats in the HCB group was the longest, followed by the HB group and BM group, while it was shortest in the NS group ($P < 0.05$; Figure 6). These results suggested that *CXCR3/HO-1* modified BMMSCs could prolong the survival time of rats after transplantation more significantly than *HO-1* modified BMMSCs and BMMSCs.

Increased expression of HO-1 in the transplanted small bowel after treatment with *CXCR3/HO-1* modified BMMSCs: The expression of HO-1 protein in the transplanted small bowel was low at instant, and there was no significant difference between the four aforementioned groups ($P > 0.05$). The relative concentration of HO-1 protein in the HCB group was higher than those in the NS group, BM group, and HB group ($P < 0.05$) at every time point except immediately after transplantation. The relative concentration of HO-1 protein in the HCB group reached its maximum at day 7 after transplantation, and remained relatively stable thereafter (Figure 7). The relative concentration of HO-1 protein in the HB group was higher than that in the NS group and BM group ($P < 0.05$; Figure 7) at every time point except immediately after transplantation (instant). The relative concentration of HO-1 protein in the BM group was comparable to that in the NS group at 1 d, 3 d, and 7 d ($P > 0.05$), and was higher than that in the NS group at 10 d and 14 d ($P < 0.05$; Figure 7). These results suggested that transfused *CXCR3/HO-1* modified BMMSCs could reach the transplanted small bowel more rapidly than transfused *HO-1* modified BMMSCs and transfused BMMSCs, leading to early expression of large quantities of HO-1 protein.

Increased expression of CXCR3 in the transplanted small bowel after treatment with *CXCR3/HO-1* modified BMMSCs: The expression of CXCR3 protein in the transplanted small bowel was low at instant, and there was no significant difference between the four aforementioned groups ($P > 0.05$). The relative concentration of CXCR3 protein in the HCB group was higher than those in the NS group, BM group, and HB group ($P < 0.05$) at every time point except immediately after transplantation (at instant). The relative concentration of CXCR3 protein in the HB group was significantly lower than those in the NS group and BM group ($P < 0.05$) at every time point except immediately after transplantation (Figure 8). This was especially demonstrable at 3 d, 7 d, 10 d, and 14 d after transplantation. The relative concentration of CXCR3 protein in the BM group was significantly lower than that in the NS group at 1 d, 3 d, 7 d, and 10 d ($P < 0.05$; Figure 8), and was higher than that in the NS group at day 14 ($P < 0.05$). These results suggested that transfused *CXCR3/HO-1* modified BMMSCs could reach the transplanted small bowel more rapidly than transfused *HO-1* modified BMMSCs and transfused BMMSCs, leading to early expression of large quantities of CXCR3 protein.

Increased co-expression of CXCR3 and HO-1 in the transplanted small bowel after treatment with *CXCR3/HO-1* modified BMMSCs: CXCR3 and HO-1 proteins were found to be co-expressed in the same cells and localized in the transplanted small

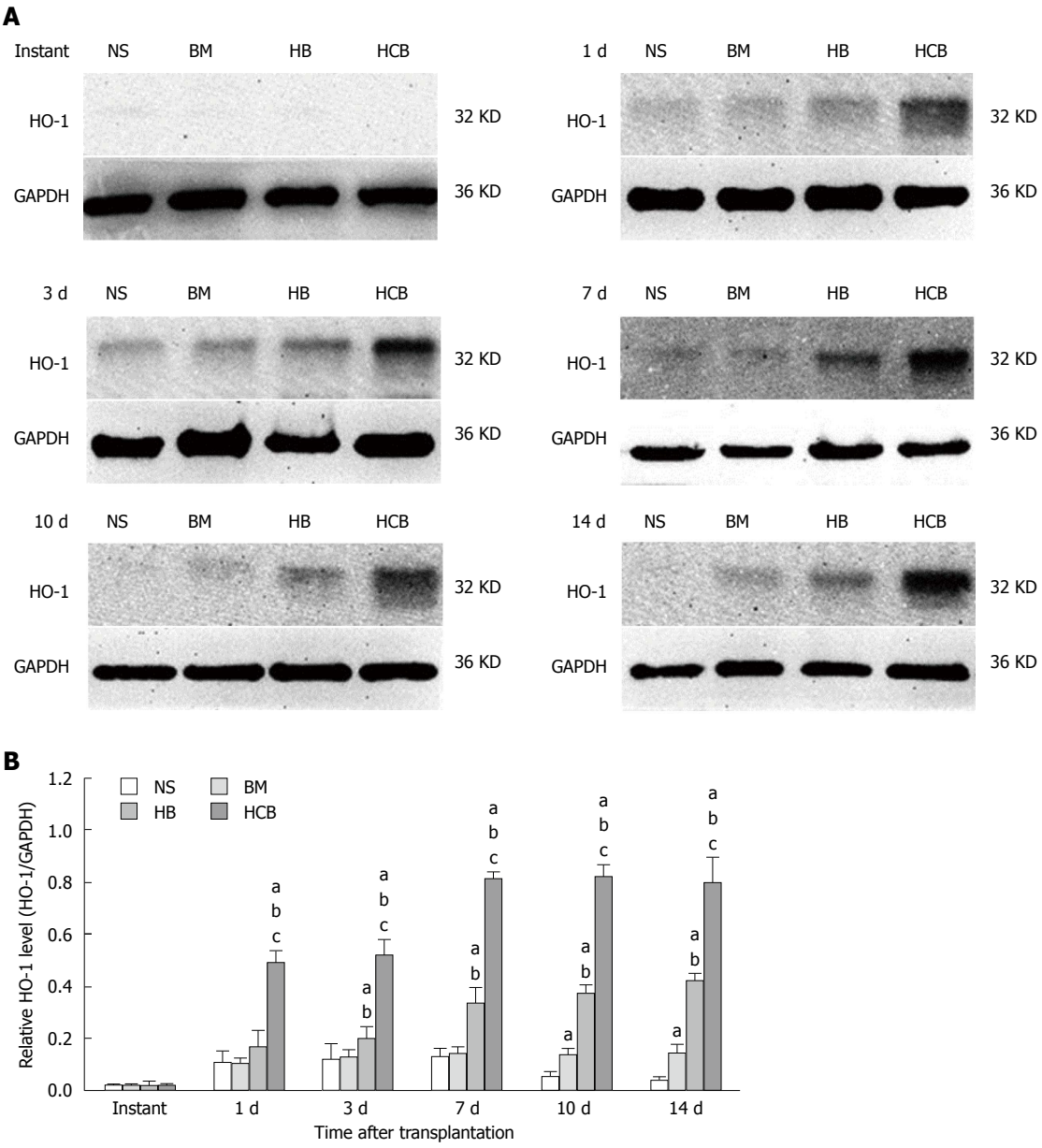


Figure 7 The expression of HO-1 protein in small intestine in different groups. $^{a}P < 0.05$ *vs* NS group, $^{b}P < 0.05$ *vs* BM group, $^{c}P < 0.05$ *vs* HB group. A: Expression of HO-1 protein in the NS group, BM group, HB group, and HCB group; B: Relative concentration of HO-1 protein in different groups. The expression of HO-1 protein in the transplanted small bowel was low at 0 h, and there was no significant difference between these four groups ($P > 0.05$). The relative concentration of HO-1 protein in the HCB group was the highest, followed by the HB group and BM group, and was lowest in the NS group at other time points ($P < 0.05$). HO-1: Heme oxygenase-1; NS: Normal saline.

bowel (shown by the arrow in Figure 9) 1 d after double fluorescent histochemical staining.

Effects of CXCR3/HO-1 modified BMMSCs on the intestinal function and apoptosis in the transplanted small bowel

Effects of *CXCR3/HO-1* modified BMMSCs on the function of transplanted small bowel: Serum concentrations of DAO in the IsoT group, NS group, BM group, HB group, and HCB group were comparable immediately after transplantation, and were all higher than that in the NSBT group ($P < 0.05$; Figure 10). Serum concentrations of DAO in the IsoT group nearly dropped to the level of the NSBT group ($P > 0.05$) at day 7 after transplantation. Serum concentration of DAO in the NS group was the highest, followed by those in the BM group and HB group, and the HCB group had the lowest level of DAO at 3 d, 7 d, 10 d, and 14 d after transplantation ($P < 0.05$). These results suggested that *CXCR3/HO-1* modified BMMSCs could improve intestinal permeability much earlier and more significantly than *HO-1* modified BMMSCs and BMMSCs.

Decreased apoptosis in the transplanted small bowel after treatment with *CXCR3/HO-1* modified BMMSCs: There was no significant difference in apoptosis between the six groups immediately after

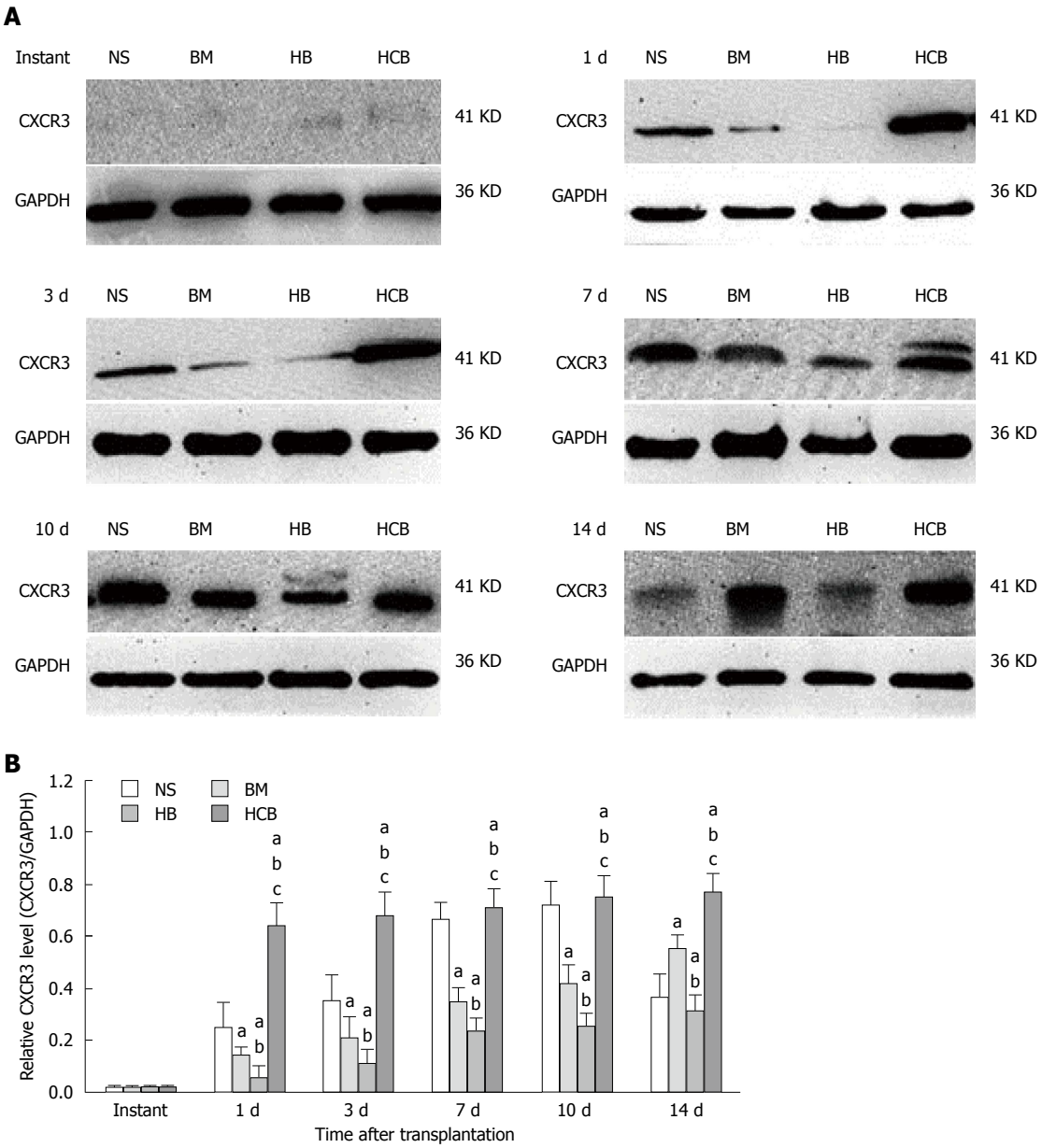


Figure 8 Expression of CXCR3 protein in small intestine in different groups. [a]*P* < 0.05 *vs* NS group, [b]*P* < 0.05 *vs* BM group, [c]*P* < 0.05 *vs* HB group. A: Expression of CXCR3 protein in the NS group, BM group, HB group, and HCB group; B: Relative concentration of CXCR3 protein in different groups. The expression of CXCR3 protein in the transplanted small bowel was low immediately after transplantation, and there was no significant difference between these 4 groups (*P* > 0.05). The relative concentration of CXCR3 protein in the HCB group was the highest, followed by the NS group and BM group, while it was lowest in the HB group at 1 d, 3 d, 7 d, and 10 d. The relative concentration of CXCR3 protein in the BM group was higher than that in the NS group at day 14, which may be due to a false increase caused by mass necrosis of transplanted small bowel in the NS group.

transplantation (*P* > 0.05; Figure 11). Apoptosis in the IsoT group increased at day 3 but nearly returned to the level of NSBT group by day 7. Apoptosis was the highest in the NS group, followed by the BM group and HB group, and was the lowest in the HCB group at 1 d, 3 d, 7 d, and 10 d (*P* < 0.05). At day 14 after transplantation, apoptosis was comparable between the NS group and BM group (*P* > 0.05), was higher in the BM group when compared with the HB group (*P* < 0.05), and was higher in the HB group when compared with the HCB group (*P* < 0.05; Figure 11). These results suggested that *CXCR3/HO-1* modified BMMSCs could reduce the level of apoptosis in the transplanted small bowel more significantly than *HO-1* modified BMMSCs and BMMSCs.

Effects of CXCR3/HO-1 modified BMMSCs on the immunologic function of rats

Inhibitory effects of *CXCR3/HO-1* modified BMMSCs on NK cells: The activity of NK cells in the BM group, HB group, and HCB group was significantly lower than that in the NSBT group, IsoT group, and NS group immediately after transplantation (*P* < 0.05; Figure 12). The NSBT group and IsoT group showed no significant change at any of the time points. The activity of NK cells in the NS group

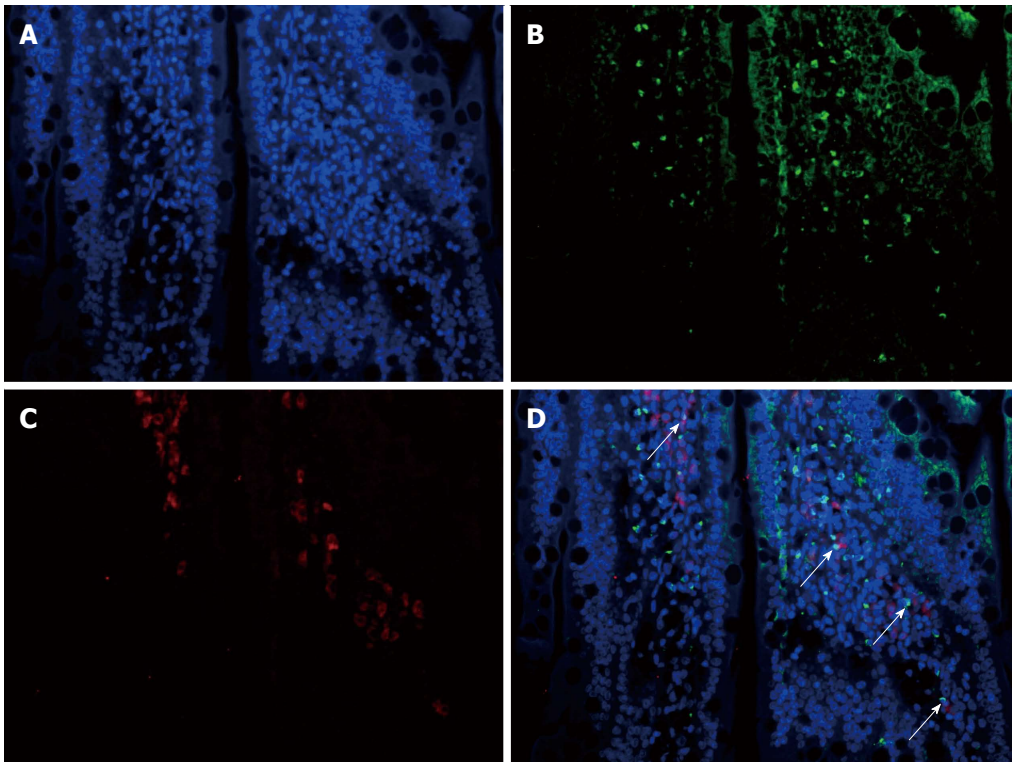


Figure 9 Expression of CXCR3/HO-1 in the transplanted small bowel in the HCB group at day 1. A: Nuclear staining (DAPI); B: Staining of CXCR3 protein; C: Staining of HO-1 protein; D: Composite image of three types of staining showed co-expression of CXCR3 and HO-1 proteins in the transplanted small bowel (shown by arrows).

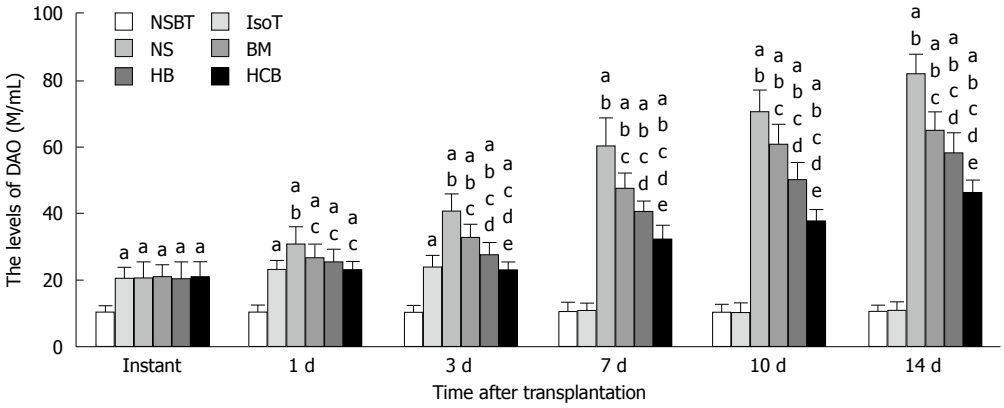


Figure 10 Concentrations of diamine oxidase in different groups. [a]$P < 0.05$ *vs* NSBT group [b]$P < 0.05$ *vs* IsoT group, [c]$P < 0.05$ *vs* NS group, [d]$P < 0.05$ *vs* BM group, [e]$P < 0.05$ *vs* HB group. The concentration of DAO in the NSBT group was significantly lower than those in the other five groups ($P < 0.05$). The concentration of DAO in the IsoT group was comparable to that in the HCB group at day 1 (23.43 ± 2.77 M/mL *vs* 23.26 ± 2.50 M/mL; $P < 0.05$) and day 3 (24.13 ± 3.51 M/mL *vs* 23.26 ± 2.50 M/mL; $P < 0.05$). Serum concentration of DAO in the IsoT group at day 7 (11.14 ± 1.25 M/mL) nearly returned to the level of the NSBT group (10.80 ± 2.78 M/mL). At day 1 after transplantation, serum concentration of DAO in the NS group was significantly higher than those in the BM group, HB group, and HCB group ($P < 0.05$), comparable in the BM group and HB group ($P > 0.05$), and was lower in the HCB group when compared with the HB group ($P < 0.05$). Serum concentration of DAO in the HCB group was significantly lower than those in the NS group, BM group, and HB group at 3 d, 7 d, 10 d, and 14 d ($P < 0.05$). DAO: Diamine oxidase.

increased significantly after transplantation, which was significantly higher than that in the NSBT group, IsoT group, BM group, HB group, and HCB group ($P < 0.05$). The activity of NK cells in the BM group, HB group, and HCB group initially increased but then decreased after transplantation. The activity of NK cells in the BM group was the highest, followed by the HB group, and was the lowest in the HCB group at every time point ($P < 0.05$; Figure 12). These results suggested that *CXCR3/HO-1* modified BMMSCs could significantly reduce the activity of NK cells in the rats after small bowel transplantation, and were able to exert their effects much earlier than *HO-1* modified BMMSCs and BMMSCs.

Increased ratio of Tregs after treatment with *CXCR3/HO-1* modified BMMSCs: The ratio of Tregs in the BM group, HB group, and HCB group

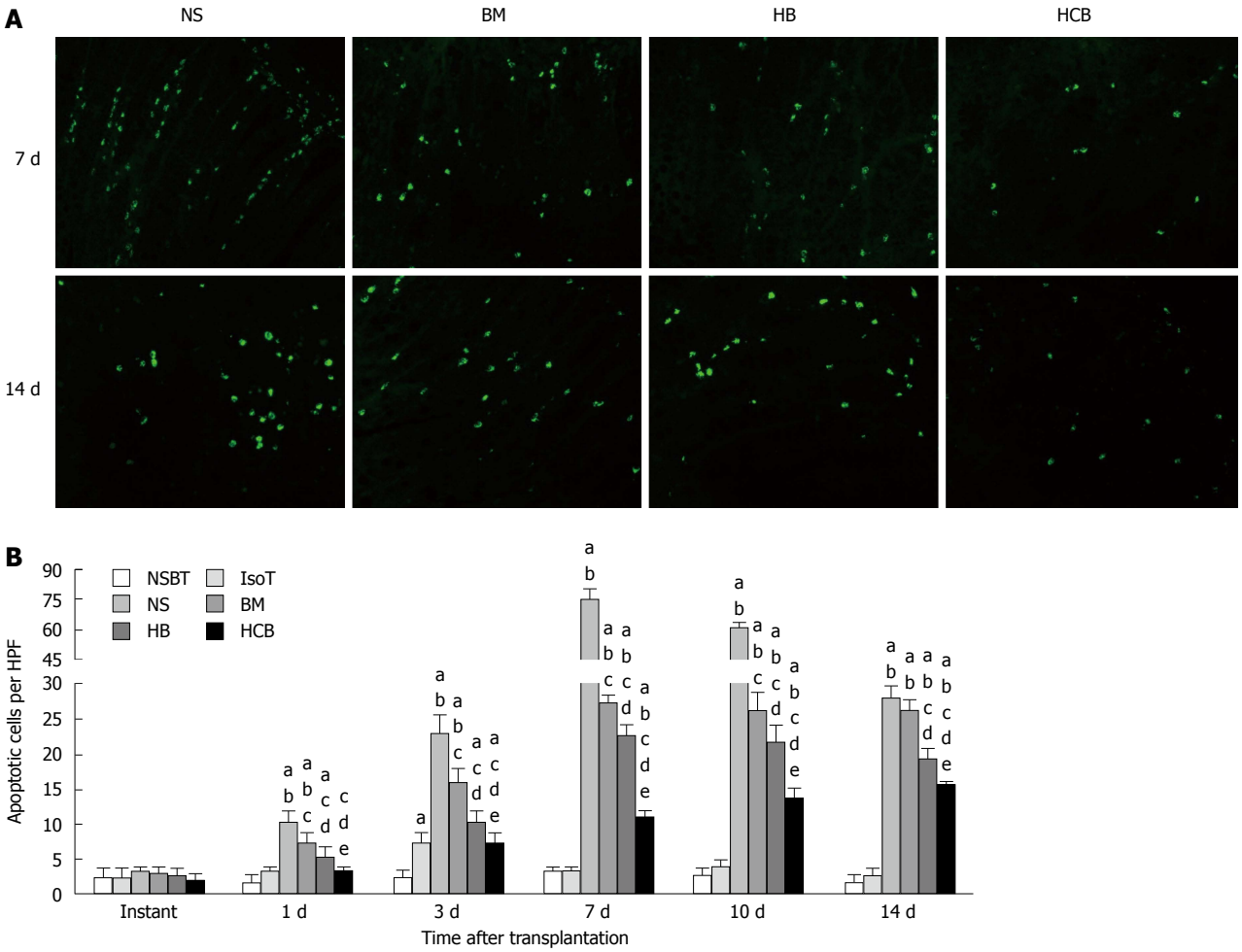


Figure 11 Apoptosis in the transplanted small bowel in different groups. A: Fluorescent histochemistry images of apoptosis in the NS group, BM group, HB group, and HCB group at 7 d and 14 d (× 200); B: Comparison of apoptotic cell numbers in different groups. [a]$P < 0.05$ *vs* NSBT group, [b]$P < 0.05$ *vs* IsoT group, [c]$P < 0.05$ *vs* NS group, [d]$P < 0.05$ *vs* BM group, [e]$P < 0.05$ *vs* HB group. There was no significant difference in apoptosis between these six groups at 0 h ($P > 0.05$). The apoptotic cell number in the IsoT group increased at day 3 (7.20 ± 1.53/HPF), and nearly returned to the level of the NSBT group at day 7 (3.30 ± 0.57/HPF *vs* 3.20 ± 0.57/HPF). The apoptotic cell number in the NS group was the highest, and was significantly higher than those in the other five groups at all time points except immediately after transplantation ($P < 0.05$). The apoptotic cell number in the NS group increased significantly, reaching its maximum at day 7 (75.00 ± 5.20/HP), before decreasing (61.00 ± 2.65/HPF at day 10, 28.00 ± 1.73/HPF at day 14). The apoptotic cell number in the BM group was higher when compared with the HB group, and was higher in the HB group when compared with the HCB group ($P < 0.05$) simultaneously.

was comparable but were all significantly higher than that in the NBST group, IsoT group, and NS group at instant ($P < 0.05$). The ratio of Tregs in the NBST group, IsoT group, and NS group showed no significant change over time ($P > 0.05$; Figure 13), while the ratio of Tregs in the BM group, HB group, and HCB group increased significantly over time ($P > 0.05$; Figure 13). The ratio of Tregs in the HCB group was higher than that in the HB group and BM group, and the ratio in the HB group was higher than the BM group at the same time points. These results suggested that *CXCR3/HO-1* modified BMMSCs could increase the ratio of Tregs in the rats after small bowel transplantation, much more significantly than *HO-1* modified BMMSCs and BMMSCs.

Effects of *CXCR3/HO-1* modified BMMSCs on serum cytokines: Serum concentrations of Th1/Th2-related and Th17/Treg-related cytokines were detected. Cytokines in the BM group, HB group, and HCB group showed significant differences when compared with those in the NBST group, IsoT group, and NS group immediately after transplantation, with a reduction in proinflammatory cytokines and an increase in anti-inflammatory cytokines ($P < 0.05$; Figure 14). The concentrations of proinflammatory cytokines in the IsoT group were significantly higher than those in the NSBT group at 1 d and 3 d ($P < 0.05$), and were comparable to those in the NSBT group at day 7 ($P > 0.05$). The concentrations of proinflammatory cytokines in the NS group were significantly higher than those in the BM group, HB group, and HCB group at 1 d, 3 d, 7 d, 10 d, and 14 d ($P < 0.05$). Proinflammatory cytokine levels in the BM group were higher than those in the HB group, and those in the HCB group were the lowest at 1 d, 3 d, 7 d, 10 d, and 14 d ($P < 0.05$). The concentrations of anti-inflammatory cytokines in the HCB group were

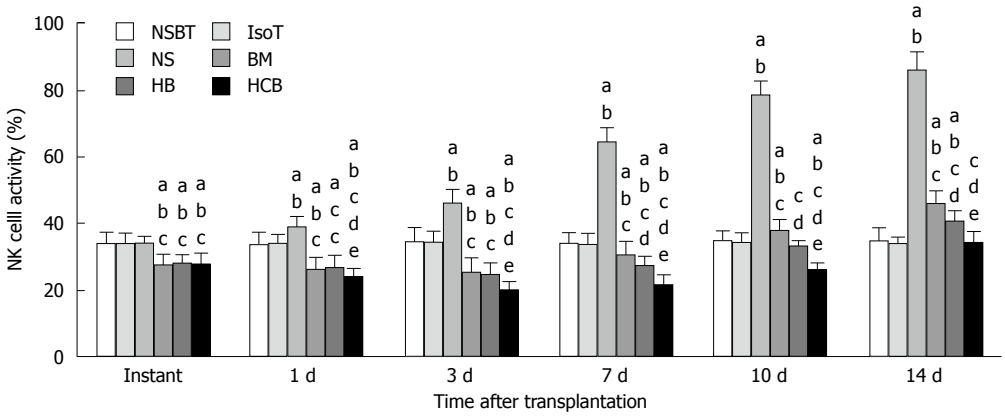


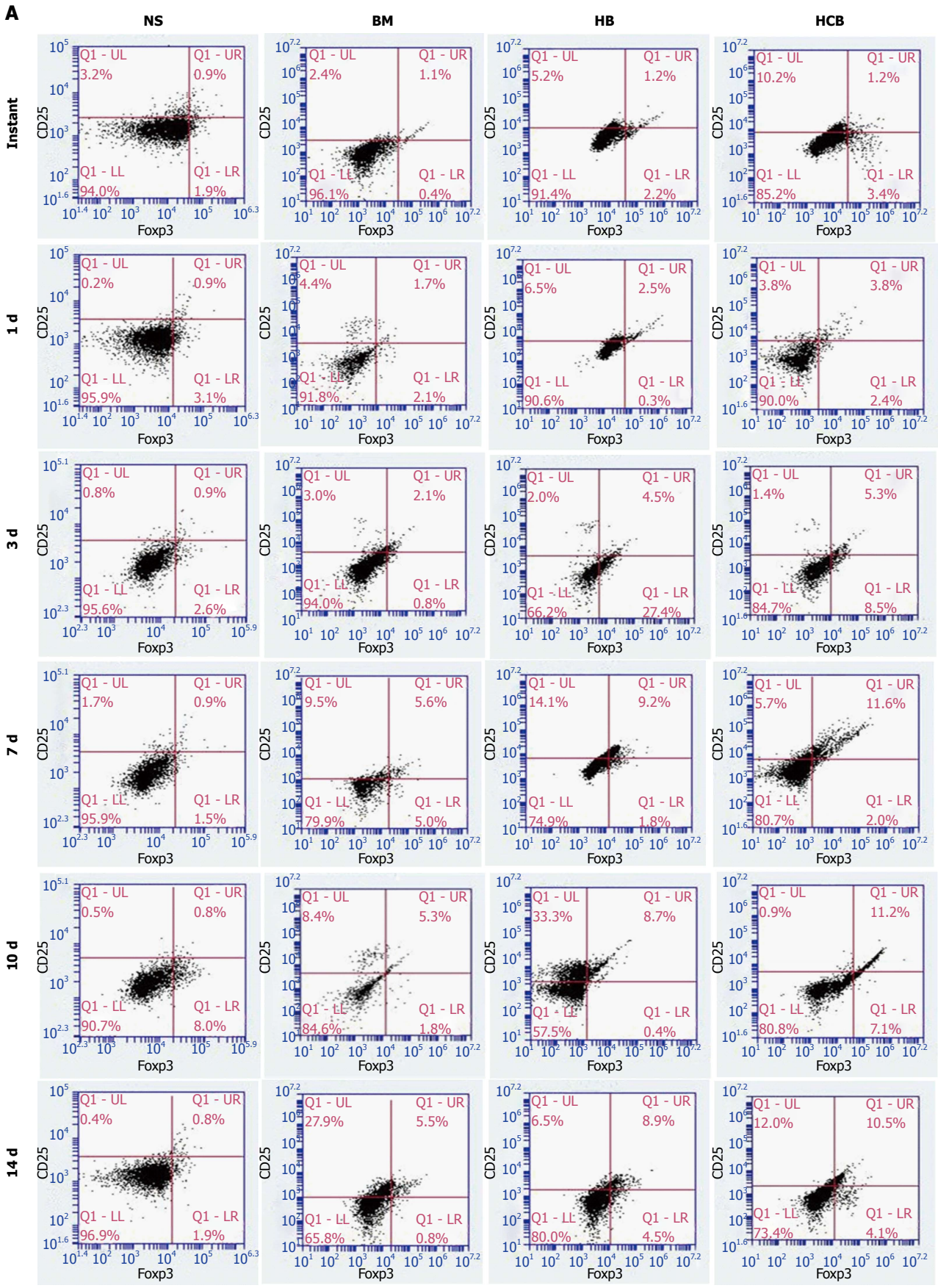
Figure 12 Activity of NK cells in different groups at each time point. [a]*P* < 0.05 *vs* NSBT group, [b]*P* < 0.05 *vs* IsoT group, [c]*P* < 0.05 *vs* NS group, [d]*P* < 0.05 *vs* BM group, [e]*P* < 0.05 *vs* HB group. The activity of NK cells in the BM group (27.70% ± 3.28%), HB group (28.05% ± 2.87%), and HCB group (27.93% ± 3.26%) was significantly lower than that in the NSBT group (34.17% ± 3.29%), IsoT group (34.05% ± 3.22%), and NS group (34.05% ± 2.19%) at instant (*P* < 0.05). The activity of NK cells in the NS group increased significantly at 1 d, 3 d, 7 d, 10 d, and 14 d, and was higher than that in the other five groups at the same time points (*P* < 0.05). The activity of NK cells in the BM group was higher than that in the HB group, and the HB group was higher than the HCB group at 1 d, 3 d, 7 d, 10 d, and 14 d after transplantation (1 d: 26.47 ± 3.65% *vs* 26.86 ± 3.65% *vs* 24.20% ± 2.54%; 3 d: 25.64% ± 4.37% *vs* 24.97% ± 3.21% *vs* 20.20% ± 2.54%; 7 d: 30.59% ± 4.02% *vs* 27.47% ± 2.94% *vs* 21.67% ± 3.02%; 10 d: 37.92% ± 3.29% *vs* 33.3%1 ± 1.76% *vs* 26.19% ± 2.02%; 14 d: 46.12% ± 3.87% *vs* 40.81% ± 3.07% *vs* 34.40% ± 3.21%; *P* < 0.05).

significantly higher than those in the NS group, BM group, and HB group (*P* < 0.05). The concentrations of anti-inflammatory cytokines in the HB group were higher than in the BM group, and those in the NS group were the lowest (*P* < 0.05). These results suggested that *CXCR3/HO-1* modified BMMSCs could induce immunological changes in rat lymphocytes after small bowel transplantation more significantly than *HO-1* modified BMMSCs and BMMSCs, resulting in increased secretion of anti-inflammatory cytokines and decreased secretion of proinflammatory cytokines.

DISCUSSION

With the progress in surgical technology, new immunosuppressive regimens, effective infection prevention, and perioperative management, the early survival rate of patients undergoing small bowel transplantation has been greatly improved[23]. However, the long-term outcome for small bowel transplantation is unsatisfactory[24]. The small bowel is an immune organ, with the small bowel mucosa being a perfect congenital immune system due to it being constantly exposed to exogenous antigens and microbial populations. As there is continual exchange of cells and antigens during congenital and acquired immune function, T cell-mediated acute cellular rejection[25] and antibody-mediated humoral rejection[26] are very likely to occur after small bowel transplantation. Studies have demonstrated that after small bowel transplantation, even under the application of a conventional immunization regimen, the incidence of rejection remains high[27,28]. The need to find a better method for induction of immune tolerance or transplantation rejection, has led to studies exploring whether BMMSCs can inhibit rejection after small bowel transplantation[29]. However, the effect of BMMSCs alone on acute immune rejection is poor, probably due to the low homing efficiency of BMMSCs after systemic infusion[30,31]. Treatment with BMMSCs results in an insufficient number of cells reaching *in vivo* lesions, and the survival time is also short when they do reach the target site.

Studies have shown that HO-1 and its metabolite system have antioxidant, anti-inflammatory, anti-proliferative and immunomodulatory effects[18]. HO-1 is the rate-limiting enzyme in the metabolism of hemoglobin. This molecule can protect cells by exerting antioxidant activity and potent antiapoptotic activity and maintaining microcirculation[32]. Modification of BMMSCs with *HO-1* enhances the ability of BMMSCs to tolerate anoxia-reoxygenation injury, thereby enhancing the survivability and proliferative capacity of these BMMSCs[33,34]. However, prolonged survival in rats is not ideal[34], and therefore this study aimed to make further improvements to the delivery of these important cells. In order to reduce the immune status of rats, we employed BMMSCs pretreatment before transplantation, which offers several advantages: (1) BMMSCs have especially poor immunogenic properties as multifunctional stem cells[35], allowing them to escape the killing effects of toxic T cells and natural killer cells[36]; (2) BMMSCs can interfere with the function of cytotoxic T cells, IFN-γ-secreting T cells, dendritic cells (DCs) and NK cells, consequently inhibiting the release of some inflammatory factors. Furthermore, BMMSCs can secrete some anti-inflammatory factors such as IL-10 and TGF-β[37], introducing a low immune state in the recipients[38]; and (3) BMMSCs can switch the immune response from that of a Th1 response to a Th2-driven response, inducing immune tolerance and a delay in rejection[39]. On this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HB
HCB
Instant
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3 d
7 d
10 d
14 d
CD25
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Q1 - UR
Q1 - LL
Q1 - LR
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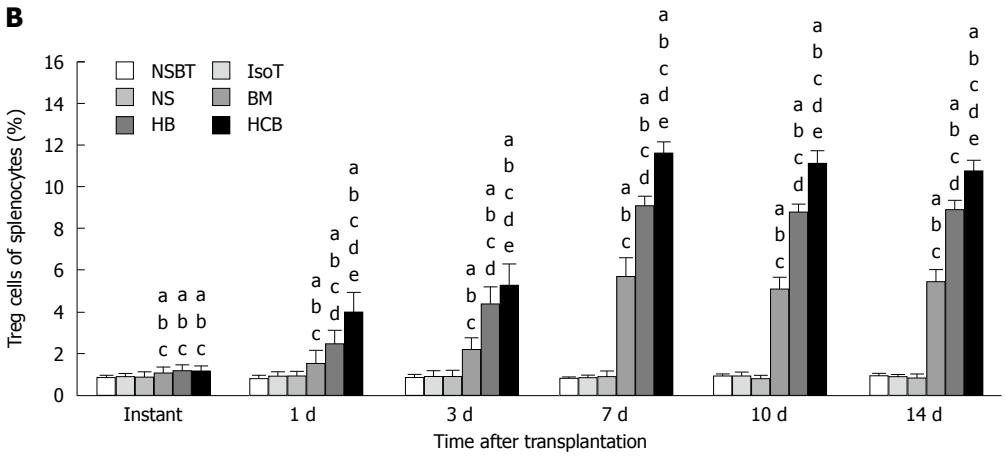

Figure 13 Ratio of regulatory T cells in different groups. A: Flow cytometry of regulatory T cells in the NS group, BM group, HB group, and HCB group; B: Column chart of the ratio of regulatory T cells in different groups. [a]*P* < 0.05 *vs* NSBT group, [b]*P* < 0.05 *vs* IsoT group, [c]*P* < 0.05 *vs* NS group, [d]*P* < 0.05 *vs* BM group, [e]*P* < 0.05 *vs* HB group. The ratio of regulatory T cells in the BM group (1.09% ± 0.28%), HB group (1.20% ± 0.29%), and HCB group (1.18% ± 0.25%) was comparable, and these ratios were all significantly higher than those in the NBST group (0.86% ± 0.12%), IsoT group (0.90% ± 0.16%), and NS group (0.88% ± 0.24%; *P* < 0.05) immediately after transplantation. The ratio of regulatory T cells in the NBST group, IsoT group, and NS group showed no significant change over time (*P* > 0.05). The ratio of regulatory T cells in the HCB group was higher than that in the HB group and BM group, and the HB group was higher than the BM group at the same time points (1 d: 3.98% ± 0.930% *vs* 2.49% ± 0.65% *vs* 1.55% ± 0.62%; 3 d: 5.29% ± 1.01% *vs* 4.38% ± 0.82% *vs* 2.19% ± 0.59%; 7 d: 11.60% ± 0.54% *vs* 9.10% ± 0.48% *vs* 5.70 ± 0.89%; 10 d: 11.14% ± 0.60% *vs* 8.79% ± 0.40% *vs* 5.13% ± 0.55%; 14 d: 10.74% ± 0.53% *vs* 8.93% ± 0.44% *vs* 5.46% ± 0.61%; *P* < 0.05).

basis, we hypothesized that the addition of a gene that could guide BMMSCs to a damaged site of tissue would further enhance the effect of *HO-1* modified BMMSCs.

Given that chemokines can be expressed and secreted by different histiocytes and immune cells under certain conditions, they bind to the corresponding chemokine receptors and affect effector cells differently[40]. The interaction of chemokines with their receptors controls the direct migration of various immune cells in the circulatory system and tissues and organs[41]. After the chemokine receptor binds to a specific chemokine, this stimulates calcium influx and results in cell chemotaxis, guiding cells to a specific part of the organism[42]. The chemokine receptor CXCR3 is a seven-subunit transmembrane G protein that is expressed in many organ lesions. They are found in parenchymal and inflammatory cells such as vascular endothelial cells, activated lymphocytes, macrophages and DCs but are not in the stationary phase of T lymphocytes, B lymphocytes and granulocytes[19].

It has been reported that CXCR3 and its ligands Mig, IP-10, and I-TAC are highly expressed during transplant rejection[43]. Th1 cells can specifically express CXCR3, which is typically a sign of activated Th1 lymphocytes[44] since the ligands of CXCR3 (Mig, IP-10, and I-TAC) can guide the activated Th1 lymphocytes to the tissue lesion[45]. Therefore, we hypothesized that modification of BMMSCs using the *CXCR3* gene with specific binding of the CXCR3 receptor to the ligand, could propel BMMSCs more quickly to the injury site and in larger numbers. At the same time, BMMSCs were modified with the *HO-1* gene to enhance the role of immunoregulation and damage repair, thereby protecting the long-term survival after small bowel transplantation. Our study was based on the chemotaxis of CXCR3 and we attempted to explore whether *CXCR3* modified BMMSCs could reach the site of inflammatory injury faster as a more pure collection of BMMSCs. Our study aimed to sacrifice the recipient rats simultaneously and observe the expression of the recipient rats at the same time point; the rejection response caused by the inflammatory response gradually augments over time, while specific rejection of the CXCR3 ligand will increase[46-48]. Therefore, we chose to sacrifice rats at multiple time points to describe the results in greater detail. The transduction of both the *HO-1* gene alone and *CXCR3*/*HO-1* genes into BMMSCs resulted in GFP expression exceeding 85%, and the protein levels of HO-1 and CXCR3 were significantly higher than those in native BMMSCs. Furthermore, the expression of *HO-1* and *CXCR3* mRNA was significantly up-regulated in modified BMMSCs compared with native BMMSCs, thus confirming that we successfully obtained *HO-1* modified BMMSCs and *CXCR3*/*HO-1* modified BMMSCs. After transplantation, we showed that the protein levels of HO-1 and CXCR3 in the HCB group were significantly higher than those of the other groups 1 d after transplantation. Furthermore, immunofluorescence double staining showed that the expression of HO-1 and CXCR3 proteins was both present 1 d after surgery, which indicated that *CXCR3*/*HO-1* modified BMMSCs could rapidly migrate to the transplanted small bowel. In addition, the levels of HO-1 and CXCR3 proteins in the HCB group were significantly higher than those in the other groups at the same time point, which indicated that *CXCR3*/*HO-1* modified BMMSCs reached the transplanted small bowel in large numbers.

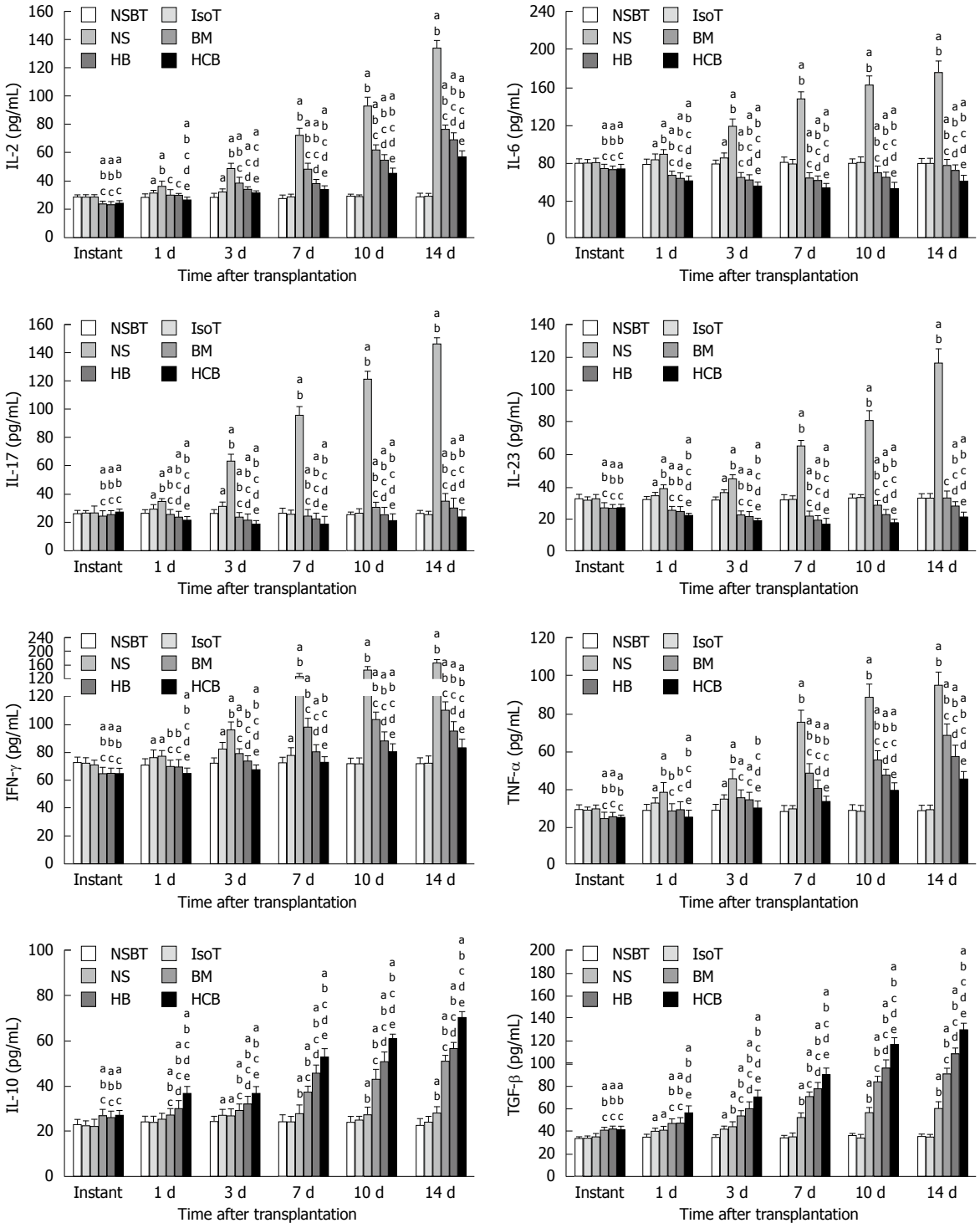

Figure 14 Proinflammatory cytokines and anti-inflammatory cytokines in different groups. [a]$P < 0.05$ *vs* NSBT group, [b]$P < 0.05$ *vs* IsoT group, [c]$P < 0.05$ *vs* NS group, [d]$P < 0.05$ *vs* BM group, [e]$P < 0.05$ *vs* HB group. Cytokines in the BM group, HB group, and HCB group were comparable ($P > 0.05$), while cytokines in the NBST group, IsoT group, and NS group were comparable immediately after transplantation ($P > 0.05$). The levels of proinflammatory cytokines (IL-2, IL-6, IL-7, IL-23, IFN-γ, and TNF-α) in the former three groups were significantly lower than those levels in the latter three groups ($P < 0.05$), and the anti-inflammatory cytokine levels (IL-10 and TGF-β) in the former three groups were significantly higher than those in the latter three groups ($P < 0.05$). Cytokines in the IsoT group changed over time, and nearly returned to normal levels at day 7 ($P > 0.05$). Concentrations of IL-2, IFN-γ, and TNF-α in the HCB group were significantly lower than those in the NS group, BM group, and HB group at each time point ($P < 0.05$), with levels in the HB group lower than in the BM group ($P < 0.05$), and highest in the NS group ($P < 0.05$). Concentrations of IL-2, IFN-γ, and TNF-α in the BM group, HB group, and HCB group were significantly higher than those in the NSBT group at 10 d and 14 d ($P < 0.05$). Concentrations of IL-6, IL-17, and IL-23 in the HCB group were significantly lower than those in the NS group, BM group, and HB group ($P < 0.05$), with levels in the HB group lower than in the BM group ($P < 0.05$), and highest in the NS group ($P < 0.05$). Concentrations of IL-6, IL-17, and IL-23 in the BM group, HB group, and HCB group were significantly lower than those in the NSBT group at 1 d, 3 d, and 7 d. Concentrations of IL-10 and TGF-β in the HCB group were higher than those in the other five groups. The HB group had the second highest level, the BM group level was higher than the NS group, and the NSBT group was comparable to the IsoT group with the lowest cytokine level, especially at 7 d, 10 d, and 14 d after transplantation.

In our study, the median survival time after transplant surgery was significantly higher in the recipient rats which were pretreated with BMMSCs and treated with *CXCR3*/*HO-1* modified BMMSCs (HCB group) (53 d) compared with the HB group (39 d), BM group (26 d), and NS group (16 d). This is likely to be related to the reduction in graft rejection, improvement in small bowel function, and a decrease in apoptosis. Pathological examination is a "gold standard" that reflects tissue damage; it scientifically and accurately reflects the degree of small bowel injury attributable to the rejection reaction[49,50]. Histopathological analysis showed that the transplanted small bowel in the HCB group had mild lymphocytic infiltration and a series of inflammatory reactions 7 d after surgery, while these phenomena in the HB group and BM group appeared 3 d after surgery. Furthermore, the infiltration of lymphocytes in the NS group was significantly different 1 d after surgery. DAO (95%) is mainly present in the cytoplasm of human and mammalian small bowel villus epithelial cells, with little or no distribution in the villi of the endometrium, or other tissues or cells. Apart from a small level of distribution in the endometrial villi, DAO is virtually absent from other tissues or cells[51]. When the bowel epithelial cells are damaged, this can lead to bowel barrier structural damage, so that villus epithelial cells will release significant quantities of DAO into the blood, leading to a rapid increase in DAO content. Thus, the DAO content in plasma directly reflects the damage to bowel epithelial cells and intestinal barrier structure, which is why it is measured[52-54]. We showed that the DAO content in plasma in the HCB group was significantly lower than those in the HB group, BM group, and NS group at the same postoperative time points, which confirmed that the degree of bowel function injury in the HCB group was ameliorated when compared with the other groups. We found that apoptosis in the IsoT group was similar to that in normal rats 7 d after transplantation, which indicated that ischemic injury had basically recovered by the 7th d after surgery; the level of apoptosis in the HCB group was lower than those of the HB group, BM group, and NS group at the same postoperative time points. However, the level of apoptosis in the NS group had clearly decreased by 10 d and 14 d after transplantation. Through pathological analysis, we found that a large number of necrotic mucosal cells had appeared. Therefore, the decrease in the NS group was not a result of an improved lesion but a false reduction caused by a large number of mucosal cells that had become necrotic and were being shed. Changes in body weight can also further reflect the rejection of the organ, *i.e.*, the less profound the rejection, the heavier the rats. We showed that HCB rats weighed the heaviest compared with other experimental groups. We also showed that *CXCR3*/*HO-1* modified BMMSCs were able to better protect transplanted small bowel, but the mechanism remains to be further elucidated.

BMMSCs regulate lymphocytes and induce the generation of regulatory T lymphocytes, which play an important role in both preventing rejection and maintaining immune tolerance[55]. Zhou *et al*[56] also found that BMMSCs have the ability to inhibit T cell responses both *in vitro* and *in vivo*, and that intravenous infusion of BMMSCs can alter the balance of Thl/Th2 cells. Th17 cells are helper T cells that can secrete IL-17, and they are iconic cells that play a positive role in immune regulation during the inflammatory response[57]. BMMSCs can inhibit the differentiation of $CD4^+$ T cells into Thl cells and Thl7 cells and upregulate the proportion of $CD4^+$ $CD25^+$ $Foxp3^+$ T cells (Tregs)[58]. Our findings showed that IL-6, IL-17, IL-23, IFN-γ, and TNF-α in serum in the preconditioned BM group, HB group, and HCB group were significantly lower than those in normal rats. The levels of IL-10 and TGF-β were significantly increased, the proportion of Tregs was significantly higher, and the activity of NK cells was also significantly lower than those observed in normal rats. These findings suggested that pretreated rats were in a low immune state.

Tregs are a regulatory T cell population distinct from Th1 and Th2 cells. They are thought to be a subset of Th3 cells that secrete TGF-β[59] and IL-10, both key in the maintenance of autoimmune tolerance[60], and important in the induction of immune tolerance after solid organ transplantation[61]. In the present study, we found that the proportion of Tregs from rats which were pretreated with BMMSCs was significantly higher than that of normal rats, and the levels of IL-10 and TGF-β were significantly higher than those of normal rats. At the same postoperative time, the proportion of Tregs in the HCB group was significantly higher than that in the HB group and BM group, while the HB group level was significantly higher than that in the BM group. These findings are similar to the results of a previous study[34] where the levels of IL-10 and TGF-β were highest in the HCB group. Thus, *CXCR3*/*HO-1* modified BMMSCs exerted their effects earlier than *HO-1* modified BMMSCs and BMMSCs.

The major Th1 cytokines are IFN-γ and IL-2[62], with such Th1 cytokines mediating proinflammatory cell immunity and acute rejection. In contrast, the major Th2 cytokine is IL-10; Th2 cells can down-regulate Th1 cells and consequently inhibit the cytotoxic effect of cytotoxic T lymphocytes, which is likely to be related to immune tolerance after transplantation[63-65]. Relevant studies have shown that IL-2 and IL-10 mutually orchestrate the final immune state[66]. Serum concentrations of IL-2 increase during acute rejection and are highly correlated with severity of rejection[67]. In contrast, high concentrations of IL-10 are associated with immune tolerance in solid organ transplantation[68]. Our results demonstrated that serum level of IL-2 in the HCB group treated with *CXCR3*/*HO-1* modified BMMSCs was the lowest of all groups and was significantly lower than those

observed in the NS group, BM group, and HB group. Furthermore, the level of IL-10 was the highest in the HCB group and significantly higher than those in the other three groups. For analysis of IFN-γ, we obtained similar results where the level of IFN-γ was significantly lower in the HCB group than in the NS group, BM group, and HB group. The cytokine profiles and trends mirror those of the pathological results of the transplanted small bowel, which is reassuring.

In recent years, studies have identified a new $CD4^+$ T cell type 17 (T helper cell 17, Th17), which is different from Th1 and Th2. This subset of T cells are independently differentiated and have different regulation mechanisms, while secreting IL-17, IL-22, IL-26, and TNF-α[69]. Therefore, IL-17 is the most characteristic cytokine that is produced by Th17 cells[70,71]. IL-23 is mainly produced by activated DCs and macrophages *in vivo*, and IL-23 plays an important role in maintaining Th17 stability and promoting Th17 proliferation[72]. IL-23 can also activate macrophages and DCs to promote the secretion of TNF-α[73]. It has been reported that IL-23 can induce activated $CD4^+$ T cells to proliferate and secrete IL-17/IL-17F, and enhance IL-23/Th17-induced autoimmune and inflammatory diseases. The binding of IL-17/IL-17F to the receptor may promote the expression of IL-1, IL-6, IL-8, TNF-α, and chemokines, thereby promoting neutrophil aggregation to the chronic inflammatory location[74]. BMMSCs can interfere with the function of DCs and macrophages, resulting in decreased levels of IL-23. TGF-β can promote the differentiation of Tregs by inducing the expression of Treg transcription factor Foxp3[75]. We demonstrated that the levels of IL-23, IL-17, and IL-6 in the pretreated BM group, HB group, and HCB group were significantly lower than those in normal rats, and also found that the levels of IL-23, IL-17, and IL-6 in the HCB group were significantly lower than those in the NS group, BM group, and HB group at the same postoperative time points. Data have shown that the concentration of TNF-α and the rejection status were positively correlated[76]. We found that the concentration of TNF-α in the HCB group was the lowest compared with those in the other groups. At the same postoperative time, those animals in the HCB group had the highest level of TGF-β, while also having similarly high levels of Tregs compared with the other groups. We speculate that *CXCR3*/*HO-1* co-transduced BMMSCs are better in modulating immunity by influencing the interaction of cytokines with a better concomitant regulatory effect.

In this study, we found that pretreatment with BMMSCs can render recipient rats into a low immune state, which is beneficial in reducing rejection after transplantation. Introduction of the *HO-1* gene may be able to solve the activity problems of BMMSCs when injected into the body, and the *CXCR3* gene can increase the homing ability of these BMMSCs. BMMSCs modified with the *HO-1* and *CXCR3* genes can quickly reach the injured site in large numbers, and thus appear to be able to reduce the rejection of small bowel transplants. We believe we have finally achieved the purpose of prolonging the survival period of rats after small bowel transplantation.

ACKNOWLEDGMENTS

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COMMENTS

Background

Small bowel transplantation is an ideal method for the treatment of end-stage intestinal failure. However, the development of small bowel transplantation has lagged behind that of other organ transplants because the incidence of rejection is high. Bone marrow mesenchymal stem cells (BMMSCs) play an important role in the regulation of immune function and are an ideal choice for cell transplantation in the treatment of autoimmune diseases and the prevention of rejection after solid organ transplantation. However, BMMSCs have a limited ability to survive and do not specifically migrate to the lesion in any meaningful capacity. Therefore, it is important to enhance the survival activity and quantity of BMMSCs at the injury site.

Research frontiers

BMMSCs play an important role in mitigating rejection and maintaining immune tolerance. However, treatment with BMMSCs tends to result in an insufficient number of cells that can target *in vivo* lesions and they have a short survival time. It is important to find a way to modify the BMMSCs, thereby increasing their activity and quantity at the injury location.

Innovations and breakthroughs

They found that BMMSCs modified with the *CXCR3* and *HO-1* genes can significantly reduce the rejection of small bowel transplantation. Thus, chemokine receptor CXCR3 and HO-1 synergistically play an important role in this process.

Applications

BMMSCs are likely to be an ideal cell therapy for the treatment of organ transplant rejection in the future. By enhancing cell activity and quantity at the injury location, this can further enhance the effect of improving the rejection of transplanted small bowel. This is a step forward for clinical application of gene modified BMMSCs.

Terminology

Diamine oxidase is a highly active intracellular enzyme that exists in human and mammalian small bowel villi. It plays a role in histamine production and a variety of polyamine metabolism processes, and its activity is closely related to the synthesis of nucleic acid and protein in mucosal cells. Its presence can reflect the intact nature of the intestinal barrier and the degree of injury.

Peer-review

The authors investigated the protective effects of BMMCCs modified with the *CXCR3* and *HO-1* genes on the rejection of small bowel transplantation. They found that *CXCR3*/*HO-1* modified BMMSCs were able to improve the survival of the recipient rats. The whole project involved a large amount of work and has a certain degree of innovation in its execution.

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ORIGINAL ARTICLE

Case Control Study

Systemic interleukin-9 in inflammatory bowel disease: Association with mucosal healing in ulcerative colitis

Malgorzata Matusiewicz, Katarzyna Neubauer, Iwona Bednarz-Misa, Sabina Gorska, Malgorzata Krzystek-Korpacka

Malgorzata Matusiewicz, Iwona Bednarz-Misa, Malgorzata Krzystek-Korpacka, Department of Medical Biochemistry, Wroclaw Medical University, 50-368 Wroclaw, Poland

Katarzyna Neubauer, Department of Gastroenterology and Hepatology, Wroclaw Medical University, 50-556 Wroclaw, Poland

Sabina Gorska, Laboratory of Medical Microbiology, Ludwik Hirszfeld Institute of Immunology and Experimental Therapy, Polish Academy of Sciences, 53-114 Wroclaw, Poland

Author contributions: Matusiewicz M and Krzystek-Korpacka M designed research; Neubauer K treated patients and collected material and clinical data from patients; Matusiewicz M, Bednarz-Misa I, Gorska S and Krzystek-Korpacka M performed the assays; Matusiewicz M and Krzystek-Korpacka M conducted statistical analyses; Matusiewicz M, Neubauer M and Krzystek-Korpacka M analysed and interpreted data; Matusiewicz M and Krzystek-Korpacka M wrote the paper; all authors critically revised the paper and approved its final version.

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Correspondence to: Malgorzata Krzystek-Korpacka, PhD, DSc (Med), Department of Medical Biochemistry, Wroclaw Medical University, ul. Chalubinskiego 10, 50-368 Wroclaw, Poland. malgorzata.krzystek-korpacka@umed.wroc.pl
Telephone: +48-71-7841395
Fax: + 48-71-7840085

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Abstract

AIM

To evaluate circulating IL9 in inflammatory bowel disease and disease-associated anemia/cachexia and assess its potential as a mucosal healing marker.

METHODS

Serum IL9 as well as other cytokines (IL1β, IL6, IL13, IFNγ, TNFα, and VEGF-A) were determined in 293 individuals: 97 patients with Crohn's disease (CD) and 74 with ulcerative colitis (UC) and in 122 apparently healthy controls. The clinical activity of CD and UC was expressed in terms of the Crohn's Disease Activity Index (CDAI) and the Mayo Scoring System (MDAI), respectively, and the severity of bowel inflammation in UC patients was assessed using Mayo endoscopic score. Cytokine concentrations were measured by a flow cytometry-

based method using Luminex xMAP® technology. High-sensitive C-reactive protein concentrations (hsCRP) were determined in CD and UC patients using the enhanced immunoturbidimetric method.

RESULTS

Systemic IL9 was significantly lower in healthy individuals [9 pg/mL (95%CI: 8.2-10)] than in patients with inflammatory bowel disease (IBD): both inactive [14.3 pg/mL (11.9-19.9)] and active [27.6 pg/mL (24.5-32), $P < 0.0001$]. Cytokine concentrations were significantly higher in active CD [27.4 pg/mL (23.4-32.2)] and in active UC [32.7 pg/mL (27-38.9)] compared to inactive diseases [15.9 pg/mL (10.8-23.4) in CD and 19.4 pg/mL (13.9-27.1) in UC, $P = 0.001$]. IL9 correlated weakly with CDAI ($\rho = 0.32$, $P = 0.003$) and MDAI ($\rho = 0.35$, $P = 0.002$) and strongly with endoscopic inflammation in UC ($\rho = 0.74$, $P < 0.0001$). As a negative marker of mucosal healing (MH), IL9 had an accuracy superior to hsCRP and IL6 [97% ($P < 0.0001$), 67% ($P = 0.071$), and 55% ($P = 0.525$), respectively]. IL9 was significantly higher in cachectic IBD patients [30.25 pg/mL (24.4-37.5) *vs* 21.88 pg/mL (18-26.5), $P = 0.026$] and negatively correlated with hemoglobin concentrations ($\rho = -0.27$, $P < 0.001$). Multiple regression showed IL1β and IL13 to be the independent predictors of circulating IL9 in healthy individuals, IFNγ or IL6 in active and inactive UC, respectively, and IL13 and VEGF-A in both active and inactive CD.

CONCLUSION

The systemic IL9 level is higher in IBD and corresponds with endoscopic inflammation, suggesting its possible application as a negative marker of mucosal healing in UC.

Key words: Interleukin 9; Mucosal healing; Biomarker; Inflammatory bowel disease; Crohn's disease; Ulcerative colitis; Cachexia; Anemia

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Core tip: Based on a large cohort of patients, our results confirm elevation of IL9 in inflammatory bowel disease (IBD). Additionally, the data demonstrate associations between IL9 and both anemia and wasting syndromes accompanying IBD. Importantly, they show that an elevation in systemic IL9 in ulcerative colitis (UC) corresponds to mucosal inflammation, with IL9 displaying a high level of accuracy as a negative marker of mucosal healing. Also, our results demonstrate IL9 to be more tightly associated with proinflammatory and Th1 cytokines in UC and with angiogenic and Th2 cytokines in Crohn's disease.

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INTRODUCTION

Inflammatory bowel disease (IBD) is a group of chronic conditions of the gastrointestinal tract encompassing Crohn's disease (CD) and ulcerative colitis (UC). The fundamental role of cytokines in the induction and perpetuation of inflammation in IBD is well established. Consequently, cytokines have attracted attention as potential goals of biological therapies, focused either on targeting proinflammatory cytokines and their signaling pathways or on administration of anti-inflammatory cytokines[1].

Recently, a new subtype of helper lymphocytes T has been described and termed Th9 due to their preferential expression of interleukin (IL)-9, a cytokine also found in the repertoire of other T cell subtypes[2,3]. IL9 is a pleiotropic cytokine affecting a variety of cells; yet, its biological activity or physiopathological relevance remains elusive. Nevertheless, the latest findings implicate IL9 in the development of autoimmune diseases[4-7]. Although IBD is not a classic autoimmune disease, IL9 and its receptor have recently been found to contribute to the pathogenesis of UC[8-10]. Inflamed gut biopsies from UC patients have been found to overexpress IL9 at both mRNA and protein levels[9]. In animal models of colitis, IL9 gene expression correlated with the severity of histological inflammation, which could be reduced by IL9-directed antibodies[11]. Evaluation of tissue expression of IL9 gene has been proposed for the monitoring of disease severity in UC. In turn, the cytokine leukocyte expression has been suggested as a systemic inflammatory marker[9].

In recent years the aims of IBD therapy have evolved from the control of symptoms to the control of inflammation as the only action that can in fact change the course of the disease and decrease the risk of complications in terms of therapy intensification, hospitalization and surgery. Evaluation of the activity of IBD remains a challenge in individual patients as well as in designing of clinical trials. Hence, mucosal healing (MH) has become a key end-point of therapy and objective markers of inflammation are intensively searched for. The significance of the optimization of IBD therapy could be even greater as an increasing incidence and prevalence of IBD is observed all over the world[11,12].

Only recently, the elevation of serum IL9 in IBD has been reported and linked with severe prognosis[13]. Supplementing this pioneering research, we aimed to assess circulating cytokines with reference to a large cohort of patients and present these data in the context of other cytokines: proinflammatory (IL1β, IL6, TNFα) and angiogenic (VEGF-A), and of Th1 (IFNγ

Table 1 Characteristics of study population (inflammatory bowel disease)

	Controls	Active IBD	Inactive IBD	*P* value
n	122	133	38	
Age (yr)	38.5 ± 14.2	37.5 ± 13.4	37.6 ± 10.5	0.825
Gender (F/M)	55/67	63/70	16/22	0.833

Data on age is presented as mean ± SD and analyzed using one-way ANOVA; data on gender distribution (F-females, M-males) was analyzed using χ^2 test. IBD: Inflammatory bowel disease.

and TNFα) and Th2 (IL13) subset signatures as well as symptoms accompanying IBD, namely, anemia and cachexia. Circulating IL9, as a serum-based marker, might be a more easily available, less invasive and less expensive indicator of IBD severity and inflammation than tissue and/or leukocyte expression of the IL9 gene. Moreover, if found to follow the pattern described for tissue and leukocyte cytokine, determination of IL9 levels in circulation might be useful as a differential marker in IBD or as a non-invasive MH marker.

MATERIALS AND METHODS

Serum IL9 was measured in 293 individuals: 97 patients with CD and 74 with UC and in 122 apparently healthy controls. IBD patients were recruited from the Department of Gastroenterology and Hepatology of Wroclaw Medical University, Poland. Individuals with unclassified colitis or the co-existence of other severe systemic diseases, malignancies, liver diseases, or pregnancies were excluded. The Crohn's Disease Activity Index (CDAI) was applied for the assessment of CD activity and the Mayo Scoring System (MDAI) for UC activity. The severity of bowel inflammation in UC patients was assessed using Mayo endoscopic score. IBD patients, with a few exceptions, were treated with 5'-aminosalicylate (5'-ASA) derivatives. Cachexia was defined as substantial and involuntary weight loss-higher than 5% of former weight during 3 mo. According to the reference values provided by the Central Hospital Laboratory conducting analyses for our patients, anemia was defined as Hb < 12 g/dL in women and < 13.5 g/dL in men.

Healthy controls were volunteers from among hospital staff or outpatients of the Research, Science, and Educational Center of Dementia Diseases, Scinawa, Poland suffering from headaches or mild cognitive disorders, but otherwise with no significant health history, or from blood donors from the Regional Center for Blood Donation and Therapeutics in Wroclaw, Poland. The following inclusion criteria were applied for the control group: age > 18 years, overall good health condition, and willingness to participate. Exclusion criteria were: pregnancy, active inflammation (based on physical examination and medical history), known severe systemic or dementive disease or depression. The demographic characteristics of the study population are given in Tables 1 and 2.

Ethical considerations

The study protocol was approved by the Medical Ethics Committee of Wroclaw Medical University and the study was conducted in accordance with the Helsinki Declaration of 1975, as revised in 1983, and informed consent was obtained from all patients.

Analytical methods

Blood was drawn following overnight fasting by venipuncture, clotted for 30 min, and centrifuged (15 min, 720 × *g*). Serum was collected, aliquoted and kept frozen at -80 ℃ until examination. IL9 as well as IL1β, IL6, IL13, IFNγ, TNFα, and VEGF-A were measured in duplicates by using a flow cytometry-based method utilizing magnetic microspheres conjugated with monoclonal antibodies using the BioPlex 200 platform with HRF (Bio-Rad, United States), incorporating Luminex xMAP® technology, and Bio-Plex Pro™ Human Cytokine, Chemokine, and Growth Factor Magnetic Bead-Based Assays according to the manufacturer's instructions, except that samples were diluted at a ratio of 1:2 in sample diluent. Standard curves were drawn using 5-PL logistic regression and the data were analyzed using BioPlex Manager 6.0 software. The concentrations of IL9 measured in our study population were within the range determined for human sera by the assay manufacturer[14].

High-sensitive C-reactive protein (hsCRP) was determined using the latex particle-enhanced immunoturbidimetric method with the CRPex-HS CRP test (Good Biotech Corp., Taichung, Taiwan) and a protein multicalibrator (ProDia International, Sharjah, UAE).

Data on hemoglobin and total protein concentrations as well as data on weight loss were retrieved from patients' medical records.

Statistical analysis

Data normality was tested using the Kolmogorov-Smirnov test with Lilliefors significance correction and homogeneity of variation using the Levene test. Log-transformation was used if appropriate. Data are presented as medians or means with 95%CI and analyzed using, respectively, the Kruskal-Wallis *H* test or one-way analysis of variance (ANOVA) with Bonferroni correction for multiple testing and *t*-test for independent samples. Two-way ANOVA was used to co-examine the influence of MH and cachexia. Logistic regression followed by the Hosmer and Lemeshow goodness of fit test and multiple regression (stepwise method; $P < 0.05$ as entrance and $P > 0.1$ as removal criteria) were used to examine IL9 associations. Correlation analysis was conducted using either the Spearman test (ρ) or the Pearson test (r). Frequency analysis was conducted using the χ^2 test or Fisher's exact test. Receiver operating

Table 2 Characteristics of study population (Crohn's disease and ulcerative colitis)

	CD active	CD inactive	UC active	UC inactive	*P* value
N	81	16	52	22	
Age (yr)	35.3 ± 12.7	35.7 ± 10.5	40.9 ± 13.8	39 ± 10.5	0.081
Gender (F/M)	42/39	5/11	21/31	11/11	0.346
Hb (g/dL)	12 ± 1.9	13.8 ± 2.1	12.2 ± 2.4	12.8 ± 1.5	0.028
PLT (× 10^3/mm^3)	397 (297-483)	283 (215-344)	314 (283-434)	264 (226-327)	< 0.001
WBC (× 10^3/mm^3)	7.89 (5.9-10.8)	6.24 (5.5-7.3)	7.91 (6.1-9)	6.2 (5.1-7.6)	0.046
Protein (g/dL)	6.97 ± 0.99	7.26 ± 0.74	6.77 ± 0.88	7.23 ± 0.54	0.152

Data on age, hemoglobin (Hb), and total protein concentration presented as mean ± SD and analyzed using one-way ANOVA; data on platelet (PLT) and leukocyte (WBC) counts presented as medians with interquartile range and analyzed using Kruskal-Wallis *H* test; data on gender distribution (F-females, M-males) was analyzed using χ^2 test. CD: Crohn's disease; UC: Ulcerative colitis.

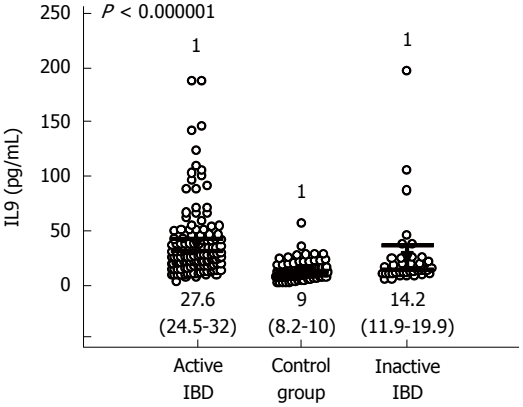

Figure 1 Systemic IL-9 in healthy individuals and patients with active and inactive inflammatory bowel disease. Data presented as medians with 95%CI and analyzed using Kruskal-Wallis *H* test. [1]Significantly different from other groups.

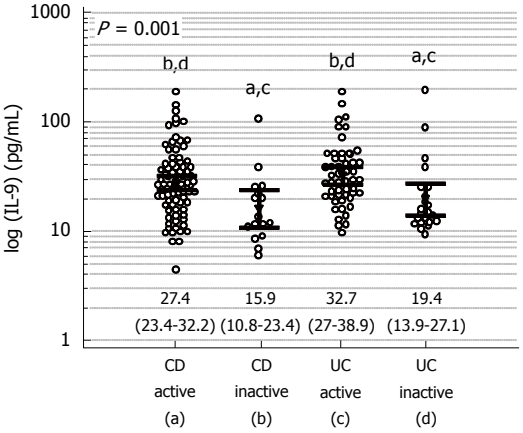

Figure 2 Systemic IL-9 in patients with active and inactive Crohn's disease and ulcerative colitis. Data presented as geometric means with 95%CI and analyzed using one-way ANOVA. Small letters indicate statistical significance of between-group differences.

characteristics (ROC) curve analysis was conducted to evaluate IL9 as a disease marker. Marker accuracy was presented as the area under the ROC curve and expressed as a percentage. For an optimal cut-off value, marker sensitivity (sens.) and specificity (spec.) as well as Youden's *J* statistic (YI, where *J* = sensitivity + specificity - 1) were calculated. All calculated probabilities were two-tailed and *P*-values ⩽ 0.05 were considered statistically significant. The analyses were conducted using MedCalc Statistical Software version 16.8.4 (MedCalc Software bvba, Ostend, Belgium; https://www.medcalc.org; 2015).

RESULTS

Interleukin-9 in IBD

The concentrations of circulating IL-9 were significantly lower in apparently healthy individuals than in patients with IBD; both inactive and active (Figure 1). Patients with active CD and active UC had significantly higher concentrations of IL-9 than patients with inactive diseases, but there was no significant difference between CD and UC (Figure 2).

The cytokine concentrations weakly correlated with disease clinical activity scores: $\rho = 0.32$, $P = 0.003$ with CDAI and $\rho = 0.35$, $P = 0.002$ with MDAI (Figure 3).

Interleukin-9 as a negative marker of mucosal healing in UC

There was a strong positive correlation between IL9 and Mayo endoscopic score: $\rho = 0.74$, $P < 0.0001$ (data available for 53 UC patients) (Figure 4). The elevated concentrations of IL9 predicted tissue inflammation on endoscopy (scores 1-3) with an accuracy of 97%. Systemic levels of IL-9 exceeding 20.5 pg/mL were both a sensitive and a specific marker of a lack of mucosal healing (Figure 5).

For comparative purposes, the concentrations of classic markers of systemic inflammation were also evaluated. Circulating IL6 and hsCRP positively correlated with the clinical activity of both CD ($\rho = 0.45$, $P < 0.0001$ for IL6 and $\rho = 0.45$, $P = 0.0001$ for hsCRP) and UC ($\rho = 0.52$, $P < 0.0001$ for IL6 and $\rho = 0.67$, $P < 0.0001$ for hsCRP). The associations were stronger than those observed for IL9. However, the association between IL6 and endoscopic findings ($\rho = 0.35$, $P = 0.011$) as well as between hsCRP and endoscopic findings ($\rho = 0.45$, $P = 0.002$) were less pronounced than that for IL9. As markers of mucosal non-healing, neither hsCRP nor IL6 displayed significant discriminative power (Figure 3).

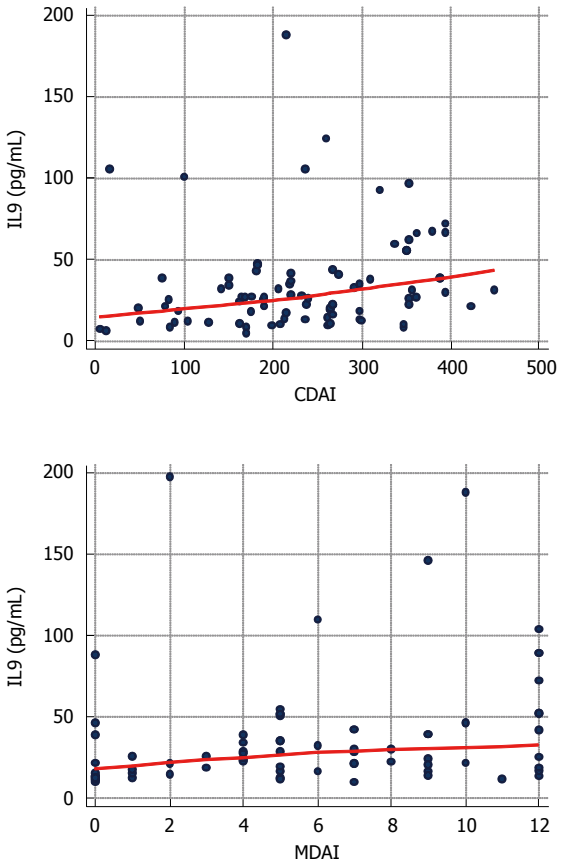

Figure 3 Correlation between the concentrations of circulating IL9 and the clinical activity of Crohn's disease and ulcerative colitis. Data analyzed using Spearman correlation test. CDAI: Crohn's Disease Activity Index; MDAI: Mayo Disease Activity Index.

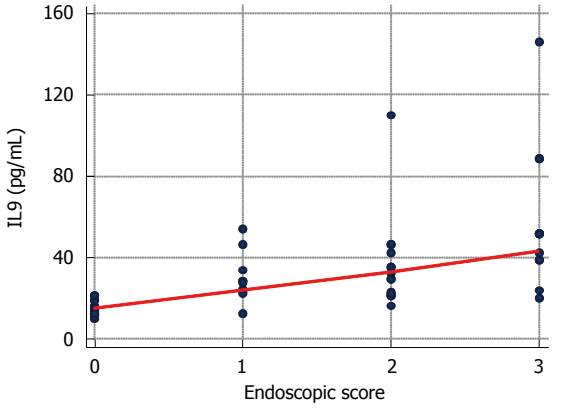

Figure 4 Correlation between the concentrations of circulating IL9 in patients with ulcerative colitis and endoscopic score. Data analyzed using Spearman correlation test.

In logistic regression, log-IL9 was an independent predictor of mucosal non-healing [b = 15.4, P = 0.002; constant-19.2, P = 0.002; goodness of fit (Hosmer and Lemeshow test): χ^2 = 2, P = 0.981], correctly classifying 88% of cases, whereas hsCRP and IL6 were not included in the regression model.

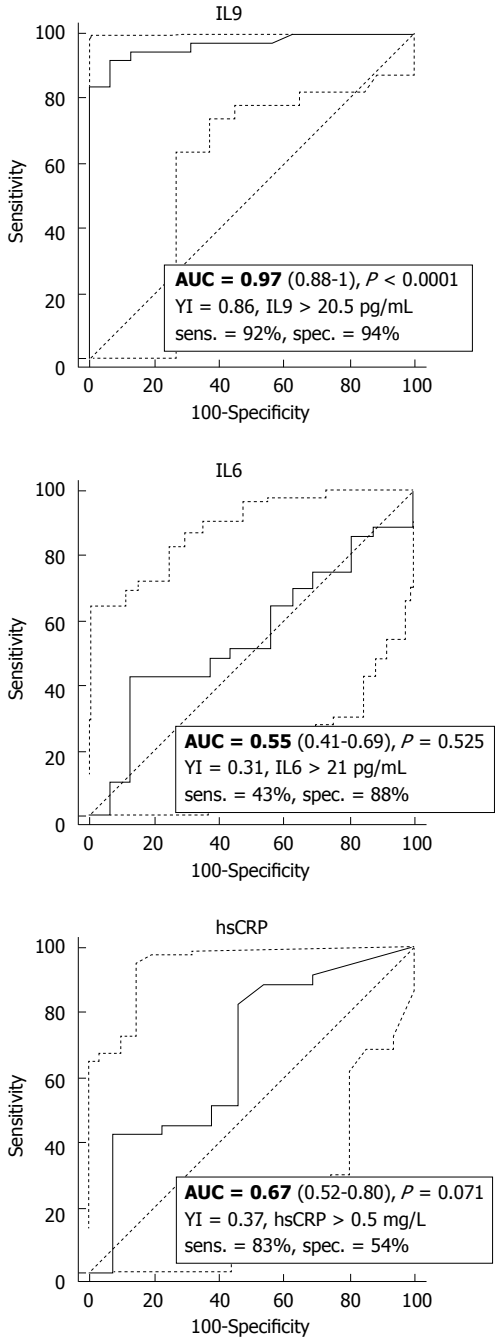

Figure 5 Comparison of IL9, hsCRP and IL6 as potential markers of mucosal non-healing. Data presented as area under ROC curve (AUC) with 95%CI and P value. For an optimal cut-off value, markers sensitivity (sens.) and specificity (spec.) as well as Youden's J statistic (YI, where J = Sensitivity + Specificity - 1) were calculated.

Interleukin-9 and cachexia and anemia

IL9 was significantly higher in IBD patients who experienced substantial weight loss than in those who did not: 30.25 pg/mL (24.4-37.5, n = 53) *vs* 21.88 pg/mL (18-26.5, n = 63), P = 0.026, respectively. Also, IL9 tended to be higher in IBD patients with anemia (29.2 pg/mL (24.8-34.5, n = 91) *vs* 23.6 pg/mL (20.1-27.6, n = 71), P = 0.069) and was negatively correlated with hemoglobin concentration (ρ

Table 3 Interleukin-9 association with proinflammatory, angiogenic and Th1 and Th2 subset-specific cytokines

	CD active	CD inactive	UC active	UC inactive	Controls
IL1β	NS	0.58, $P = 0.020$	0.42, $P = 0.002$	0.76, $P < 0.0001$	0.51, $P < 0.0001$[1]
IL6	NS	0.58, $P = 0.018$	0.38, $P = 0.005$	0.77, $P < 0.0001$[1]	0.33, $P < 0.001$
IL13	0.48, $P < 0.0001$[1]	0.62, $P = 0.010$[1]	NS	NS	0.46, $P < 0.0001$[1]
IFNγ	0.29, $P = 0.008$	NS	0.51, $P = 0.0001$[1]	0.74, $P = 0.0001$	NS
TNFα	NS	NS	NS	0.65, $P = 0.001$	0.19, $P = 0.045$
VEGFA	0.37, $P < 0.001$[1]	0.77, $P < 0.001$[1]	0.38, $P = 0.006$	0.46, $P = 0.033$	0.36, $P = 0.0001$

[1]Independently associated with IL9 in multivariate analysis (stepwise method). CD: Crohn's disease; UC: Ulcerative colitis.

= -0.27, $P < 0.001$).

Cachectic and non-cachectic UC patients differed in their degree of endoscopic inflammation ($P = 0.049$). When co-examined, both cachexia ($P = 0.016$) and endoscopic ($P < 0.001$) inflammation were independently associated with IL9. However, IL9 lost significance as a cachexia predictor when co-examined with classic procachectic cytokines (IL1β, IL6, TNFα), hsCRP and total protein concentration.

Interplay between IL9 and other cytokines in IBD

In healthy individuals, in univariate analysis, circulating IL9 was positively correlated with inflammatory cytokines (IL1β and IL6), with IL13, a T helper 2-type cytokine, and VEGF-A, a key angiogenic growth factor. In IBD, the correlation pattern was affected by both the disease type and its activity. Circulating IL9 was correlated with inflammatory cytokines in inactive IBD, more pronouncedly in UC than CD. A correlation with IL13 was observed exclusively in CD, and this was more pronounced in patients in remission. Circulating IL9 correlated with VEGF-A in all IBD patients, but the association was more pronounced in inactive CD (Table 3).

Multiple regression showed IL1β and IL13 to be the independent predictors of circulating IL9 in healthy individuals, IFNγ or IL6 in active and inactive UC, respectively, and IL13 and VEGF-A in both active and inactive CD (Table 2).

DISCUSSION

The recent discovery of Th9 cells has rekindled interest in IL9 and their targeting has emerged as a potential therapeutic option in UC[15,16]. In turn, IL9 gene expression in bowel tissue and leukocytes has been proposed as a marker of local and systemic inflammation, respectively[10]. The strict association between IL9 and local inflammation is of particular relevance in the light of mucosal healing becoming a key treatment goal in IBD, a new measure of IBD activity, an outcome predictor, and an endpoint in clinical trials[17,18]. However, evaluation of tissue-based markers is invasive, expensive, and time-consuming, not easily accessible and related to some risks for patients. Such an evaluation may also be unavailable for CD patients with lesions situated in the small intestine. Clinical indices of IBD activity correlate with endoscopic findings rather poorly and as such do not allow for effective treatment modification. Therefore, surrogate markers of mucosal healing are needed[18].

Defendenti *et al*[13] were the first to report on an elevation of circulating IL9 in IBD and link this finding to severe prognosis. Using a more sensitive, fluorescence-based assay for cytokine determination, our research corroborates their findings on a larger set of patients, but focuses on IL9 as a possible MH marker. Although non-invasive and easier to assess, it is not clear to what extent serum-based markers can accurately reflect local immune response. Indeed, we observed IL6 and CRP to positively correlate with IBD clinical activity rather than Mayo endoscopic score. In contrast, IL9 predominantly mirrored the endoscopic activity of UC and was only weakly correlated with a clinical one. As a marker of mucosal non-healing (defined as scores other than 0 on the Mayo Clinic endoscopy scoring system), systemic IL9 is highly accurate with near perfect sensitivity and specificity. Our findings are consistent with the pathogenic role attributed to IL9 in wound healing[9,10]. In animal models of colitis, *Il9* expression correlated with the severity of histological inflammation, which could be reduced with antibodies against IL9[10]. Functionally, IL9 altered the expression of tight junction proteins, inducing a notable bacteria translocation[10]. It also perpetuated inflammatory response *via* up-regulation of IL8, facilitating leukocyte trafficking and survival[9].

Unlike the cytokine tissue expression, preferential in UC[9,10], circulating IL9 did not differ between CD and UC in either Defendenti *et al*[13] or our cohorts. However, we observed a divergent association pattern with inflammatory and angiogenic indices that might translate into functional differences in IL9 between both conditions. An elevation in circulating IL9 was related to systemic inflammation in UC rather than CD, as evidenced by stronger correlations with proinflammatory cytokines. IL9, more so in CD, was correlated with VEGF-A, which might imply an association between IL9 and IBD angiogenesis. Correspondingly, in atopic dermatitis, IL9 and VEGF-A mRNA expressions were positively correlated and IL9 induced VEGF-A expression in cultured keratinocytes[19]. In IBD, IL9 might also be indirectly associated with angiogenesis by being a growth factor for mast cells, the source of VEGF-A, FGF2, and IL8[20].

IL9 in CD was also tightly correlated with IL13, an important Th2 cytokine. Traditionally, CD has been referred to as a Th1 condition and UC as a Th2 disease. However, this classic paradigm has recently been challenged, as the cytokines considered specific signatures for Th1 and Th2 subsets display diverse and often opposing activities[21]. Moreover, the discovery of the Th17 subset has further changed our understanding of IBD pathogenesis. Analysis of IL9 correlation patterns in CD and UC exemplifies the complexity of cytokine interactions. IL9 correlation with IL13 is in line with their co-expression by Th2 lymphocytes and the role of IL9 in maintaining IL13 production by innate lymphoid cells[22,23]. However, since this association was observed exclusively in our CD cohort, encompassing patients with the disease located in the small intestine, this may reflect the effect of IL9 on Paneth cells. Paneth cells play a critical role in resistance against enteric bacterial pathogens and in the maintenance of the normal composition of the gut microbiota[24,25]. IL9 induces their hyperplasia *via* up-regulation of IL13 expression[26]. Interestingly, both at a systemic level in the current study and at an mRNA level in inflamed bowel tissue[9], IL9 positively correlated with IFNγ. This association was particularly pronounced in UC patients, although IFNγ serves as a subset specific signature for Th1 cells and has been reported to inhibit IL9 production (reviewed in[3]).

IBD can ultimately lead to malnutrition, which, unaddressed, might lead to unfavorable outcomes[27,28]. In the face of the overweight/obesity epidemic, BMI has lost some of its credibility as a marker of malnutrition[29]. Accordingly, the vast majority of our cachectic patients had normal BMI (70%) and some (7%) remained overweight. Biochemical markers of poor nutritional status might facilitate prompt classification of IBD patients for dietary intervention. However, the application of traditional markers such as CRP and albumins has recently been criticized. These represent inflammation, which is an etiologic factor in cachexia, and are the main reason for reduced visceral protein levels[29]. The rationale for evaluating IL9 as a potential marker of cachexia was provided by Gerlach *et al*[10], who demonstrated that IL9-deficient mice responded to oxazolone challenge with less pronounced weight loss than wild type animals expressing IL9. We validated IL9's association with weight loss in a clinical setting. However, this seems to be mediated by IL9's correlation with classic procachectic cytokines, hampering IL9's suitability as a cachexia marker.

Our results confirm IL9 elevation in IBD in a large cohort of patients and demonstrate IL9's association with anemia and wasting syndromes accompanying IBD. Importantly, the data show that an elevation in systemic IL9 in UC corresponds with mucosal inflammation, with IL9 displaying a high level of accuracy as a negative marker of mucosal healing. Also, our results demonstrate IL9 to be more tightly associated with proinflammatory and Th1 cytokines in UC and with angiogenic and Th2 cytokines in CD.

COMMENTS

Background

Recently, a new subtype of helper lymphocytes T that overexpress cytokine IL9, termed Th9, has been described and involved in the pathogenesis of ulcerative colitis. Targeting IL9 signaling has emerged as a potential new therapeutic option in ulcerative colitis and evaluation of *IL9* gene expression in bowel tissue and leukocytes has been proposed as a marker of respectively local and systemic inflammation. These findings were followed by an observation on the elevation of systemic IL9 in inflammatory bowel disease (IBD) patients, regardless the type of the disease, and linked with poor prognosis.

Research frontiers

In the last years the aims of the IBD therapy evolved from the control of symptoms to control of inflammation, the only mode of action capable of altering the disease course. As such, mucosal healing become a key treatment goal in IBD, a new measure of IBD activity, an outcome predictor, and an endpoint in clinical trials and non-invasive methods of its evaluation are intensively searched for. IBD leads to malnutrition, worsening patient's quality of life, increasing the disease severity or risk of relapse, negatively affecting patient's response to treatment, and facilitating the development of systemic manifestations of the disease. Markers of poor nutritional status might facilitate prompt classification of IBD patients for dietary intervention. However, the suitability of traditional ones like BMI, C-reactive protein concentrations or albumin concentrations has recently been questioned.

Innovations and breakthroughs

The authors confirm findings of previous study showing an elevation of systemic IL9 in IBD in a large cohort of patients and expand it on a link between cytokine elevation and local inflammation in ulcerative colitis and cachexia and anemia of chronic diseases. The authors also observed that although there is no difference in the degree of IL9 elevation between two main types of IBD, there are dissimilarities in the pattern of interplay between IL9 and other cytokines manifested by more pronounced association with proinflammatory and Th1 cytokines in ulcerative colitis and with angiogenic and Th2 cytokines in Crohn's disease.

Applications

IL9 measurement might be considered, as an adjunct to endoscopy, for non-invasive evaluation of mucosal healing in patients with ulcerative colitis.

Peer-review

The present study adds relevant news to the current literature and could be of practical interest for clinicians experienced in IBD.

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ORIGINAL ARTICLE

Case Control Study

Association of keratin 8/18 variants with non-alcoholic fatty liver disease and insulin resistance in Chinese patients: A case-control study

Rui Li, Xian-Hua Liao, Jun-Zhao Ye, Min-Rui Li, Yan-Qin Wu, Xuan Hu, Bi-Hui Zhong

Rui Li, Xian-Hua Liao, Jun-Zhao Ye, Min-Rui Li, Yan-Qin Wu, Xuan Hu, Bi-Hui Zhong, Department of Gastroenterology, The First Affiliated Hospital of Sun Yat-Sen University, Guangzhou 510080, Guangdong Province, China

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Correspondence to: Bi-Hui Zhong, MD, PhD, Department of Gastroenterology, The First Affiliated Hospital of Sun Yat-Sen University, No. 58 Zhongshan Road II, Guangzhou 510080, Guangdong Province, China. sophiazhong@medmail.com.cn
Telephone: +86-20-87755766
Fax: +86-20-87332916

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Abstract

AIM

To test the hypothesis that K8/K18 variants predispose humans to non-alcoholic fatty liver disease (NAFLD) progression and its metabolic phenotypes.

METHODS

We selected a total of 373 unrelated adult subjects from our Physical Examination Department, including 200 unrelated NAFLD patients and 173 controls of both genders and different ages. Diagnoses of NAFLD were established according to ultrasonic signs of fatty liver. All subjects were tested for population characteristics, lipid profile, liver tests, as well as glucose tests. Genomic DNA was obtained from peripheral blood with a DNeasy Tissue Kit. K8/K18 coding regions were analyzed, including 15 exons and exon-intron boundaries.

RESULTS

Among 200 NAFLD patients, 10 (5%) heterozygous

carriers of keratin variants were identified. There were 5 amino-acid-altering heterozygous variants and 6 non-coding heterozygous variants. One novel amino-acid-altering heterozygous variant (K18 N193S) and three novel non-coding variants were observed (K8 IVS5-9A→G, K8 IVS6+19G→A, K18 T195T). A total of 9 patients had a single variant and 1 patient had compound variants (K18 N193S+K8 IVS3-15C→G). Only one R341H variant was found in the control group (1 of 173, 0.58%). The frequency of keratin variants in NAFLD patients was significantly higher than that in the control group (5% *vs* 0.58%, P = 0.015). Notably, the keratin variants were significantly associated with insulin resistance (IR) in NAFLD patients (8.86% in NAFLD patients with IR *vs* 2.5% in NAFLD patients without IR, P = 0.043).

CONCLUSION

K8/K18 variants are overrepresented in Chinese NAFLD patients and might accelerate liver fat storage through IR.

Key words: Keratin; Variant; Non-alcoholic fatty liver disease; Insulin resistance; Chinese population

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Core tip: This study presents the first investigation of the association between keratin 8 and 18 (K8/K18) variants and non-alcoholic fatty liver disease (NAFLD) in a Chinese population. We found an increased frequency of variants in NAFLD patients *vs* controls. We also identified a new amino acid-altering variant of K18. The results demonstrate that keratin variants are overrepresented in Chinese NAFLD patients and might accelerate liver fat storage through insulin resistance.

Li R, Liao XH, Ye JZ, Li MR, Wu YQ, Hu X, Zhong BH. Association of keratin 8/18 variants with non-alcoholic fatty liver disease and insulin resistance in Chinese patients: A case-control study. *World J Gastroenterol* 2017; 23(22): 4047-4053 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i22/4047.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i22.4047

INTRODUCTION

Keratin (K) proteins form intermediate filaments (IFs) in epithelial cells, which constitute the cytoskeleton along with actin filaments and microtubules[1,2]. Keratin proteins are primarily expressed in epithelial tissues, hair and skin appendages, and they are classified into relatively acidic type Ⅰ (K9-K28, K31-K40) and relatively basic type Ⅱ keratins (K1-K8, K71-K86)[3,4]. Type Ⅰ and type Ⅱ keratins exist as paired polymeric filaments that display a tripartite structure containing a conserved α-helical central rod domain flanked by less conserved N-terminal head and C-terminal tail domains[3,5]. For example, K5/K14 and K1/K10 are found in basal and suprabasal keratinocytes, whereas K8/K18 are found in adult hepatocytes and other simple epithelial cells[6]. Moreover, human association studies have identified mutations in keratins that can cause or predispose carriers to a broad range of human diseases, including multiple liver diseases[1,7]. Finally, the importance of K8/K18 in protecting hepatocytes from apoptosis was clearly demonstrated in keratin-related genetically engineered animal models[6,8].

Non-alcoholic fatty liver disease (NAFLD), which is regarded as a hepatic manifestation of metabolic syndrome, has become the most common chronic liver disease worldwide, as it affects 20%-50% of the general population in affluent countries[9,10], and 15% of individuals in China[11]. NAFLD is strongly concomitant with obesity, hypertriglyceridemia, type 2 diabetes mellitus, and insulin resistance (IR)[12]. It is generally accepted that the initiating events in NAFLD depend on the development of obesity and IR in adipose tissue and the liver.

There are accumulating examples of K8/K18 involvement in the glucose-insulin cross-talk, which support the impact of K8/K18 IFs on insulin-dependent glucose metabolism regulation in the liver and its implication in glucose- or insulin-associated diseases[12,13]. Lower fasting glucose levels, increased glucose tolerance and insulin sensitivity, reduced glucose-stimulated insulin secretion and decreased pancreatic insulin content have been shown to occur in K8 knockout mice[14]. The mislocalization of glucose transporter (GLUT) and hexokinase (HK) status may contribute to the modulation of IFs in hepatocytes and hepatoma cells that lack K8/K18[14,15].

Subsequent studies have established that keratin mutation plays an important role in liver diseases and glucose metabolism. Therefore, we hypothesized that K8/K18 variants may contribute to susceptibility to NAFLD. No studies have been reported on the association between K8/K18 mutations and NAFLD. Therefore, we tested our hypothesis by sequencing the K8/K18 coding regions in genomic DNA from 200 NAFLD subjects. Herein, we report our positive findings.

MATERIALS AND METHODS

Subjects

A total of 373 unrelated adult subjects were selected from our Physical Examination Department from January 2011 to December 2012, including 200 unrelated Chinese patients of both genders and different ages (164 males, 36 females; mean age, 40.85 ± 9.9 years) and 173 healthy controls who were matched for sex and age (130 males, 43 females; mean age, 39.55 ± 9.9 years). Diagnoses of NAFLD were performed under standard clinical evaluation conditions by ultrasonography according to the AASLD

Table 1 Demographics and clinical information of non-alcoholic fatty liver disease patients and controls

	Controls (n = 173)	NAFLD patients (n = 200)	P value
Sex (male, %)	130 (75.14)	164 (82.00)	0.107
Age (yr)	39.55 ± 9.92	40.85 ± 9.89	0.101
ALT (U/L)	21.65 ± 12.69	43.63 ± 29.16	< 0.0001
AST (U/L)	24.74 ± 6.52	33.85 ± 12.93	< 0.0001
FBG (mmol/L)	4.89 ± 0.62	5.40 ± 1.45	< 0.0001
TC (mmol/L)	4.71 ± 0.77	5.68 ± 1.09	< 0.0001
TG (mmol/L)	1.29 ± 0.99	2.86 ± 2.14	< 0.0001
HDL (mmol/L)	1.22 ± 0.29	1.07 ± 0.23	< 0.0001
LDL (mmol/L)	2.80 ± 0.61	3.32 ± 0.95	< 0.0001
UA (μmol/L)	331.33 ± 88.61	412.42 ± 96.60	< 0.0001

Data are presented as mean ± SD. ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; FBG: Fasting blood glucose; TC: Total cholesterol; TG: Triglyceride; HDL: High-density lipoprotein; LDL: Low-density lipoprotein; NAFLD: Non-alcoholic fatty liver disease.

criteria[16]. Other causes of liver disease were excluded, including increased alcohol intake (> 210/140 g weekly for males/females), viral and autoimmune hepatitis, hereditary hemochromatosis, Wilson's disease and alpha1-antitrypsin deficiency. Controls were confirmed as healthy based on a medical history, general examinations and laboratory examinations at the same hospital. Each participant underwent an anthropometric assessment, including measurements of weight and stature. Body mass index (BMI) was calculated as weight (kg)/stature (m^2). All subjects received blood tests after an overnight 12 h fast, including serum triglycerides (TG), total cholesterol (TC), high-density lipoprotein (HDL), low-density lipoprotein (LDL), serum uric acid (UA), fasting blood glucose (FBG), fasting insulin (FIN), aspartate aminotransferase (AST), and alanine aminotransferase (ALT). IR was defined by the homeostasis model assessment-IR (HOMA-IR) index. As reported in an epidemiology survey carried out in China, IR was defined as HOMA-IR > 2.69 (*i.e.*, exceeding the 75% percentile of HOMA-IR in normal glucose tolerance subjects)[17,18].

Genomic DNA extraction and genotyping

Genomic DNA was obtained from peripheral blood (EDTA anticoagulation) using a DNeasy Tissue Kit (Tiangen, Biotech, Beijing, China). The entire K8/K18 coding regions (15 exons and their adjacent exon-intron boundaries) of the DNA fragments were analyzed, which had been amplified with a Touchdown polymerase chain reaction protocol using Premix PrimeSTAR HS (TaKaRa, Biotechnology, Dalian, China) and previously described primers[19] to obtain a high amplification specificity. Messenger RNA sequences of K8 (NM002273) and K18 (NM000224) were used to localize coding variants, while genomic sequences (hKRT8 [M34482] and hKRT18 [AF179904]) were employed for noncoding variants.

Statistical analysis

Statistical analyses were performed using SPSS statistical software, version 20.0 for Windows (SPSS Inc., Chicago, IL, United States). Continuous variables are expressed as mean ± SD. For continuous variables, a two-tailed t-test was used for two-group comparisons, whereas Kruskal-Wallis nonparametric one-way analysis of variance was used to compare qualitative variables. K8/K18 variant frequencies in the NAFLD patient and control groups were compared by the two-tailed Fisher exact probability test. P values less than 0.05 were considered statistically significant.

RESULTS

Demographics and characteristics of NAFLD patients

Demographics and clinical information for the NAFLD patients and control groups are summarized in Table 1. A total of 200 patients with NAFLD were included. As expected, most (82%) patients were male. Compared with the control group, NAFLD patients showed higher ALT, AST, FBG, TC, TG, LDL and UA levels and lower levels of HDL.

Keratin variants in NAFLD patients and controls

We identified keratin heterozygous variants in 10 (5%) of 200 NAFLD patients, including 5 carriers of amino-acid-altering heterozygous variants and 6 carriers of non-coding heterozygous variants (Table 2). Among 200 NAFLD patients, 2 (1%) carried R341H heterozygous exonic variants (Figure 1A), which is lower than the previously reported frequency. Additionally, one novel amino acid-altering heterozygous variant (K18 N193S) and three novel non-coding variants (K8 IVS5-9A→G, K8 IVS6+19G →A, K18 T195T) were observed (Figure 1B). A total of 9 patients had a single variant and 1 patient had compound variants (K18 N193S+K8 IVS3-15C→G). A previously described silent and common heterozygous K8 L227L variant, which we detected in 43% of patients (not shown), is not included in Table 2 or any subsequent analysis.

Keratin variant is associated with biochemical parameters in patients with NAFLD

We found an increased frequency of variants in NAFLD patients *vs* controls (5% *vs* 0.58%, P = 0.015). To explore whether K8/K18 variants could affect the biochemical parameters, we compared non-carriers with variant carriers in all NAFLD patients. We found that there was no significant difference in clinical features, including BMI, liver biochemistry, glucose, lipids or HOMA-IR (Table 3). However, after dividing patients into those with or without IR, keratin variants showed a significant association with IR in NAFLD patients (Table 2; 8.86% in NAFLD patients with IR *vs* 2.5% in NAFLD patients without IR, P = 0.043).

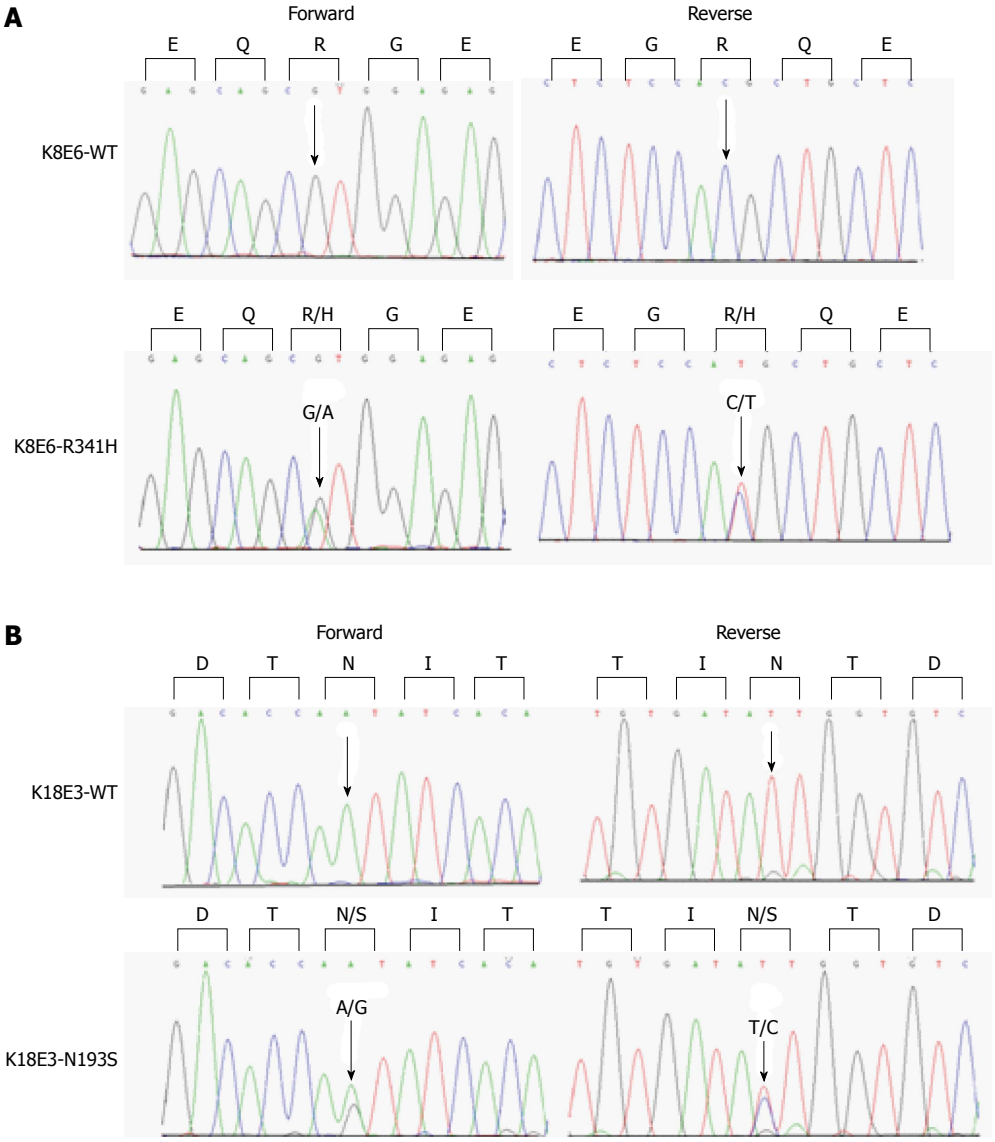

Figure 1 Detection of keratin variants in non-alcoholic fatty liver disease patients and controls. Genomic DNA was sequenced in the forward and reverse directions. The nucleotide locations of the variants were determined as described in "Patients and Methods". The heterozygous K8 exon 6-R341H (A) and novel heterozygous variant K18 exon 3-N193S (B) are presented in comparison with their corresponding wild type (WT) samples.

DISCUSSION

Previous reports suggested that K8/K18 variants predispose carriers to various types of liver injury, but it is not known whether K8 or K18 variants show an association with NAFLD. For the first time, our present study investigated the association between K8/K18 variants and NAFLD in a Chinese population. We analyzed K8/K18 variants in NAFLD patients and observed the potential association of keratin variants with NAFLD.

Our results demonstrate that keratin variants are overrepresented in NAFLD patients. Moreover, K8 has the most keratin variants, which is consistent with data from patients with chronic hepatitis C, primary biliary cirrhosis and acute liver failure. This finding highlights the importance of *K8/K18* gene variants in NAFLD. The frequency of keratin variants presented here is somewhat lower compared with those in patients who suffer from other types of liver disease (*e.g.*, 13.1% and 12.4%[20,21]), as previously reported, but it is close to that observed in an Asian cohort in a United States study[22]. Multiple K8/K18 variants have been verified and R341H represents the most common amino acid-altering K8/K18 variant in Chinese populations. As confirmed herein, the K8 variant R341H also exclusively associates with the intronic K8 IVS7+10delC deletion[19]. We also found a new amino acid-altering variant N193S of K18 in a patient who simultaneously carried an intronic variant IVS3-15C→G. It remains to be determined whether this nucleotide error at one site acts in conjunction with the other.

Hepatic steatosis occurs when insulin signaling is impaired, with the development of IR driven by adipose tissue and the liver, along with the sustained excess delivery of fatty acids to the liver. Here, we

Table 2 Distribution of keratin variants in non-alcoholic fatty liver disease patients and controls

Keratin gene	Variant		NAFLD			
	Nucleotide	Amino acid	Control (n = 173)	Total (n = 200)	NAFLD with IR (n = 79)	NAFLD without IR (n = 121)
K8	1022G→A	R341H	1	2	2	0
	904C→T	R302C	0	1	1	0
	1381G→A	V461M	0	1	0	1
	IVS5-9A→G (new)		0	1	1	0
	IVS3-26 C→T		0	1	1	0
K18	IVS6+19G→A (new)		0	1	1	0
	IVS6+17C→T		0	1	0	1
	IVS3-15C→G		0	1[1]	1[1]	0
	1590C→G	N193S (new)	0	1[1]	1[1]	0
	1448A→G	T195T (new)	0	1	0	1
Total (%)			1 (0.58)	10 (5.00)	7 (8.86)	3 (2.48)

[1]One patient carries two K8/K18 variants (K18 N193S+K8 IVS3-15C→G). The table displays the number of patients with NAFLD and controls harboring the listed keratin variants. IR: Insulin resistance; NAFLD: Non-alcoholic fatty liver disease.

Table 3 Clinical features of non-alcoholic fatty liver disease patients harboring significant keratin variants

	Keratin variant carriers		*P* value
	No (n = 190)	Yes (n = 10)	
Sex (male, %)	155 (81.58)	9 (90.00)	0.436
Age (yr)	40.75 ± 9.87	42.60 ± 10.63	0.603
Diastolic pressure (mmHg)	82.67 ± 10.47	85.80 ± 9.38	0.330
Systolic pressure (mmHg)	130.79 ± 14.82	134.20 ± 17.07	0.550
BMI (kg/m^2)	26.26 ± 2.91	26.10 ± 3.09	0.877
ALT (U/L)	35.93 ± 18.35	38.40 ± 25.30	0.767
AST (U/L)	34.03 ± 13.08	30.40 ± 9.31	0.265
TC (mmol/L)	5.67 ± 1.02	6.10 ± 1.69	0.453
TG (mmol/L)	2.85 ± 2.14	3.00 ± 2.35	0.850
HDL (mmol/L)	1.09 ± 0.24	1.10 ± 0.18	0.828
LDL (mmol/L)	3.31 ± 0.94	3.65 ± 1.12	0.374
FBG (mmol/L)	5.38 ± 1.43	5.91 ± 1.44	0.281
FINS (mmol/L)	11.17 ± 4.52	12.73 ± 6.00	0.439
HOMA-IR	2.64 ± 1.14	3.25 ± 1.50	0.237
UA (μmol/L)	414.06 ± 96.89	381.50 ± 89.88	0.292

Data are presented as mean ± SD. BMI: Body mass index; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; TC: Total cholesterol; TG: Triglyceride HDL: High-density lipoprotein; LDL: Low-density lipoprotein; FBG: Fasting blood glucose; FINS: Fasting insulin; UA: Uric acid.

observed a correlation of IR with keratin variants. This association suggests that K8/K18 mutations result in increased liver fat, probably through the hepatic IR, in accordance with other studies. For example, early work using K8-null mice found reduced fasting blood glucose levels, increased glucose tolerance and insulin sensitivity, altered insulin vesicle morphology, and reduced pancreatic insulin levels[14]. Similarly, a study conducted in Finland shows that K8/K18 loss in hepatoma cells leads to distinct alterations in HK status, which are associated with a differential modulation of insulin signaling-dependent regulation of glucose-mediated glycogen formation and proliferative capacity[23]. However, differences in K8/K18 IF loss cause the mislocalization of the GLUT 1-4 transport proteins. Additionally, it is important to consider the cellular context, namely, cultured hepatic cells[23] *vs in vivo* embryonic[24] conditions. This reveals distinctive increases in glucose uptake, glucose-6-phosphate formation, lactate release, and glycogen formation in K8/K18 IF- deficient hepatocytes *vs* respective IF-containing counterparts. Moreover, compelling evidence suggests that the shape and distribution of mitochondria are modulated by cytoskeletal proteins, including keratins[25]. Together, these findings suggest that hepatocyte keratins have a central role in the systemic regulation of glucose. Although the molecular mechanisms that underlie these metabolic perturbations are unclear, K8/K18 IFs likely modify liver fat content via the insulin signaling pathway[26,27].

This study was limited by the absence of liver biopsies to evaluate the severity of hepatic steatosis and fibrosis stage. The absence of histological evidence may weaken the clinical relevance of these genetic effects, although abdominal ultrasound is generally applied in larger surveys as a noninvasive and convenient tool to diagnose NAFLD[12]. Moreover, cell-based and transgenic mouse studies with genetic K8/K18 mutants or knockouts are needed to verify that keratin variants can cause or predispose carriers to the development of pathologies.

As studies of Asian populations have been limited, we are the first to discuss an association between keratin variants and NAFLD. Our findings provide novel evidence for keratin proteins as modulators in the development of NAFLD and in the pathophysiology of IR. Further studies of K8/K18 mutations as drivers of IR and liver fat accumulation are needed. These observations raise the question of how naturally occurring keratin mutations affect liver fatty in humans, and whether such mutations may similarly affect *in vitro* models or animals.

COMMENTS

Background

Keratins 8 and 18 (K8/K18) protect hepatocytes from various types of injury, and previous reports have suggested that K8/K18 variants predispose carriers

to various forms of liver injury. However, it is not known whether K8 or K18 variants have an association with non-alcoholic fatty liver disease (NAFLD). Thus, we tested the hypothesis that K8/K18 variants predispose humans to NAFLD progression and its metabolic phenotypes.

Research frontiers

The relationship between keratin variants and liver disease varies in different populations. Therefore, studies enrolling histological evidence and more ethnic groups are required. Further studies are also needed to determine the associations and functional consequences of these variants in NAFLD.

Innovations and breakthroughs

This is the first published study to investigate the relationship between keratin variants and NAFLD. These findings also provide novel evidence for keratin proteins as modulators in the development of NAFLD and in the pathophysiology of insulin resistance.

Applications

The association of keratin variants with NAFLD suggests a prominent gene influence on human NAFLD. They should pay more attention to individuals who carries keratin variant, and provide early intervention before disease progression.

Terminology

Keratins constitute the largest subgroup of intermediate filaments and are expressed primarily in epithelial tissues, hair and skin appendages. NAFLD is the most common cause of liver dysfunction in the Western world. It is a spectrum of liver disease that includes simple steatosis, fatty infiltration plus inflammation, and hepatocellular ballooning degeneration, progressing to fibrosis and ultimately cirrhosis.

Peer-review

This is a very interesting human study that suggests an association between keratin 8/18 variants and NAFLD. Also the authors successfully showed that IR is a potential mediator of this association. These are completely novel viewpoints in NAFLD research. This study is a good start for future research in this field.

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ORIGINAL ARTICLE

Retrospective Study

Barcelona Clinic Liver Cancer outperforms Hong Kong Liver Cancer staging of hepatocellular carcinoma in multiethnic Asians: Real-world perspective

James Weiquan Li, Boon-Bee George Goh, Pik-Eu Chang, Chee-Kiat Tan

James Weiquan Li, Boon-Bee George Goh, Pik-Eu Chang, Chee-Kiat Tan, Department of Gastroenterology and Hepatology, Singapore 169856, Singapore

Boon-Bee George Goh, Pik-Eu Chang, Chee-Kiat Tan, Duke-NUS Medical School, 8 College Road, Singapore 169857, Singapore

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Correspondence to: Chee-Kiat Tan, MBBS, MRCP, Associate Professor, Department of Gastroenterology and Hepatology, Level 3, Academia, 20 College Road, Singapore 169856, Singapore. tan.chee.kiat@singhealth.com.sg
Telephone: +65-62223322
Fax: +65-62273623

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Abstract

AIM

To compare the Barcelona Clinic Liver Cancer (BCLC) and Hong Kong Liver Cancer (HKLC) classification systems when applied to HCC patients from the largest tertiary-level centre in Singapore.

METHODS

One thousand two hundred and seventy hepatocellular carcinoma (HCC) patients prospectively enrolled in a tertiary-level centre registry in Singapore since 1988 were studied. Patients were grouped into their respective BCLC and HKLC stages. Data such as demography, aetiology of HCC and type of treatment were collected. Survival data was based on census with the National Registry of Births and Deaths on 31st October 2015. Statistical analyses were done using SPSS version 21 (Chicago, IL, United States). Survival analyses were done by the Kaplan-Meier method. Differences in survival rates were compared using the log-rank test.

RESULTS

The median age at presentation was 63 years (range 13-94); male 82.4%; Chinese 89.4%, Malay 7.1%, Indian, 2.8%. Hepatitis B was the predominant aetiology (75.0%; Hepatitis C 7.2%, Hepatitis B and C co-infection 3.8%, non-viral 14.0%). Both BCLC and HKLC staging systems showed good separation with overall log rank test confirming significant survival differences between stages in our cohort ($P < 0.001$). 206 out of the 240 patients (85.8%) assigned for curative treatment by the BCLC treatment algorithm received curative therapy for HCC [Stage 0 93.2% (68/73); Stage A 82.6% (138/167)]. In contrast, only 341/558 (61.1%) patients received curative treatment despite being assigned for curative treatment by the HKLC treatment algorithm [Stage Ⅰ 72.7% (264/363); Stage Ⅱ 40.2% (66/164); Stage Va 35.5% (11/31)]. Patients who were assigned to curative treatment by HKLC but did not receive curative treatment had significantly poorer ECOG ($P < 0.001$), higher Child-Pugh status ($P < 0.001$) and were older (median age 66 *vs* 61, $P < 0.001$) than those who received curative therapy. Median overall survival in patients assigned to curative treatment groups by BCLC and HKLC were 6.1 and 2.6 years respectively ($P < 0.001$). When only patients receiving curative treatment were analyzed, BCLC still predicted overall median survival better than HKLC (7.1 years *vs* 5.5 years, $P = 0.037$).

CONCLUSION

BCLC performs better than HKLC in our multiethnic Asian population in allocating patients to curative treatment in a real-life situation as well as in predicting survival.

Key words: Hepatocellular carcinoma; Barcelona Clinic Liver Cancer; Hong Kong Liver Cancer; Staging systems; Prognosis; Survival

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Core tip: This is a retrospective study of Barcelona Clinic Liver Cancer (BCLC) and Hong Kong Liver Cancer (HKLC) staging systems when applied to a multiethnic Asian cohort, where Chinese ethnicity and hepatitis B aetiology are predominant. BCLC was more accurate in directing therapy, with a significantly higher proportion of patients assigned to curative therapy receiving the recommended curative treatment (85.8% *vs* 61.1%, $P < 0.001$). Median overall survival in patients assigned to curative therapy by the BCLC and HKLC staging systems was 6.1 and 2.6 years respectively ($P < 0.001$). Thus, overall, BCLC performed better than HKLC for staging our cohort of patients.

Li JW, Goh BBG, Chang PE, Tan CK. Barcelona Clinic Liver Cancer outperforms Hong Kong Liver Cancer staging of hepatocellular carcinoma in multiethnic Asians: Real-world perspective. *World J Gastroenterol* 2017; 23(22): 4054-4063 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i22/4054.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i22.4054

INTRODUCTION

Hepatocellular carcinoma (HCC) is the second leading cause of cancer-related death in men and the sixth leading cause of cancer death in women worldwide[1]. HCC in the context of a cirrhotic patient is unique as many factors have an impact on the type of treatment modality suitable for the individual patient, which in turn influence patient survival. These include tumor-related factors such as tumor size and extent (number and size of lesions) as well as patient-related factors such as underlying liver function and performance status (PS). The Barcelona Clinic Liver Cancer (BCLC) staging system has been widely used since its inception for this purpose[2], and is the preferred approach by many clinicians for more than a decade due to its treatment recommendations based on stage and its ability to offer predictions on patient survival[3,4]. More recently, the Hong Kong Liver Cancer (HKLC) staging system[5] was developed. Like the BCLC staging system, it also incorporates PS, underlying liver function and tumor stage in its treatment recommendations.

Two main criticisms of the BCLC staging system are that it was derived from a relatively small cohort of patients and that it was derived from a predominantly Western population. This has resulted in a limited applicability in some settings[6,7]. In comparison, the HKLC staging system was developed from a larger cohort of patients with predominantly viral etiology, in particular hepatitis B. The HKLC staging system is arguably more aggressive in its treatment recommendations, assigning intermediate stage tumors (for example HKLC stage IIb) to curative treatment, whereas these patients would have been assigned to palliative treatment under the BCLC staging system. Intuitively, more aggressive treatment of HCC will lead to better survival rates for patients stratified by the HKLC staging system. However, this hypothesis requires external validation, and there are limited large scale studies comparing the two staging systems in terms of prognostication and treatment recommendations[8].

Singapore is a multiethnic Asian country where HCC is the fourth most common cancer in males and the second most fatal cancer[9]. Most clinicians have adopted the BCLC staging system as it is more well-established[10], although the predominant etiology of HCC in Singapore is hepatitis B. The aim of our study was to compare the abilities of the BCLC and HKLC staging systems to correctly assign patients to curative

treatment groups in real life and to prognosticate survival when applied to patients with HCC in Singapore.

MATERIALS AND METHODS

Patients

One thousand two hundred and seventy patients with HCC seen in the Department of Gastroenterology and Hepatology in the Singapore General Hospital, a tertiary-level medical center in Singapore, were studied. These patients were prospectively enrolled into a HCC registry since January 1988. The patients were enrolled upon diagnosis of HCC and were treatment-naïve at the time of enrollment. These patients were classified according to the BCLC and HKLC staging systems (Figure 1A and B). Patients assigned to the various tumor stages according to the BCLC and HKLC algorithms were divided into those who received treatment as recommended by the respective staging systems and those who did not.

The study protocol was approved by the Institutional Review Board and was conducted in accordance with the Declaration of Helsinki.

Data collection

Data of patients enrolled in the HCC registry were prospectively collected. Patient characteristics include age at diagnosis, gender, etiology, PS by Eastern Cooperative Oncology Group (ECOG) stage, and Child Pugh status. Tumor characteristics such as number of lesions, size of individual lesions, presence of vascular invasion and extra-hepatic spread were collected. In addition, treatment modalities received by the patient such as surgical resection, radiofrequency ablation, liver transplantation, transarterial chemoembolization (TACE), and TACE with drug-eluding beads were also captured in the HCC registry. Survival census with the Singapore National Registry of Births and Deaths was performed on 31st October 2015.

Statistical analysis

Statistical analyses were done using SPSS version 21 (Chicago, IL, United States). Patients were grouped into the different stages according to the BCLC and HKLC staging systems. Survival rates for patients assigned to curative treatment by the respective staging systems were compared. Survival analyses were done by the Kaplan-Meier method. Differences in survival rates were compared using the log-rank test.

RESULTS

The median age at presentation of HCC was 63 years (range 13 to 94 years). 82.4% of the patients were male, and the majority were of Chinese ethnicity (89.4%), followed by Malays (7.1%), Indians (2.8%), and other ethnic groups (0.7%). Hepatitis B was the predominant etiology, accounting for 75% of cases.

Table 1 Baseline characteristics *n* (%)

Variable	All Patients (*n* = 1270)
Age, yr, median (range)	63 (13-94)
Male	1046 (82.4)
Ethnicity	
Chinese	1135 (89.4)
Malay	90 (7.1)
Indian	36 (2.8)
Others	9 (0.7)
Aetiology	
Hepatitis B	953 (75.0)
Hepatitis C	91 (7.2)
Hepatitis B and C co-infection	48 (3.8)
Others	178 (14.0)
Child-Pugh class	
A:B:C	641 (50.5): 452 (35.6): 177 (13.9)

7.2% of HCC cases had hepatitis C as the etiology, while hepatitis B and C co-infection comprised 3.8% of patients. Non-viral etiologies accounted for 14% of the patients. These findings, together with the Child Pugh class, are summarized in Table 1.

Both the BCLC and HKLC staging systems showed good differentiation in survival between the various stages, with the overall log-rank test showing significant survival differences between the stages (Figure 2A and B, respectively).

Survival based on assignment to curative treatment in the BCLC staging system

240 out of 1270 patients (18.9%) were assigned to curative treatment by the BCLC algorithm (Stage 0 5.7%, n = 73; Stage A 13.2%, n = 167). Within this group of patients, 206 out of the 240 patients (85.8%) received curative treatment, while the remaining patients (14.2%, n = 34) did not receive curative treatment despite being assigned as such. Between the BCLC stages recommending curative treatment, a higher proportion of patients in Stage 0 [93.2% (68/73)] received curative treatment compared to those assigned to Stage A [82.6% (138/167)], $P < 0.05$ (Figure 3).

Patients who received the recommended curative treatment according to their respective assignments had a better median survival than those who did not (7.1 years *vs* 1.1 years, $n < 0.001$). This observation was consistent when the survival analysis was applied to individual BCLC stage 0 and stage A.

Survival based on assignment to curative treatment in the HKLC staging system

In contrast to the BCLC staging system, more patients (43.9%, n = 558) in our study were assigned to curative treatment by the HKLC algorithm (Stage I 28.6%, n = 363; Stage II 12.9%, n = 164; Stage Va 2.4%, n = 31). Of these 558 patients, 341 (61.1%) received curative treatment as recommended by HKLC algorithm, while the remaining patients did not receive

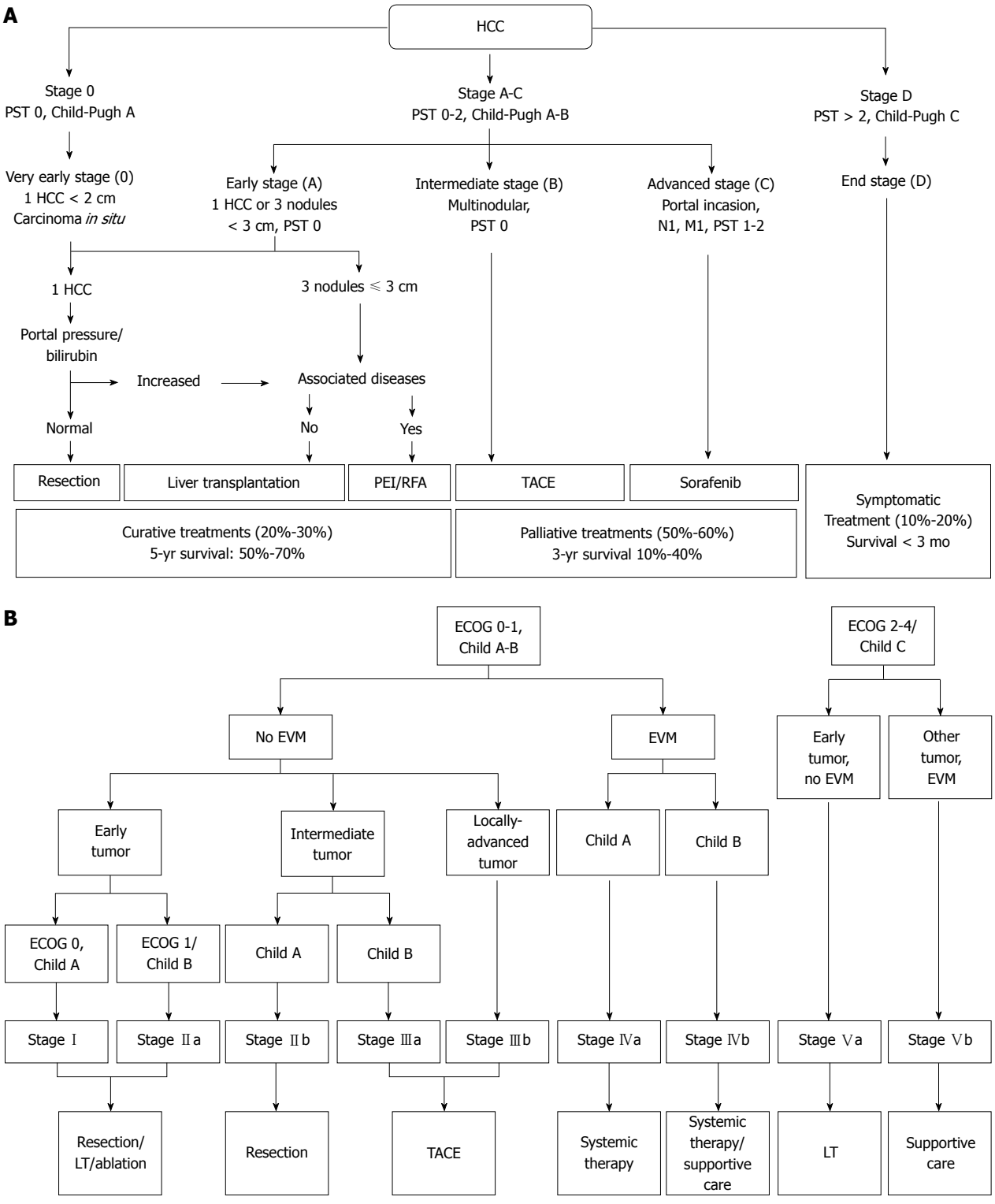

Figure 1 Barcelona Clinic Liver Cancer and Hong Kong Liver Cancer. A: Barcelona Clinic Liver Cancer (BCLC) staging system[2]; B: Hong Kong Liver Cancer (HKLC) staging system[5].

curative treatment. Within these HKLC stages, 264 out of 363 patients (72.7%) in HKLC Stage Ⅰ received curative treatment, compared to 66 out of 164 patients (40.2%) in HKLC Stage Ⅱ and 11 out of 31 patients (35.5%) in HKLC Stage Va (Figure 3).

Patients assigned to curative treatment by the HKLC staging system but who did not receive the recommended treatment were significantly older (median age 66 years *vs* 60 years, $P < 0.001$), had significantly poorer ECOG status ($P < 0.001$), and were also of poorer Child-Pugh status ($P < 0.001$) at the time of diagnosis of HCC, compared to patients

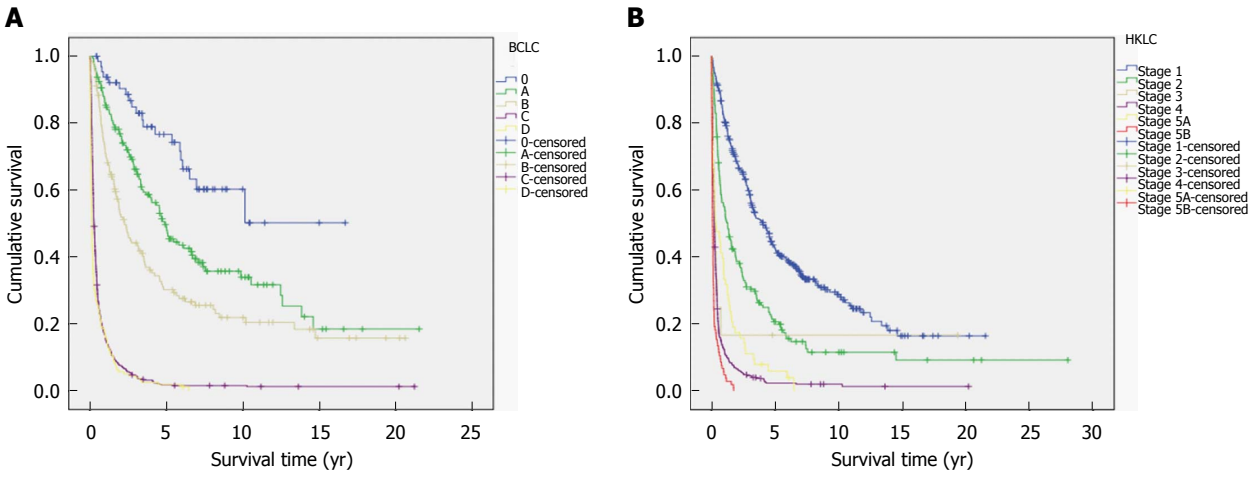

Figure 2 Kaplan-Meier survival curve. A: Kaplan-Meier survival curve according to Barcelona Clinic Liver Cancer (BCLC) staging system; B: Kaplan-Meier survival curve according to Hong Kong Liver Cancer (HKLC) staging system.

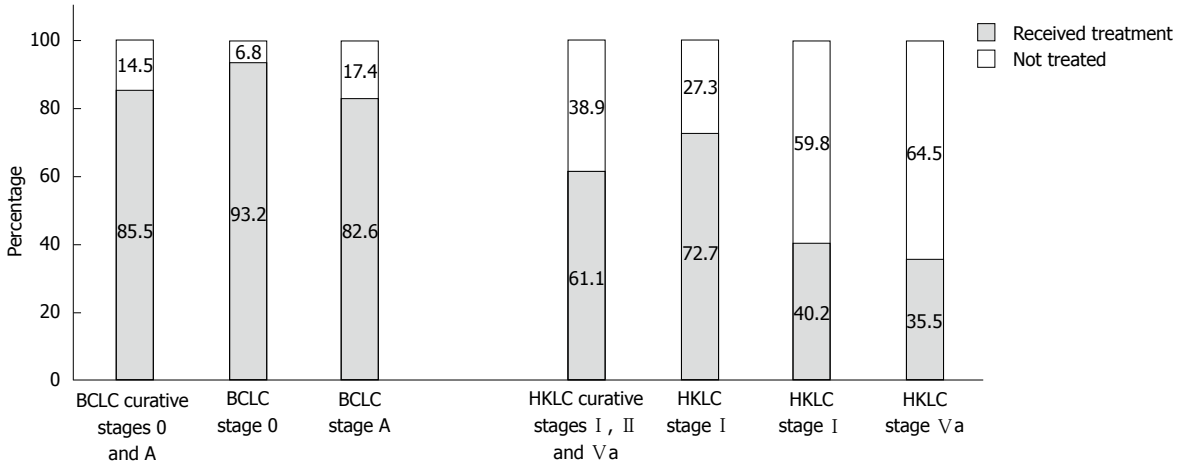

Figure 3 Patients receiving curative treatment after assignment to curative treatment groups by Barcelona Clinic Liver Cancer and Hong Kong Liver Cancer staging systems.

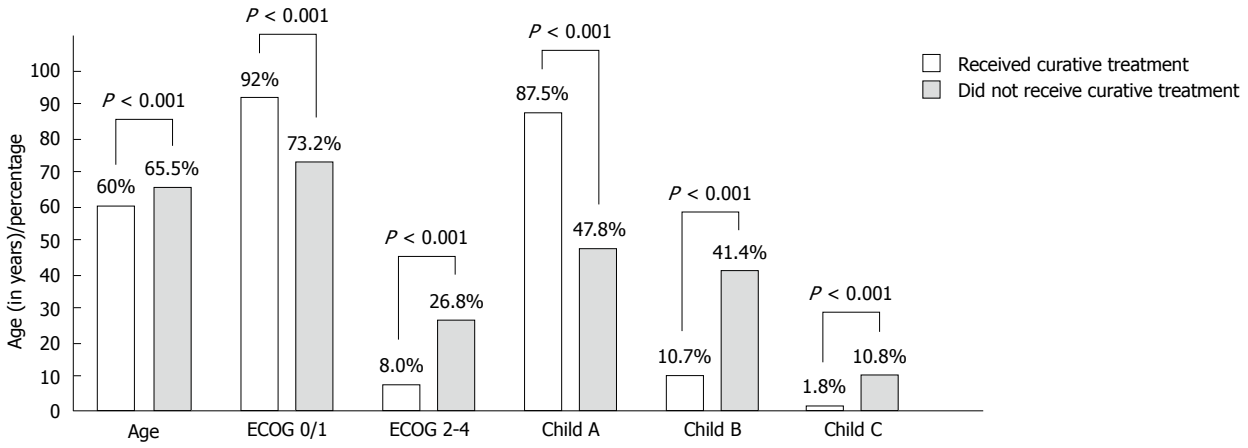

Figure 4 Comparison of characteristics of patients assigned by Hong Kong Liver Cancer staging system to curative treatment that did and did not receive recommended treatment.

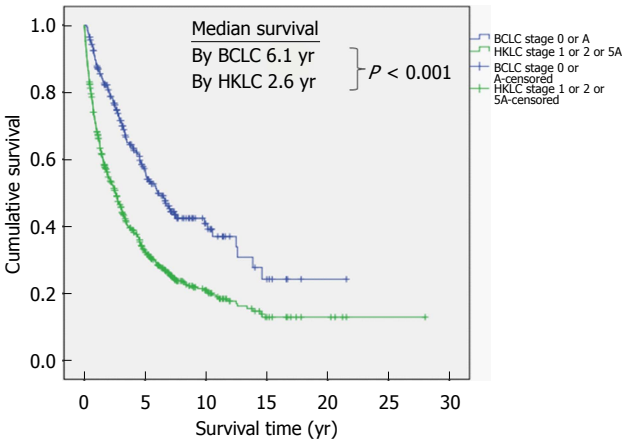

Figure 5 Median survival of patients assigned to curative treatment by Barcelona Clinic Liver Cancer and Hong Kong Liver Cancer staging systems.

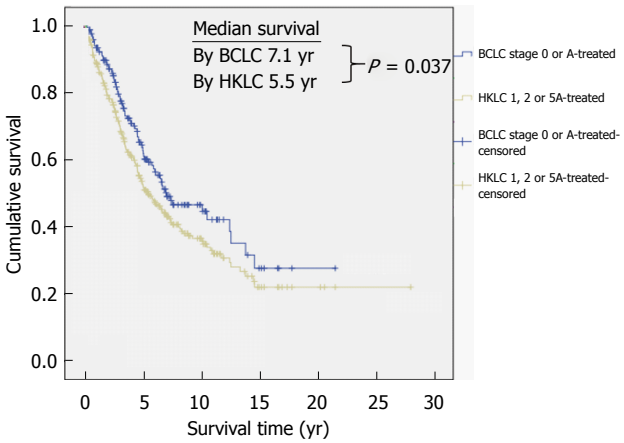

Figure 6 Median survivals of patients who received the assigned curative treatment according to the Barcelona Clinic Liver Cancer and Hong Kong Liver Cancer staging systems.

within the same group who received the recommended curative treatment (Figure 4).

Prediction of survival based on the BCLC and HKLC staging systems for patients assigned to curative treatment groups

Median overall survival in patients assigned to curative treatment by the BCLC and HKLC staging systems were 6.1 and 2.6 years respectively ($P < 0.001$) (Figure 5). Among patients who received the recommended curative treatment by the respective staging systems, the BCLC system also predicted better overall survival than the HKLC system (7.1 *vs* 5.5 years respectively, $P = 0.037$) (Figure 6).

DISCUSSION

HCC treatment in the setting of liver cirrhosis is not always straightforward, given the need to consider the underlying liver function and ECOG status of the patient in addition to tumor extent to determine the most appropriate treatment modality. Both the BCLC and HKLC staging systems were developed in different cohorts with the intention of taking all these factors into account when recommending the most appropriate therapy for HCC. Besides the different ethnicities and HCC etiologies of the populations upon which the BCLC and HKLC staging systems were derived, the BCLC staging system has often been criticized for being too heterogeneous in its definition of stage B as well as being overly conservative[11,12]. This study adds further validation to both staging systems, as evidenced by the good separation of the survival curves when applied to our cohort of patients. In particular, it validates the BCLC system for prognostication of a multiethnic Asian HCC population with hepatitis B as the predominant etiology, a finding seen in few other studies to date[13].

Our study addressed the limitation of patients not receiving the treatment recommended. We examined the survival separately in patients who received and in those who did not receive the recommended curative treatment. We found significantly better survival in patients receiving the recommended curative treatment compared to those who did not despite assignment to the same stage in both staging systems. This demonstrated the validity of the curative treatment recommendations by both BCLC and HKLC staging systems in our population.

Of note, a significantly higher proportion of patients in our study were assigned to curative treatment by the HKLC staging system compared to BCLC (43.9% *vs* 18.9% respectively, $P < 0.01$). This is explained by the more aggressive approach by the HKLC staging system in the assignment of patients to curative therapy. For instance, the BCLC staging system utilizes PS as a veto factor to determine if a patient with HCC should be assigned to undergo curative treatment, and this has been criticized as one of its weaknesses. In contrast, the HKLC staging system collapses ECOG 0 and 1 patients into a single entity. In patients with early tumor, Child-Pugh B liver cirrhosis is also not a barrier to curative treatment. This effectively increases the pool of HCC patients being considered for curative treatment under HKLC staging, who would otherwise not receive such an assignment by the BCLC algorithm. These findings are similar to a recent study conducted in a separate Singapore center, where 57.3% (439/766) of patients were classified by HKLC into stages I, II and Va, while only 38.5% (295/766) were classified by BCLC into stages 0 and A[14].

However, despite the assignment, a significantly lower proportion of patients in our cohort classified by the HKLC staging system to receive curative therapy actually received the recommended curative treatment compared to BCLC (61.1% *vs* 85.8%, $P < 0.001$). This highlights important practical limitations in the application of the HKLC system in a real world setting.

Certain forms of curative therapy, such as surgical resection, carry significant risk and morbidity, especially in patients with poorer ECOG status and more advanced cirrhosis. We postulate that this influenced both the managing physician's ultimate recommended treatment option and the patient's reluctance to accept treatment risks, resulting in a discrepancy between patients being assigned to curative treatment and those who actually received the recommended treatment. This is supported by the finding that patients who did not receive the recommended treatment were of older age, poorer ECOG status and worse Child-Pugh status within the patients assigned to curative treatment by the HKLC staging system. In addition, HKLC stage Va is curative liver transplantation therapy. Our liver transplantation program was set up only in 2014 and it was a small program in terms of numbers of transplantation done. Thus this also accounted for the less than expected patients receiving the assigned curative therapy. Nevertheless, even if we excluded the 31 patients who were assigned to HKLC stage Va (liver transplantation), there is still a significantly lower proportion of patients receiving curative treatment as recommended by HKLC staging compared to those similarly staged by BCLC (330/527 (62.6%) *vs* 206/240 (85.8%), $P < 0.001$).

At first glance, this difference in the proportion of patients receiving the curative treatment recommended by the respective staging systems could explain the significant differences in median overall survival between patients in the BCLC and HKLC curative stages (6.1 years and 2.6 years, $P < 0.001$). However, even when we looked only at patients who received curative treatment, patients assigned by BCLC still had significantly better median survival than HKLC (7.1 years *vs* 5.5 years, $P = 0.037$). This calls for further studies comparing the BCLC and HKLC staging systems to determine not just the prognostic predictability of the stages in each system, but the accuracy of the assignments as well.

In a review article by Maida *et al*[7], studies investigating the performance of different HCC staging systems then available in the literature were compared. The general trend was that staging systems developed in Western centers performed better when applied to Western populations. In particular, the BCLC staging system was the best prognostic model in studies conducted in Italy[15-18], Spain[19] and the United States[20]. These studies compared the BCLC against other staging systems, including those from Asia such as the Okuda staging system. Indeed, the converse was also true, as staging systems derived from Asian populations tended to be the best prognostic model when tested in cohorts from Asian countries[21-23]. The HKLC staging system represents a staging system developed from a large Asian population. Since its development, there have been studies evaluating its validity. Similar to the trends illustrated HKLC tends to perform better in Asian populations[24,25]. This was partly attributed to the predominance of hepatitis B in these Asian cohorts, as reflected in the study by Liu *et al*[25] that showed HKLC had better prognostic accuracy and therapeutic efficacy in hepatitis B-related HCC but not to hepatitis C-related HCC. Similarly, a study conducted by Adhoute *et al*[26] across two French centers, where hepatitis C and alcohol were the predominant etiologies, failed to show a better predictive value of HKLC compared to BCLC staging. In contrast, our study shows that the BCLC system is a better prognostic model than the HKLC system even though our population was of Asian ethnicity with predominantly hepatitis B-related HCC (Table 1).

A more recent study by Kim *et al*[8] looked individually at the BCLC and HKLC staging systems in a treatment-naïve Korean cohort, with the aim of investigating if survival is better if patients followed the recommended treatment by the respective staging systems. The findings in this study concurred with our study on 2 aspects: (1) the HKLC staging system could not direct therapy for a large proportion of patients; and (2) survival was better in general when patients in the early stages of either staging system received the recommended treatment, that is, curative therapy for HCC. However, our findings differ in that the BCLC staging system was able to direct curative treatment accurately for the vast majority (85.8%) of patients assigned to curative therapy groups, leading to drastically improved median survival times as demonstrated in Figure 5. This is in contrast to the findings by Kim *et al*[8], where only 49.5% and 55.6% of the population studied received the recommended therapy by BCLC and HKLC respectively. Moreover, our study compared the median survival of patients between the 2 staging systems when they were assigned to curative therapy, and further analyzed this trend when only patients who actually received the recommended curative treatment by either staging system were studied (Figure 6). In both instances, the BCLC staging system proved to be superior in predicting survival.

Our study has several limitations. Firstly, given the retrospective nature of this study, it was not possible to determine the exact reason why patients did not adhere to the treatments recommended by the BCLC and HKLC staging systems. We attempted to elucidate possible causes by examining differences in age, ECOG and Child-Pugh status between patients receiving and not receiving recommended curative treatment as assigned by the HKLC staging system. However, this cannot accurately pinpoint if the discrepancy was due to hesitancy on the part of the physicians to follow the treatment recommendations due to the clinical condition of the patient or patients declining curative treatment for other reasons, such as an

unwillingness to accept the risks, financial constraints or social considerations. Moreover, it was also not possible to tell if other co-morbidities precluded certain curative treatment options, for example a patient with significant pulmonary hypertension or valvular heart disease would be a high risk candidate for surgery but still have a good ECOG and Child-Pugh status at the time of diagnosis of HCC. This was also the conclusion in the study by Selby *et al*[14], where nearly half of the patients did not follow treatment recommendations by either staging system due to patients' personal and physicians' professional decision. Secondly, therapies have evolved and so have the techniques and experience of physicians, surgeons and interventional radiologists over the three decades of our HCC registry. This could mean that the morbidity and mortality from the same treatment diminishes with time, given improvement in techniques and experience of the managing multidisciplinary team over the years, making accurate comparisons in survival difficult.

The strengths of this study are a large population of HCC patients that are well-characterized and studied over a long follow-up period. In addition, the survival data was robust as it is based on census by a national registry of deaths.

In summary, our study showed that the BCLC staging system performed better in predicting overall survival compared to the HKLC staging system in our cohort. This difference could be explained by the limitations in applying the more aggressive HKLC treatment recommendations in real life, as a lower proportion of patients than otherwise predicted by HKLC staging eventually received curative treatment. Moreover, the differences in survival when the survival analysis was performed according to patients receiving the recommended curative treatment highlight the need for continued refinement of these staging systems to ensure patients are appropriately directed to curative therapy, especially as new treatment modalities evolve and our collective experience in treating HCC increases.

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COMMENTS

Background

Hepatocellular carcinoma (HCC) is one of the leading causes of cancer-related mortality worldwide. It is important to accurately stage tumours according to patient characteristics, liver function and tumour extent, to determine the appropriate therapy. Both the Barcelona Clinic Liver Cancer (BCLC) and and newer Hong Kong Liver Cancer (HKLC) staging systems were developed for this purpose, although there are significant differences in the cohorts from which they were derived and the variations in treatment recommendations for different stages, in particular intermediate tumours. There have been limited large scale studies comparing the two staging systems in terms of their abilities to assign treatment recommendations accurately and for prognosis of survival.

Research frontiers

The BCLC staging system has been criticised for being too heterogeneous in its definition of Stage B, as well as being overly conservative in its treatment recommendations. In contrast, the newer HKLC staging system offers a finer stratification into 5 main stages with Stages II to V having 2 sub-stages each, the treatment recommendations being tied closely to the 9 eventual stages from this algorithm. It is also arguably more aggressive in its curative treatment recommendations. With a more detailed algorithm derived from a larger cohort of patients and more aggressive treatment recommendations which have taken into account advances made in HCC research since the introduction of the BCLC staging system, it is intuitive to expect that the HKLC staging system will lead to better survival rates. However, this hypothesis still requires external validation. This study aimed to compare the abilities of the BCLC and HKLC staging systems to correctly assign patients to curative treatment groups in real life and to prognosticate survival when applied to patients with HCC from a multi-ethnic population with hepatitis B as the predominant etiology in the largest tertiary-level centre in Singapore.

Innovations and breakthroughs

The general trend from published studies investigating prognostic models in HCC shows that staging systems developed in Western centres tend to perform better when applied to Western study populations. The converse is also true, with more recent studies showing that HKLC tends to perform better in Asian populations but not necessarily in Western populations. This was partly attributed to the predominance of hepatitis B as the underlying etiology in Asian populations, which corresponds to the study population from which the HKLC staging system was derived. This study investigated a large cohort from an Asian population which was well-characterised and studied over a long follow-up period, with robust survival data based on an updated census by the national registry of deaths. It showed that the median overall survival in patients assigned to curative treatment by the BCLC staging system was significantly higher than those assigned to curative treatment by HKLC (6.1 and 2.6 years respectively, $P < 0.001$). Although a significantly higher proportion of patients in our study were assigned to curative treatment by HKLC compared to BCLC (43.9% *vs* 18.9% respectively, $P < 0.01$), BCLC was more accurate in directing therapy, with a significantly higher proportion of patients assigned to curative therapy receiving the recommended curative treatment compared to HKLC (85.8% *vs* 61.1%, $P < 0.001$). Thus, BCLC performed better than HKLC in terms of accuracy in assigning curative treatment and for prognosticating survival in a real-world setting. This could be due to the limitations in applying the more aggressive HKLC recommendations in real life, as reflected by the lower proportion of patients who eventually received curative treatment after being assigned to receive curative therapy by this staging system. Moreover, when only patients who received the recommended curative treatment were compared, BCLC still predicted better overall survival than HKLC (median survival 7.1 and 5.5 years respectively, $P = 0.037$).

Applications

This study highlights the need for continued refinement of the different staging systems for HCC to ensure that patients most likely to benefit from curative therapy are appropriately as such. This is especially true as new treatment modalities evolve and our collective understanding of HCC increases.

Peer-review

The topic is one of great interest today. They would insist more about the fact that the criteria raised in the HKLC system remain to be further adjusted and verified. The BCLC system, although widely used was created based on several small Western cohorts of patients with predominant alcoholic liver disease and hepatitis C related HCC. The system which links stage stratification with corresponding therapeutic recommendations was criticized for being too

restrictive, having in mind for example, that liver resection is recommended only to the patients with early stage tumors, or the fact that patients with cancer-related symptoms should be classified as advanced HCC. More, recent papers argue that this system is indeed able to provide accurate outcome prediction and treatment recommendations for HCC patients with hepatitis B virus as the predominant etiology. The HKLC staging system recently proposed has been show to achieve better prognostic ability and to identify subsets of patients for more aggressive treatment (intermediate and advanced stage patients) in Eastern population, with hepatitis B as main etiology. The improved stratification of the patients with intermediate-to-advanced stage using the triad of tumor size, number of nodules and tumor thrombus seems to offer to this patients with multiple tumors the possibility to achieve a better outcome if they receive hepatic resection following HKLC criteria. It worth mentioning that this is still a controversial point, if they keep in mind regarding the patients with multiple tumors the problem of cancer genetic heterogeneity.

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ORIGINAL ARTICLE

Observational Study

Single-operator cholangioscopy for biliary complications in liver transplant recipients

Anna Hüsing-Kabar, Hauke Sebastian Heinzow, Hartmut Hans-Jürgen Schmidt, Carina Stenger, Hans Ulrich Gerth, Michele Pohlen, Gerold Thölking, Christian Wilms, Iyad Kabar

Anna Hüsing-Kabar, Hauke Sebastian Heinzow, Hartmut Hans-Jürgen Schmidt, Carina Stenger, Christian Wilms, Iyad Kabar, Department of Transplant Medicine, University Hospital Muenster, 48149 Muenster, Germany

Hans Ulrich Gerth, Gerold Thölking, Department of Medicine D, Division of General Internal Medicine, Nephrology and Rheumatology, University Hospital Muenster, 48149 Muenster, Germany

Michele Pohlen, Department of Medicine A, Hematology and Oncology, University Hospital Muenster, 48149 Muenster, Germany

Author contributions: Hüsing-Kabar A, Heinzow HS and Kabar I designed the study; Hüsing-Kabar A, Heinzow HS and Kabar I performed the study; Hüsing-Kabar A, Heinzow HS, Stenger C, Wilms C and Kabar I collected the patients' data; Hüsing-Kabar A, Heinzow HS, Schmidt HHJ, Stenger C, Gerth HU, Pohlen M, Thölking G, Wilms C and Kabar I analyzed the data; Hüsing-Kabar A, Heinzow HS, Schmidt HHJ, Gerth HU, Pohlen M, Thölking G and Kabar I wrote the manuscript; Hüsing-Kabar A and Heinzow HS contributed equally to this work and are co-first-authors.

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Correspondence to: Iyad Kabar, MD, Department of Transplant Medicine, University Hospital Muenster, Albert-Schweitzer-Campus 1, Gebäude A14, 48149 Muenster, Germany. iyad.kabar@ukmuenster.de
Telephone: +49-251-8344957
Fax: +49-251-8357771

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Abstract

AIM

To evaluate cholangioscopy in addition to endoscopic retrograde cholangiopancreatography (ERCP) for management of biliary complications after liver transplantation (LT).

METHODS

Twenty-six LT recipients with duct-to-duct biliary reconstruction who underwent ERCP for suspected biliary complications between April and December

2016 at the university hospital of Muenster were consecutively enrolled in this observational study. After evaluating bile ducts using fluoroscopy, cholangioscopy using a modern digital single-operator cholangioscopy system (SpyGlass DS™) was performed during the same procedure with patients under conscious sedation. All patients received peri-interventional antibiotic prophylaxis and bile was collected during the intervention for microbial analysis and for antibiotic susceptibility testing.

RESULTS

Thirty-three biliary complications were found in a total of 22 patients, whereas four patients showed normal bile ducts. Anastomotic strictures were evident in 14 (53.8%) patients, non-anastomotic strictures in seven (26.9%), biliary cast in three (11.5%), and stones in six (23.1%). A benefit of cholangioscopy was seen in 12 (46.2%) patients. In four of them, cholangioscopy was crucial for selective guidewire placement prior to planned intervention. In six patients, biliary cast and/or stones failed to be diagnosed by ERCP and were only detectable through cholangioscopy. In one case, a bile duct ulcer due to fungal infection was diagnosed by cholangioscopy. In another case, signs of bile duct inflammation caused by acute cholangitis were evident. One patient developed post-interventional cholangitis. No further procedure-related complications occurred. Thirty-seven isolates were found in bile. Sixteen of these were gram-positive (43.2%), 12 (32.4%) were gram-negative bacteria, and Candida species accounted for 24.3% of all isolated microorganisms. Interestingly, only 48.6% of specimens were sensitive to prophylactic antibiotics.

CONCLUSION

Single-operator cholangioscopy can provide important diagnostic information, helping endoscopists to plan and perform interventional procedures in LT-related biliary complications.

Key words: Cholangioscopy; Endoscopic retrograde cholangiopancreatography; Liver transplantation; Biliary complications; Biliary strictures

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Core tip: Biliary complications represent a leading cause of morbidity and mortality in liver transplant recipients. To date, endoscopic retrograde cholangiopancreatography still remains the gold standard for diagnosing and treating such complications. The present study examined the benefit of complementary single-operator cholangioscopy. Our results are encouraging and demonstrate strong evidence for a diagnostic and therapeutic advantage of additional cholangioscopy for management of biliary disorders following liver transplantation.

Hüsing-Kabar A, Heinzow HS, Schmidt HHJ, Stenger C, Gerth HU, Pohlen M, Thölking G, Wilms C, Kabar I. Single-operator cholangioscopy for biliary complications in liver transplant recipients. *World J Gastroenterol* 2017; 23(22): 4064-4071 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i22/4064.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i22.4064

INTRODUCTION

Over the last few decades, the long-term outcome, morbidity, and mortality of liver transplant (LT) recipients have markedly improved because of advances in surgical techniques, modern immunosuppressive medication, and close follow-up. However, biliary complications after LT are still common[1,2]. Biliary complications affect 10%-25% of adult LT recipients[3-5]. In the majority of cases, patients present with biliary leakage and bile duct strictures. The latter can be subdivided into anastomotic and non-anastomotic strictures[6], with anastomotic strictures responding well to endoscopic treatment[3,7]. Additional biliary complications are biliary stones, cast, and sludge. Because of several disadvantages of first generation direct peroral cholangioscopy (*e.g.*, high costs, fragility, and the prerequisite of two experienced endoscopists), introduction of this technique in 1976 initially failed to gain widespread acceptance. However, currently, cholangioscopy has become an established modality in diagnosing and treating pancreaticobiliary diseases[8,9]. In 2007, the digital single-operator per oral cholangioscopy system SpyGlass™ (Boston Scientific Corp., Natick, MA, United States) was introduced. This system featured crucial improvements in visualization and technical tools, leading to revived interest in the field of cholangioscopy for diagnosis and management of biliary disorders[10,11]. In 2015, high-resolution cholangioscopy (SpyGlass DS™) was introduced by Boston Scientific (Boston Scientific Corp.), enabling high-definition imaging of bile ducts. The wide range of potential indications and therapeutic procedures for SpyGlass DS, such as diagnosis of indeterminate biliary strictures, lithotripsy of bile duct stones, ablative techniques for intraductal malignancies, removal of foreign bodies, and gallbladder drainage, have led to more widespread use of this procedure. A study by Chen *et al*[12] showed that single-operator cholangioscopy using SpyGlass was feasible and safe for diagnosis and therapy of biliary disorders. Because of the possibility of direct high-resolution imaging of bile ducts, single-operator cholangioscopy has recently attracted attention in the field of management of biliary complications after LT[13]. However, only a few case reports and small case series have analyzed the role of single-operator cholangioscopy for management

of biliary complications after LT[14-16]. Moreover, most of these case series were performed using the earlier generation of the SpyGlass system. To the best of our knowledge, data on the effect of cholangioscopy using the high-resolution SpyGlass DS system on management of biliary complications in LT recipients are still unavailable.

Therefore, this study aimed to examine the role of complementary single-operator cholangioscopy using the SpyGlass DS system during endoscopic retrograde cholangiopancreaticography (ERCP) for management of biliary complications following LT.

MATERIALS AND METHODS

This prospective, observational study was performed at the University Hospital Muenster, Germany. The study was performed in accordance with the guidelines of the Declaration of Helsinki and was approved by the local ethics committee. All patients gave prior written informed consent. Patients with LT and duct-to-duct biliary anastomosis who presented with clinical or biochemical signs of biliary complications, and/or suspected biliary complications based upon imaging and/or histology between April and December 2016 were consecutively included in the study. Initial imaging included transabdominal Ultrasound in all cases. In case of inconclusive findings on transabdominal ultrasound and absence of clinical evident cholangitis, additional endoscopic ultrasound was performed followed by ERCP in case of documented biliary tract alterations. During the procedures, patients received conscious sedation using propofol and piritramide with or without midazolam. All of the patients first received ERCP followed by cholangioscopy during the same procedure. ERCP was performed using a large-diameter channel duodenoscope (TJF-180V, Olympus Corp., Tokyo, Japan). Intubation of the bile duct was guidewire-assisted (0.025 inches, VisiGlide™, Olympus Corp.) using either a catheter (StarTip2V™, Olympus Corp.) or a sphincterotome (CleverCut2V™, Olympus Corp.). If necessary, biliary sphincterotomy was performed.

Cholangioscopy was carried out using a single-operator cholangioscopy device (SpyGlass DS; Boston Scientific Corp.) that was pushed along the guidewire through the working channel of the duodenoscope into the bile duct. The guidewire was then removed and cholangioscopy was continued under visual guidance. A biopsy was performed in case of unclear bile duct mucosal lesions. After the intervention, patients remained at least 3 d in hospital.

The interventions were performed by two investigators rated as highly experienced with a case volume above 200 endoscopic biliary interventions/year. Procedure related complications were evaluated according to the ASGE guidelines[17].

Peri-interventional antibiotics

Standard antibiotic prophylaxis included intravenous piperacillin/tazobactam at least 2 h before the procedure, and up to 3 d thereafter. During ERCP/cholangioscopy, bile was collected for microbial analysis and for antibiotic susceptibility testing.

Immunosuppression

All of the patients were maintained on a calcineurin inhibitor only or in combination with either an m-TOR-inhibitor or mycophenolate mofetil.

Interpretation of ERCP findings

Strictures were determined as an abrupt narrowing of the bile duct with delayed outflow of contrast media through the stricture. Bile strictures were fluoroscopically subdivided into anastomotic strictures at the site of biliary anastomosis and non-anastomotic strictures affecting donor bile ducts that were proximal to the biliary anastomosis. Bile duct stones and biliary cast were evident as intraluminal filling defects of contrast media.

Interpretation of cholangioscopy findings

Strictures were determined as above and were visible as an abrupt substantial narrowing of bile ducts compared with distal and proximal segments of the bile duct. Biliary cast was determined as dark smooth foreign bodies mostly adhering to the bile wall, whereas stones were determined as free-moving, hard, foreign bodies in the bile duct.

Statistical analysis

Statistical analysis was conducted using SPSS 24 (SPSS Inc., Chicago, IL, United States). All data are presented as absolute and relative frequencies and reported as median (minimal-maximal) values. Categorical variables were compared using Fisher's exact test. P values $\leqslant 0.05$ were considered statistically significant.

RESULTS

Over the period covered by our study, 26 consecutive patients underwent ERCP followed by cholangioscopy. Their median age was 54.5 years (25-75 years), and 14 (53.8%) patients were women. Procedures were carried out after a median of 18.5 mo (1-159 mo) after LT. The patients' clinical and demographic data, their primary underlying disease, as well as the findings of ERCP and cholangioscopy, are shown in Table 1.

A total of 33 biliary tract complications were diagnosed in 22 patients. Anastomotic strictures were observed in 14 (53.8%) patients, non-anastomotic strictures in seven (26.9%), biliary cast in three (11.5%), and stones in six (23.1%).

In four patients, no bile tree abnormalities were

Table 1 Patients' characteristics and findings of endoscopic retrograde cholangiopancreatography/SpyGlass DS

Patient no.	Age (yr)	Sex	Indication for LT	Findings of ERCP	Findings of cholangioscopy	Endoscopic Intervention
1	64	M	Caroli syndrome	AS, non-AS	AS, non-AS	Stent insertion
2	65	M	Cryptogenic liver cirrhosis	AS	AS	Balloon dilation
3	28	M	Cryptogenic liver cirrhosis	AS, non-AS	AS, non-AS	Balloon dilation
4	48	M	Transplant dysfunction	Normal	Bile duct erythema	None
5	30	M	Alcoholic liver cirrhosis	AS, non-AS	AS, non-AS	Balloon dilation
6	63	M	Hepatocellular carcinoma, alcoholic liver cirrhosis	Normal	Normal	None
7	56	F	Alcoholic liver cirrhosis	AS	AS	Balloon dilation
8	48	F	Autoimmune hepatitis and primary sclerosing cholangitis	Non-AS	Non-AS	Balloon dilation
9	46	M	Acute liver failure	Stones	Stones	Extraction of stones
10	70	M	Hepatocellular carcinoma/hepatits C	AS	AS, stones	Balloon dilation, extraction of stones
11	75	F	Autoimmune hepatitis and primary biliary cholangitis	AS	AS, stones	Extraction of stones, stent insertion
12	51	F	Cryptogenic liver cirrhosis	AS, non-AS	AS, non-AS	Balloon dilation, bougienage of stricture
13	57	M	Alcoholic liver cirrhosis	AS	AS	Balloon dilation
14	30	F	Transplant dysfunction after LT for Wilson disease	AS, stones	AS, stones	Balloon dilation, extraction of stones
15	60	F	Drug-induced liver injury	None	None	None
16	57	F	Hepatitis C	Stones	Stones	Extraction of stones
17	52	F	Hepatocellular carcinoma/hepatitis B	None	None	None
18	44	F	Acute liver failure	AS	AS	Balloon dilation
19	60	M	Alcoholic liver cirrhosis	AS, non-AS	AS, non-AS, biliary cast	Balloon dilation, extraction of cast
20	53	F	Alcoholic liver cirrhosis	Non-AS	Non-AS, biliary cast	Extraction of cast
21	25	F	Acute liver failure	None	None	None
22	63	M	Non-alcoholic steato-hepatitis	AS	AS	Balloon dilation
23	37	M	Hepatitis C/Wilson disease	AS	AS, stones	Balloon dilation, extraction of stones
24	63	F	Non-alcoholic steato-hepatitis	None	Hiliar ulcer	None
25	66	F	Primary biliary cholangitis	None	Biliary cast	Extraction of cast
26	34	F	FAP	Bile duct kinking	Bile duct kinking	Stent insertion

AS: Anastomotic stricture; non-AS: Non-anastomotic stricture; ERCP: Endoscopic retrograde cholangiopancreaticography; FAP: Familiar amyloid polyneuropathy; LT: Liver transplant.

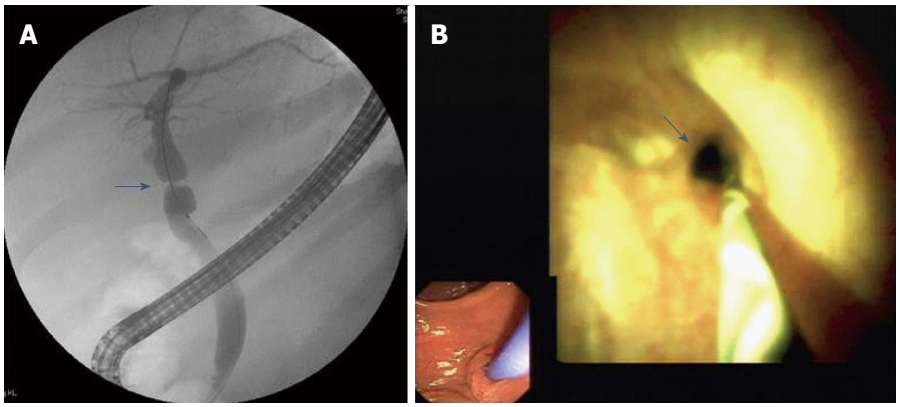

Figure 1 Anastomotic stricture (arrows) as shown by fluoroscopy (A) and a corresponding cholangiographic image (B) showing high-grade fibrotic stenosis that initially could not be passed by the cholangioscope.

detected. Final diagnoses were confirmed by clinical follow-up, further liver biopsies, and biochemical tests. In these cases, graft rejection, drug-induced liver injury, and infections were the causative disorders.

Findings of ERCP

During ERCP, anastomotic strictures were observed in 14 patients, non-anastomotic in seven, and stones in three. One patient showed bile duct kinking. In seven patients, ERCP showed no pathological results.

Findings of cholangioscopy

Cholangioscopy showed anastomotic strictures in 14 patients (Figure 1), non-anastomotic strictures in

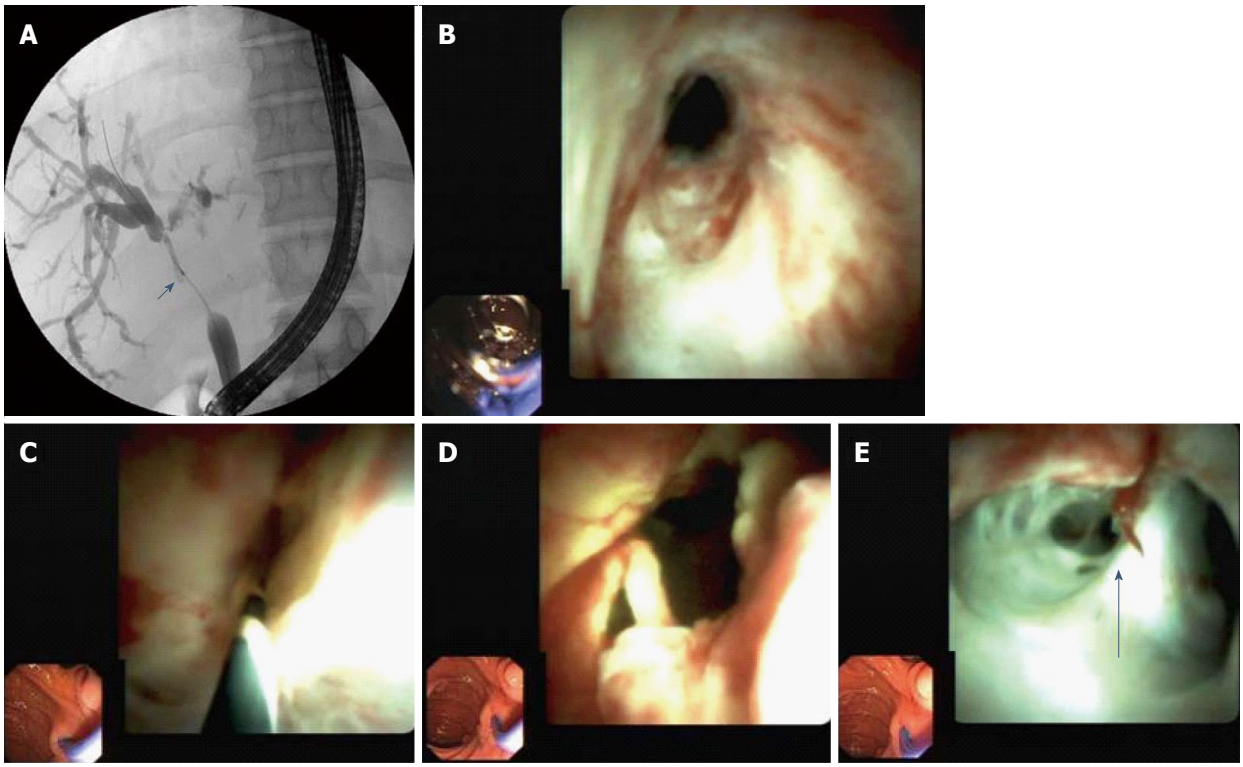

Figure 2 Non-anastomotic strictures in seven. A: High-grade, long-segment stenosis of the main bile duct between biliary anastomosis and the hilus region. The biliary hilus appears to be involved in the stenosis; B: Cholangioscopic image of biliary anastomosis with slight stenosis; C: High-grade stricture of the bile duct beyond the biliary stricture that could not be passed using a cholangioscope; D: Bile duct above the biliary anastomosis after balloon dilation with marked erythema and polypoid growth of the bile duct wall, and signs of pronounced inflammation; E: Bile duct at the height of the hilus (arrow) with inflammation involving the left hepatic bile duct (right side of the image).

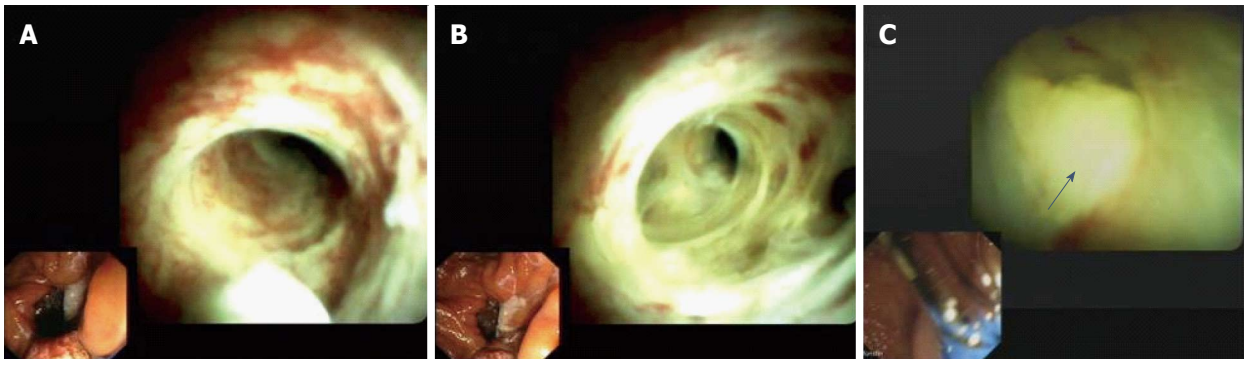

Figure 3 Bile duct hyperemia of intrahepatic bile ducts. A and B: Marked bile duct erythema caused by cholangitis; C: White plaque-coated bile duct ulcer.

seven (Figure 2), biliary cast in three, and stones in six. All cases of non-anastomotic strictures showed a fibrotic obstruction and signs of inflammation, such as hyperemia, erosions, and polyp-like tissue growth. In one case, a fungal ulcer confirmed by microbiology was detected. In another case, bile duct hyperemia of intrahepatic bile ducts was found (Figure 3).

Comparison between ERCP and cholangioscopy

Benefits of cholangioscopy over ERCP were found in 12 of 26 (46.2%) patients. In four cases of failed cannulation of biliary strictures during ERCP, selective guidewire placement was successful by cholangioscopy under direct vision. Furthermore, cholangioscopy was superior to ERCP for detecting stones in three patients ($P < 0.008$) and cast in three patients ($P < 0.001$) that ERCP failed to detect in these patients. In one patient, a fungal bile duct ulcer that was confirmed by microbiology (candidiasis) was detected, and this resulted in targeted antifungal therapy. In another patient, strong hyperemia of intrahepatic bile ducts due to acute cholangitis was observed.

Histological findings

A total of 11 biopsies were obtained. Histological samples showed fibrotic shrinkage without inflammation

Table 2 Species found in bile

Microorganism	Frequency	Percentage
Stenotrophomonas	1	2.7%
Enterococcus faecium	7	18.9%
Escherichia coli	5	13.5%
Streptococcus viridans	2	5.4%
Micrococcus luteus	1	2.7%
Enterococcus faecalis	2	5.4%
Klebsiella oxytoca	2	5.4%
Enterobacter cloacae	2	5.4%
Raultella ornithinolytica	1	2.7%
Enterobacter aerogenes	1	2.7%
Staphylococcus haemolyticus	1	2.7%
Pedicoccus pentosaceus	1	2.7%
Enterococcus avium	2	5.4%
Candida albicans	6	16.2%
Candida duliniensis	1	2.7%
Candida tropicalis	1	2.7%
Candida glabrata	1	2.7%
Total	37	100%

in case of anastomotic strictures. In a patient with non-anastomotic strictures, signs of acute and chronic inflammation with mixed infiltration of lymphocytes, plasmacytes, and granulocytes, as well as granulation tissue and scars, were observed. In a patient with hyperemia, inflammation with predominantly granulocytes was observed. In this patient, bacterial cholangitis was confirmed by the clinical course and microbiologically.

Endoscopic treatment

In patients with biliary complications, a total of 13 balloon dilatations, six extractions of stones, three stent insertions, one bougienage of a tight stricture, and three extractions of biliary cast were performed. Only one procedure-related complication (cholangitis) occurred and was managed antibiotically.

Microbial analysis of bile

Bile was collected from 23 patients. In seven (30.4%) patients, the bile showed no microbial growth. In 16 (69.6%) patients, bacteria and/or fungi were detected. In nine (39.1%) patients, Candida species were observed (*Candida albicans* in six cases, and *C. duliniensis*, *C. glabrata,* and *C tropicalis* in one patient each).

A total of 37 microorganisms were isolated from the bile. Of these, 16 were gram-positive (43.2%), whereas 12 (32.4%) were gram-negative bacteria. Candida species accounted for 24.3% of all isolates. The microorganisms that were found in bile are listed in Table 2.

In patients with evidence of bile colonization, standard antibiotic prophylaxis using piperacillin/tazobactam was effective in only 48.6% of all isolates. Susceptibility testing for ciprofloxacin showed that 36.1% of isolates were sensitive, whereas 58.3% were resistant and 5.6% were intermediate susceptible. For ceftriaxone, 70.3% of microorganisms were resistant and 29.7% were sensitive. Among all specimens, 54.1% were sensitive to carbapenems. For gentamicin, 32.4% were resistant, 55.6% were sensitive, and 11.1% were intermediate susceptible to this antibiotic. For vancomycin, 87.5% of the tested gram-positive bacteria were sensitive. For tigecycline, 62.5% of all tested specimens were also sensitive.

Of all fungal isolates, eight were sensitive to triazoles, while one was intermediate susceptible. All isolated Candida species were susceptible to amphotericin B and echinocandins.

DISCUSSION

Despite improvements in surgical techniques, biliary complications are common and still considered the "Achilles heel" of LT[18,19]. To date, ERCP represents the gold standard for diagnosis and treatment of biliary complications after LT[20,21]. However, increasing evidence is currently emerging of the major benefits of using cholangioscopy in management of these biliary disorders.

In our study, an advantage of cholangioscopy over ERCP was observed in 12 of 26 (46.2%) patients. In one case series by Woo *et al*[13] SpyGlass cholangioscopy showed a high performance in visualization of strictures, but a poor performance of cholangioscopy-assisted guidewire placement in 60% of cases. In contrast to these findings, we were able to selectively steer the guidewire over the stricture in every patient prior to planned treatment. An explanation of the poor success of guidewire placement in Woo *et al*[13] study could be that their study was performed in patients after living donor LT, with special and sometimes complex anatomy of bile ducts. However, most of our patients underwent whole cadaveric LT prior to the procedure (only one patient in our cohort had a living donor LT). The use of the new generation of high-resolution cholangioscopy (SpyGlass DS™) in our study may have provided an additional advantage.

In our study, cholangioscopy was superior to ERCP in detection of stones and biliary cast. Several previous studies showed that the sensitivity of ERCP in detecting bile duct stones ranged between 89% and 93%. Especially in cases of small stones and dilated bile ducts, false negative ERCP has been observed[22-24]. In a previous study at our center, we found that biliary cast appear to be underdiagnosed using ERCP only[21]. Biliary stones and cast can be masked by dense contrast media during ERCP. Furthermore, stones and cast in bile ducts may be easily misinterpreted as biliary air. In the present study, ERCP failed to identify biliary cast in three patients and biliary stones in a further three patients. In two of the three patients with failed diagnosis of cast, further biliary tract disorders were coincident. In one of these patients,

anastomotic stricture and multiple non-anastomotic strictures were found. In another of these patients, multiple non-anastomotic strictures were observed. These additional conditions could have made it even more difficult to identify biliary cast. In the third patient, biliary cast was detected only in the intrahepatic bile ducts and was adhered to the bile duct wall without completely occluding the whole bile duct lumen. In all patients in whom ERCP failed to detect stones, anastomotic stricture was also present, and small stones were detected by cholangioscopy directly proximal to the anastomosis. This was most likely as a consequence of bile stasis caused by stenosis. The coincident anastomotic stricture and bile duct dilation could have masked the small stones in these special cases. One further advantage for cholangioscopy was found in patients in whom pathological changes in bile ducts (*e.g.,* hyperemia and bile duct ulcers; Figure 1) were only detectable by direct visualization of bile duct walls, but not by fluoroscopy. In these special cases, there were therapeutic consequences. In the current study, ERCP was highly effective in detecting bile duct strictures. In these cases, cholangioscopy offered no further benefits.

In our study, additional histological information that was obtained by tissue samples of the bile duct was of minor clinical relevance. In the case of non-anastomotic strictures, fibrosis and inflammation were simultaneously evident in every patient. This finding supports the role of ongoing inflammation for the pathogenesis of non-anastomotic strictures. Our findings are consistent with the results of previous studies suggesting immunological aspects in the pathogenesis of non-anastomotic strictures[3,25].

During endoscopic procedures, we obtained bile samples for microbial analysis in the majority of patients. Several studies have identified LT as a risk factor for increasing microbial colonization of bile. In these studies, an increased incidence of gram-positive bacteria with increasing antibiotic resistance, such as enterococci and Candida species, in patients with LT was found[26-29]. In our study, we found a remarkably high prevalence of Candida species (39.1%), whereas the prevalence of enterococci was 29.7%. In a minority of isolates (48.6%), specimens that were found in bile were sensitive to administered prophylactic antibiotics. These findings are important because microbial colonization of bile has been identified to be the origin of post-ERCP cholangitis and cholangiosepsis[26]. Even though microbial analysis was not the focus of the present study, these findings suggest that bile colonization during endoscopic interventions in the bile duct of LT recipients should be monitored. The resulting resistogram can be helpful in choosing effective antibiotics in case of endoscopy-related septic complications. Because of the high prevalence of enterococci and Candida species, empirical antimicrobial treatments should include vancomycin/linezolid and an antifungal agent in case of post-procedural septic complications. Isolated Candida species showed no relevant resistance to common antifungal agents in our study. However, selection of an antibiotic regimen should always be based on local microbial resistance patterns.

In conclusion, high-resolution cholangioscopy using the SpyGlass DS system is safe and feasible in LT recipients with biliary complications, and offers useful diagnostic information in addition to ERCP. Cholangioscopy is superior to ERCP in diagnosing biliary cast and stones, and optimizes treatment in the patients concerned. Therefore, we recommend performing cholangioscopy in LT recipients who have negative ERCP results and suspected biliary complications.

Use of a general peri-interventional antibiotic prophylaxis in patients with LT based on the general increase in microbial resistance should be critically examined in future studies. Furthermore, microbial analysis of bile collected during bile duct interventions should be regularly performed to adapt anti-infective treatments in case of post-ERCP septic complications.

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COMMENTS

Background

Despite improvements in surgical techniques, biliary complications still remain common in liver transplant (LT) recipients influencing their morbidity and mortality. Nowadays, endoscopic retrograde cholangiopancreatography (ERCP) represents the gold standard for diagnosis and treatment of biliary complications following LT. Until now, data evaluating the utility and value of cholangioscopy for management of biliary disorders after LT are still lacking.

Research frontiers

The present study explores the benefit of modern digital single-operator cholangioscopy (SpyGlass DS™) in addition to ERCP for the management of biliary complications after LT.

Innovations and breakthroughs

The results of our study indicate that use of cholangioscopy provides valuable diagnostic information which is able to improve the management of biliary complications after LT.

Applications

These data demonstrate that cholangioscopy is effective for selective guidewire placement in high-grade biliary strictures that failed to be cannulated using ERCP alone. Furthermore, cholangioscopy offers diagnostic superiority in comparison to ERCP in detecting biliary cast and stones and therefore should be used in LT recipients with clinical signs of biliary complications despite negative ERCP results.

Terminology

Single-operator cholangioscopy is an innovative tool that enables high-resolution imaging of bile ducts.

Peer-review

The manuscript is fine and well written. In addition the manuscript is useful for physician facing with post liver transplant complication clearly documenting the superiority of cholangioscopy with respect to ERCP. Interestingly the superiority is clearly documented for biliary stones, casts and unusual, but diverse finding as micotic ulcer.

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ORIGINAL ARTICLE

Observational Study

Efficacy and safety of combined directly acting antivirals for treatment of Chinese chronic hepatitis C patients in a real-world setting

Jian-Hong Chen, Zheng Zeng, Xia-Xia Zhang, Yu Zhang, Ren-Wen Zhang, Shuai Wang, Chi-Hong Wu, Min Yu, Dan Liu, Hong-Li Xi, Yi-Xing Zhou, Yao-Yu An, Xiao-Yuan Xu

Jian-Hong Chen, Zheng Zeng, Xia-Xia Zhang, Yu Zhang, Ren-Wen Zhang, Chi-Hong Wu, Min Yu, Dan Liu, Hong-Li Xi, Yi-Xing Zhou, Yao-Yu An, Xiao-Yuan Xu, Department of Infectious Diseases, Peking University First Hospital, Xicheng District, Beijing 100034, China

Shuai Wang, Department of Hepatology, General Hospital of Beijing Military Region, Beijing 100700, China

Author contributions: Xu XY designed the research; Chen JH performed the research; Chen JH, Zeng Z, Zhang XX, Zhang Y, Zhang RW, Wang S, Wu CH, Yu M, Liu D, Xi HL, Zhou YX and An YY contributed to data collection; Chen JH analysed the data and wrote the paper.

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

Conflict-of-interest statement: The authors declare that no conflict of interest exists.

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Correspondence to: Xiao-Yuan Xu, Professor, Department of Infectious Diseases, Peking University First Hospital, Xicheng District, Beijing 100034, China. xiaoyuanxu6@163.com
Telephone: +86-10-83575787
Fax: +86-10-83575787

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Abstract

AIM

To assess the efficacy and safety of combined directly acting antivirals (DAAs) for the treatment of Chinese chronic hepatitis C (CHC) patients in a real-world setting.

METHODS

Hospitalized CHC patients who were treated with DAAs at Peking University First Hospital between January 2015 and December 2016 were enrolled. Samples and clinical data were collected at 0 wk, 2 wk, 4 wk, 8 wk, 12 wk, or 24 wk during DAAs treatment and at 4 wk, 12 wk, and 24 wk after the end of treatment.

RESULTS

Fifty-four patients who underwent DAAs treatment were included in our study, of whom 83.3% (45/54) achieved rapid virological response at 2 wk after treatment initiation (RVR 2) and 94.4% (51/54)

achieved sustained virological response at 24 wk after the end of treatment (SVR 24). Serum creatinine and uric acid levels at the end of treatment were significantly increased compared with baseline levels (83.6 ± 17.9 *vs* 88.8 ± 19.4, $P_{01} < 0.001$; 320.8 ± 76.3 *vs* 354.5 ± 87.6, $P_{01} < 0.001$), and no significant improvements were observed at 24w after the end of treatment (83.6 ± 17.9 *vs* 86.8 ± 19.1, $P_{02} = 0.039$; 320.8 ± 76.3 *vs* 345.9 ± 89.4, $P_{02} = 0.001$). The total frequency of adverse events (AEs) during treatment was 33.3% (18/54), with major AEs being fatigue (16.7%), headache (7.4%), anorexia (7.4%), and insomnia (5.6%).

CONCLUSION

Though based in a small cohort of patients, the abnormal changes in renal function indices and relative high frequency of AEs during combined DAAs treatment should be taken as a note of caution.

Key words: Chronic hepatitis C; Directly acting antivirals; Efficacy; Safety; China

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Core tip: Treatment of hepatitis C virus infection has reached a new era with the approval of directly acting antivirals (DAAs), while there had been limited data on the use of DAAs treatment in a real-world setting in China. We explored the changes of hepatorenal function indices before and after DAAs treatment and found that serum creatinine and uric acid levels at the end of treatment were significantly increased compared with baseline levels, and no significant improvements were observed at 24 wk after the end of treatment. This study may serve as a reminder to clinicians to implement close renal function monitoring in patients receiving combined DAAs treatment.

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INTRODUCTION

Chronic hepatitis C virus (HCV) infections affect approximately 130-150 million people worldwide and is a major cause of liver cirrhosis and hepatocellular carcinoma[1-3]. Treatment of HCV infection has reached a new era with the approval of first generation directly acting antivirals (DAAs) in 2011 and the subsequent development of interferon (IFN)-free, all-oral DAAs combination regimens[4]. All-oral DAAs combination regimens simplified the treatment and improved compliance of patients who disliked injections or were intolerable to them[5]. Combined DAAs regimens shortened treatment durations from 48 wk to 12 wk or 24 wk; Sofosbuvir (SOF)/Velpatasvir also attained a promising efficacy at 8 wk, and some studies tried to shorten treatment times even further[6]. Similar to cocktail therapies against human immunodeficiency virus, combination therapies that target different stages of the HCV life cycle have been conceived to avoid cross-resistance[7]. Importantly, all-oral combination regimens increased sustained virological response (SVR) rates to more than 90% with fewer contraindications and adverse events (AEs) in patients infected with different HCV genotypes (GT) and in those with different liver conditions, treatment experiences and concomitant diseases[8-14]. Sofosbuvir (SOF)/Velpatasvir combination regimen might even provide complete pan-genotypic treatment for patients with HCV infection[15,16]. Though price decreases for HCV drugs have already been announced for some DAAs, most of them are currently too expensive for governments worldwide to deliver on their promise to cure and eliminate the disease, especially in low- and middle-income countries[17].

China has the greatest number of chronic hepatitis C (CHC) cases worldwide, with an estimated 29.8 million patients infected. GT 1b and GT 2a are the two major HCV subtypes, accounting for 62.78% (95%CI: 59.54%-66.02%) and 17.39% (95%CI: 15.67%-19.11%), respectively[18,19]. The traditional treatment for patients with CHC in China is peginterferon in combination with ribavirin (PegIFNα-2a/RBV, PR), which was found to be associated with lower SVR rates and more AEs[20]. Refractory CHC patients and patients with contraindications and intolerances to the AEs associated with PR treatment try to initiate DAAs treatment.

So far, there have been limited data on the use of combined DAAs treatment in a real-world setting in China. This study aimed to show the efficacy of DAAs for the treatment of Chinese CHC patients and explore the effects of DAAs on hemogram and hepatorenal function indices, and the frequency of AEs during treatment in a real-world setting.

MATERIALS AND METHODS

Patients

CHC patients who were treated with DAAs while hospitalized at Peking University First Hospital between January 2015 and December 2016 and met the following criteria were enrolled in this study: (1) infected with HCV GT 1b or 2a; (2) negative for hepatitis A virus immunoglobulin (Ig) M, hepatitis B surface antigen, hepatitis E virus IgM and human immunodeficiency virus; (3) no severe heart disease; (4) no active drug use; (5) no severe renal function

damage or renal failure (eGFR < 30 mL/min); (6) no pregnancy; (7) appropriate DAAs treatment regimens; and (8) complete clinical information. A total of 16 patients were excluded, including one HBV/HCV co-infected patient, three patients with severe renal function damage, one patient treated with inappropriate DAAs regimens, and 11 patients with incomplete clinical information. All study participants provided informed written consent prior to study enrollment. Ethical approval was given by the Ethics Committee of Peking University People Hospital.

Clinical data collection and assessment

Hematological, biochemical, and urine tests were performed at 0 wk, 2 wk, 4 wk, 8 wk, 12 wk, or 24 wk during DAAs treatment, as well as 4 wk, 12 wk, and 24 wk after the end of treatment at clinical laboratory[21]. White blood cell (WBC) count, red blood cell (RBC) count, hemoglobin concentration (HGB), and blood platelet (PLT) count were used to assess the changes of hemogram; alanine aminotransferase (ALT), aspartate aminotransferase (AST), fibrosis-4 (FIB-4) score, and liver stiffness measurement (LSM) were used to assess the degree of liver inflammation and fibrosis; estimated glomerular filtration rate (eGFR), serum creatinine (Scr), uric acid (UA), and blood urea nitrogen (BUN) were used to assess renal function.

LSM was measured by transient elastography (Fibroscan, Echosens, Paris). Presence of cirrhosis was determined by LSM > 17.5 kPa[22-24]. FIB-4 score was calculated with the equation: FIB-4 = [AGE * AST (U/L)]/[PLT (10^9/L) * ALT (U/L) ^ (1/2)][25]. The eGFR was calculated with the Modification of Diet in Renal Disease Study equation adjusted for the Chinese population: eGFR = 175 * (serum creatinine)$^{-1.234}$ * age$^{-0.179}$ * 0.79 (if female)[26].

HCV RNA quantitation and genotyping were measured at the virus laboratory in department of infectious disease. Serum HCV RNA quantitation was measured using a COBAS Taqman HCV Test kit (Roche Molecular Systems Inc., Pleasanton, CA, United States) according to the manufacturer's instructions, with COBAS AmpliPrep instrument used for automated specimen processing and COBAS Taqman analyser for automated amplification and detection[27]. HCV genotypes were determined by restriction fragment length polymorphism (RFLP) analysis of the amplified 5'-noncoding genome region[28]. Briefly, HCV RNA was extracted from 140 μL serum samples using a QIAamp viral RNA mini kit (Qiagen, Hilden, Germany). Reverse transcription and polymerase chain reaction (PCR) amplification were performed using BG1 (5'-CTGTGAGGAACTACTGTCTT-3') and BG2 (5'-AACACTACTCGGCTAG CAGT-3') as upstream and downstream primers, respectively, for the first round reaction in a reaction system containing 15 μL of 2 × Buffer, 1 μL of BG1 and BG2 each, 10 μL of cDNA, and 3 μL of H_2O. The cycling parameters were denaturation at 95 ℃ for 2 min, 30 cycles of denaturation at 94 ℃ for 30 s, annealing at 55 ℃ for 30 s, and extension at 72 ℃ for 60 s, and final extension at 72 ℃ for 7 min. This was followed by the second round reaction using BG3 (5'-TTCACGCAGAAAGCGTCTAG-3') and BG4 (5'-GTTGATCCA AGAAAGGACCC-3') as upstream and downstream primers, respectively, in a reaction system consisting of 15 μL of 2 × Buffer, 1 μL of BG3 and BG4 each, 10 μL of cDNA, and 3 μL of H_2O. The cycling parameters were denaturation at 95 ℃ for 2 min, 30 cycles of denaturation at 94 ℃ for 30 s, annealing at 60 ℃ for 30 s, and extension at 72 ℃ for 60 s, and final extension at 72 ℃ for 7 min. The PCR products were purified using QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) and digested with Hae III at 37 ℃ for 2 h in a reaction system containing 2 μL of 10 × Buffer, 2.3 μL of Hae III 2.3 μL, 9.7 μL of ddH_2O, and 6 μL of PCR production. Subsequently, agarose gel electrophoresis was performed to analyse the RFLP of the digestion products.

Statistical analysis

Microsoft Excel (Microsoft, Redmond, Washington, United States) was used for data collection and analysis. Data are expressed as mean ± SD or count number. We used Student's *t*-test, Fisher's exact test or χ^2 test to calculate the statistical difference in baseline characteristics between different HCV GT infected patients. Repeated measures analysis of variance was used to give comparisons among different groups or different time points and calculate the interaction effect between treating factors and time factors. Mauchly's test of sphericity was used to judge whether there were relations among the repeatedly measured data. If any (P < 0.05), Greenhouse-Geisser corrected results should be taken; Bonferroni or Fisher's Least Significant Difference tests (when Epsilon < 0.7, Bonferroni test) were used to do pairwise comparisons of the repeatedly measured data in different measurement times. We carried out statistical analyses with SPSS version 16.0. P < 0.05 was considered statistically significant.

RESULTS

Baseline characteristics of enrolled patients

A total of 54 patients who underwent DAAs treatment were enrolled in our study, including 40 HCV GT 1b infected patients and 14 HCV GT 2a infected patients. Their mean age was 55.4 ± 16.6 years. Of the 54 patients included, 29 were male, 21 had experienced PR treatment, and 20 had cirrhosis. The mean score of LSM was 15.9 ± 14.1 (kPa), and the mean PLT count was 147.1 ± 65.1 (10^9/L). Baseline characteristics for all the 54 CHC patients are shown in Table 1. The distribution of PR treatment experienced patients and cirrhotic patients and all other baseline characteristics did not differ significantly between HCV GT 1b infected

Table 1 Baseline characteristics of enrolled patients

Characteristic	All (n = 54)	GT1b (n = 40)	GT2a (n = 14)	$P_{(1b\ vs\ 2a)}$
Age	55.4 ± 16.6	57.2 ± 15.9	50.1 ± 18.1	0.175
Male/Female	29/25	21/19	8/6	0.764
HCV RNA log_{10} (IU/mL)	6.48 ± 0.97	6.63 ± 0.89	6.06 ± 1.1	0.058
PR(experienced/naive)	21/33	17/23	4/10	0.358
Non-cirrhotic/cirrhotic	34/20	24/16	10/4	0.446
LSM (kPa)	15.9 ± 14.1	17.5 ± 15.1	11.4 ± 9.8	0.162
FIB-4 score	4.07 ± 4.35	4.14 ± 3.53	3.87 ± 6.29	0.847
ALT (IU/L)	54.6 ± 36.3	57.4 ± 38.7	46.5 ± 27.9	0.339
AST (IU/L)	50.8 ± 33.1	50.6 ± 26.8	46.3 ± 37.1	0.555
eGFR(mL/min per 1.73 m^2)	87.1 ± 19.5	87.2 ± 20.9	86.5 ± 15.7	0.908
Scr (μmol/L)	83.6 ± 17.9	83.3 ± 19.5	84.4 ± 12.9	0.848
UA (μmol/L)	320.8 ± 76.3	315.2 ± 78.5	337.4 ± 72.9	0.349
BUN (mmol/L)	5.17 ± 1.50	5.21 ± 1.52	5.12 ± 1.55	0.881
WBC (10^9/L)	4.85 ± 1.67	4.67 ± 1.65	5.35 ± 1.70	0.192
RBC (10^{12}/L)	4.42 ± 0.64	4.40 ± 0.67	4.49 ± 0.59	0.652
HGB (g/L)	140.8 ± 17.2	139.9 ± 17.9	143.6 ± 15.6	0.492
PLT (10^9/L)	147.1 ± 65.1	143.0 ± 68.4	158.8 ± 55.0	0.439

HCV: Hepatitis C virus; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; Scr: Serum creatinine; UA: Uric acid; BUN: Blood urea nitrogen; LSM: Liver stiffness measurement; eGFR: Estimated glomerular filtration rate.

patients and HCV GT 2a infected patients (Table 1).

Treatment efficacy

Among the 40 HCV GT1b infected patients, 15 were treated with SOF + Daclatasvir (DAC) and 25 were treated with SOF/Ledipasvir (LDV). Among the 14 HCV GT2a infected patients, 6 were treated with SOF + DAC and 8 were treated with SOF + Ribavirin (RBV) (< 75 kg, 1000 mg/d; > 75 kg, 1200 mg/d). All non-cirrhotic patients were treated for 12 wk, HCV GT 1b infected patients with cirrhosis were treated for 24 wk, HCV GT 2a infected patients with cirrhosis were treated with SOF + DAC for 12 wk or SOF + RBV for 20 wk[29].

The majority of patients [83.3% (45/54)] achieved rapid virological response at 2 wk after treatment initiation (RVR 2), while nine patients, including four HCV GT1b infected patients treated with SOF + DAC, three HCV GT1b infected patients treated with SOF/LDV, one HCV GT2a infected patient treated with SOF + DAC, and one HCV GT2a infected patient treated SOF + RBV, had detectable HCV RNA. Of these nine patients, four had experienced PR treatment and five were treatment naïve; four were cirrhotic and five were non-cirrhotic. At the end of treatment, 96.3% (52/54) patients achieved virological response, while one HCV GT1b infected patient treated with SOF/LDV for 12 wk and one HCV GT2a infected patient treated with SOF + DAC for 12 wk still had detectable HCV RNA. SVR rate at 24 wk after the end of treatment (SVR 24) was 94.4% (51/54), and one GT2a patient treated with SOF + RBV for 20 wk relapsed at 12 wk after the end of treatment (Figure 1).

When patients were classified by HCV GT, PR treatment experience, DAAs regimens and liver condition, RVR 2 rates were 73.3% (11/15) in HCV GT 1b infected patients treated with SOF + DAC, 88.0% (22/25) in HCV GT 1b infected patients treated with SOF/LDV, 83.3% (5/6) in HCV GT 2a infected patients treated with SOF + DAC, 87.5% (7/8) in HCV GT 1b infected patients treated with SOF + RBV, 84.8% (28/33) in PR treatment naive patients, 81.0% (17/21) in PR treatment experienced patients, 85.3% (29/34) in non-cirrhotic patients, and 80.0% (16/20) in cirrhotic patients; SVR 24 rates were 97.5% (39/40) in HCV GT 1b infected patients, 85.7% (12/14) in HCV GT 2a infected patients, 93.9% (31/33) in PR treatment naive patients, 95.2% (20/21) in PR treatment experienced patients, 94.1% (32/34) in non-cirrhotic patients, and 95.0% (19/20) in cirrhotic patients.

Changes of clinical indices before and after combined DAAs treatment

The changes of clinical indices among different observing points at the end of treatment and at 24w after the end of treatment compared with baseline data in 54 included patients are shown in Table 2. For all patients, ALT and AST levels at the end of treatment and at 24 wk after the end of treatment were significantly decreased compared with baseline levels (ALT: 54.6 ± 36.3 *vs* 20.3 ± 13.3, $P_{01} < 0.001$; 54.6 ± 36.3 *vs* 17.1 ± 6.9, $P_{02} < 0.001$. AST: 50.8 ± 33.1 *vs* 24.4 ± 10.4, $P_{01} < 0.001$; 50.8 ± 33.1 *vs* 22.4 ± 7.0, $P_{02} < 0.001$). Post-treatment FIB-4 score exhibited a continued reduction (4.07 ± 4.35 *vs* 2.94 ± 2.76, $P_{01} < 0.001$; 2.94 ± 2.76 *vs* 2.61 ± 2.21, $P_{12} = 0.003$) compared with that at baseline (Table 2). At the end of treatment, eGFR level had a significant decrease (eGFR: 87.1 ± 19.5 *vs* 81.2 ± 20.0, $P_{01} = 0.001$), serum creatinine (Scr) and uric acid (UA) levels were significantly increased compared with baseline levels (Scr: 83.6 ± 17.9 *vs* 88.8 ± 19.4, $P_{01} < 0.001$; UA: 320.8 ± 76.3 *vs* 354.5 ± 87.6, $P_{01} <$

Table 2 Change of clinical indices before and after combined directly acting antivirals treatment

	T0	T1	T2	P_{01}	P_{12}	P_{02}	$P_{(DAAs*Time)}$
FIB-4 score	4.07 ± 4.35	2.94 ± 2.76	2.61 ± 2.21	0.001	0.003	< 0.001	0.399
ALT (IU/L)	54.6 ± 36.3	20.3 ± 13.3	17.1 ± 6.9	< 0.001	0.061	< 0.001	0.594
AST (IU/L)	50.8 ± 33.1	24.4 ± 10.4	22.4 ± 7.0	< 0.001	0.006	< 0.001	0.733
eGFR(mL/min/1.73 m^2)	87.1 ± 19.5	81.2 ± 20.0	83.6 ± 21.2	0.001	0.174	0.097	0.646
Scr (μmol/L)	83.6 ± 17.9	88.8 ± 19.4	86.8 ± 19.1	< 0.001	0.137	0.039	0.481
UA (μmol/L)	320.8 ± 76.3	354.5 ± 87.6	345.9 ± 89.4	< 0.001	0.212	0.001	0.299
BUN (mmol/L)	5.17 ± 1.50	5.12 ± 1.40	5.65 ± 1.80	0.757	0.003	0.009	0.858
WBC (10^9/L)	4.85 ± 1.67	4.91 ± 1.54	5.00 ± 1.34	0.725	0.595	0.342	0.536
RBC (10^{12}/L)	4.42 ± 0.64	4.43 ± 0.68	4.50 ± 0.68	0.822	0.345	0.223	0.023
HGB (g/L)	140.8 ± 17.2	139.4 ± 20.9	141.1 ± 21.1	0.467	0.443	0.860	0.026
PLT (10^9/L)	147.1 ± 65.1	153.6 ± 67.5	158.2 ± 65.9	0.053	0.117	0.008	0.540

T0: Baseline; T1: End of treatment; T2: 24 wk after the end of treatment. P01: Significance of difference between T0 and T1; P12: Significance of difference between T1 and T2; P02: Significance of difference between T0 and T2; P(DAAs*Time): Interactive effects of DAAs regimens and time points on the changes of renal function indices. ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; Scr: Serum creatinine; UA: Uric acid; BUN: Blood urea nitrogen; LSM: Liver stiffness measurement; eGFR: Estimated glomerular filtration rate; DAAs: Directly acting antivirals.

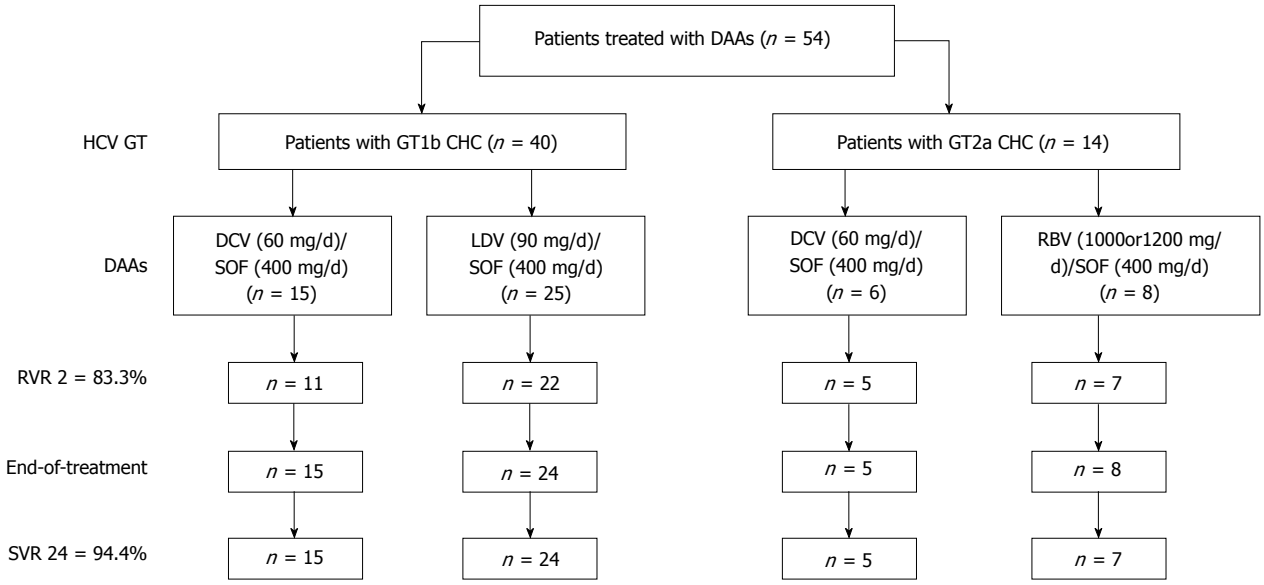

Figure 1 Diagram of detailed treatment regimens for 54 chronic hepatitis C patients and the treatment efficacy. CHC: Chronic hepatitis C; RVR 2: Rapid virological response at 2 wk after treatment initiation; SVR 24: Sustained virological response at 24 wk after the end of treatment.

0.001), although no significant improvements were observed at 24 wk after the end of treatment (Scr: 83.6 ± 17.9 *vs* 86.8 ± 19.1, P_{02} = 0.039; UA: 320.8 ± 76.3 *vs* 345.9 ± 89.4, P_{02} = 0.001). BUN level at the end of treatment had no significant changes compared with baseline level, while an increased BUN level was observed at 24 wk after the end of treatment (5.17 ± 1.50 *vs* 5.65 ± 1.80, P_{02} = 0.009) (Figure 2). DAAs regimens and time points had no interactive effects on the changes of hepatorenal function indices, and the interactive effects on changes of RBC and HGB may be caused by RBV (Table 2). Combined DAAs treatment had no significant effect on the WBC count, RBC count, or HGB concentration; however, the PLT count had a remarkable increase at 24 wk after the end of treatment compared with that at baseline (147.1 ± 65.1 *vs* 158.2 ± 65.9, P_{02} = 0.008) (Table 2).

AEs during combined DAAs treatment

The frequency of AEs during treatment was 33.3% (18/54). The major AEs were fatigue (16.7%), headache (7.4%), anorexia (7.4%), and insomnia (5.6%), and most of them were mild and tolerable. One GT1b patients treated with SOF + DAC discontinued the treatment at 8 wk due to the development of renal area pain (Table 3). The common AEs during traditional PR treatment, like fever, anemia, neutropenia, and thrombocytopenia, rarely occurred during DAAs treatment, and only two patients treated with SOF + RBV were observed with mild anemia.

DISCUSSION

The availability and development of DAAs revolutionized the management of HCV infection. In America,

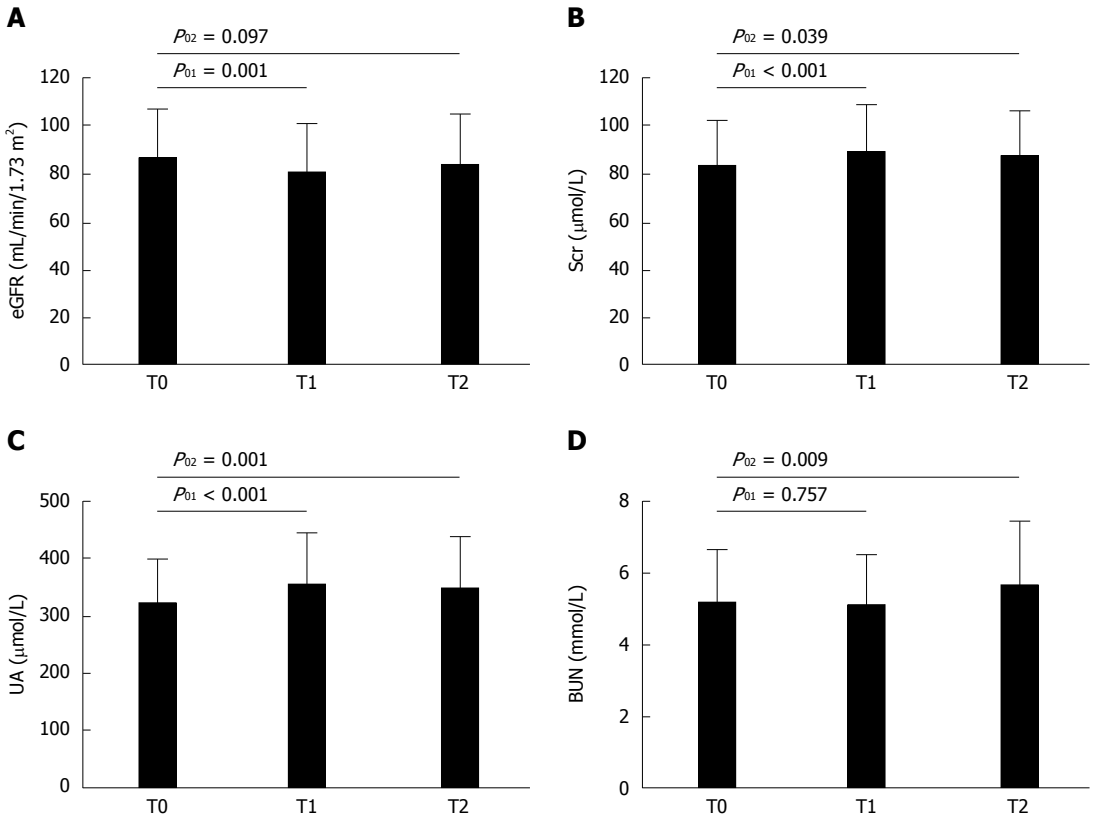

Figure 2 Changes of renal function indices among different observing points. A: eGFR; B: Scr; C: UA; D: BUN. T0: Baseline; T1: End of treatment; T2: 24 wk after the end of treatment. eGFR: Estimated glomerular filtration rate; Scr: Serum creatinine; UA: Uric acid; BUN: Blood urea nitrogen.

Table 3 Frequency of adverse events during combined directly acting antivirals treatment

Adverse event	*n* (%)
Fatigue	9 (16.7)
Headache	4 (7.4)
Anorexia	4 (7.4)
Insomnia	3 (5.6)
Anemia	2 (3.7)
Pruritus	1 (1.9)
Anxiety	1 (1.9)
Renal area pain	1 (1.9)
Treatment discontinuation	1 (1.9)
Total	18 (33.3)

Europe, Japan, and many other countries, DAAs achieved high SVR rates with a low frequency of AEs in clinical trials and real-world cohorts[7], while limited data were available in China. Considering the ethnic, regional, and virological differences, we analyzed the efficacy and safety of combined DAAs for treatment of 54 Chinese CHC patients in a real-world setting.

This study showed a promising SVR rate as those in other countries and areas, while abnormal changes in renal function indices and relative more AEs were unexpected. In this study, 83.3% (45/54) of patients achieved RVR 2 and 94.4% (51/54) of patients achieved SVR 24 which had no significant difference with those reported in previous studies[30-33]. With the application of DAAs, some cases were reported with nephrotoxicity and hepatotoxicity due to DAAs treatment[34,35]. Thus, our study analyzed the changes of hemogram and hepatorenal function indices and the frequency of AEs associated with combined DAAs treatment. After the treatment, liver function indices and FIB-4 score reflecting the liver fibrosis stage had significant improvements. Different with traditional PR treatment, DAAs had no significant effect on hemogram, and along with the improvement of liver function, PLT count at 24 wk after the end of treatment was significantly increased compared with the baseline value. However, the mean Scr and UA levels at the end of treatment had a significant elevation compared with those at baseline, and there was no trend toward improvement at 24 wk after the end of treatment. The specific reasons for changes of renal function indices are unknown, considering that no abnormal changes in renal function indices were found in clinical trials[29,36,37]. The potential drug-drug interactions between combined DAAs regimens and complicated concomitant medications in this real-world cohort may be the major reason. Our study showed relatively more AEs associated with the use of combined DAAs treatment, and the major AEs were fatigue, headache, anorexia, and insomnia. Although most of them were mild and tolerable, more attention should be paid during the treatment.

Though based on a small cohort of patients, the abnormal changes in renal function indices and relative more AEs during treatment should be taken as a note of caution. Clinical physicians should implement close renal function monitoring and attach importance to AEs occurring in patients receiving combined DAAs treatment.

COMMENTS

Background

Treatment of hepatitis C virus (HCV) infection has reached a new era with the approval of directly acting antivirals (DAAs). All-oral DAAs combination regimens have achieved high sustained virological response (SVR) rates with minor contraindications and adverse events. China has the greatest number of chronic hepatitis C (CHC) cases worldwide, with an estimated 29.8 million patients infected, while there had been limited data on the use of combined DAAs treatment in a real-world setting in China.

Research frontiers

In China, there have been limited data on the use of combined DAAs treatment in a real-world setting. The research hotspot is to show the efficacy of DAAs for treatment of Chinese CHC patients and explore the effects of DAAs on hemogram and hepatorenal function indices, and the frequency of adverse events (AEs) during treatment in a real-world setting.

Innovations and breakthroughs

The changes of clinical indices among different observing points during combined DAAs treatment were analysed. At the end of treatment, eGFR level had a significant decrease, serum creatinine and uric acid levels were significantly increased compared with baseline levels, although no significant improvements were observed at 24 wk after the end of treatment. On the other hand, the current data also showed a high frequency of AEs during combined DAAs treatment (33.3%), and the major AEs were fatigue, headache, anorexia, and insomnia.

Applications

The data in this study showed the abnormal changes in renal function indices and relative high frequency of AEs during combined DAAs treatment. This study would remind clinical physicians of implementing close renal function monitoring and focusing on AEs occurring in patients receiving combined DAAs treatment.

Terminology

DAAs are inhibitors directly acting on different viral targets, including NS3 protease inhibitors, NS5A inhibitors, nucleoside/nucleotide analogues, and non-nucleoside inhibitors of the RNA-dependent RNA polymerase. In 2011, Telaprevir and Boceprevir opened a new area for HCV therapy, while these two NS3/4 protease inhibitors were given in combination with pegylated interferon and ribavirin. Subsequent all-oral DAAs combination regimens have achieved high SVR rates with fewer contraindications and AEs.

Peer-review

The chosen topic is currently one of the hot topics and of great interest to a lot of clinicians. The last few years have witnessed significant progress in HCV therapy by replacing of IFN + ribavirin combined therapy with oral compounds acting directly to inhibit HCV replication (DAAs). DAAs target multiple steps in the HCV life cycle and are currently used in combination to treat HCV infection without need of IFN.

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ORIGINAL ARTICLE

Observational Study

Observation of the effect of targeted therapy of 64-slice spiral CT combined with cryoablation for liver cancer

Qiao-Huan Yan, Dian-Guo Xu, Yan-Feng Shen, Ding-Ling Yuan, Jun-Hui Bao, Hai-Bin Li, Ying-Gang Lv

Qiao-Huan Yan, Department of Geriatrics, the First Hospital of Handan, Handan 056001, Hebei Province, China

Dian-Guo Xu, Department of Anatomy, Medical College of Hebei University of Engineering, Handan 056001, Hebei Province, China

Yan-Feng Shen, Hai-Bin Li, Department of Oncology, Affiliated Hospital of Hebei University of Engineering, Handan 056001, Hebei Province, China

Ding-Ling Yuan, Jun-Hui Bao, Ying-Gang Lv, Department of Imaging, Affiliated Hospital of Hebei Medical University, Shijiazhuang 056002, Hebei Province, China

Ying-Gang Lv, Department of Radiology, Affiliated Hospital of Hebei University of Engineering, Handan 056001, Hebei Province, China

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Correspondence to: Ying-Gang Lv, Deputy Chief Physician, Department of Radiology, Affiliated Hospital of Hebei University of Engineering, Jianshe Street No. 80, Fuxing District, Handan 056001, Hebei Province, China. lvyinggang@126.com
Telephone: +86-310-8577555
Fax: +86-310-8577555

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Abstract

AIM

To observe the effect of targeted therapy with 64-slice spiral computed tomography (CT) combined with cryoablation for liver cancer.

METHODS

A total of 124 patients (142 tumors) were enrolled into this study. According to the use of dual-slice spiral CT or 64-slice spiral CT as a guide technology, patients were divided into two groups: dual-slice group (n = 56, 65 tumors) and 64-slice group (n = 8, 77 tumors). All patients were accepted and received targeted

therapy by an argon-helium superconducting surgery system. The guided scan times of the two groups was recorded and compared. In the two groups, the lesion ice coverage in diameter of ⩾ 3 cm and < 3 cm were recorded, and freezing effective rate was compared. Hepatic perfusion values [hepatic artery perfusion (HAP), portal vein perfusion (PVP), and the hepatic arterial perfusion index (HAPI)] of tumor tissues, adjacent tissues and normal liver tissues at preoperative and postoperative four weeks in the two groups were compared. Local tumor changes were recorded and efficiency was compared at four weeks post-operation. Adverse events were recorded and compared between the two groups, including fever, pain, frostbite, nausea, vomiting, pleural effusion and abdominal bleeding.

RESULTS

Guided scan times in the dual-slice group was longer than that in the 64-slice group (t = 11.445, P = 0.000). The freezing effective rate for tumors < 3 cm in diameter in the dual-slice group (81.58%) was lower than that in the 64-slice group (92.86%) (χ^2 = 5.707, P = 0.017). The HAP and HAPI of tumor tissues were lower at four weeks post-treatment than at pre-treatment in both groups (all P < 0.05), and those in the 64-slice group were lower than that in the dual-slice group (all P < 0.05). HAP and PVP were lower and HAPI was higher in tumor adjacent tissues at post-treatment than at pre-treatment (all P < 0.05). Furthermore, the treatment effect and therapeutic efficacy in the dual-slice group were lower than the 64-slice group at four weeks post-treatment (all P < 0.05). Moreover, pleural effusion and intraperitoneal hemorrhage occurred in patients in the dual-slice group, while no complications occurred in the 64-slice group (all P < 0.05).

CONCLUSION

64-slice spiral CT applied with cryoablation in targeted therapy for liver cancer can achieve a safe and effective freezing treatment, so it is worth being used.

Key words: 64-slice spiral computed tomography; Cryoablation; Liver cancer

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Core tip: One hundred and twenty-four patients with liver cancer were accepted therapy by argon-helium superconducting surgery system. Compared with 64-row group, guided scan times was longer and freezing effective rate for tumors < 3 cm was lower in the dual-slice group. Four weeks after treatment, compared with dual-slice group, the hepatic artery perfusion and hepatic arterial perfusion index of tumor tissues were lower, the treatment effect and therapeutic efficacy in the 64-row group were higher. Complications were higher in the double-row group than in the 64-row group. 64-slice spiral computed tomography applied with cryoablation in targeted therapy for liver cancer can achieve a safe and effective freezing treatment.

Yan QH, Xu DG, Shen YF, Yuan DL, Bao JH, Li HB, Lv YG. Observation of the effect of targeted therapy of 64-slice spiral CT combined with cryoablation for liver cancer. *World J Gastroenterol* 2017; 23(22): 4080-4089 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i22/4080.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i22.4080

INTRODUCTION

The incidence of liver cancer in China accounts for third place malignant tumors, and there has been an increasing trend in its incidence in recent years[1,2]. In clinical practice, the best treatment for early liver cancer is the surgical resection of lesions. However, early symptoms of liver cancer are not obvious. Therefore, most patients diagnosed with liver cancer already developed and their tumors cannot be surgically removed or have poor surgical outcomes, and miss the opportunity for conventional radical surgery[3-6]. For patients with advanced tumors, if local treatment was available to delay the growth and spread of the tumor, it would help extend survival time and improve the quality of life of patients. With the development of minimally invasive treatments for tumors, these patients can be clinically given advanced cryoablation therapy at present[7-10]. Cryoablation targeted treatment has features such as a precise curative effect, easy to operate, safe and effective. This has gradually become an important method of minimally invasive treatment for liver cancer. Furthermore, imaging technology has been combined with the application of cryoablation during the course of treatment to observe tumor position and size, develop a preoperative puncture path, and for postoperative evaluation, of which computed tomography (CT) is widely used[11-14]. However, dual-slice spiral CT scanning technology has a long scan time and can't accurately position smaller tumors. As a guide during the cryosurgical treatment process, it may result in a smaller tumor omission. 64-slice spiral CT has a fast scanning speed and can provide clearer tumor positioning[15]. In order to better understand the application of 64-slice spiral CT during cryoablation, this study compared and analyzed the dual-slice spiral CT and 64-slice spiral CT guided cryosurgery treatments performed for liver cancer patients in our hospital; provide a reference for the clinical application of 64-slice spiral CT guided cryoablation therapy.

MATERIALS AND METHODS

General information

A total of 124 patients with primary liver cancer, who were treated in our hospital from January 2014 to June

2016, were enrolled in this study. A total of 142 tumors were found from these patients. Among these patients, 83 patients were males and 41 patients were females; and the age of these patients ranged between 31-78 years, with a mean age of 54.5 ± 9.9 years. The diameter of the tumors ranged from 1.1 cm to 9.3 cm (6.3 ± 3.4 cm). In this study, the 124 patients were divided into two groups: dual-slice group and 64-slice group. Patients in the dual-slice group comprised of 56 patients (65 tumors) who underwent dual-slice spiral CT guided cryosurgery from January 2014 to March 2015. Among these patients, 38 were male and 18 were female, and the mean age of these patients was 52.4 ± 10.4 years. Furthermore, the tumor diameters ranged from 1.1 cm to 8.2 cm (6.2 ± 3.1 cm). Patients in the 64-slice group comprised 68 patients (77 tumors) who underwent 64-slice spiral CT guided cryosurgery from April 2015 to June 2016. Among these patients, 45 were male and 23 were female, and the mean age of these patients was 57.4 ± 9.5 years. Furthermore, the tumor diameters ranged from 1.3 to 9.3 cm (6.5 ± 3.6 cm). The difference in age, tumor diameter and other general information between these two groups of patients was not statistically significant ($P > 0.05$).

Inclusion criteria: (1) patients with pathological examination-confirmed primary liver cancer; (2) elderly patients with heart and lung function not suitable for open surgery; (3) patients with liver function Child-Pugh grade A or B; (4) patients without severe hepatocellular jaundice and had a large number of ascites; and (5) patients with no serious coagulation dysfunction. This study was approved by the hospital ethics committee. All patients voluntarily chose cryoablation therapy, understood its importance, and provided a signed informed consent.

Methods

In this study, 64-slice spiral CT guidance technology was Light Speed VCT; GE Company, United States, while the dual-slice spiral CT machine guidance instrument was Prospeed F-II; GE Company, United States. The two groups used the Magnetic Resonance-Compatible Cryotherapy System (Cryo-HIT) manufactured by Galileo, Israel. The surgical operations were performed by the same group of physicians. The patient's preoperative CT and other test results were used to determine the entry route, probe combined model and treatment range. Local anesthesia was performed on the puncture site after conventional disinfection, and intermittent CT scans were performed to observe the probe position and for timely adjustment. To determine the expected location of the needle on the tumor to start the ablation, first, the helium is frozen for 10-15 min. Then, re-warm the helium after three minutes. Repeat freezing and thaw once. Perform regular CT scans to detect iceball formation. Withdraw the frozen knife, hemostasis, and suture and bandaged up the puncture site.

A full perfusion examination of the liver was performed on patients three days before cryoablation treatment and four weeks after surgery in the Radiation Department of our hospital using Light Speed VCT (GE Company, United States). Preoperative preparation of CT perfusion include prothrombin time, blood biochemistry and other routine preoperative examinations, fasting for 4-6 h, and drinking 1000 mL of warm water 10-15 min before examination. An 18G standard intravenous catheter was placed at the right arm elbow anterior vein. The patient was encapsulated with an abdominal bandage and given breathing exercises, which were mainly chest breathing, supplemented by abdominal breathing. Precautions during the examination were explained to the patient.

Patients were placed in the supine foot first scan position. Before perfusion, patients were first given a routine abdominal plain scan to observe the liver and location of lesions, as well as the extent of the disease. Then, a dynamic volume scan was performed. Next, 40 mL of non-ion contrast agent iohexol and 30 mL of physiological saline (rate 5 mL/s) was administered through the intravenous indwelling needle in front of the elbow using a double-barreled high-pressure syringe. Then, the injection of the contrast agent was performed after eight seconds. The liver perfusion imaging picture obtained through the body perfusion software used dual input mode for analysis. Furthermore, hepatic artery perfusion (HAP), portal vein perfusion (PVP), and the hepatic arterial perfusion index (HAPI) perfusion parameter pseudo-color images were also obtained. Three region of interest (ROI) parameters of perfusion in the axial position, in the coronal and sagittal positions were randomly selected. Notice that the preoperative and postoperative selection of the region should be consistent, and mean values were calculated for the tumor, adjacent tumor and normal liver tissues. After four weeks, liver perfusion examination was carried out and an enhanced scan was performed, enhancement of the lesion was observed, and the local ablation of the tumor was evaluated.

Observation index

CT-guided scan times were recorded and compared between the two groups of patients. Furthermore, the postoperative ice coverage range of $\geqslant$ 3 cm and < 3 cm diameter tumors in the two groups were recorded. Then, the freezing effective rate was compared between the two groups. The liver perfusion values of tumor tissues, adjacent tumor tissues, and normal liver tissues between the two groups at preoperative and four weeks after cryoablation treatment were compared. Four weeks after surgery, local changes in the tumor were observed. The treatment was divided as follows: (1) complete ablation, complete necrosis of

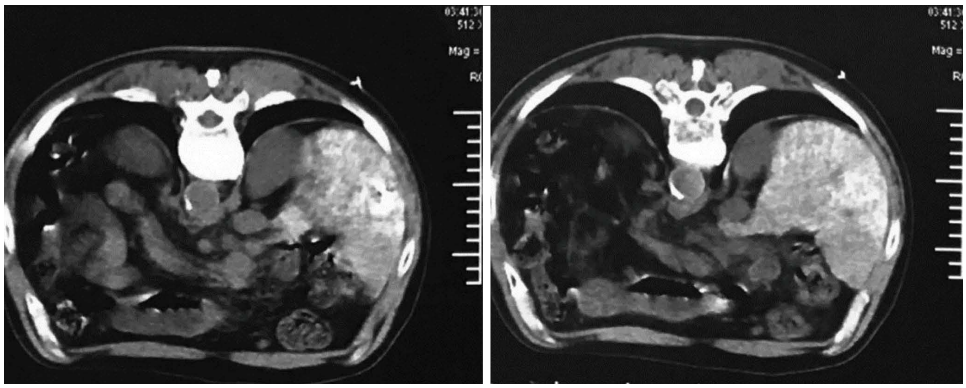

Figure 1 Before cryoablation, patients were placed in the prone position. Liver cancer chemoembolization after interventional therapy is shown, the lipiodol deposition area was the lesion tissue.

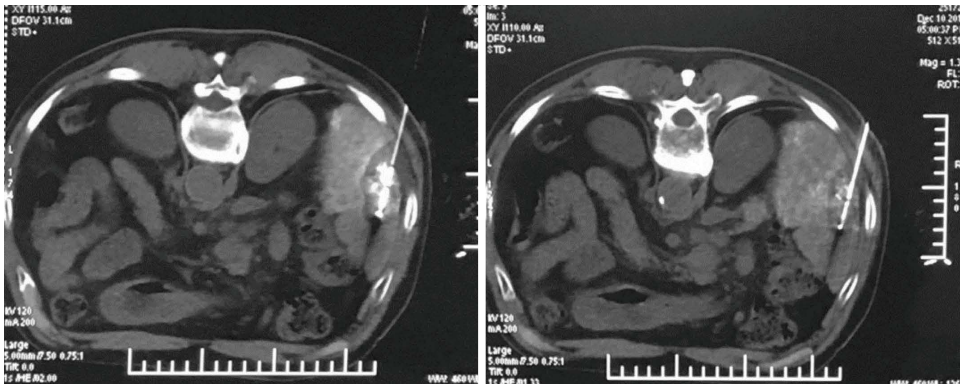

Figure 2 During cryoablation therapy, patients were placed in the prone position. The needle was placed to the right side of the abdominal wall and the puncture needle was positioned to the lesion bottom wall. The effective freezing area covered the lesions.

tumor tissues; (2) mostly ablated: lesion necrosis was > 80%; (3) stable: tumor necrosis was > 50%; and (4) progressive: increased mass or the emergence of newborn foci. The effective rate of the two groups was compared (complete ablation and major ablation). The clinical situation of these patients was closely observed. The following adverse reactions were recorded and compared between the two groups: fever, pain, skin frostbite, nausea, vomiting, pleural effusion, and abdominal bleeding.

Statistical analysis

The data were analyzed using SPSS 17.0 software. Scanning time, hepatic perfusion (HAP, PVP and HAPI), and other measurement data were expressed as mean ± SD. *t*-test was used to compare the differences between the two groups. The freeze effective rate, effective rate of treatment after four weeks and the incidence of various adverse reactions that occurred and other count data were compared using the χ^2-test between the two groups. The local therapeutic effects between the two groups were compared using the Mann-Whitney rank sum test. $P < 0.05$ was considered statistically significant.

RESULTS

Freezing effect of different diameter tumors in two groups

In the two groups, a total of 142 lesions successfully underwent puncture and cryotherapy. Before treatment, the location and size of the tumor were observed by scanning to establish a reasonable puncture path. During cryosurgery, scanning was performed to guide the positioning of the puncture needle and to observe the postoperative changes of the tumor. As shown in Figures 1, 2 and 3, the guide-scan time for the dual-slice group (5.7 ± 1.8 min) was greater than that in the 64-slice group (2.8 ± 1.2 min); and the difference was statistically significant (t = 11.445, P = 0.000). Furthermore, the freeze effective rate of patients with tumor diameters < 3 cm in the dual-slice group was significantly less than that in patients in the 64-slice group; and the difference was statistically significant (χ^2 = 5.707, P = 0.017). Moreover, there was no significant difference in the freeze effective rate of patients with tumor diameters > 3 cm between the two groups (χ^2 = 0.236, P = 0.627); Table 1.

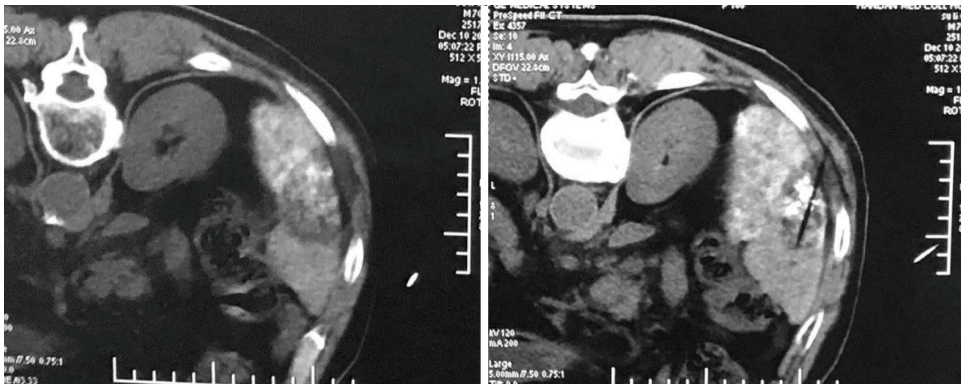

Figure 3 After the cryoablation treatment, the patient was placed in the prone position. The circular low-density area was the frozen necrotic area, which shows the lesions were within the range and it has good treatment effect.

Table 1 Postoperative ice coverage of different tumor sizes

Groups	Tumor diameter (cm)	Number of tumors (count)	ice coverage (%)			Effective rate
			100	80-99	< 80	
Dual-slice	< 3	38	19	12	7	81.58%
group	≥ 3	27	17	8	2	92.59%
64-slice	< 3	42	30	9	3	92.86%[a]
group	≥ 3	35	26	7	2	94.29%

Effective rate = (ice coverage ≥ 80% of the tumor volume in the number of tumors)/(the total number of tumors) × 100%. [a]$P < 0.05$, *vs* the dual-slice group.

Comparison of liver perfusion values between the two groups

At four weeks after cryoablation treatment, CT perfusion examination was performed again to observe the change in liver perfusion value. The difference in HAP, PVP and HAPI before treatment in two groups was not statistically significant. In the two groups, HAP and HAPI levels in tumor tissues were lower after treatment than before treatment ($P < 0.05$), and the difference was statistically significant ($P < 0.05$). Furthermore, HAP and HAPI levels in the 64-slice group were lower than in the dual-slice group; and the difference was statistically significant (all $P < 0.05$). HAP and PVP in the two groups were lower after treatment than before treatment, while HAPI increased; and the differences were statistically significant (all $P < 0.05$). There was no significant difference in HAP, HAPI and PVP between the two groups after treatment ($P > 0.05$). Furthermore, there was no significant difference in HAP, PVP and HAPI in normal liver tissues before and after treatment (all $P > 0.05$; Table 2).

Comparison of postoperative local treatment effect between the two groups

Patients in the two groups underwent CT perfusion imaging of the liver after four weeks to assess the therapeutic effect. In comparing these two groups, the treatment effect of patients in the 64-slice group was better than that observed in the dual-slice group; and the difference was statistically significant ($z = -2.325$, $P = 0.020$). Furthermore, the effective rate in the dual-slice double row group (76.93%) was lower than that in the 64-slice group (92.21%); and the difference was statistically significant ($\chi^2 = 8.946$, $P = 0.003$) (Table 3).

Incidence of post-operative adverse reactions

In terms of post-operative adverse reactions, three patients had pleural effusion and two patients had intraperitoneal hemorrhage in the dual-slice group, however, these two complications did not occur in the 64-slice group, the difference between these two groups was statistically significant ($P < 0.05$). Furthermore, fever rate was slightly higher in the 64-slice group than in the dual-slice group; but the difference was not statistically significant ($P > 0.05$). The difference in pain, skin frostbite, nausea and vomiting between the two groups was not significant ($P > 0.05$; Table 4).

DISCUSSION

For patients who cannot receive radical resection of liver cancer due to poor cardiopulmonary function or combined with extrahepatic metastasis or other factors, ablation therapy can not only removes the cancer cells, but also saves more normal liver function as much as possible. Furthermore, this treatment induces small physical trauma to patients[16-18]. Cryoablation therapy is a kind of local ablation therapy. With the development of technology in recent years, it has become one of the important means of minimally invasive treatment for liver cancer and other tumors[8,19]. Cryoablation therapy is the combined application of ultra-low temperature and re-warming technology, in which tumor tissues are rapidly frozen and melted to damage tumor cells[20,21]. With the right guidance technology, the frozen knife was placed in the tumor tissue; and high pressure argon and helium was successively input at room temperature. Argon at the tip of the knife was rapidly expanded, the tumor tissue was rapidly frozen to -140 ℃, and

Table 2 Comparison of liver perfusion values before and after treatment (mean ± SD)

Tissue		64-slice group			Dual-slice group		
		HAP (mL/min·100 mg)	PVP (mL/min·100 mg)	HAPI (%)	HAP (mL/min·100 mg)	PVP (mL/min·100 mg)	HAPI (%)
Tumor tissue	Pre-treatment	47.82 ± 16.71	8.51 ± 3.71	84.31 ± 13.22	48.42 ± 12.85	9.16 ± 3.75	82.27 ± 14.26
	Post-treatment	20.21 ± 9.42[a,c]	8.13 ± 3.22	48.93 ± 9.42[a,c]	26.21 ± 9.36[a]	8.53 ± 3.22	38.93 ± 9.42[a]
Adjacent tumor tissues	Pre-treatment	35.95 ± 15.25	47.81 ± 8.51	38.92 ± 16.91	35.95 ± 15.25	45.81 ± 8.51	37.92 ± 13.91
	Post-treatment	21.34 ± 9.95[a]	39.82 ± 14.33[a]	47.01 ± 9.71[a]	20.34 ± 9.95[a]	37.82 ± 14.33[a]	46.01 ± 9.71[a]
Normal liver tissue	Pre-treatment	25.65 ± 11.86	57.90 ± 18.93	28.62 ± 11.72	24.87 ± 13.48	58.93 ± 16.75	27.75 ± 14.68
	Post-treatment	26.02 ± 10.13	58.23 ± 16.94	27.43 ± 12.23	25.89 ± 10.78	59.78 ± 13.76	26.63 ± 12.25

[a]$P < 0.05$, post-treatment *vs* pre-treatment in the group; [c]$P < 0.05$, *vs* the dual-slice group post-treatment. HAP: Hepatic artery perfusion; PVP: Portal vein perfusion; HAPI: Hepatic arterial perfusion index.

Table 3 Follow-up observation of treatment effect

Groups	Number of tumors (*n*)	Treatment effect (%)				Effective rate
		Complete ablation	Mostly ablated	Stable	Progressive	
Dual-slice group	65	42 (64.62)	8 (12.31)	10 (15.38)	5 (7.69)	76.93%
64-slice group	77	62 (80.52)	9 (11.69)	4 (5.19)	2 (2.60)	92.21%
Test value	-		z = -2.325			χ^2 = 8.946
P value	-		0.02			0.003%

Effective rate = (the number complete ablation cases + the number of mostly ablated cases)/ total number of tumors × 100%.

Table 4 Postoperative adverse reactions *n* (%)

Group	Number of cases	Fever	Pain	Skin frostbite	Nausea and vomiting	Pleural effusion	Intraperitoneal hemorrhage
Dual-slice group	56	33 (53.57)	5 (8.93)	1 (1.79)	5 (8.93)	3(5.36)	2(3.57)
64-slice group	68	43 (63.24)	7 (10.29)	1 (1.47)	6 (8.82)	0	0
χ^2	-	1.925	0.106	0.032	0	5.508	3.635
P value	-	0.165	0.744	0.858	0.978	0.019	0.057

helium which re-entered into the tumor tissue rapidly rise to 40-45 ℃. During cryoablation, rapid freezing resulted in the production of intracellular ice crystals. This would destroy the osmotic balance inside and outside the cell membrane. Furthermore, due to cell volume expansion, extracellular ice crystals are formed, resulting in mutual extrusion and increased cell damage[22-24]. The melting immediate after the freezing causes the ice crystal balls to expand and burst, destroying the tumor cells. Then, after helium re-warms it to a certain extent, frozen ablation was carried out once again. This again damages tumor cells that have not been destroyed by the first cryoablation due to the dehydration of cells, increasing the destruction of tumor tissue[25,26]. In the treatment of liver cancer, cryosurgery allows the accurate display of the tumor location and peripheral vascular conditions, which is important for an accurate puncture for cryotherapy and the right cryoablation area. Therefore, seeking for a good imaging technology to accurately display the location of the tumor and its surrounding tissue blood vessels can provide guidance in cryoablation treatment. That can significantly reduce damage to the surrounding tissues of the lesion and reduce postoperative complications[27-32]. At present, CT guidance has been widely used in the clinic. With the development of CT technology, 64-slice spiral CT has a high scanning speed, provides better resolution images, has a powerful 3D reconstruction capability that provides more intuitive images for the doctor, and can more clearly show the situation of blood vessels. This would help in understanding the relationship between the lesion and the surrounding blood vessels, observe whether blood vessels are within the liver variation, and avoid intraoperative variability vascular puncture accidental injury[33-36]. Therefore, this study compares and analyzes the cryosurgical treatment effects and complications between 64-slice spiral CT and double-slice spiral CT guidance, and try to observe whether 64-slice spiral CT-guided cryosurgery of the liver cancer can achieve better results.

Effect of cryotherapy in the two groups of patients

Comparing the scan time between the two groups of patients, it can be seen that the scan time of the 64-slice spiral CT is shorter, since the adjustment of the needle position during surgery and the observation of the formation of ice balls needs to be scanned several

times, 64-slice spiral CT scan can help reduce scan waiting time; which speeds up the surgical process. The difference in freezing efficiency rate in tumor lesions ⩾ 3 cm in diameter between the two groups was not statistically significant. However, the freezing efficiency rate was significantly greater in tumors < 3 cm in diameter in the 64-slice group. 64-slice spiral CT-guided cryoablation have better results for smaller lesions.

This is because 64-slice spiral CT has higher spatial resolution, compared to dual-splice spiral CT. Furthermore, the 64-slice spiral CT enables the simultaneous collection of a 64-layer sub-millimeter thick image, and covers a long revolution of nearly 40 mm when it rotates in a circular course. In addition, it enables the reconstruction of the cross-section, coronal plane and other arbitrary plane images. The collected images are multi-planar imaging (MPR). That is, scanning is performed once, which can be adjusted in multiple directions. This enables an arbitrary slice image to be obtained, and helps in observing the details of the lesion and its spatial anatomy relationship. These advantages allow the 64-slice spiral CT to more clearly show the edge of the tumor and the formation of the edge of the ice ball during the surgery, help physicians control the ice ball range, and more clearly show the tumor tissue and its surrounding tissue. For smaller lesions, it can also clearly show the edge of the lesion and its adjacent vascular structure. Particularly near the diaphragm of the lesion, hilar large vessels and other special locations, the 64-slice spiral CT provides a clear image that enables physicians reduce intraoperative time, avoid damage to the surrounding normal tissues, and limits the freezing range; which affects the influence of the ablation[37-40]. 64-slice spiral imaging can directly reflect the internal hemodynamics of the liver, and has good reference value for understanding tumor angiogenesis and its biological characteristics [11,41-44].

Evaluation of effects after four weeks of treatment

CT perfusion examination after four weeks revealed that HAP and HAPI decreased in tumor tissues in the two groups. However, these decreased more significantly in the 64-slice group, while the difference among HAP, HAPI and PVP between the two adjacent tissues and normal liver tissues was not statistically significant. The significant reduction in HAP and HAPI in the liver after treatment was an indicator of efficacy. This proves that lesion ablation treatment was better in patients in the 64-slice group. The presence of HAP in the tumor may represent the presence of arterial blood supply in the tumor, or the formation of postoperative granulation tissues with small blood vessels. In addition, liver cancer can easily recur and metastasize. Therefore, patients still need to be examined through continuous follow-ups. The effective rate in the 64-slice group was higher than that in the dual-slice group. The reason is that 64-slice spiral CT can provide good coverage of the ice ball for lesion with ⩾ 3 cm and < 3 cm diameters, and improve the ablation effect. Its three-dimensional imaging technology enables physicians to view liver blood vessels and lesions that are more solid, clear, more accurate, and more comprehensive; allowing a reasonable needle puncture and optimizing the freezing effect[11,29,45,46]. Its rapid scan imaging during surgery allows the detection of the formation of ice ball and freezing conditions, provides an accurate lesion and surrounding tissue image, help in the timely adjustment of the ablation procedure, and prevents damage in surrounding tissues, while improving the effectiveness of tumor tissue ablation[9,35,47].

Incidence of postoperative complications in the two groups

In the dual-slice group, three patients had pleural effusion and two patients had intraperitoneal hemorrhage. However, none of these complications occurred in patients in the 64-slice group. Furthermore, the difference between fever, pain, skin frostbite, nausea and vomiting occurring in patients in these two groups was not statistically significant. That proves 64-slice spiral CT can be used as a means of guidance, it has fewer postoperative complications, and its absence of pleural effusion and abdominal bleeding may be due to better liver blood vessel imaging technology. Compared to dual-slice spiral CT, 64-slice spiral CT can display a clearer visualization of the distribution of small blood vessels, allows the observation of the presence of variant blood vessels, and reduced the incidence of intraoperative vascular injury. Pleural effusion may occur due to tumors near the diaphragm, the intraoperative frozen stimulation to the diaphragm, or failure to monitor the extent of the frozen lesions in time; stimulating the pleura. The 64-slice spiral CT provides clear imaging, helps the surgeon accurately observe and control the freezing range, and reduces stimulation to the diaphragm and pleura[48-50].

However, in this study, only the treatment effect between 64-slice spiral CT-guided and dual-slice spiral CT-guided cryoablation were compared. At present, there is a need to further observe and compare the clinical applications of spiral CT, ultrasound, MRI and other guidance imaging technologies; and determine whether there is a difference in the guidance effect of these techniques when compared with 64-slice spiral CT. Furthermore, there is also a need to analyze the clinical applications the current guidance technologies, which has its own advantages; particularly in determining which technology has a better guiding treatment for patients with certain characteristics. In addition, this study followed-up patients up to four weeks after surgery. Future research should extend this follow-up time, in order to observe the recurrence

and metastasis of liver cancer, as well as the survival time of patients of patients after treatment.

In conclusion, 64-slice spiral CT in the cryosurgical treatment of liver cancer targeting process provides a safe and effective freezing treatment. It is a reasonable choice worthy for clinical applications.

COMMENTS

Background

Liver cancer is the third most common malignant tumour in China, and there has been an increasing trend in its incidence in recent years. For patients with advanced tumors, if local treatment is available to delay the growth and spread of the tumor, it would help extend survival time and improve the quality of life of patients. Cryoablation targeted treatment has gradually become an important method of minimally invasive treatment for liver cancer. The guide technology used in argon-helium cryoablation therapy includes double-row spiral computed tomography (CT) and 64-slice spiral CT and so on. To compare the dual-slice spiral CT and 64-slice spiral CT guided cryosurgery treatments performed for liver cancer patients can provide a reference for the clinical application of 64-slice spiral CT guided cryoablation therapy.

Research frontiers

In the treatment of liver cancer, cryosurgery allows the accurate display of the tumor location and peripheral vascular conditions, which is important for an accurate puncture for cryotherapy and the right cryoablation area. The 64-slice spiral CT has a high scanning speed, provides better resolution images, and has a powerful 3D reconstruction capability that provides more intuitive images for the doctor. Therefore, the authors hope that this technology in the guidance of argon helium cryo-knife treatment has a good effect.

Innovations and breakthroughs

The 64-slice spiral CT has higher spatial resolution. For smaller lesions, it can also clearly show the edge of the lesion and its adjacent vascular structure. The 64-slice spiral CT provides a clear image that enables physicians to reduce intraoperative time, avoiding damage to the surrounding normal tissues. This means that guidance of argon-helium cryosurgery treatment of small diameter liver cancer also has a good freezing effect. It has an important clinical application value and is worthy of promoting.

Applications

In this study, compared with the double-row group, the guide scan time is short and more efficient in tumors < 3 cm in diameter in the 64-slice group. Four weeks after treatment, the treatment effect and treatment efficiency is better, less complications in the 64-slice group. Therefore, it is recommended to use 64-slice spiral CT in the treatment of liver cancer by argon helium cryoablation.

Peer-review

This an interesting manuscript. In this manuscript, the effect of targeted therapy of 64-slice spiral CT combined with cryoablation for liver cancer was observed. A total of 124 patients were enrolled into this study. According to the use of dual-slice spiral CT or 64-slice spiral CT as a guide technology, patients were divided into two groups: dual-slice group and 64-slice group. All the patients were accepted for the targeted therapy by argon-helium superconducting surgery system. The guided scan times of the two groups was recorded and compared. The authors found that the treatment effect and therapeutic efficacy in the dual-slice group were lower than the 64-slice group at four weeks post-treatment.

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ORIGINAL ARTICLE

Prospective Study

Inflammatory bowel disease incidence in Czech children: A regional prospective study, 2000-2015

Jan Schwarz, Josef Sýkora, Dominika Cvalínová, Renáta Pomahačová, Jana Klečková, Martin Kryl, Petr Včelák

Jan Schwarz, Josef Sýkora, Dominika Cvalínová, Renáta Pomahačová, Department of Paediatrics, Charles University, Faculty of Medicine in Pilsen, Faculty Hospital, 30460 Pilsen, Czech Republic

Jana Klečková, Martin Kryl, Petr Včelák, Department of Computer Science and NTIS - Research Centre, Faculty of Applied Sciences, University of West Bohemia, 30614 Pilsen, Czech Republic

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Correspondence to: Jan Schwarz, MD, Researcher in Pediatric Gastroenterology, Department of Paediatrics, Charles University, Faculty of Medicine in Pilsen, Faculty Hospital, Alej Svobody 80, 30460 Pilsen,
Czech Republic. schwarzj@fnplzen.cz
Telephone: +420-377-104330
Fax: +420-377-104693

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Abstract

AIM

To examine the incidence and trends in pediatric inflammatory bowel diseases (IBDs) over 2000-2015 and project the incidence to 2018.

METHODS

A 16-year prospective study of IBD patients < 19 years of age was conducted in the Czech Republic (the Pilsen region). All incident IBD cases within a well-defined geographical area were retrieved from a prospectively collected computerized clinical database. Historical Czech data were used for comparison (1990-2001). Our catchment population was determined from the census data. We calculated the incidence by relating the number of newly diagnosed cases to the size of the

pediatric population-at-risk in each calendar year. Age/sex, disease type, place of residence, and race/ethnicity were identified.

RESULTS

In total, 170 new IBD cases [105 Crohn's disease (CD), 48 ulcerative colitis (UC), and 17 IBD-unclassified (IBD-U)] were identified. The median age at IBD diagnosis was 14.2 years, 59.4% were males, and 97.1% were Caucasians. A male preponderance of IBD (P = 0.026) and CD (P = 0.016) was observed. With 109209 person-years in the catchment area, the average incidence of IBD per 100000 person-years was 10.0 (6.2 for CD, 2.8 for UC, and 1.0 for IBD-U) for children aged 0 to 19 years; for those aged 0 to 15 years, the incidence rate was 7.3 (4.6 for CD, 2.0 for UC, and 0.7 for IBD-U). An increase in incidence with age was observed (P = 0.0003). Over the 16-year period, the incidence increased for IBD patients (P = 0.01) and CD in particular (P < 0.0001), whereas the incidence for UC (P = 0.09) and IBD-U (P = 0.339) remained unchanged. IBD-projected data from 2016 to 2018 were 12.1, 12.3 and 12.6 per 100000 person-years, respectively.

CONCLUSION

Pediatric-onset IBD incidence is around its highest point. The increase, which is particularly pronounced for CD, may be challenging to relate to causes of pediatric disease.

Key words: Inflammatory bowel disease; Incidence; Children; Czech Republic; Pilsen region; Projections; Crohn's disease; Ulcerative colitis

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Core tip: The incidence of inflammatory bowel diseases (IBDs) is around its highest point to date. It has been markedly rising over a 16-year period and is especially pronounced for Crohn's disease (CD), such that CD is now more common than ulcerative colitis and IBD-unclassified. The changes in IBD incidence in developed countries cannot be explained by changes in genetic background, but the influence of environmental hazards on incidence may be involved in the pathogenesis of IBD. Analyses of time trends and the implications of environmental determinants are required to unravel concurrent factors and causal relationships with the ultimate goal of improving the current care of these patients.

Schwarz J, Sýkora, J, Cvalínová D, Pomahačová R, Klečková J, Kryl M, Včelák P. Inflammatory bowel disease incidence in Czech children: A regional prospective study, 2000-2015. *World J Gastroenterol* 2017; 23(22): 4090-4101 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i22/4090.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i22.4090

INTRODUCTION

The group of life-long, chronic relapsing inflammatory bowel diseases (IBDs) of unknown origin encompasses three separate disorders: Crohn's disease (CD), ulcerative colitis (UC), and IBD-unclassified (IBD-U). Genetic and environmental factors should be considered for IBD in childhood[1]. Approximately 25% of all diagnoses are made during childhood or adolescence[2], but a shift toward a younger age (*i.e.*, < 6 years old) has been observed[3].

IBD is unevenly distributed throughout the world. Studies worldwide have shown a rising but variable incidence of IBD, including large increases in incidence in pediatric populations[4,5]. Pediatric IBD incidence rates are higher in North America, the United Kingdom, and northern and western Europe than in southern latitudes[6-10]. Studies that evaluated only the incidence of pediatric-onset IBD with a wide range of incidence rates have been published previously[2]. A recent analysis also identified an east-west gradient in the incidence of IBD in Europe[11].

Although no firm conclusions can be drawn with respect to addressing the rising incidence of IBD, differences in geographic distribution, and particularly in changes in incidence over time within one area, may provide new insights into concurrent etiological factors[12-15]. However, this is possible only when enough pediatric projects within one defined geographical area, in relatively homogeneous populations, provide a fundamental basis for a better understanding of the epidemiology and environmental influences in any geographically restricted pediatric population and for the assessment of IBD.

Despite a rising worldwide incidence of IBD, there is a paucity of recent information regarding the exact incidence of IBD in Czech children[16,17]. Thus, it is of great interest to explore temporal time trends with an emphasis on children in central and eastern Europe and to describe differences among geographical regions across Europe. Thus, there are conflicting data on the rate at which IBD is diagnosed and whether its incidence has declined, stabilized, or even continued to rise in Czech children. Furthermore, no studies of IBD incidence have been published using data from the past 15 years; therefore, current time trends in Czech children remain entirely unknown.

Building on our previous experience[16], the objectives of this study were: (1) to discern the current incidence of IBD using a pediatric population (< 19 years of age at diagnosis) residing in a well-defined geographical region of the Czech Republic (Figure 1); (2) to characterize differences by age and sex; and (3) to gain insights into recent trends from 2000 to 2015 and compare our results to historical incidence data from comparable Czech studies (1990-2001)[16]. A further aim was to recognize projections for future occurrence rates from the same geographical area.

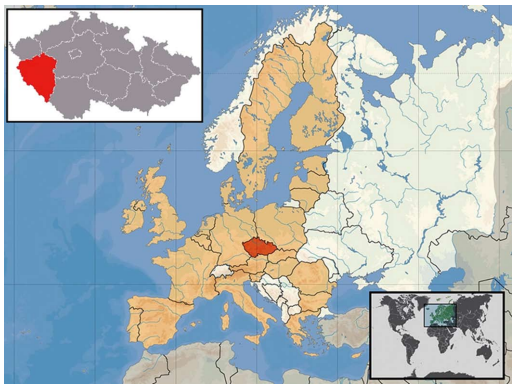

Figure 1 Map of the catchment area, the geographical area under investigation for the Czech pediatric inflammatory bowel disease study over the period of 2000-2015. Outline of the Pilsen region (red) in the Czech Republic (orange) situated in central Europe.

MATERIALS AND METHODS

Study setting

The study area covered the Pilsen region (western Bohemian region of the Czech Republic) as the catchment area (Figure 1), corresponding well to the geographical distribution of the pediatric population. The Pilsen region is one of the 14 regional administrative units in the Czech Republic (http://en.plzensky-kraj.cz/en/kategorie/pilsen-region). The child population was estimated to account for approximately 14.3% of the total population in that region (January 2015). The catchment area is the third largest region in the Czech Republic and the ninth most populous. The region area encompasses 7125 km^2, having a population of approximately 577538 inhabitants (approximately 6% of the total population of the Czech Republic, 2015, Czech census data), including seven counties. The mean population density (population per square kilometer) in the entire region ranged from 52 to 1178 among the counties, with the pediatric population distributed evenly throughout all counties.

The structural settlement of the region is unbalanced. The metropolitan area of Pilsen is connected to small rural areas, whereas mid-sized towns are largely lacking. Approximately one-third of the population resides in Pilsen, and the rest is in semi-urban and rural areas. The large number of small settlements is a typical feature of the area. More than four out of five municipalities in the region have fewer than 2000 residents, and more than 30% of the region's population resides in such small towns and villages. However, the city of Pilsen is the natural center of the region; it is currently the fourth largest city in the Czech Republic, with approximately 200000 inhabitants.

All children in our country are insured and thus have free access to health care. The Pilsen region is included in the Czech National Health System and provides universal health insurance for its residents, including free-of-charge coverage for general practitioners and hospital services. In the Czech Republic, the treatment of pediatric IBD is conducted in a tertiary hospital setting and is free for all inhabitants. Thus, the study area was well delineated as consistent with the health care system in the Czech Republic, which dictates that all children and adolescents in a specific municipality should be referred to a tertiary pediatrician gastroenterology center for the diagnosis/treatment of IBD.

Thus, as a part of a tertiary referral teaching hospital, our gastroenterology center services the whole pediatric population (< 19 years) in the catchment area. Very few, if any, children are diagnosed or treated by adult gastroenterologists in the catchment area by 19 years of age. We believe that the vast majority of pediatric IBD cases would have been referred to our IBD single center, particularly older adolescents, who were probably not diagnosed by adult gastroenterologists.

Study population and data collection

This clinical population-based prospective regional cohort consisted of children and adolescents with new onset IBD (< 19 years old at the time of diagnosis) whose residential addresses were in the Pilsen region. The study cohort included children from the same geographic region who were living in different settings (rural *vs* urban). Only unequivocal IBD cases were captured upon diagnosis over a 16-year period (between January 1, 2000 and December 31, 2015).

The following demographic elements were available: age, sex, race/ethnicity, place of residence, and Czech census region. Patient data were entered consistently with the first diagnosis; thus, if subjects were diagnosed initially with IBD-U and subsequently reclassified to CD and UC, the former was considered the most accurate diagnosis. Any surgical intervention needed at the time of diagnosis was also recorded. The geographical unit of a person's residence was used to determine the place of county residence; all participating children were presumably of Czech ethnicity, which is a Caucasian population. Patients were excluded if they originated from a city outside the catchment area.

We sampled a geographically restricted pediatric population with documented IBD who were referred to a tertiary referral teaching hospital in the Czech Republic (Charles University, Faculty Hospital in Pilsen, Department of Pediatrics, Pediatric Gastroenterology Unit). This is the only tertiary pediatric center in the Pilsen region that can treat this population, and all pediatric gastroenterologists in the catchment area throughout the period were based in this hospital. Additionally, all pediatric departments within the area collaborated on the project.

Detailed information from the diagnostic work-up data for all subjects, including data from the first attendance and from the hospital discharge records, were prospectively collected from an in-house computerized clinical database (patient registries). The

Table 1 Demographics of newly diagnosed pediatric patients (n = 170) less than 19 years of age in the Pilsen area from January 1, 2000 to December 31, 2015

	CD	UC	IBD-U	IBD
Total patients, *n*	105	48	17	170
Male	68	24	9	101
Female	37	24	8	69
Age in years, median (range)	14.1 (1.4-18.1)	14.6 (2.7-18.3)	14.1 (2.5-17.7)	14.2 (1.4-18.3)

Data reported for the entire IBD population, CD, UC and IBD-U. IBD: Inflammatory bowel disease; CD: Crohn's disease; UC: Ulcerative colitis; IBD-U: Inflammatory bowel disease-unclassified.

data on patients were collected using a data form completed by gastroenterologists. The system has been developed in cooperation with the University of West Bohemia in Pilsen, Faculty of Applied Sciences. The application, which has a common client service architecture, provides the means to collect the data both manually *via* structured web forms and automatically from other medical systems using the Czech data standard for the exchange of medical data (DASTA). Fully automatic data interchange between the application and hospital information system reduces the possibility of human error during data entry, thus improving the data accuracy and quality.

All data from the work-ups were present in the central dataset, forming the data network with high quality, and complete detailed information was linked at a subject level. For this reason, there was presumably no bias in the sampling or selection procedure. The size of the pediatric population (< 19 years old) and age- and sex-stratified population data were determined from census data collected between 2000 and 2015.

Diagnosis and disease subclassification

All IBD patients were diagnosed according to clinical history, physical examination, laboratory and serological testing, radiologic studies, and endoscopic appearance with stepwise biopsy for review by clinical pathologists[18]. The date of initial diagnosis was set as the day of the definitive diagnosis made during our study period, as evidenced by diagnostic evaluation or surgical findings consistent with IBD rather than symptom onset. The new IBD group was subdivided into three main clinical types: CD, UC, and IBD-U. If a distinction between UC and CD could not be determined based on these criteria, cases that were not otherwise specified in the presence of nonspecific inflammation were designated as IBD-U. The subjects without firm evidence of IBD were excluded from further analyses.

Statistical analysis

Standard descriptive statistics were used to summarize the characteristics of the entire population. The incidence rates were expressed as new cases per 100000 pediatric persons per year by dividing the observed number of ascertained new cases in a specified time period (numerator) by the size of the resident pediatric populations under effective observation from January 1, 2000 to December 31, 2015 (denominator). The data were analyzed for each time period and stratified by age, sex, and Czech census region. The IBD patients were analyzed in two separate categories with respect to age: < 10 years and 11-19 years. The age of the IBD patients was expected to have a skewed distribution because the median was used. Incidence trends and time projections of IBD and its subtypes with the number of cases as the dependent variable, and population size as the offset variable, were estimated using linear regression analyses and correlation coefficients. Student's *t*-test (unpaired, 2-tailed) and stratified analysis reflecting age and sex were used to measure differences between the groups. A P-value < 0.05 and precise 95%CI were calculated to evaluate the significance and precision of the estimate.

RESULTS

Demographics and IBD incidence

The demographic characteristics are shown in Table 1. As of 2015, the data from 170 incident patients (< 19 years), including 96 subjects (< 15 years of age at diagnosis) with pediatric-onset IBD in the Pilsen region (since 2000), were entered into the electronic databases. Of the entire group, 59.4% were males. The median age in years was 14.2 (range: 1.4-18.3) for IBD, 14.1 (1.4-18.1) for CD, 14.6 (2.7-18.3) for UC, and 14.1 (2.5-17.7) for IBD-U. The median age at diagnosis did not differ between CD and UC, and there were no significant differences between the age at onset in boys and girls. The self-reported racial/ethnic distributions were 97.1% Caucasian and 2.9% other.

The number of cases diagnosed each year in the inclusion period is shown in Figure 2. Of the new cases, 105 (61.8%) were CD, 48 (28.2%) were UC and 17 (10%) were IBD-U. There was a predominance of CD (the CD to UC ratio was 2.2:1). Of the 17 subjects with IBD-U, the initial IBD-U diagnosis was changed in 13 (76.5%), with 7 (41.2%) reclassified to UC and 6 (35.3%) reclassified to CD during the follow-up. During the 16-year study period, there were 1709209 person-year observations aged < 19 years in the catchment area, summarizing the source population

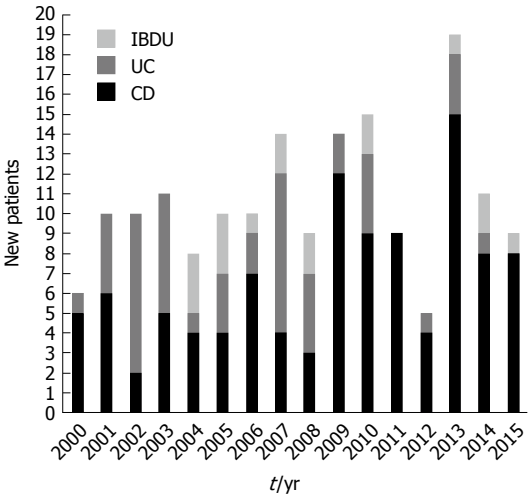

Figure 2 Bar graph showing the total annual numbers of newly diagnosed Crohn's disease, ulcerative colitis and inflammatory bowel disease-unclassified individuals (< 19 yr) in the Pilsen region, 2000-2015. CD: Crohn's disease; UC: Ulcerative colitis; IBD-U: Inflammatory bowel disease-unclassified.

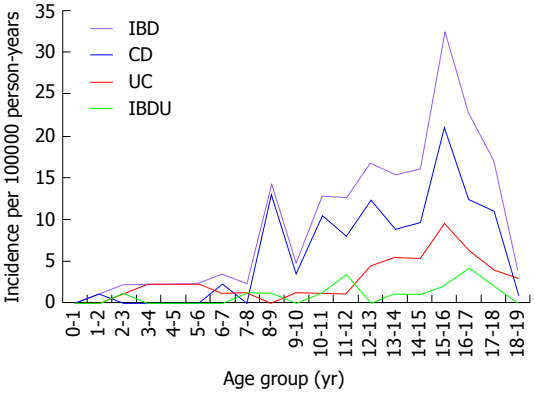

Figure 3 Age-specific incidence of all new-onset pediatric inflammatory bowel disease, Crohn's disease, ulcerative colitis, and inflammatory bowel disease-unclassified per 100000 person-years (< 19 yr) in the Pilsen region, 2000-2015. The incidence of IBD significantly increased with age (P = 0.0003). IBD: Inflammatory bowel disease; CD: Crohn's disease; UC: Ulcerative colitis; IBD-U: Inflammatory bowel disease-unclassified.

corresponding to 170 children who received diagnoses of IBD. Among the 0- to 15-year-olds, the overall incidence was 7.3 per 100000 person-years (95%CI: 6.1-8.5) for IBD, 4.6 (95%CI: 3.7-5.5) for CD, 2.0 (95%CI: 1.3-2.7) for UC and 0.7 (95%CI: 0.1-1.3) for IBD-U. Among 0- to 19-year-olds, the respective incidences were 10.0 (95%CI: 9.2-10.9), 6.2 (95%CI: 4.7-7.7), 2.8 (95%CI: 1.5-4.1) and 1.0 (95%CI: 0.2-1.8).

IBD type by age group

Figure 3 shows the age-related incidence rates of IBD, CD, UC and IBD-U. The diagnosis was most commonly made after the age of 10 years for IBD; the age at diagnosis was less than 10 years in 30 children (17.6%). Early presentation, before the age of 6 years, was observed in 0.58%, 4.1%, 0.58% and 5.29% of children with CD, UC, IBD-U and IBD respectively. The overall incidence of IBD rose from 3.5 (95% CI: 2.5-4.3) per 100000 person-years among 0- to 10-year-olds to 16.6 (95%CI: 12.5-20.1) per 100000 person-years in 11- to 19-year-olds (P = 0.0003).

The highest age-related occurrence for IBD was observed in the 15-year-old age group. Likewise, significantly higher incidence rates were observed for CD (P = 0.0006), UC (P = 0.002) and IBD-U (P = 0.01) respectively in 11- to 19-year-olds. The age-standardized incidence was significant for both males and females in IBD (P = 0.001 and P = 0.01 respectively), CD (P = 0.006 and P = 0.01 respectively) and UC (P = 0.02 and P = 0.001 respectively), whereas it reached marginal statistical significance for IBD-U (P = 0.047 and P = 0.058 respectively). No differences were found in the age distribution of subjects among CD, UC and IBD-U in 0-to 10-year-olds, whereas the older (11 to 19 years old) group had a significantly increased incidence of CD compared with UC and IBD-U (P < 0.001).

Effect of sex

Over the 2000-2015 period, 105 patients (males/females: 68/37) were diagnosed with pediatric-onset CD, and 48 subjects with UC (males/females: 24/24) and 17 patients with IBD-U (males/females: 9/8) were diagnosed at < 19 years of age. Among all cases of IBD, 59.4% were males. The differences in sex were significant only for IBD (especially CD). More boys than girls suffered from CD. The average IBD incidence rate per 100000 person-years was 11.6 (95%CI: 9.4-14.1) for males and 8.3 (95%CI: 6.8-10.1) for females (P = 0.026). The incidence of CD was 7.8 (95%CI: 6.2-9.8) for males and 4.5 (95%CI: 2.8-4.1) for females (P = 0.016). We found an equal sex ratio in UC and IBD-U. The incidence of UC was 2.7 (95%CI: 2.1-3.1) for males and 2.9 (95%CI: 2.3-3.4) for females (P = 0.44). The incidence of IBD-U was 1.0 (95%CI: 0.8-1.5) for males and 1.0 (95%CI: 0.8-1.5) for females (P = 0.45).

IBD incidence trends between 2000 and 2015

Trends in the population under observation over the 16-year period were determined (Figure 4A-C). Incidence rates were analyzed in 1-year blocks. When analyzing the 0- to 15-year-old group, we observed no statistically significant changes in incidence for the whole group of IBD (r = 0.15, P = 0.09), IBD-U (r = 0.04, P = 0.213) or CD (r = 0.13, P = 0.109). Despite the consistent decreasing trends in incidence rates of UC, this analysis failed to reveal any statistical significance (r = - 0.01, P = 0.810). The change in incidence over the 16 years of the study period was statistically significant in the 0- to 19-year-old group in the examined region.

Over the study period, the overall incidence rate

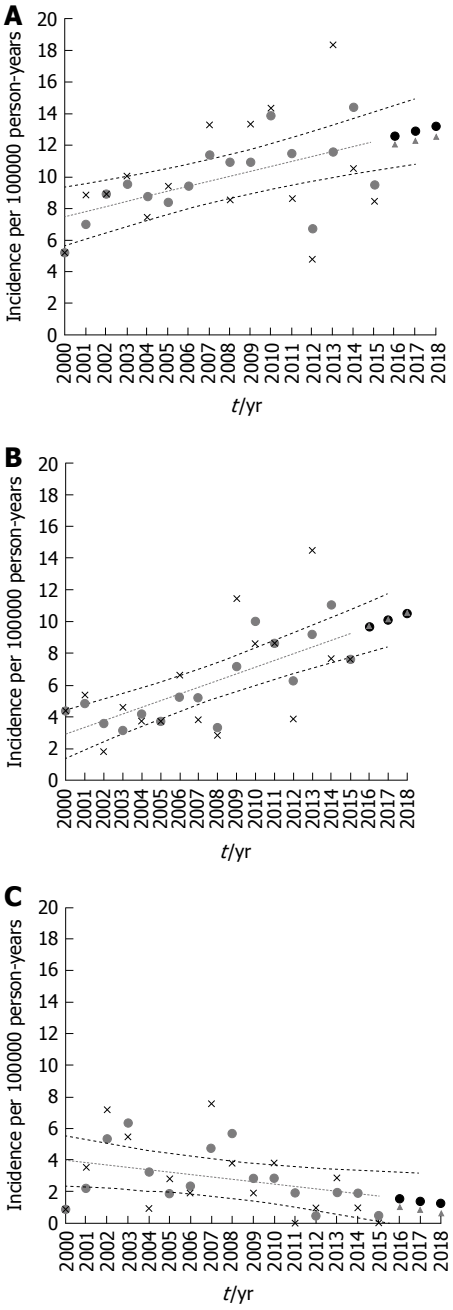

Figure 4 Scatter plots of the data. The average annual age-standardized incidence rate per 100000 person-years by age group (< 19 years) and the 16-year trend for inflammatory bowel disease (A), Crohn's disease (B), and ulcerative colitis (C) in the Pilsen region over the period of 2000-2015 in the population under observation and the incidence rate projected for 2016-2018. The points are the actual data; Stars indicate the incidence of IBD (A), CD (B) and UC (C) per 100000 person-years; Blue points indicate calculating the moving average; Dotted blue line indicates a fitted linear regression model to predict trends; Triangles indicate the incidence rate projected for 2016-2018; Red points indicate calculating the moving average. There was a significant increase in incidence over the 16-year period (A: $r = 0.32$, $P = 0.012$; B: $r = 0.42$, $P < 0.0001$; C: $r = -0.15$, $P = 0.134$).

of IBD and CD rose significantly ($r = 0.32$, $P = 0.012$ and $r = 0.42$, $P < 0.0001$ respectively) and showed a male preponderance. Apart from a non-significantly higher point estimate for 2000-2004, the incidence of UC decreased without reaching statistical significance ($r = -0.15$, $P = 0.134$), and the overall rates of IBD-U remained fairly stable ($r = 0.05$, $P = 0.341$). The incidence rate of IBD increased over the study period to 5.8 (95%CI: 3.6-8.6) per 100000 person-years for girls and 11.1 (95%CI: 7.1-14.5) for boys in 2015. Similarly, the incidence rate of CD increased over the study period to 5.8 (95%CI: 3.4-9.1) per 100000 person-years for girls and 9.4 (95%CI: 7.1-13.4) for boys in 2015. In contrast, the incidence of UC decreased to 0 per 100000 person-years for both girls and boys in 2015. The most pronounced increase was observed in CD among adolescents aged 12-19 years ($P = 0.001$). For children less than 10 years of age, the rate remained low, limited by too few young children for the analysis in consecutive years of observations (2000-2015).

Compared with historical pediatric data in the Czech Republic from 1990 to 2001, the incidence of CD rose from 1.26 per 100000 person-years in 2001 to 5.8 per 100000 person-years in 2015 in children under 15 years of age. The incidence of UC gradually decreased from 1.84 per 100000 person-years in 1999 to 0 per 100000 person-years in 2015.

IBD projections to 2018

The IBD projections to 2018 are shown in Figure 4. The IBD incidence rates projected for 2016, 2017 and 2018 were 12.1 (95%CI: 10.6-14.6), 12.3 (10.3-15.0) and 12.6 (11.0-15.4) (Figure 4A). The incidence rates of CD projected for 2016-2018 were 9.7 (8.1-11.3), 10.2 (8.7-12.3) and 10.2 (8.7-12.3) respectively (Figure 4B). The projected UC incidence rates (Figure 4C) were 1.0 (0-3.4), 0.8 (0-3.19) and 0.6 (0- 3.2) respectively. The projected IBD-U incidence rates were 1.3 (0.5-2.2), 1.3 (0.5-2.3) and 1.4 (0.5-2.4) respectively.

DISCUSSION

Here, we report the incidence of IBD over a period of 16 years from 2000 to 2015 in Czech children (< 19 years) in a large, geographically well-defined population. To our knowledge, this is the first Czech prospective regional cohort of IBD, covering a wide area in the Pilsen region within the Czech Republic. We substantiated several important conclusions: (1) some of the highest incidence rates of pediatric IBD reported to date; (2) the higher incidence (more than twice that of UC) and male predominance of CD; (3) the significantly increased incidence of IBD (in particular CD) over time; and (4) the gradual increase in incidence rates with age. We further compared our findings with historical Czech data and showed that IBD-projected data were enhanced substantially until 2018.

We have added new insights to a global map of the incidence of IBD. We identified one of the highest incidences of IBD reported to date. The rate of IBD over the time period covered by the research was 10.0 per 100000 person-years for CD 6.2, 2.8 for

UC and 1.0 for IBD-U up to 19 years of age. The respective incidences were 7.3, 4.6, 2.0 and 0.7 when considering an age of 15 years as the upper limit. We identified one of the highest incidences of childhood IBD reported to date. Our results well resemble those from areas showing similarly high IBD incidences, such as reported from studies in England[19,20], Sweden[21,22], Finland[23,24], Norway[25] and Canada[3,26]. Against this backdrop, our findings confirm high rates of IBD (primarily CD) in our country that are comparable to the rates in high-incidence Western populations[2]. IBD incidence was considerably lower in studies conducted in the United States according to data from Wisconsin[27] and northern California[28], as well as in Iceland[10], France[29], Italy[30], Scotland[9], Italy[30] and the Netherlands[15].

Our findings should be considered while bearing in mind that incidence rates are challenging due to the age limit used for pediatric patients and the heterogeneity of data collection techniques. Another important finding was a relatively low contribution of UC and IBD-U compared with the overall IBD incidence rate. In our study, the incidence of CD was more than twice that of UC. Thus, our results are similar to those of Buderus *et al*[31], Ashton *et al*[19] and the European pediatric registry EUROKIDS[32] that also showed CD accounting for most IBD cases. In contrast, Castro *et al*[30] showed that UC was more prevalent than CD in Italian children. An equal incidence of UC and CD occurs among adult populations[33], whereas the incidence of UC was approximately 2-fold higher than the incidence of CD in the Lazio region in Italy[34].

Next, we calculated time trends in the incidence of IBD for all three pathologies, as it is still debated whether there are temporal aspects in IBD. In a systematic pediatric review by Benchimol *et al*[2], only 20.1% of studies used statistical analyses to determine trends over time, while 77.8% reported a statistically significant increase in incidence of pediatric IBD. Among the studies calculating trends in the incidence of CD, 60% reported a significantly increased incidence. Among similar UC projects, 20% noted significantly increased rates[2]. Our data suggest a clear indication of the rising trend of newly diagnosed IBD, especially for CD due primarily to an increase in the incidence of CD in males. The incidence of CD more than doubled from 2000 to 2015. In contrast, the incidence of UC and IBD-U was altered very little throughout the study period. Thus, the results of our study are consistent with recent reviews[13].

Similarly, the increased incidence of IBD, with a predominance of CD over UC in recent decades, was reported in Australia[35], France[29], Sweden[22], Canada[3], Denmark[7], Scotland[36] and other countries[25,27]. In Australia, recent Victorian studies clearly showed increasing rates in children, with a greater than 10-fold increase in CD over the 30-year period to 2001. In addition, the incidence of UC also remained relatively stable in other studies, despite the increasing evidence of CD[2,29]. These figures are not fully comparable to those obtained in other pediatric studies. In northern California, Abramson *et al*[28] observed a several-fold rise in UC incidence, and the incidence of CD remained relatively stable over a period of 11 years, similar to the report by Orel *et al*[37] in Slovenia. In contrast, a Canadian study from Ontario[26], and the French EPIMAD registry between 1988 and 2007, reported a striking decrease in UC despite a persistent increase in CD[38]. An explanation for the differing trends observed in CD and UC remains elusive[39]. According to these results, we may speculate that this geographic variability is probably due to genetic and environmental implications; however, the exact triggers may not be easily identified.

We observed that IBD-U was diagnosed most frequently among the oldest group of children. The finding of 10% of subjects with IBD-U is among the lowest reported. The proportion of patients was similar to that previously reported in other pediatric observations in whom IBD-U accounts for 10% to 15% of newly diagnosed patients with IBD[8,15,20,25,30,31,36,40], but other researchers have reported a somewhat lower incidence of IBD-U[22,29,41]. Undoubtedly, our data parallel observations obtained in adults[11,34,42,43]. This is a partially unexplained phenomenon. Heyman *et al*[44] speculated that IBD-U might represent an evolving form of IBD that presents before a definitive diagnosis of IBD. In our series, the proportion of patients who initially presented with IBD-U decreased over time. There were still 23.5% of subjects with IBD-U, but 41.2% of cases had already evolved toward UC and 35.3% toward CD.

We observed the presenting features and compared them with the rates from the 1990s. We suggest that the incidence of pediatric IBD is increasing in the Czech Republic. These results indicate that the incidence of CD has markedly increased, while the incidence of UC has decreased substantially in the Pilsen region compared with the historical figures in the Czech Republic over the past 25 years. Indeed, an increased pediatric IBD incidence has been reported by Kolek *et al*[17] in a prospective population-based study from Moravia (a northern region in the Czech Republic). The incidence of CD increased from 0 to 2.7/100000 person-years between 1999 and 2001, and the incidence of UC increased from 0.68 to 1.84 per/100000 person-years between 1990 and 1999.

In the first nationwide study in Czech pediatric subjects, Pozler *et al*[16] published the results of a partly retrospective study exploring CD incidence in Czech children (< 15 years) between 1990 and 2001. A marked (5-fold) increase in the incidence of CD was found from 0.25/100000/year in 1990 to 1.26/100000/year in 2001[16]. The incidence of CD in children under 15 years of age increased 4.6-fold between 2001 and 2015 (1.26-5.8/100000/year), but the incidence of UC was lower than that of the hitherto published studies in the Czech Republic[16,17], remaining almost unchanged with little difference between the

studies (1.84-0.9/100000/year). We also assumed that parallel increases are also occurring in adult Czech populations[11]. The reasons for these increases are largely unknown.

Although given a 25-year period, the increased risk of IBD tracks with the effects of external factors, such as environmental features that are constantly changing, rather than of shifts in the frequency of susceptibility genes alone that may change slowly[3,13]. In our population, there were important changes in higher socio-economic status and in demographic or environmental health conditions over time, which may have altered the frequency of disease. However, even in low-incidence countries, the occurrence of IBD seems to be rising. Thus, increased awareness by physicians and advancements in the diagnosis of IBD, homogeneity of registration databases, data collection methods, and possibly the completeness of case ascertainment may partially contribute to the striking increase in the incidence of IBD and geographic differences. We consider this a very plausible explanation for the precipitation of the clinical illness, although there are no clear answers from this research in terms of the relevance of the explanation in the Czech Republic. More detailed studies are warranted.

Unfortunately, data are lacking on the incidence in the pediatric population in eastern Europe because most of the published data were conducted in the United States and western Europe. However, in the past few years, recent papers have indicated a sharp increase in incidence in various parts of eastern Europe[39,45]. A recent study from western Hungary (the Veszprem province) revealed that the incidence of pediatric IBD has rapidly increased over the 35-year period. The incidence of CD and UC increased from 0 and 0.7 in 1977-1981 to 7.2 and from 5.2 in 2007-2011 per 100000 person-years[46]. By contrast, the results from our study revealed no changes in UC over time, although the incidence rates of UC in the Hungarian study increased approximately 7-fold. Very recently, the Hungarian nationwide pediatric registry data were published, with a mean incidence for the 3-year observation period from 2007 to 2009 of 7.48, 4.72, 2.32 and 0.45 for IBD, CD, UC and IBD-U respectively. The incidence of pediatric IBD in Hungary was at the higher end of the reported range[47]. Thus, the situation in our country is comparable to that in Hungary.

These findings collectively indicated that the incidence of IBD in central and eastern European countries is increasing in childhood. Consequently, these findings do not concur with the previously described west-east gradient decrease in the incidence of IBD in Europe[11], reflecting the possible geographic impact of the diagnosis of IBD. We suggest that a comparison of these studies may be indicative of the loss of this gradient in European children and exceptions to that rule. In addition, these findings reporting low incidence rates in eastern Europe contrast with the findings of a previous study in Polish children that suggested that the incidence of UC was higher than that of CD (1.3 *vs* 0.8/100000 person-years)[48].

In an analysis stratified by age, we uncovered a distinct contrast in the distribution of IBD with a gradual increase in older age groups, in agreement with other studies. We observed that approximately 17% of children were diagnosed with IBD by the age of 10 years, corresponding to the results reported by Buderus *et al*[31] in Germany and Austria. The highest rates were observed in the 11- to 19-year-old group in our study, which is consistent with previous reports[27,38,46]. In the population aged 0-10 years, the incidence of UC and CD was similar, with no differences by sex. As age increased, the incidence of IBD rose and peaked in children around the age of 15 years, with CD exhibiting a steeper increase compared with UC around puberty, although it was rare in a younger age category. The age-specific peak for diagnosis was not consistent with the findings of earlier studies by Sawczenko *et al*[49], van der Zaag-Loonen *et al*[15], Buderus *et al*[31] and Orel *et al*[37]. These discordant data are probably related to differences in the methods applied for case ascertainment, disease registries and the demographic differences of the underlying populations.

We reported a male preponderance of CD but no significant differences in sex for UC and IBD-U as previously reported[26,49] because of a doubled and significantly higher incidence of CD in males. Similar proportions regarding male susceptibility to CD have been recognized for many years[19,22,27,50], as opposed to a slight female predominance in adults[33]. Some data suggest that pediatric UC is more common in girls[29,37,51]. A systematic review by Molodecky *et al*[5] failed to obtain consistent results, suggesting that the disease occurred equally between the sexes in adults. Together with the different findings of the inverted male-to-female ratio in adult studies, these data suggest that biological age-related factors may contribute to predisposing people to and triggering of IBD, especially CD in Czech children. Given that our population consisted mainly of native Caucasian children, we could not estimate the specific incidence because almost all participating subjects were of similar ethnic origin. However, past studies have shown a high incidence of IBD in Caucasian children[27].

We based projections for future incidence rates on our knowledge. Our estimates for the projected rate of IBD to 2018 were alarming, which may reflect the increasing incidence of IBD over time. Our results indicate that the incidence estimates for IBD in 2018 are somewhat higher than most reported in our current study. Regarding IBD incidence rate projections, based on the most likely scenario and on the estimated trend from the linear analysis, we projected a model showing that the number of new cases of Czech children with diagnosed IBD (in particular CD) will continue to increase over an extended time period. Conversely,

our projections clearly show that the incidence of UC will decrease.

To our knowledge, no study has yet focused on IBD incidence projections in children, thus making a direct comparison problematic. However, in our opinion, the fitted model actually created appraisals that seemed to be similar to those observed. Although it remains to be further confirmed, the disturbing incidence rate of 12.6 per 100000 person-years projected for 2018 appears to be realistic if current circumstances persist. The simplest explanation for this finding could be that the IBD incidence projected herein could be attributable to demographic factors and may be indicative of the persistence of previous conditions, with no major or slow changes in pediatric IBD. It will be interesting to follow these temporal trends in the coming years, as we may learn more about the role of environmental factors in the pathogenesis of IBD[12].

The strength of this study is its robust, prospective manner because the available prospective observation shows more homogeneity. In addition, we used the same defined geographic region with an ethnically homogeneous study sample, establishing well-defined selection criteria. Furthermore, we used an appropriate time period and case ascertainment identification of IBD cases, and we included patients at or shortly after diagnosis. Since newly enrolled individuals were included only once, despite meeting the inclusion criteria over multiple years, a bias may not exist for including those with more chronic or severe disease, requiring additional visits.

These strengths allowed us to provide up-to-date estimates of the incidence and trends and to depict the main differences by time period, age and sex, thus not affecting the time trend analyses. An observational survey and the appropriate type of sampling frame for study recruitment, from which subjects were selected, was critical for the sample representativeness by covering the entire population of interest with unlimited access to subspecialist care to obtain reproducible results.

The Czech Republic is a country with centralized health care for IBD, and pediatric IBD subjects are cared for only in our tertiary hospital in the catchment area. Therefore, we trust that the sample is representative due to the easily surveyable area of the Pilsen region. Due to the centralization of pediatric gastroenterology care, we believe that all pediatric patients residing in the Pilsen region were included in the database, reducing the probability of recall bias. Thus, it is highly probable that all children with IBD in the Pilsen region sought medical attention in our clinical setting. In the assessment of time trends, we reported 16 years of data with figures specifying the numeric incidence rates over a long time period. This study was also strengthened by reliable data from the files and our registry of pediatric IBD in the included area. The census data provide data sets from which we can identify a sample that is deemed to have minimal bias.

A potential weakness of our study is that our registry was based on data from one large regional referral center encompassing a specific child population, and the generalizability of the presented findings regardless of the setting may be of concern. Another possible limitation is an underestimation of rates of IBD because we were unable to exclude the possibility that the IBD subjects may have been treated in other institutions, which may have led to an underestimation of the results of the analysis. However, in the Czech pediatric population at time of this study, there was an almost universal admission policy for investigation of IBD; unlike adults, the disease is not diagnosed in outpatient settings by primary care providers or pediatricians. Our facility is the only referral center in the catchment area that provides inpatient/outpatient care, and all children with IBD are concentrated in our center for pediatric gastroenterology. Of note, in the Pilsen region, probable IBD cases in childhood are treated exclusively in our single facility. In general, adult gastroenterologists in the Czech Republic do not provide medical care for IBD-affected children until they reach 19 years of age (older teenagers with a new diagnosis of IBD are not usually referred directly to adult specialists). Therefore, we probably did not underestimate the true incidence of IBD, and the present data provide a reasonably accurate evaluation of the incidence of IBD because the major portion of the subjects would have been referred to our referral center.

Furthermore, our data did not demonstrate the apparent decline in the incidence rate after the age of 15 years, which could represent a spurious event caused by pediatric subjects being diagnosed by adult specialists and thus failing to be captured in our study. Consequently, we trust that there was no selection bias in favor of younger subjects, the capture of pediatric cases is trustworthy even in the 15- to 19-year-old age class, and the limit of 19 years better mirrors the usual situation in the pediatric population in the Czech Republic. Because our data revealed significant time trends, we regard any misclassification as stable over the studied periods and thus did not influence the time trend analyses or the actual epidemiologic characteristics.

In conclusion, the incidence of pediatric IBD, especially CD, is among the highest reported to date. This study revealed a marked increase in the incidence of CD over the 16-year period, such that CD is now much more common than UC and IBD-U in children in the Pilsen region and exhibits a persistent male preponderance. The reasons for these trends are unknown, and further studies in different regions of the country would be helpful to determine whether these trends are present in other areas of the Czech Republic. Further database projects may be attempted with software data, which should permit future research due to greater region specificity.

As the changes in the past decades in industrialized countries cannot be interpreted by changes in genetic background, the influence of environmental hazards on incidence appears to be a crucial area of study. This study reaffirms the need for analyses of time trends and subsequent research to better understand the combination of genetic/family history and environmental influences, to unravel concurrent factors in the etiology of IBD and to determine causal relationships in pediatric disease.

COMMENTS

Background

Studies worldwide have shown a rising but variable incidence of inflammatory bowel disease (IBD), including in pediatric populations. Despite a rising worldwide incidence of IBD, limited data are available on the exact incidence of IBD in Czech children. The current research was designed to evaluate the incidence and overall time trends of IBD in children aged 0- to 19-years-old over the period 2000-2015 and to project incidence to 2018 in the Czech Republic.

Research frontiers

In this study, it is suggested that although no firm conclusions can be drawn to address the rising incidence of IBD, differences in the geographic distribution, and particularly changes in incidence over time within one area, may provide new insights into concurrent etiological factors. This is possible only when enough pediatric projects within one defined geographical area with a relatively homogeneous population provide a fundamental basis for a better understanding of the epidemiology and environmental influences of any geographically restricted pediatric population and for the assessment of IBD.

Innovations and breakthroughs

The findings in this and other studies suggest an increasing trend in IBD incidence. The current research adds to that literature, with the suggestion of increased rates of IBD, especially of Crohn's disease (CD), in the time period under study from 2000 to 2015 in this pediatric population in central Europe. However, ulcerative colitis (UC) and IBD-unclassified (IBD-U) are not common forms of IBD in Czech children.

Applications

The results of this study serve as additional evidence supporting the investigation of different environmental and triggering factors in the development of IBD and its subtypes in children and adolescents.

Terminology

UC, CD and IBD-U are three primary forms of IBD. The incidence rates are expressed as new cases per 100000 pediatric persons per year.

Peer-review

The manuscript presents an interesting study, which examined the incidence in pediatric IBD during 2000-2015 in the Pilsen Region of the Czech Republic and revealed a marked increase in the incidence of CD over the 16-year period.

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ORIGINAL ARTICLE

Prospective Study

Drug-induced liver injury in inflammatory bowel disease: 1-year prospective observational study

Tomas Koller, Martina Galambosova, Simona Filakovska, Michaela Kubincova, Tibor Hlavaty, Jozef Toth, Anna Krajcovicova, Juraj Payer

Tomas Koller, Martina Galambosova, Simona Filakovska, Michaela Kubincova, Tibor Hlavaty, Jozef Toth, Anna Krajcovicova, Juraj Payer, 5th Department of Internal Medicine, Gastroenterology and Hepatology, Comenius University in Bratislava Faculty of Medicine, University Hospital Bratislava Ruzinov, 82606 Bratislava, Slovakia

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Correspondence to: Tomas Koller, MD, PhD, Associate Professor, 5th Department of Internal Medicine, Gastroenterology and Hepatology, Comenius University in Bratislava Faculty of Medicine, University Hospital Bratislava Ruzinov, Ruzinovska 6, 82606 Bratislava, Slovakia. tomas.koller@ru.unb.sk
Telephone: +42-190-5118692

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Abstract

AIM

To analyze 1-year liver injury burden in inflammatory bowel disease (IBD) patients.

METHODS

During a 6-mo inclusion period, consecutive IBD cases having a control visit at IBD center were included. Basic demographics, IBD phenotype and IBD treatment were recorded on entry. Aminotransferase (AT) activities of ALT, AST, ALP and gamma-glutamyl transpeptidase (GGT) were measured at baseline, 3 mo prior to study entry and prospectively every 3 mo for 1 year. Liver injury patterns were predefined as: Grade 1 in ALT 1-3 × upper limit of normal (ULN), grade 2 in ALT > 3 × ULN, hepatocellular injury in ALT > 2 × ULN, cholestatic injury in simultaneous GGT and ALP elevation > ULN.

Persisting injury was reported when AT elevations were found on > 1 measurement. Risk factors for the patterns of liver injury were identified among demographic parameters, disease phenotype and IBD treatment in univariate and multivariate analysis. Finally, implications for the change in IBD management were evaluated in cases with persisting hepatocellular or cholestatic injury.

RESULTS

Two hundred and fifty-one patients were included having 917 ALT and 895 ALP and GGT measurements. Over one year, grade 1 injury was found in 66 (26.3%), grade 2 in 5 (2%) and hepatocellular injury in 16 patients (6.4%). Persisting hepatocellular injury was found in 4 cases. Cholestasis appeared in 11 cases (4.4%) and persisted throughout the entire study period in 1 case. In multivariate analysis, hepatocellular injury was associated with BMI (OR = 1.13, 1.02-1.26), liver steatosis (OR = 10.61, 2.22-50.7), IBD duration (1.07, 1.00-1.15) and solo infliximab (OR = 4.57, 1.33-15.7). Cholestatic liver injury was associated with prior intestinal resection (OR = 32.7, 3.18-335), higher CRP (OR = 1.04, 1.00-1.08) and solo azathioprine (OR = 10.27, 1.46-72.3). In one case with transient hepatocellular injury azathioprine dose was decreased. In 4 cases with persisting hepatocellular injury, fatty liver or alcohol were most likely causes and IBD treatment was pursued without change. In the case with persisting cholestatic injury, no signs of portal hypertension were identified and treatment with infliximab continued.

CONCLUSION

Liver injury was frequent, mostly transient and rarely changed management. Infliximab or azathioprine were confirmed as its risk factors indicating the need for regular AT monitoring.

Key words: Drug-induced liver injury; Risk factors; Inflammatory bowel disease; Infliximab; Adalimumab; Azathioprine

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Core tip: We evaluated liver injury in consecutive inflammatory bowel disease (IBD) patients followed for one year in whom aminotransferase activities (AT) were measured at baseline and at 3 mo intervals. We found AT elevations frequently, but they were mostly mild and transient. Even persisting abnormalities had rarely an effect on IBD management. However, ALT elevations and cholestasis appeared more commonly among patients treated with infliximab (ALT) or azathioprine (cholestasis). This finding points to their potential for hepatotoxicity and the need for regular AT monitoring.

Koller T, Galambosova M, Filakovska S, Kubincova M, Hlavaty T, Toth J, Krajcovicova A, Payer J. Drug-induced liver injury in inflammatory bowel disease: 1-year prospective observational study. *World J Gastroenterol* 2017; 23(22): 4102-4111 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i22/4102.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i22.4102

INTRODUCTION

Inflammatory bowel disease (IBD) is a chronic inflammatory condition of the digestive tract. Over past decades, significant changes in the treatment of this condition have occurred. Most patients are currently treated with long-term immunosuppression, which has been shown to be effective in improving patients' symptoms and quality of life[1]. The treatment has a potential for various adverse events including a drug-induced liver injury (DILI). Recently, several reports have raised a concern that hepatotoxicity of IBD treatment might be underestimated. The DILI network has listed infliximab and azathioprine in category A, with more than one hundred well documented cases of hepatotoxicity[2]. A population-based study from Iceland reported that azathioprine and infliximab were among five most common drugs causing liver injury[3]. Furthermore, chronic drug induced liver injury is increasingly being recognized[4]. Drug induced liver injury may range from mild aminotransferase elevations to symptomatic hepatitis or acute liver failure. Finding causes of mild or persisting aminotransferase (AT) elevations in immunosuppressed IBD patients is challenging. Apart from DILI, many other comorbid conditions and therapies could be involved[5]. Analogically to other causes of liver injury, such abnormalities might indicate an ongoing liver injury[6]. However, clinical relevance and evolution of these findings remain unclear. We are lacking studies from a real-life setting reporting on how often this potential risk actually interferes with IBD management.

We aimed to analyze a real-life burden of liver related adverse events in IBD patients over one year. First, we aimed to estimate the prevalence of liver injury among treated IBD patients. Second, to assess its prevalence according to IBD treatment. Third, to analyze evolution of liver injury and its independent risk factors. Fourth, to evaluate its implications on further IBD management.

MATERIALS AND METHODS

Study population

Our study was carried out in a single IBD center where we prospectively included all consecutive IBD patients having a control visit between January 2nd 2014 and June 30th 2014. Inclusion criteria for the study population were the diagnosis of ulcerative colitis or Crohn's disease and a control visit at the center during the inclusion period. All studied parameters

were prospectively entered into an electronic hospital database and patient folders according to the pre-defined protocol. At entry to the study, we recorded basic demographics (age, gender, body mass index), characteristics of IBD phenotype [IBD duration, IBD type, diagnosis of primary sclerosing cholangitis (PSC)], past surgeries (intestinal resections), current inflammatory activity (serum CRP and fecal calprotectin), quality of life (short IBDQ questionnaire) and current IBD therapy and dosing.

Patients with the history of known chronic liver disease other than PSC and patients with the history of known cirrhosis were not included in the study. Alcohol abuse was estimated during the baseline visit. In cases with significant AT elevations [> 3 times upper limit of normal (ULN)] it was also estimated in the process of evaluation for possible DILI. All patients were tested for HBs antigen and antibodies against HCV prior to any IBD treatment with negative results in all cases. Previous or incident infections with other hepatotropic viruses as well as other possible hepatotoxic drug were not assessed in the data analysis.

Liver injury

Serum activities of alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma-glutamyl transpeptidase (GGT), alkaline phosphatase (ALP) and serum bilirubin concentration were used as markers of liver injury as recommended by the regulatory authorities[7]. Blood sampling for AT activities was carried out on entry to the study and was pre-planned at 3 mo intervals for up to 12 mo. We also recorded aminotransferase activities from electronic records 3 mo prior to the study entry. This allowed us to evaluate the evolution of AT elevations even in cases having abnormal baseline AT values. Overall, each patient had a baseline AT measurement, four prospective AT and bilirubin measurements and one retrospective AT measurement from electronic records.

Liver injury was graded as defined by the Common terminology criteria for adverse events v. 4.03[8]. For hepatocellular injury enzymes ALT and AST, a grade 1 injury was defined as an increase in activity up to three times the upper limit of normal (3 × ULN), grade 2 injury as an increase superior to 3 × ULN. We also evaluated a more conservative and clinically relevant ALT cut-off of > 2 × ULN further referred to as "hepatocellular injury". For cholestatic enzymes GGT and ALP, a grade 1 injury was defined as an increase in activity up to 2.5 × ULN and Grade 2 injury as an increase superior to 2.5 × ULN. Since GGT elevation is not considered a specific marker for liver injury, we used it in parallel with alkaline phosphatase. An event of cholestasis was defined as an increase in GGT and ALP > ULN. An increase in bilirubin concentration was recorded when superior to 2 × ULN (> 34 μmol/L). A subgroup patients (n = 155) had undergone abdominal ultrasound for the presence or absence of liver steatosis, but severity of liver steatosis was not assessed. The following liver injury events were pre-defined: grade 1 and 2 ALT and AST increase, hepatocellular injury with ALT increase > 2 × ULN, grade 1 and 2 ALP increase, cholestasis and bilirubin increase > 2 × ULN.

We report the prevalence of liver injury by counting all particular events of an enzyme increase among all measurements of the enzyme. Second, we report the injury events separately for each of the following treatment groups: no immunosuppression, solo azathioprine, solo infliximab, solo adalimumab, azathioprine and infliximab, azathioprine and adalimumab. These treatment groups were defined according to the IBD treatment received at baseline. The prevalence of the liver event in the treatment group was compared to the prevalence of the same event in the entire study population. For graphical illustration of the findings we constructed an evaluation of drug induced serious hepatotoxicity (eDISH) plot[9]. Third, an evolution of liver injury is reported by counting how many times a liver injury event was observed in patients (from once up to five times). Two patterns of evolution were pre-defined: a transient increase was defined as one grade 1 event, a persisting increase as two or more grade 1 events.

We assessed risk factors for the following events and patterns of liver injury: persisting grade 1 ALT and AST increase, hepatocellular injury with ALT increase > 2 × ULN and any event of cholestasis. Finally, all hepatocellular injury cases (ALT > 2 × ULN) were analyzed for further evolution and change of treatment.

All fasting blood samples were analyzed in a single local laboratory by the standard automated analyzers. All suspected DILI events were investigated for possible alternative causes of increased AT activities and were followed more closely.

Statistical analysis

Data were assessed by the statistical software package MedCalc v. 14, Ostende, Belgium. For normality testing we used Kolmogorov-Smirnov test and variables are expressed as a mean ± SD, median and interquartile range or as a relative count and percentage. AT activities were expressed as multiples of an upper limit of normal. We used chi-square test to compare a relative proportion of an event in the treatment group with its proportion in the entire study population. Risk factors for liver injury events and patterns were identified using a logistic regression. All available parameters were assessed as independent variables and the liver injury event or pattern as the dependent variable. Results are given as odds ratios (OR) with 95% confidence intervals and P values. Risk factors were considered significant when P values were inferior to 0.05. All identified risk factors were entered into a stepwise multivariate logistic regression model to identify independently associated risk factors.

Table 1 Summary statistics table of the study group of 251 inflammatory bowel disease patients

n = 251	Median; IQR, *n* (%)
Age	39; 30.0-52.75
Female gender	129 (51.4)
Body mass index	24.298; 21.19-27.34
Crohn's disease	154 (61.4)
Ulcerative colitis	97 (38.6)
Inflammatory bowel disease duration (yr)	8 (5-13
Primary sclerosign cholangitis	2 (0.8)
Prior intestinal resection	73 (29.4)
Short IBDQ questionaire score	58 (50-64)
Fecal calprotectin on study entry (mg/g)	83.195 (23.45-331.8)
C-reactive protein on study entry (mg/L)	5 (2.4-8.2)
Inflammatory bowel disease therapy on entry	
Mesalamine	173 (69.5)
Antibiotics	17 (6.9)
No immunosupression	66 (26.3)
Steroids	42 (16.9)
Azathioprine solo	47 (18.7)
anti TNF therapy (all)	138 (55)
anti TNF therapy solo	74 (29.5)
Infliximab solo	41 (16.3)
Adalimumab solo	33 (13.1)
Combination therapy (anti-TNF and azathioprine)	64 (25.5)
Days between 1st and 5th AT sampling	382 (353-439.8)
Liver steatosis on ultrasound (*n* = 155)	34 (21.9)

Ethical issues

Our study was non-interventional and was carried out in accordance with the Helsinki declaration. Data acquisition was approved by our University hospital ethics committee. Due to the noninterventional nature of our study, patients were exempted from signing an informed consent form.

RESULTS

We included 251 IBD patients fulfilling the inclusion criteria. Summary statistics of the entire study population is displayed in Table 1. One hundred and fifty-four cases had Crohn's disease and ninety-seven had ulcerative colitis. The prevalence of all predefined liver injury events is displayed in Table 2.

Hepatocellular injury

We assessed 917 ALT and AST measurements. Grade 1 ALT and AST elevation was observed in 112 and 55 measurements (12.2% and 6%). Hepatocellular injury with ALT superior to 2 × ULN was observed 26 times (2.84%), Grade 2 ALT and AST increase was observed 6 and 8 times (0.65 and 0.87%). We did not observe any grade 3 or 4 liver injury.

Cholestasis

We assessed 897 ALP and 895 GGT measurements. Grade 1 and 2 ALP elevation was observed in 34 and 0 measurements (3.79 and 0%). Cholestasis was observed 11 times (1.23%).

Table 2 Prevalence of liver injury events among all aminotransferase measurements in 251 inflammatory bowel disease patients over 1 year *n* (%)

Liver injury event	All measurements *n*	Abnormal Number of events
ALT increase Grade 1 (0-3 × ULN)	917	112 (12.21)
ALT increase > 2 × ULN (hepatocellular injury)	917	26 (2.84)
ALT increase Grade 2 (> 3 × ULN)	917	6 (0.65)
AST increase Grade 1 (0-3 × ULN)	917	55 (6.0)
AST increase Grade 2 (> 3 × ULN)	917	8 (0.87)
GGT increase Grade 1 (0-2.5 × ULN)	895	80 (8.94)
GGT increase Grade 2 (> 2.5 × ULN)	895	24 (2.68)
ALP increase Grade 1 (0-2.5 × ULN)	897	34 (3.79)
ALP increase Grade 2 (> 2.5 × ULN)	897	0 (0)
Cholestasis (parallel ALP and GGT elevation)	895	11 (1.23)
Total bilirubin > 2 × ULN	370	1 (0.27)

ULN: Upper limit of normal; ALT: Alanine aminotransferase; AST: Aspratate aminotransferase; GGT: Gamma-glutamyl transpeptidase; ALP: Alkaline phosphatase.

Treatment

Among 251 study patients, 66 had no maintenance therapy, 47 were on solo azathioprine, 74 on solo anti TNF therapy (infliximab 41, adalimumab 33) and 64 had combination therapy of anti-TNF (infliximab 48, adalimumab 16) with azathioprine. Proportions of liver injury according to IBD treatment are displayed in Table 3. Grade 1 ALT increase was more common in patients treated with solo adalimumab (18.5% *vs* 12.2%) compared with the entire study population. In patients treated with solo infliximab: grade 2 ALT increase (2.5% *vs* 0.65%), hepatocellular injury with ALT > 2 × ULN (9.2% *vs* 2.84%) and grade 1 AST increase (12.3% *vs* 6.0%) were more common. The DISH plot showing ALT/total bilirubin and ALT/ALP relationship for all measurements and treatment groups is shown in Figures 1 and 2. Cholestasis was more common among patients treated with solo azathioprine (3.4% *vs* 1.23 %). There were no differences among other treatment groups (combination therapy and no-immunosuppression) for other liver injury events.

Evolution and patterns of liver injury

ALT elevation was the most common liver injury event. Grade 1 increase was observed in 66 (26.3%) patients, it was unique in 38 patients and persisted in 28 cases (11.2%). Grade 2 increase was found in 5 cases, it was unique in 4 cases. Hepatocellular injury (ALT > 2 × ULN) was observed in 16 (6.3%) cases and was unique in 12 patients.

For AST, grade 1 increase was found in 34 (13.5%) cases, it was unique in 25 and persisted in 9 cases (3.6%). Grade 2 increase was observed in 6 cases, it was unique in 4 cases.

Table 3 Prevalence of liver injury events according to the inflammatory bowel disease treatment groups

Liver injury event	IBD treatment					
	Solo azathioprine		Solo adalimumab		Solo infliximab	
	All measurements	Abnormal	All measurements	Abnormal	All measurements	Abnormal
	n	*n* (%)	*n*	*n* (%)	*n*	*n* (%)
ALT increase Grade 1 (0-3 × ULN)	154	16 (10.4)	135	25 (18.5)[a]	163	26 (16)
ALT > 2 × ULN (hepatocellular injury)		3 (1.9)		2 (1.5)		15 (9.2)[a]
ALT increase Grade 2 (> 3 × ULN)		2 (1.3)		0 (0)		4 (2.5)[a]
AST increase Grade 1 (0-3 × ULN)	154	6 (3.9)	135	7 (5.2)	162	20 (12.3)[a]
AST increase Grade 2 (> 3 × ULN)		0 (0)		1 (0.1)		2 (1.2)
ALP increase Grade 1 (0-2.5 × ULN)	148	6 (4.1)	132	2 (1.5)	163	11 (6.7)
ALP increase Grade 2 (> 2.5 × ULN)		0 (0)		0 (0)		0 (0)
Cholestasis (parallel ALP and GGT > ULN)	146	5 (3.4)[a]	132	1 (0.1)	162	2 (1.2)
	Azathioprine + infliximab		Azathioprine + adalimumab		No immunosupression	
	All measurements	Abnormal	All measurements	Abnormal	All measurements	Abnormal
ALT increase Grade 1 (0-3 × ULN)	199	15 (7.5)	59	8 (13.6)	207	22 (10.6)
ALT > 2 × ULN (hepatocellular injury)		2 (1)		1 (1.7)		3 (1.4)
ALT increase Grade 2 (> 3 × ULN)		0 (0)		0 (0)		0 (0)
AST increase Grade 1 (0-3 × ULN)	200	11 (5.5)	59	2 (3.4)	207	9 (4.3)
AST increase Grade 2 (> 3 × ULN)		1 (0.5)		2 (3.4)		2 (0.1)
ALP increase Grade 1 (0-2.5 × ULN)	195	0 (0)	58	3 (5.2)	201	12 (6)
ALP increase Grade 2 (> 2.5 × ULN)		0 (0)		0 (0)		0 (0)
Cholestasis (parallel ALP and GGT > ULN)	195	0 (0)	58	1 (1.7)	201	2 (1)

[a]$P < 0.05$ for comparison with the prevalence of the liver injury event among all measurements (Table 2).

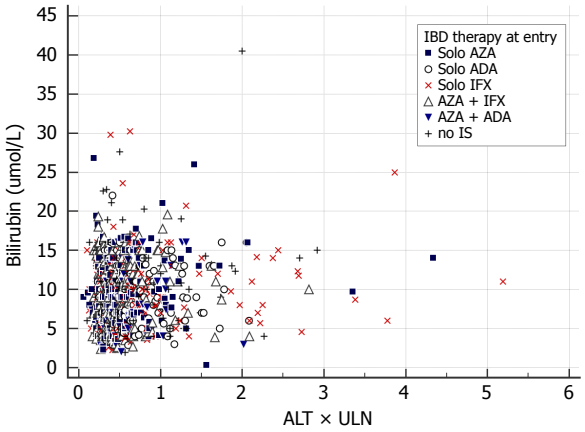

Figure 1 Serum alanine aminotransferase and bilirubin plot in 251 consecutive IBD patients (all measurements in all patients, values expressed in times the upper limit of normal. ADA: Adalimumab; AZA: Azathioprine; IFX: Infliximab; no-IS: No Immunosuppression.

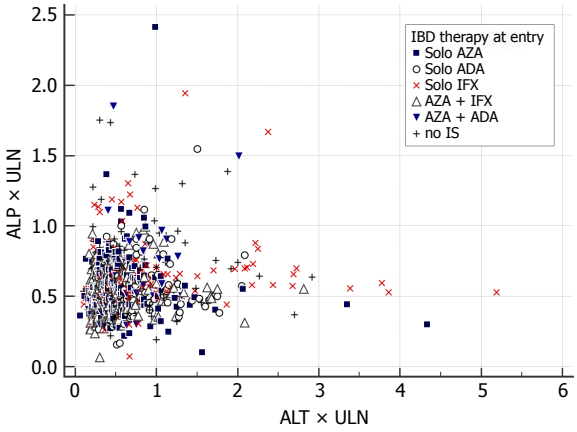

Figure 2 Serum alanine aminotransferase and alkaline phosphatase plot in 251 consecutive inflammatory bowel disease patients (all measurements in all patients, values expressed in times the upper limit of normal. ADA: Adalimumab; AZA: Azathioprine; IFX: Infliximab; no-IS: No immunosuppression.

For ALP, grade 1 increase was found in 19 (7.6%) patients, it was unique in 11 cases. We recorded no case of grade 2 ALP increase. Cholestasis was found in 11 (4.4%) cases, and was unique in 7 cases. Total bilirubin superior to 2 × ULN was observed once and it was not paralleled with AT elevation suggesting a Gilbert syndrome. Numbers of cases for each liver injury event and pattern of evolution are summarized Table 4.

Risk factors for liver injury

Univariate regression identified five risk factors for persisting grade 1 ALT increase: female gender (OR = 0.22), body mass index (OR = 1.13), solo infliximab (OR = 2.8) and liver steatosis (OR = 7.77). For hepatocellular injury (ALT > 2 × ULN): body mass index (OR = 1.13), duration of IBD (OR = 1.08), solo infliximab (OR = 3.43) and liver steatosis (HR = 7.14). The exact same pattern of risk factors was observed for persisting AST increase. Finally, risk factors for cholestasis were prior intestinal resection (HR = 7.06), the level of CRP on entry (HR = 1.04) and solo azathioprine (HR = 3.93). IBD phenotype was not found to be a risk factor for any type of liver injury. Odds ratios for all demographic parameters, IBD

Table 4 Numbers of cases with liver injury events and patterns among 251 inflammatory bowel disease patients over 1 year *n* (%)

Liver injury event	Numbers of cases with liver injury events						
			Transient	Persisting injury			
	No event	Any event	1 event	2 events	3 events	4 events	5 events
ALT increase Grade 1 (0-3 × ULN)	185 (73.7)	66 (26.3)	38 (15.1)	16 (6.4)	6 (2.4)	6 (2.4)	0 (0)
ALT increase > 2 × ULN (hepatocellular injury)	235 (93.63)	16 (6.4)	12 (4.8)	1 (0.4)	1 (0.4)	1 (0.4)	1 (0.4)
ALT increase Grade 2 (> 3 × ULN)	246 (98.0)	5 (2)	4 (1.6)	1 (0.4)	0 (0)	0 (0)	0 (0)
AST increase Grade 1 (0-3 × ULN)	217 (86.45)	34 (13.5)	25 (10)	4 (1.6)	1 (0.4)	1 (0.4)	3 (1.2)
AST increase Grade 2 (> 3 × ULN)	245 (97.6)	6 (2.3)	4 (1.6)	2 (0.8)	0 (0)	0 (0)	0 (0)
ALP increase Grade 1 (0-2.5 × ULN)	232 (92.43)	19 (7.5)	11 (4.4)	3 (1.2)	4 (1.6)	0 (0)	1 (0.4)
ALP increase Grade 2 (> 2.5 × ULN)	251 (100)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Cholestasis (parallel ALP and GGT elevation)	240 (95.61)	11 (4.4)	7 (2.8)	2 (0.8)	1 (0.4)	0 (0)	1 (0.4)
Total bilirubin > 2 × ULN	250 (99.6)	1 (0.4)	1 (0.4)	0 (0)	0 (0)	0 (0)	0 (0)

ULN: Upper limit of normal; ALT: Alanine aminotransferase; AST: Aspratate aminotransferase; GGT: Gamma-glutamyl transpeptidase; ALP: Alkaline phosphatase.

Table 5 Univariate analysis of risk factors for liver injury events and patterns among 251 inflammatory bowel disease patients

	Risk factors for selected liver injury events and patterns							
	Persisting ALT increase		ALT > 2 × ULN		Persisting AST increase		Cholestasis	
	OR; 95%CI	*P* value	OR; 95%CI	*P* value	OR; 95%CI	*P* value	OR; 95%CI	*P* value
Age[1]	1.01; 0.98-1.04	0.628	1.02; 0.98-1.05	0.317	1.03; 0.99-1.08	0.185	1.02; 0.98-1.06	0.300
Female gender	0.22; 0.87-0.57	0.002	0.55; 0.19-1.55	0.260	0.75; 0.20-2.86	0.672	2.62; 0.68-10.13	0.160
Body mass index[1]	1.13; 1.04-1.23	0.004	1.13; 1.03-1.23	0.011	1.18; 1.06-1.32	0.003	0.95; 0.82-1.08	0.413
Crohn's disease	2.03; 0.83-4.97	0.122	2.89; 0.80-10.41	0.105	1.27; 0.31-5.20	0.739	2.95; 0.62-13.94	0.173
Ulcerative colitis	0.49; 0.20-1.21	0.122	0.35; 0.10-1.25	0.105	0.79; 0.19-3.22	0.739	0.34; 0.07-1.60	0.173
Inflammatory bowel disease duration (yr)[1]	1.04; 0.98-1.09	0.180	1.08; 1.02-1.15	0.007	1.10; 1.03-1.18	0.006	1.04; 0.97-1.11	0.295
Prior intestinal resection	0.95; 0.40-2.28	0.915	1.10; 0.37-3.27	0.869	0.68; 0.14-3.33	0.631	7.06; 1.81-27.41	0.005
Short IBDQ questionaire score[1]	1.00; 0.97-1.04	0.935	0.99; 0.95-1.04	0.703	0.99; 0.94-1.06	0.925	0.98; 0.93-1.04	0.545
Fecal calprotectin on study entry (mg/g)[1]	0.999; 0.99-1.00	0.909	1.00; 0.99-1.00	0.895	0.99; 0.99-1.00	0.424	1.00; 0.99-1.00	0.346
C-reactive protein on study entry (mg/L)[1]	0.98; 0.94-1.03	0.520	0.98; 0.93-1.04	0.590	1.00; 0.95-1.06	0.907	1.04; 1.01-1.07	0.024
Inflammatory bowel disease therapy on entry								
Mesalamine	0.64; 0.29-1.45	0.288	0.54; 0.19-1.51	0.241	0.34; 0.09-1.29	0.112	0.76; 0.22-2.67	0.667
No immunosupression	0.58; 0.21-1.59	0.287	0.38; 0.08-1.73	0.211	0.34; 0.41-2.77	0.314	0.61; 0.13-2.9	0.535
Steroids	0.16; 0.02-1.22	0.078	0.69; 0.15-3.13	0.627	0.60; 0.07-4.96	0.640	1.90; 0.48-7.50	0.357
Azathioprine solo	0.49; 0.14-1.69	0.258	1.00; 0.27-3.67	0.998	0.53; 0.07-4.36	0.557	3.93; 1.15-13.47	0.030
Anti-TNF therapy solo	2.72; 1.22-6.03	0.014	2.56; 0.923-7.10	0.071	5.11; 1.24-21.05	0.024	0.89; 0.23-3.46	0.870
Infliximab solo	2.80; 1.18-6.80	0.020	3.43; 1.17-10.03	0.030	4.43; 1.14-17.28	0.032	1.15; 0.24-5.50	0.866
Adalimumab solo	1.51; 0.53-4.31	0.437	0.94; 0.20-4.33	0.937	1.94; 0.39-9.79	0.420	0.65; 0.08-5.25	0.686
Combination therapy (anti-TNF and azathioprine)	0.776; 0.3-2.01	0.601	0.66; 0.18-2.39	0.525	0.36; 0.04-2.90	0.330	0.28; 0.04-2.24	0.231
Liver steatosis on ultrasound (*n* = 155)	7.77; 3.03-19.9	< 0.0001	7.14; 2.15-23.59	0.001	6.78; 1.53-30.03	0.012	1.45; 0.27-7.83	0.666

[1]Modeled as continuous variables. ULN: Upper limit of normal; ALT: Alanine aminotransferase; AST: Aspratate aminotransferase.

related phenotype, inflammatory activity, the quality of life and IBD treatment are displayed in Table 5.

Multivariate logistic regression identified the following independent risk factors for persisting grade 1 ALT increase: female gender (OR = 0.221; 95%CI: 0.07-0.67), BMI (OR = 1.15, 1.05-1.27), and liver steatosis (OR = 31.0, 6.76-142.1), for hepatocellular injury (ALT > 2 × ULN): IBD duration (OR = 1.07, 1.00-1.15), BMI (OR = 1.13, 1.02-1.26), solo infliximab (OR = 4.57, 1.33-15.7) and steatosis (OR = 10.61, 2.22-50.7), for cholestasis: prior IBD resection (OR = 32.7, 3.18-335); CRP (1.04, 1.00-1.08) and solo azathioprine (OR = 10.266, 1.46-72.3).

Implications for further management

Sixteen patients (6.3%) with observed hepatocellular injury (ALT > 2 × ULN) were closely managed by a treating physician. ALT normalized in 12 cases with subsequent follow-up. In 4 cases, ALT elevation persisted. Analysis of possible causes of this persisting elevation identified other possible conditions: alcohol abuse in one case, type 2 diabetes with liver steatosis twice, obesity with liver steatosis once. Fifteen subjects had no change in IBD treatment, azathioprine dose was halved in one patient. Cholestasis was transient in 7 from 11 cases. One patient had persisting cholestasis at all measurements and was evaluated for possible NRH. Upper endoscopy and ultrasound did not suggest signs of portal hypertension and the patient continues the treatment with infliximab. We did not observe worsening of liver injury among subjects pursuing the treatment.

DISCUSSION

Our prospective study reports on the liver injury burden among treated IBD patients over one year. We found mild ALT elevation in 26.3% of patients, hepatocellular injury 6.4% and persisting elevation in 11.2% of patients. Events of hepatocellular injury were more common with higher BMI and steatosis, with longer duration of IBD and on treatment with solo infliximab. Cholestasis was observed in 4.4% of patients and was more common after intestinal resection, higher inflammatory activity at baseline and on therapy with solo azathioprine. Most events of liver injury were transient and rarely required any change in management.

Hepatocellular injury

Clinical interpretation of hepatocellular injury occurring in IBD patients remains a challenge. In most cases, competing etiologies do not allow identification of a single one. Alcohol abuse, fatty liver disease, de-novo viral infection, concomitant medication and comorbidities could all be involved. Specific etiological models such as RUCAM model[10], might help in confirming or excluding DILI. However, AT abnormalities are frequently transient and stopping IBD therapy is not feasible at the moment of first appearance of liver injury. Therefore, this model does not help in cases with mild elevation. Moreover, it has been shown that several causes occurring in one patient might have a synergistic effect. Schröder *et al*[11] reported that patients with fatty liver were more susceptible to liver injury when treated with non-anti TNF immunosuppression. For now, it is not clear whether this finding also applies to anti-TNF therapy. In an observation study, Cappello *et al*[12] reported mild hepatocellular injury in 20.9% of 335 IBD patients. Liver injury was transient and most commonly due to fatty liver or DILI. Authors did not give details on IBD treatment and the most commonly observed pattern of injury was mild cholestasis. Parisi *et al*[13] report abnormal ALT in 39.2% and grade 2 increase in 7.9% of 176 patients treated with infliximab. In this study, authors identified several risk factors for liver injury: previous abnormal ALT suggesting a preexisting liver disease, immunomodulatory use and duration of infliximab therapy. Shelton *et al*[14] report a new transient increase in ALT > 2 × ULN following anti-TNF induction in 102 (6%) of 1753 patients. Liver injury could be linked to alternative etiologies in 54 of these cases (antibiotics, thiopurines, alcohol, fatty liver), leaving 48 (2.7%) directly linked to anti-TNF therapy. In 34 cases, ALT abnormalities were transient and anti-TNF therapy could continue. Fourteen patients with persisting abnormalities had to stop therapy. In our study, we included patients regardless of the timing of IBD induction and we did not exclude patients with fatty liver or preexisting ALT elevation. Nevertheless, our results appear consistent with previous reports showing ALT elevations in roughly the same proportion of cases. It appears, that transient liver injury occurs during induction, but also during maintenance therapy with a thiopurine as well as anti-TNF[13-15].

In our study, BMI, fatty liver, solo infliximab and longer duration of therapy have been identified as independent risk factors for hepatocellular injury. For BMI and fatty liver, the findings are consistent with the previous reports. However, ALT elevation was also more frequent among patients on solo infliximab compared to other treatment groups (adalimumab, azathioprine, combination therapy, no immunosuppression). This observation might be interpreted as a specific safety signal for solo infliximab therapy[5,7]. It has been previously shown that severe liver injury occurs on the background of less severe ALT elevations occurring much more commonly. Björnsson *et al*[16] showed that solo infliximab therapy had higher risk of DILI compared to adalimumab or combination therapy. In theory, solo infliximab therapy could be more immunogenic than a combination therapy in triggering an immune response. Parisi *et al*[13] also found that immunomodulatory therapy increased the risk of infliximab induced liver injury. This might be explained by hepatotoxicity of the immunomodulatory therapy itself, but the true reason for this finding is unclear. Observed normalization of ALT in most cases does not appear to exclude drug-induced immune mediated injury. It might actually support it, since presumed induction of immune tolerance to the drug might lead to the resolution of liver injury. Normalization of ALT in cases of fatty liver without any intervention appears less likely. For now, there appears to be a background of common causes of hepatocellular injury (steatosis, obesity, diabetes, alcohol). In our study, it might be observed in patients with no immunosuppression. On top of this, there is likely a higher risk of the injury in cases with solo infliximab therapy. Concomitant immunomodulator and liver steatosis probably serve as modulators, but we might also anticipate other influencing factors: genetic, ethnic, co-treatment and its dosing, infliximab administration protocol *etc.* We also report that longer duration of IBD increases the risk of hepatocellular injury. Some studies have found a similar association with the duration of infliximab therapy.

In view of the observed safety signals and the fact that liver injury occurs at distinct time points, our results support the need for regular AT monitoring regardless of the treatment duration. There is apparently a growing need for predictive models being able to identify patients at risk for DILI[17]. However, to date there are no clinically usable biomarkers that could preclude IBD patients from aminotransferase monitoring.

Cholestasis

Cholestasis, a parallel ALP and GGT elevation, was observed in 4.4% of patients. Cholestasis was

more frequently observed in cases treated with solo azathioprine compared with other treatment groups. Moreover, independent risk factors for cholestasis were solo azathioprine therapy, prior intestinal resection and higher CRP. Clinical interpretation of cholestasis in IBD patients should start with excluding possible biliary obstruction and the primary sclerosing cholangitis[18]. In our cohort, two cases had diagnosed PSC, but none had cholestasis during the study period. Drug induced cholestatic liver injury has been described in IBD patients treated with azathioprine or less commonly by anti-TNF therapy[19,20]. Symptomatic cholestatic hepatitis has been reported in patients on high-dose azathioprine[21]. Milder liver injuries might also present as cholestasis. In fact, a very recent study shows that mild transient cholestasis is actually the most common pattern of azathioprine induced liver injury[22]. Our findings are consistent with the report, since we found transient cholestasis in 7 of 11 cases. In contrast, persisting cholestasis during azathioprine therapy could be caused by PSC, IBD drugs and nodular regenerative hyperplasia (NRH). NRH is asymptomatic in most subjects and no specific NRH markers have been identified. The exact risk of this condition has not been accurately established[23,24]. Nevertheless, thiopurine therapy, prior intestinal resection and male gender have been previously reported as risk factors for NRH[25,26].

Another interesting finding of our study is that prior intestinal resection was an independent risk factor for cholestasis. Seventy-three patients in our study cohort had prior intestinal resection, 68 of whom had Crohn's disease. Azathioprine therapy was equally distributed between groups with or without resection, excluding the effect of the drug itself. It was therefore likely, that cholestasis was a consequence of shortening the terminal ileum. Extensive intestinal resection with intestinal failure have been shown to cause severe cholestasis[27]. Moreover, terminal ileum resection in Crohn's disease decreases the amount of biliary acids reabsorbed into the enterohepatic pool[28], causing diarrhea due to bile acid malabsorption. One proposed mechanism of this condition could be associated with cholestasis. Impaired bile acid reabsorption decreases farnesoid X receptor signaling (FXR) with decreased production of fibroblast growth factor 19 (FGF19). This might have an stimulatory effect on bile acid synthesis in the liver[29]. Indeed, levels of FGF19 have been reported decreased in patients with diarrhea due to bile acid malabsorption[30]. The role of FGF19 in cases with NRH remains unclear. Furthermore, cholestasis in patients with prior resection could also point to the NRH, as discussed above. For now, it appears that azathioprine treated patients are at risk for cholestatic hepatitis and NRH and should be monitored for liver adverse events while on treatment.

Our study has several strengths. We report a real-life burden of liver injury, not excluding patients with preexisting liver disease or other comorbidities. Our study observed various well defined liver injury events, their severities and patterns. On entry to the study, the study cohort was well defined for IBD phenotype and IBD therapy. We also included patients not treated with immunosuppression. This enabled us to identify the background of liver injury in IBD and the comparison among treatment groups. Our study completes the mosaic of reports on the burden of liver injury in IBD patients coming from clinical trials, registries and cohort studies on azathioprine or infliximab induction.

There are several limitations of our study. The size our cohort was not designed to capture the prevalence of severe DILI. Not surprisingly, we have not observed a single case of severe liver injury. However, in accordance with previous reports, some of our results could be viewed as safety signals for solo infliximab or azathioprine. Our study cohort consisted of patients regardless of the treatment timing. Although most patients were on stable maintenance therapy, some patients could have induction treatment. Our treatment groups were assigned according to the treatment at baseline. Later changes in therapy due to treatment efficacy on IBD were possible, but were infrequent. Inclusion of one retrospective value of AT 3 mo prior to study entry was designed to minimize an effect of this change. Finally, cases with incident mild AT elevations were not investigated for other possible hepatotoxic drugs taken, nor changes in alcohol intake or infections with various hepatotropic viruses. By missing these factors, the real DILI prevalence could have been overestimated.

In conclusion, our study shows that aminotransferase abnormalities are common in IBD patients. They are caused by preexisting liver diseases as well as by the IBD treatment. We found no case of severe DILI, but we observed higher risk of hepatocellular injury in patients on solo infliximab and cholestasis in patients on solo azathioprine. These findings could be viewed as safety signals. The real burden of liver injury on IBD appeared low with most cases resolving spontaneously without any change in disease management. However, our findings point to the potential for hepatotoxicity indicating the need for regular aminotransferase monitoring.

COMMENTS

Background

There is a growing concern, that drug-induced liver injury of inflammatory bowel disease (IBD) therapies might be underestimated. Therefore the authors aimed to analyze a real-life burden of liver injury and its impact of IBD management in a prospective observation during 1 year.

Research frontiers

There are no valid predictive models or biomarkers which would allow us to predict liver injury in IBD treated patients. Therefore, all patients should be monitored for liver tests during treatment. Liver test monitoring during long-term therapy is costly and time consuming. The authors observation was designed to estimate the burden of liver injury and to identify its risk factors (from demographics, IBD phenotype and the particular treatment). Possibly, to

identify patients with high-risk of liver injury in which the liver test monitoring should be close. Or, to identify a low risk group in which monitoring would not be necessary. This observation study was the first step in this research project.

Innovations and breakthroughs

They showed that liver injury was common, but in the great majority of cases it was mild and did not require any change in IBD management. However, patients with longer IBD duration, on solo immunosupressive therapy with infliximab or solo therapy with azathioprine had higher risk of liver injury. It appears therefore, that this group of patients would require regular monitoring of liver tests. These observations might lead in the future, to the development of a predictive model for liver injury in IBD.

Applications

For now, all patients on IBD therapy should be monitored. However, the risk is significantly higher in patients on solo immunosuppressive therapy, with prior intestinal resection and with longer IBD duration and these cases should be closely monitored for liver injury. In contrast, they have no arguments which would allow us to preclude other patients from liver test monitoring. Predictive models based on the current finding would probably allow us to identify low risk groups in the future.

Terminology

They use common terms and definitions. Hepatocellular injury was defined as ALT elevation superior to 2 x the upper limit of normal (ULN) and cholestasis as ALP and GGT elevation above the ULN.

Peer-review

The paper is well written and provides new insights about drug-induced liver injury occurrence and features in IBD patients.

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SYSTEMATIC REVIEWS

Can fecal microbiota transplantation cure irritable bowel syndrome?

Sofie Ingdam Halkjær, Anders Watt Boolsen, Stig Günther, Alice Højer Christensen, Andreas Munk Petersen

Sofie Ingdam Halkjær, Andreas Munk Petersen, Department of Gastroenterology, Copenhagen University Hospital Hvidovre, 2650 Copenhagen, Denmark

Sofie Ingdam Halkjær, Stig Günther, Alice Højer Christensen, Department of Gastroenterology, Aleris-Hamlet Hospitals Copenhagen, 2100 Søborg, Denmark

Anders Watt Boolsen, Andreas Munk Petersen, Department of Clinical Microbiology, Copenhagen University Hospital Hvidovre, 2650 Copenhagen, Denmark

Andreas Munk Petersen, Faculty of Health and Medical Sciences, University of Copenhagen, 2650 Hvidovre, Denmark

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Correspondence to: Andreas Munk Petersen, MD, PhD, Department of Gastroenterology, Copenhagen University Hospital Hvidovre, 2650 Copenhagen, Denmark. andreas.munk.petersen@regionh.dk

Telephone: +45-38-625960
Fax: + 45-38-623777

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Abstract

AIM

To verify the utility of treatment with fecal microbiota transplantation (FMT) in patients with irritable bowel syndrome (IBS).

METHODS

We searched EMBASE, Cochrane Library and PubMed in March, 2017. The reviewed literature was based on two systematic searches in each of the databases. The MeSH terms used were IBS and fecal microbiota transplantation and the abbreviations IBS and FMT. Reference lists from the articles were reviewed to identify additional pertinent articles.

RESULTS

A total of six conference abstracts, one case report, one letter to the editor, and one clinical review were included. In the final analysis, treatment of 48 patients was evaluated. Treatment revealed an improvement in 58% of cases. The varying structure of the nine included studies must be taken into consideration.

CONCLUSION

Data on FMT and IBS are too limited to draw sufficient

conclusions. Standardized double blinded randomized clinical trials need to be carried out to evaluate the effect of FMT on IBS.

Key words: Fecal microbiota transplantation; Microbiota; Irritable bowel syndrome

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Core tip: In humans, the gastrointestinal tract represents a large microbial ecosystem, housing several trillion microbial cells named the gut microbiota. Recent advances in sequencing methods have increased our understanding of the role of the gut microbiota in health and disease. Worldwide, interest is growing rapidly for fecal microbiota transplantation (FMT) as an "ecological" therapy for several diseases. Evidence suggests that a disturbance in the gut microbiota may be responsible for the initiation and persistence of symptoms in patients with irritable bowel syndrome. FMT could, therefore, be an ideal treatment option.

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INTRODUCTION

There is a growing interest in fecal microbiota transplantation (FMT) therapy for several gastrointestinal (GI) disorders. Treating GI disorders with FMT has been attempted as early as the 4th century, where a Chinese physician named Ge Hong advised patients suffering from severe diarrhea to consume fresh stool from a healthy neighbor as a form of treatment[1]. It is thought that the microbiome in our GI system plays an important role in health and disease. Literature in this area has increased markedly over recent years. More than 90% of the nearly 4000 articles indexed by PubMed on the subject have been published in the past five years (data from 2014)[2], indicating the rapidly growing interest within this field. Our knowledge of the microbiome is expanding due to, amongst other things, the development of new genetic technologies that allow us to identify and quantify the organisms of the microbiome at a faster rate[2]. Treatment with FMT has been a huge success when treating *Clostridium difficile* infection (CDI), with 33 case studies and a single randomized clinical trial (RCT) showing efficacy rates ranging from 81%-94%[1]. An increasing number of studies demonstrate an aberrant gut microbiota composition in irritable bowel syndrome (IBS)[3-6] and raise the question of whether FMT has a place in the treatment of this condition. The microbial pathophysiology of IBS is, however, not clearly understood, as microbiota alterations in IBS might either be a cause of IBS or a consequence of intestinal secretion and motility altered by IBS. FMT may play a significant role in future treatment of several other diseases which are thought to be linked to an abnormal gut microbiota, such as metabolic syndrome[1], inflammatory bowel disease (IBD)[1], obesity, type 2 diabetes mellitus, colonization of the gastrointestinal tract by pathogenic and multi-resistant microorganisms[7], depression, autism spectrum disorders[8], and chronic stress[9].

IBS is the most prevalent functional GI disorder in developed countries. It is estimated that IBS affects 10%-15% of the adult population[10] and strongly impairs quality of life, work productivity, and social function as well as inflicting substantial costs to health care systems[11,12].

The pathogenesis of IBS is complex and not yet fully understood. Accumulating evidence indicates that the gut microbiota plays a significant role[13], and alterations in gut microbiota among IBS patients have been described frequently[14]. It is also postulated that gut motility, enhanced visceral hypersensitivity, post infectious states[15], food sensitivity[2], genetics[16,17], and psychosocial disturbances[14] play a role in the pathophysiology of IBS.

IBS symptoms are characterized by chronic abdominal pain and altered bowel habits, including diarrhea and/or constipation, in the absence of organic or structural causes[2]. To receive the diagnosis IBS, symptoms must concur with the Rome Ⅲ criteria[18]. Furthermore, IBS can be subcategorized into diarrhea predominant (IBS-D), constipation predominant (IBS-C), and alternating (IBS-A) or mixed (IBS-M), where the last two are sometimes considered synonymous[19]. In most patients, IBS is a chronic relapsing disease in which symptoms and IBS subtype may vary over time. IBS affects women more often than men[10].

The treatment of IBS remains challenging due to the heterogeneity of the disorder, a lack of reliable outcome measures, and high placebo response rates. At present, there is no cure for IBS and, while there are a number of pharmacological therapies available to treat IBS symptoms, they are not effective in many patients[20].

An improved understanding of microbiota in IBS is important not only with regard to its pathogenesis but also in enabling therapeutic modulation of the microbiota. Many medical and alternative therapies have been tested without convincing effects.

Some studies indicate that moderate effects can be achieved by probiotics and prebiotics[21,22]. These

products must, however, be taken continuously to obtain a lasting effect[23]. Also, treatment with tricyclic antidepressants, antibiotics, anti-cholinergic drugs, motility regulatory drugs, selective serotonin reuptake inhibitors, melatonin, non-steroid anti-inflammatory drugs, opioids, and even Chinese herbs are suggested in severe IBS cases and underlines the fact that we do not yet know the etiology of the disease.

Many patients report that their symptoms are related to various food items and two-thirds of IBS patients report dietary restrictions on this basis[24]. Many different dietary approaches for the management of IBS symptoms have been tested over the years. Although dietary interventions for IBS are frequently recommended, there is, however, a lack of data to support their use[25]. This impact highlights the need for more effective IBS treatments as current therapies are not successful in many patients. This review aims to investigate current evidence about FMT and its use in IBS.

MATERIALS AND METHODS

Literature search

The reviewed literature was based on two systematic searches performed on March 13th, 2017 in the databases PubMed, Cochrane Library and EMBASE. The MeSH terms used were irritable bowel syndrome and fecal microbiota transplantation and the abbreviations IBS and FMT.

One hundred and fifty-eight papers were discovered in the 6 searches and 9 additional records were found by going through reference lists and other sources. Seventy-two papers were assessed; where one unobtainable and 63 others were excluded since they were reviews and referred to the same 3 original studies. Of these 3 studies, 2 were recovered but the 3rd could not be found[26]. The authors of this 3rd study have made a clinical review, in which they mention their study. This review has been included instead[27]. A total of 9 papers were found from scrutinizing references, 3 articles were retrieved from the 6 searches made, and 3 were found from other sources. Nine papers were, therefore, included in the final review. Of the 9 articles, 6 are conference abstracts[28-33], 1 is a case report[34], 1 is a letter to the editor[35], and 1 is a review[27]. A PRISMA (http://www.prisma-statement.org/statement.htm) flow diagram illustrates the outcome of the search (Figure 1).

All studies evaluating the effects of FMT in IBS patients are included in this paper. To estimate the overall effect of FMT in IBS patients, we decided to include studies with a follow-up longer or equal to 3 mo. Due to the restricted number of FMT studies in IBS patients, we have also decided to include conference abstracts.

RESULTS

A limited number of studies (summarized in Table 1) have examined the therapeutic role of FMT in IBS. The 9 publications reviewed in this paper consisted of 6 conference abstracts, 1 case report, 1 letter to the editor, and 1 clinical review. The studies are all case reports or case series and include a total of 127 patients. Borody *et al*[35] describe the treatment of 55 patients with FMT. The administration route of the FMT is not specified. What is stated is that the original bowel flora was removed by gastrointestinal lavage and replaced with bowel bacteria from a healthy donor. The 55 patients were suffering from constipation, diarrhoea, abdominal pain, ulcerative colitis, or Crohn's disease. Patients were included if other forms of therapy had failed to ease their symptoms. Unfortunately, a distinction between patient groups was not made. Of the 55 patients, 20 (36.4%) were described as "cured", 9 (16.4%) experienced a decrease of symptoms, and 26 (47.3%) experienced no improvement in symptoms.

Andrews *et al*[34] published a case report of a patient with chronic constipation who they, in a separate publication, diagnosed with constipation-predominant IBS[36]. The patient presented with a history of constipation spanning 3 years after an uncomplicated hysterectomy. Defaecation was once per week and required the use of laxatives. Associated symptoms included abdominal bloating, daily nausea, mild oesophageal reflux symptoms, and frequent headaches. The patient was given a treatment of vancomycin 250 mg thrice daily for 4 wk. Her constipation and associated symptoms disappeared promptly, but returned within 3 d of seponating treatment. Andrews *et al*[34] then decided to continue treatment with FMT. A fresh suspension of faeces was collected from her spouse and infused by enema for 2 d. Within 3 d of treatment, her stool frequency had shifted to 1-2 times per day without the use of laxatives. The headaches, abdominal bloating, and reflux symptoms had also disappeared. Symptom improvement was sustained at 18 mo follow-up.

The work of Borody *et al*[26] has not been acquired, despite extensive searching. Instead, the clinical review by Borody *et al*[27] is included as a substitute and describes the same case series from 2001. In 2001, Borody *et al*[27] published a case series study of ulcerative colitis and chronic constipation with 3 patients in each series. Chronic constipation was described as constipation-predominant IBS. Patients received single daily retention enemas for 5 d with donor stool suspended in 200 mL water with NaCl and a tablespoon of Psyllium. The patients with chronic constipation experienced a restoration of normal bowel function with a frequency of defaecation of 1-2 times per day. The follow-up period ranged from 8 to 28 mo

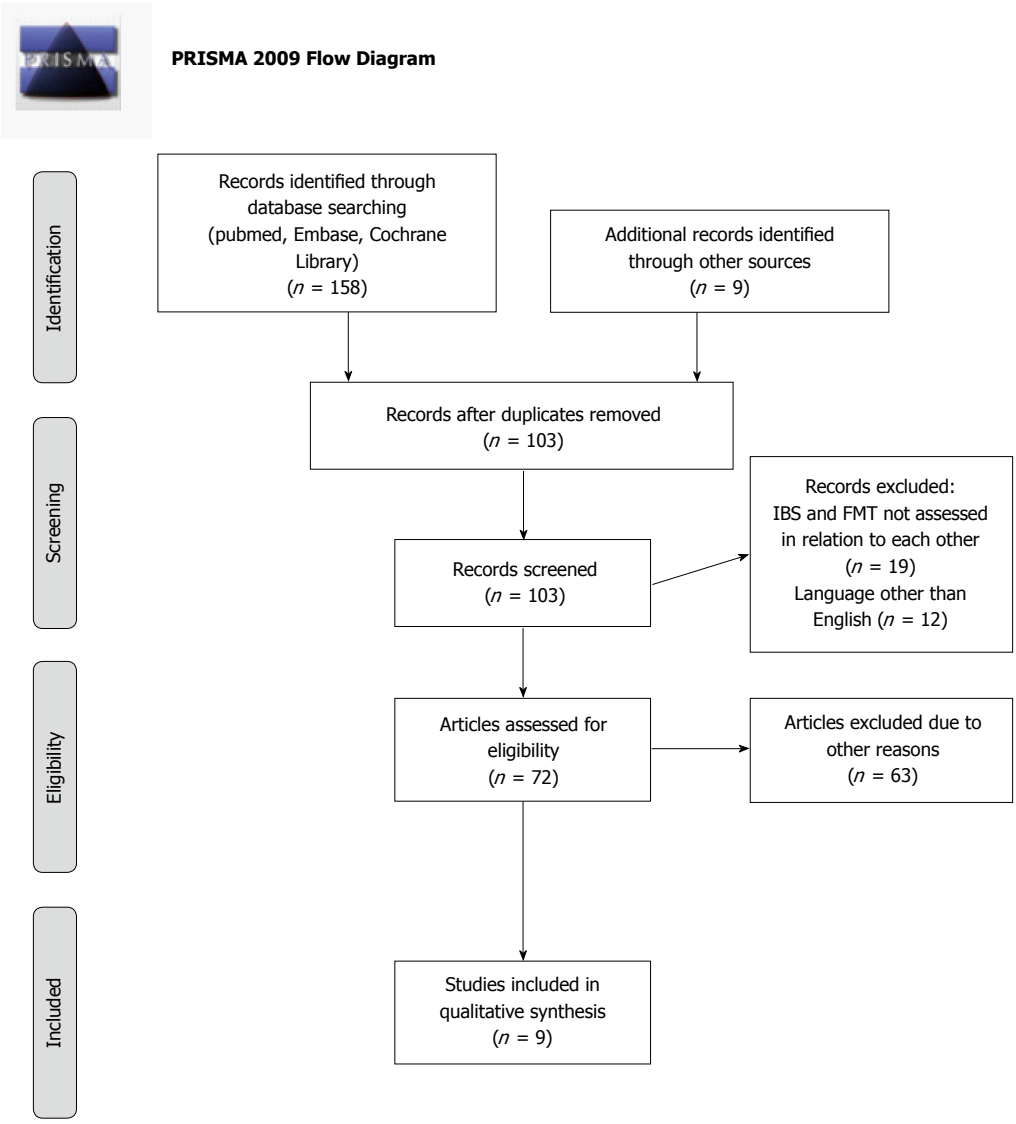

Figure 1 Results from the literature search for studies describing fecal microbiota transplantation in irritable bowel disease patients.

in both case series.

In 2013, Pinn *et al*[28] carried out a follow-up study of IBS treatment with FMT. Diagnosis of participants was based on the Rome Ⅲ criteria and patients, who were otherwise unresponsive to traditional treatment, were included in the study. Traditional treatment included probiotics, antibiotics, dietary changes, and other therapeutic modalities[28,37]. Thirteen of 15 eligible patients completed the study and were grouped in diarrhoea or constipation predominant IBS or mixed IBS. A questionnaire with 41 items addressing demographic data, pre- and post-FMT data, severity of abdominal pain, bloating, flatus, dyspepsia, diarrhoea, constipation, and overall well-being were filled out by the participants. Nine patients had IBS-D, 3 had IBS-C and 1 had IBS-M. The abstract does not include a description of how the FMT was administered[28]. Average time from FMT to data collection was 11 mo (range: 6-18 mo). FMT resolved or improved symptoms in 70% of the included patients. Factors which improved or resolved included abdominal pain (72%), changes in bowel habit (69%), dyspepsia (67%), bloating (50%), flatus (42%), and improved quality of life (42%). Transient increase in flatus was the only adverse effect reported[28,37].

Holvoet *et al*[29] carried out a prospective pilot study where 12 patients with refractory IBS symptoms underwent FMT. Patients with symptoms of severe bloating were included in the study. Fresh stool, under 6 hours after donation, were administered to the right colon *via* colonoscopy. The treatment was considered effective if the patient experienced an adequate relief of symptoms at week 12 post-transplantation. As secondary end points, the authors monitored IBS symptom scores and quality of life *via* questionnaires. At week 4 and week 12, 67% and 75% of patients reported an adequate relief of general IBS symptoms and, in particular, improvements

Table 1 List of articles included in the review examining the treatment of irritable bowel syndrome with fecal microbiota transplantation

Ref.	Year	Type	*n*	N in regard to IBS	Subcategory		
					IBS-D	IBS-C	IBS-M
Borody *et al*[35]	1989	Letter to the editor	55	Not specified	-	-	-
Andrews *et al*[34]	1992	Case report	1	1	-	1	-
Borody *et al*[27]	2004	Review	6	3	-	3	-
Pinn *et al*[28]	2013	Conference abstract	13	13	9	3	1
Holvoet *et al*[29]	2015	Conference abstract	12	12	-	-	-
Cruz Aguilar *et al*[30]	2015	Conference abstract	9	9	5	4	0
Hong *et al*[31]	2016	Conference abstract	10	10	-	-	-
Syzenko *et al*[32]	2016	Conference abstract	12	12	6	5	1
Mazzawi *et al*[33]	2016	Conference abstract	9	9	-	-	-

IBS: Irritable bowel syndrome.

in bloating, respectively. Furthermore, 16s rRNA amplicon sequencing was carried out on stool samples taken at different time points before and following transplantation. No 16s RNA results were presented in this paper[29]. In a subsequent journal letter written by Holvoet *et al*[38] further results were, however, presented. They found that the positive effects on IBS-related symptoms were linked to changes in the microbiota as a result of FMT treatment. Their stool sample analysis showed no microbiota community differences between patients and donors. Furthermore, no difference in microbial dissimilarity between patient-donor responders and non-responder pairs at baseline was found. A trend of higher *Streptococcus* counts was seen in donors compared to patients, and successful donors tended to have higher counts of *Streptococcus* compared with donors without success. In responders, a trend of higher enrichment potential compared with non-responders was also observed. Furthermore, the median number of successfully transferred phylotypes was higher in responders in relation to non-responders. The responders to FMT were assessed at a 1 year follow-up, where 7/9 (78%) still reported a significant relief of symptoms. The use of the Rome Ⅲ criteria was utilized in this work[38].

In 2015, Cruz Aguilar *et al*[30] published an abstract in which they summarise the treatment and results of 9 patients suffering from IBS. Five patients had IBS-D and 4 had IBS-C. Patients received a pre-treatment of rifaximin and, 3 wk later, a single FMT was performed during a colonoscopy. Evaluation of the treatment was performed 3 mo after FMT using a standardized questionnaire [Rome Ⅲ, Patient Health Questionnaires, Short Form Health Survey (36 items)] and clinical evaluation. Furthermore, deep sequencing analysis was performed on the microbiome before and 12 wk after FMT. The IBS-D patients immediately experienced a reduction of 2.5 points in the Bristol Stool Scale (BSS) score. No change in BSS score was reported in the IBS-C patients. A 50% reduction of abdominal pain was reported by 66% of the patients. A 50% reduction in bloating was reported by 16% of the participants. Reduction of symptoms lasted only 8 wk after FMT before a gradual reinstatement of symptoms occurred. Changes of the microbiome were seen in both IBS-D and IBS-C patients. In The IBS-D patients, a more diverse flora were discovered after treatment[30].

In 2016, Hong *et al*[31] published an abstract on FMT treatment in 10 patients with moderate IBS that did not respond to traditional treatment. Diagnosis was based on the Rome Ⅲ criteria and healthy donors were selected from family members and screened for infectious diseases before donation. It is not specified through which route the FMT was administrated or how the FMT was performed. Patients answered the IBS severity score before as well as 1 and 3 mo after FMT. Study outcomes included the length of symptom-free intervals, bloating, flatus, abdominal pain, frequency of bowel movements, dyspepsia, and overall well-being before and after FMT. Eighty percent of the study participants experienced resolution or improvement of symptoms after FMT. According to their IBS severity score (231 ± 110), patients' symptoms did, however, tend to return to their pre-treatment state within 3 mo after FMT. Clinically significant improvements in IBS severity score were observed at only 1 mo follow-up after FMT (132 ± 100) compared to baseline (252 ± 122) (P = 0.027). No long-term side-effects was reported by patients[31].

Mazzawi *et al*[33] conducted a study with FMT in IBS-D patients in order to investigate the effect of FMT on symptoms and the density of duodenal enteroendocrine cells. Nine patients were included according to the Rome Ⅲ criteria. The FMT consisted of freshly donated stool from relatives. Details concerning how the FMT was administrated and performed were not included. Apart from the IBS severity score, the IBS symptom questionnaire and Bristol stool form scale were completed before and 3 wk after FMT. IBS symptom scores were significantly reduced 3 wk after FMT treatment; abdominal pain (P = 0.005), diarrhea (P = 0.0002), constipation (P = 0.02), nausea (P =

0.004), and anorexia (P = 0.096). Furthermore, total IBS severity scores and Bristol stool scale scores were significantly reduced 3 wk after FMT (P = 0.0002 and P = 0.02)[33].

Syzenko *et al*[32] published an abstract in 2016 on a study evaluating the effect of FMT in "treatment resistant" IBS patients. Twelve patients were enrolled according to the Rome Ⅲ criteria, including 6 with IBS-D, 5 with IBS-C, and 1 with IBS-M. Treatment resistance was defined as continuous GI symptoms after adequate lifestyle modification, as well as antibiotic, pre- and probiotic, and antipsychotic treatment. FMT was accomplished *via* colonoscopy with or without consecutive enemas. To quantify the severity of GI symptoms, all patients registered eventual abdominal pain, bloating, and flatus according to the VAS scale. Bowel habits were evaluated using the Bristol stool scale and through frequency assessment. The results showed an abdominal pain resolution or significant improvement in 9 (75%) patients ($P \leqslant 0.01$). Only 1 patient reported no change in pain level. Normalization of stool frequency and consistency was reported in all IBS-M and IBS-D patients. In IBS-C patients, a significant reduction in frequency of laxative using was reported ($P \leqslant 0.01$). They also observed a significant improvement or complete resolution of symptoms in 7 (58.3%) and 4 (33.3%) patients, respectively. No date for time of assessment of the study's data has been provided[32].

In total, this review includes 9 published abstracts that describe 118 patients treated with FMT. Since the criteria for the diagnosis of the 55 included patients were not specified in the study by Borody *et al*[35], the results have been excluded from this paper. The results from Syzenko *et al*[32] and Mazzawi *et al*[33] have also been excluded. Syzenko *et al*[32] did not describe when the outcome data was measured. Mazzawi *et al*[33] had their follow-up after 3 wk - a period rated too short according to our criteria for evaluation of treatment effect. Therefore, the total number of treated patients with IBS included in this review is 48. Andrews *et al*[34], Borody *et al*[27], Pinn *et al*[28], and Holvoet *et al*[29] reported improvements in symptoms in 1, 3, 9, and 9 patients, respectively. Hong *et al*[31] reported no effect in 10 patients. Because of the manner in which Cruz Aguilar *et al*[30] present their results, it is difficult to give an exact number of patients who experienced symptom improvement. Since 6 (66%) patients experienced a 50% reduction in abdominal pain, abdominal pain has been chosen as the most significant parameter in evaluating FMT effect. A total of 28 of 48 patients (58%) experienced an improvement of symptoms upon review of the existing literature. No serious adverse effects were reported in any of the 9 included studies.

DISCUSSION

Current evidence suggests that the microbiota of the GI tract is a significant factor in the aetiology of IBS. Several aspects support this: the onset of IBS after infectious gastroenteritis[15], transient relief of symptoms after antibiotic treatment[15], previous reports of the successful treatment of *Clostridium difficile* infection with FMT[1], improvements of symptoms in combination with probiotic treatment[22], and findings of an altered gut microbiome combined with improvement in IBS-D patients after FMT[30]. Holvoet *et al*[38] ascribed the positive effects of FMT on IBS-related symptoms to changes in the microbiota. Furthermore, orally administered antibiotic drugs that are poorly absorbed through the GI tract result in a temporarily reduction of symptoms[39]. The aetiology of IBS is complex and, though it is not certain that it is of bacterial origin, the treatment with FMT appears to be beneficial with an improvement in 58% of patients treated. Several factors make a comparison of existing studies difficult, however. Holvoet *et al*[29] included patients with refractory IBS symptoms and severe bloating, Hong *et al*[31] included IBS patients who were moderately or fully unresponsive to traditional treatment, Cruz Aguilar *et al*[30] and Mazzawi *et al*[33] included patients with diarrhoea predominant IBS, Pinn *et al*[28] and Syzenko *et al*[32] divided their patients into 3 groups of IBS-D, IBS-C and IBS-M, and Borody *et al*[27] included 3 patients with chronic constipation. None of the included studies specify how patient subgroups were categorised or diagnosed. Additionally, the method used to evaluate symptom relief, if specified at all, varied. Borody *et al*[27] failed to clarify how improvement was assessed. In the abstract from 1989[35], the group describe their patients as "cured" but the definition of cure was not specified. In the study by Pinn *et al*[28], a 41-point-questionnaire was used with an average time from FMT to data collection of 11 mo, while Cruz Aguilar *et al*[30] used clinical evaluation and a standardized questionnaire with data collection 3 mo after FMT. Holvoet *et al*[29] also used questionnaires for quality of life but did not specify how patients reported relief of IBS symptoms 12 wk post FMT[29]. Cruz Aguilar *et al*[30], Pinn *et al*[37], Holvoet *et al*[29,38] Hong *et al*[31], Syzenko *et al*[32], and Mazzawi *el al*[33] used the ROME Ⅲ criteria. These criteria are, however, not mentioned in Andrews *et al*[34] or the articles by Borody[27] and his group.

Differences between the 9 studies make it difficult to verify findings and reproduce results. The absence of the Rome Ⅲ criteria from some of the included studies[27,34,35] could be explained by the fact that the Rome Ⅲ criteria were only first published in 2006[19]. The included studies in our review do, however, lay the ground work for larger scale clinical trials by attempting FMT in several subgroups of IBS.

Consensus over the use of internationally accepted guidelines is needed to ensure that patients are sorted into predefined groups according to symptoms and diagnosis in order to accurately elucidate subgroup differences and FMT effect. Additional research should

also include the microbiome of donors so that donors with advantageous microbiomes can be matched with a specific subtype of IBS or any other FMT-treatable GI disorder. The RCTs of the future should implement standard criteria, such as the ROME Ⅲ criteria, when diagnosing patients. Six out of the 8 RCTs currently listed on clinicaltrials.gov (15/3-2017) use or refer to the Rome Ⅲ criteria.

Holvoet *et al*[29] and Cruz Aguilar *et al*[30] made efforts to map the GI-microbiome associated with IBS before, during, and after their trials. This has not been accomplished earlier most likely because of lack of or access to necessary technology. Access to such technology is now becoming more widespread and rapid identification and quantification of the highly diverse organisms that comprise the human microbiome is becoming a reality[2]. In the case of Cruz Aguilar *et al*[30], their 5 IBS-D patients displayed a higher degree of microbiome diversity after FMT, but it is not specified what kind of bacteria this diversity included. The IBS-C patients also showed a change in gut microbiota, but this change was not further elaborated on[30]. Holvoet *et al*[29] failed to mention any results regarding the sequencing of 16s rRNA in their trial. In a later letter, they did, however, link the positive effects on IBS-related symptoms to changes in the microbiota as a result of FMT treatment[38]. Further sequencing of the microbiome is needed from larger groups to ascertain a broader picture of what a healthy microbiome consists of. This could be done through screening of healthy stool donors.

In only 1[38] of the 9 core studies, a placebo effect was mentioned. Placebo has a large effect in clinical trials concerning IBS and ranges from 16%-71%[40]. The currently ongoing clinical trials listed above are all placebo controlled and mainly include patients with diarrhoea predominant IBS. Furthermore, there is a risk of positive outcome bias when dealing with small trials and case reports in which researchers only publish cases where improvement was reported.

Several other factors could influence the effect of FMT, such as the route used for FMT, duration of treatment and quantity of fecal microbiota transplanted to the patient. There are many different ways in which FMT can be administered, including capsules, enemas, and colonoscopy. No clinical trials have compared FMT delivery routes in IBS and further trials are needed to determine the ideal route of FMT treatment in these patients[14]. Furthermore, it is unknown whether 1 FMT treatment is enough or if FMT should be repeated for best effect. In the study by Hong *et al*[31], results suggest that FMT may only be beneficial for 1 mo. The positive effects seemed to decrease over time and symptoms tended to return to their pre-FMT state within 3 mo after FMT treatment.

A systematic review by Wang *et al*[41] found that FMT could result in serious adverse effects, even death. Looking into the actual cases, however, the adverse effects were mostly related to the mode of delivery rather than the actual FMT, *e.g.,* one death was due to sedation issues before colonoscopy. Using an endoscope to administer the FMT is widely used and will always include the risk of perforation of the intestine. The use of encapsulated FMT as mode of delivery can circumvent this problem and is, therefore, an attractive alternative. There is a general opinion that thorough donor screening is necessary in order to avoid possible transfer of disease or pathogens[42]. No standardized donor screening protocol has yet emerged, however.

Currently, 8 FMT and IBS studies (15/3-2017) are registered on clinicaltrials.gov. These ongoing trials primarily focus on the beneficial effects of FMT on IBS. Secondary goals of these trials include research into the possible bacterial aetiology behind IBS. Several of these studies have included sequencing of bacteria before and after FMT which hopefully will reveal a pattern or give us clues as to where to look next.

Few studies examining FMT in the treatment of IBS have been published. Despite the small number of patients reviewed in this paper and differences in study design between the included studies, it seems that there is an - at least temporary- improvement in a large proportion of FMT treated patients. An improvement was seen in 58% of participating IBS patients. Randomized, double-blinded placebo controlled trials are, however, still lacking. Currently, 8 studies (15/3-2017) fulfilling these aforementioned criteria (clinicaltrials.gov) are underway, and considerable leaps in knowledge on the effect of FMT in IBS is expected within the near future.

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CASE REPORT

Application of novel magnified single balloon enteroscopy for a patient with Cronkhite-Canada syndrome

Masaki Murata, Shigeki Bamba, Kenichiro Takahashi, Hirotsugu Imaeda, Atsushi Nishida, Osamu Inatomi, Tomoyuki Tsujikawa, Ryoji Kushima, Mitsushige Sugimoto, Akira Andoh

Masaki Murata, Shigeki Bamba, Kenichiro Takahashi, Hirotsugu Imaeda, Atsushi Nishida, Osamu Inatomi, Akira Andoh, Department of Medicine, Shiga University of Medical Science, Seta-Tsukinowa, Otsu 520-2192, Japan

Tomoyuki Tsujikawa, Comprehensive Internal Medicine, Shiga University of Medical Science, Seta-Tsukinowa, Otsu 520-2192, Japan

Ryoji Kushima, Department of Clinical Laboratory Medicine, Shiga University of Medical Science, Seta-Tsukinowa, Otsu 520-2192, Japan

Mitsushige Sugimoto, Division of Digestive endoscopy, Shiga University of Medical Science, Seta-Tsukinowa, Otsu 520-2192, Japan

Author contributions: Murata M, Bamba S, Takahashi K, Imaeda H, Nishida A, Inatomi O, Tsujikawa T, Kushima R, Sugimoto M and Andoh A collected and analyzed the patient's clinical data and wrote the paper.

Institutional review board statement: Approval of this case report was not given in advance by the Institutional Review Board of Shiga University of Medical Science.

Informed consent statement: A patient of this case provided informed written consent prior to the endoscopic procedure and treatment.

Conflict-of-interest statement: The magnifying single-balloon enteroscope (SIF Y-0007) was loaned free of charge by the Olympus Corporation.

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Correspondence to: Shigeki Bamba, MD, PhD, Department of Medicine, Shiga University of Medical Science, Seta-Tsukinowa, Otsu 520-2192, Japan. sb@belle.shiga-med.ac.jp
Telephone: +81-77-5482217
Fax: +81-77-5482219

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Abstract

We present a case of Cronkhite-Canada syndrome (CCS) in which the entire intestine was observed using a prototype of magnifying single-balloon enteroscope (SIF Y-0007, Olympus). CCS is a rare, non-familial gastrointestinal polyposis with ectodermal abnormalities. To our knowledge, this is the first report showing magnified intestinal lesions of CCS. A 73-year-old female visited our hospital with complaints of diarrhea and dysgeusia. The blood test showed mild anemia and hypoalbuminemia. The esophagogastroduodenoscopy and colonoscopy revealed diffuse and reddened sessile to semi-pedunculated polyps, resulting in the diagnosis of CCS. In addition to the findings of conventional balloon-assisted enteroscopy or capsule endoscopy, magnifying observation revealed tiny granular structures, non-uniformity of the villus, irregular caliber of the loop-like capillaries, scattered white spots in the villous tip, and patchy redness of the villus. Histologically, the scattered white spots and patchy redness of the villus reflect lymphangiectasia and bleeding to interstitium, respectively.

Key words: Balloon-assisted enteroscopy; Magnified endoscopy; Narrow band imaging; Small intestine

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Core tip: While the endoscopic findings using esophagogastroduodenoscopy or colonoscopy of Cronkhite-Canada syndrome (CCS) are common, there have been few reports visualizing the small intestinal lesions of CCS. We have used a prototype of magnifying single-balloon enteroscope and have shown the detailed image of the small intestinal lesions of CCS. We also find some novel findings of CCS, some of which were confirmed by histological analysis. We also present a detailed video of this case.

Murata M, Bamba S, Takahashi K, Imaeda H, Nishida A, Inatomi O, Tsujikawa T, Kushima R, Sugimoto M, Andoh A. Application of novel magnified single balloon enteroscopy for a patient with Cronkhite-Canada syndrome. *World J Gastroenterol* 2017; 23(22): 4121-4126 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i22/4121.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i22.4121

INTRODUCTION

Cronkhite-Canada syndrome (CCS) is characterized by gastrointestinal polyposis accompanied by hair loss, nail abnormalities, chromatosis, dysgeusia, and other ectodermal abnormalities. Pathophysiology of CCS also includes protein-losing enteropathy and malabsorption[1]. While the endoscopic findings using esophagogastroduodenoscopy or colonoscopy of CCS are common, there have been few reports visualizing the small intestinal lesions of CCS. We herein present a case of CCS in which the entire intestine was observed using a prototype of magnifying SBE (SIF Y-0007, Olympus). We believe the findings of the present study are of particular importance as no previous reports have identified small intestinal lesions in CCS using magnifying enteroscopy.

CASE REPORT

A 73-year-old woman developed diarrhea, dysgeusia and loss of appetite in January 2015. Her past medical history included benign lung tumor at age 60 and type II diabetes at age 67. In March 2015, she presented to our hospital because the diarrhea worsened (7 bowel movements per day). She also experienced body weight loss of 4 kg over 3 mo, pedal edema and epigastric pain. Physical examination revealed painful superficial ulcers on the tongue in addition to chromatosis on the dorsum of the hands and nail abnormalities. Edema was present in both legs, and there were loss of head hair, eyebrows, and eyelashes. The laboratory results indicated anemia (hemoglobin 11.6 g/dL) and hypoalbuminemia (serum albumin 2.5 g/dL). Urine albumin was normal. Scintigraphy indicated diffuse protein leakage throughout the entire small bowel. Alpha-1 antitrypsin clearance was elevated at 134 mL/d (normal, < 20 mL/d), and protein leakage from the gastrointestinal tract was observed.

The esophagogastroduodenoscopy revealed no abnormal findings in the esophagus. In the stomach, diffuse sessile and semipendunculated polyps were observed predominantly in the antrum. The polyp surfaces were smooth and displayed intense reddening (Figure 1A). On the other hand, in the fundus and upper body of the stomach, there are relatively few polyps (Figure 1C). Biopsies were taken from the polypoid lesion at antrum and the inter-polypoid lesions in the fundus (Figure 1B and D). Histopathological findings of the antral polyp indicated an edematous and myxomatous lesion (Figure 1B). Similar findings were also observed from the biopsy taken from the inter-polypoid mucosa (Figure 1D).

Colonoscopy revealed diffuse sessile and semipundunculated polyps with intense reddening in the entire large bowel (Figure 2A). Histology of the colonic biopsy showed inflammatory cell infiltration, predominantly eosinophilic infiltration, and ductal cystic dilatation (Figure 2B).

Using a magnifying SBE, we performed an endoscopic examination *via* both trans-oral and trans-anal routes (Supplemental video). Structural differences in the villi were observed in the jejunum and the ileum. In the jejunum, the villi were predominantly elongated and exhibited scattered white spots (Figure 3A-C). In the ileum, the villi exhibited prominent reddening and swelling with a salmon roe appearance (Figure 4A-C). Magnified observation with narrow-band imaging enabled us to observe villi structure in detail and the presence of loop-like capillaries within villi. Specifically, we were able to clearly observe an irregular villous structure, scattered white spots within the villi, fine granular structures at the tips of villi, irregular caliber of the loop-like capillaries, and spotted villous erythema (Figure 3B, C and Figure 4B, C). Histopathological findings were as follows: jejunal biopsy showed the presence of twisted crypts and interstitial edema (Figure 3D). Ileal biopsy demonstrated elongated crypts and atrophied villus and inflammatory cell infiltration predominantly consisting of eosinophils (Figure 4D). The presence of scattered white spots and spotty erythema was consistent with lymphangiectasia and interstitial bleeding.

Based on the history of the current illness characterized by ectodermal symptoms, the presence of gastrointestinal polyposis, and the results of histopathological assessments, the patient was diagnosed with CCS. Oral administration of prednisolone 30 mg/d and tranexamic acid 1000 mg/d

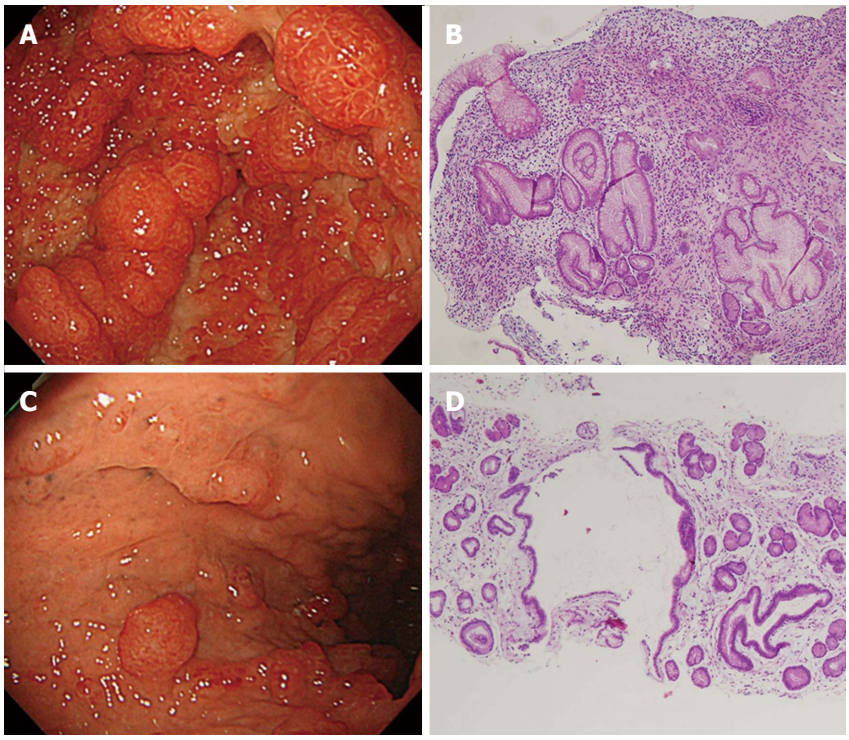

Figure 1 Esophagogastroduodenoscopy and histopathological findings. A: Diffuse sessile and semipendunculated polyps were observed in the antrum; B: Biopsies of the polyps in the antrum indicated interstitial edematous and myxomatous lesions; C: Mild polyposis was observed in the upper body of the stomach; D: Biopsies of the interpolypoid lesions indicated the presence of the same interstitial edematous and myxomatous lesions as observed in polyp biopsies.

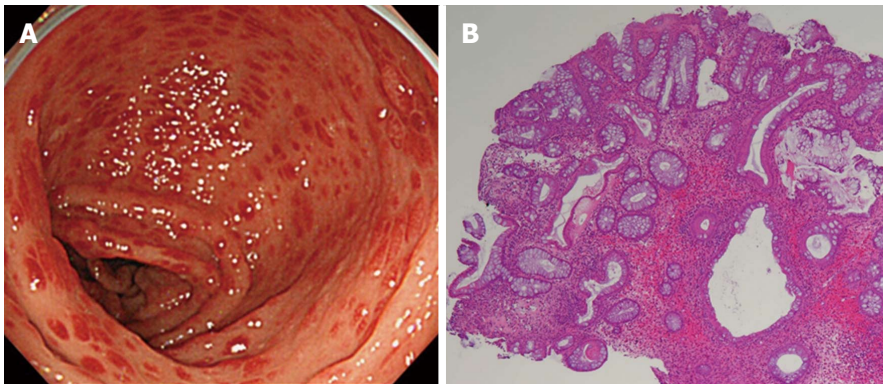

Figure 2 Colonoscopy and histopathological findings. A: White light observation demonstrated flat elevated lesions with intense reddening throughout the entire large bowel; B: We observed both inflammatory cell infiltration into the large bowel mucosal epithelium and cystic ductal dilatation.

was initiated on day 7 of hospitalization. The patient subsequently reported improvement in symptoms, including a marked reduction in the frequency of diarrhea. The prednisolone dose was gradually reduced, and she was discharged on hospital day 29. However, she was re-admitted to our hospital on day 74 since initial hospitalization, because diarrhea frequency and general malaise worsened. A stool sample taken on the same day indicated the presence of *Clostridium difficile* (CD) toxin. The patient was diagnosed with worsening due to CD enteritis. On day 79, she was administered metronidazole, resulting in rapid remission of clinical symptoms. She was then discharged on day 113. During subsequent outpatient visits, the prednisolone dose was gradually reduced and eventually discontinued without leading to any recurrence of symptoms. Post-treatment upper and lower GI endoscopic examination and an α1 antitrypsin clearance test were conducted on day 211. Marked improvement in polyposis was noted, particularly in the large bowel, and the presence of adenoma was revealed. No reddening or swelling of the villi in the jejunum and ileum was observed, and the scattered white spots were seen to have disappeared. Gastric biopsy indicated hyperplasia of the ductal epithelium and remnant interstitial swelling; however, the inflammatory cell infiltration had resolved. The result of the α1 antitrypsin clearance test was 9.3 mL/d,

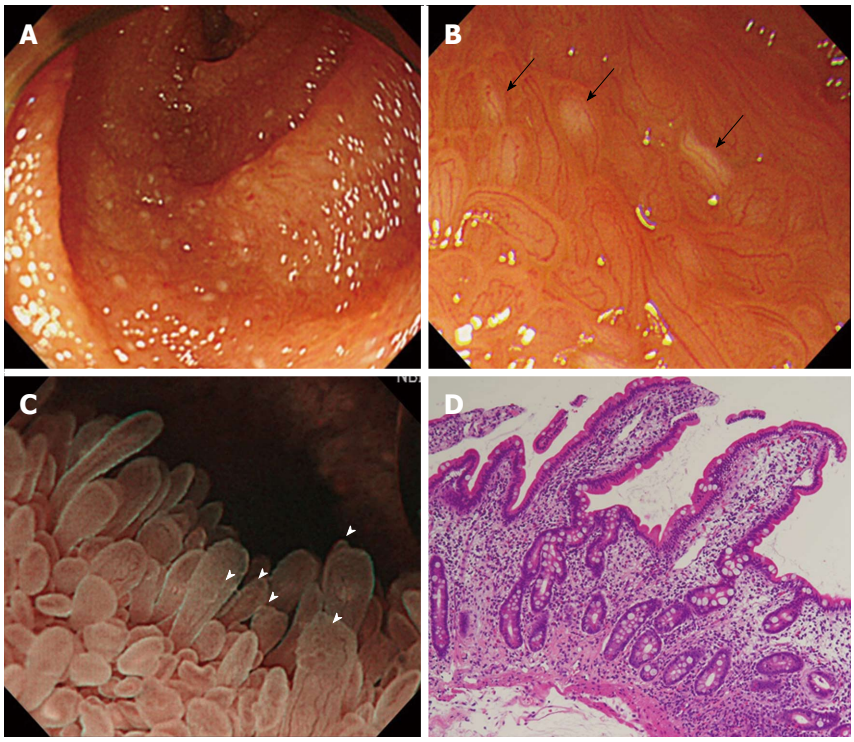

Figure 3 Trans-oral single balloon enteroscopy (jejunum) and histopathological findings. A: White light observation demonstrated scattered white spots and reddening; B: Magnified observation under white light demonstrated scattered white spots (black arrows) as well as irregular caliber of the loop-like capillaries; C: Underwater magnifying enteroscopy demonstrated elongated villi and fine granular structures at the tips of villi (white arrowheads); D: Histopathological findings demonstrated twisted crypts and interstitial edema.

indicating marked improvement.

DISCUSSION

CCS is a relatively rare disease in which ectodermal symptoms are accompanied by gastrointestinal polyposis[1]. CCS tends to occur in middle-aged men and approximately 400 cases have been reported worldwide[2]. As approximately three-quarters of those reports are from Japan, it appears that racial background factors might affect the onset of this disease[3]. Although the pathogenesis of CCS remains unknown, genetic abnormalities[4], abnormal proliferation and differentiation of intestinal epithelium[5], immune-related abnormality[6], and stress[7] have been implicated.

Polyposis in CCS is non-neoplastic, and non-atypical ductal proliferation, cystic dilatation, swelling of the lamina propria mucosa, and marked inflammatory cell infiltration predominantly consisting of eosinophils are observed. Biopsy from the polypoid lesion is not sufficient to differentiate between juvenile polyps and inflammatory polyps[8,9]. Additional biopsies from inter-polypoid lesions also yield the above findings, which are characteristic of CSS and have diagnostic value.

While CCS is thought to cause polyposis throughout the entire gastrointestinal tract except for the esophagus, polyposis in the small bowel is rare. However, some form of small bowel lesion is seen in over half of cases[10,11]. There are few studies reporting the use of endoscopic observation for small bowel lesions. Review of small number of previous reports indicates that typical endoscopic findings in CCS include the following: (1) mucosal edema and enlarged villi[12,13]; (2) reddened mucosa[12,13]; (3) white villus or scattered white spots[12,14]; (4) flat protuberances or small polyps (herpes-like lesions at jejunum[12-15]; and strawberry-like lesions at ileum[12,13,16]; (5) elongated villi[14,16]; and (6) atrophied villi[2,17].

The above findings (1), (2), (3), (5) and (6) were observed in the present case. In addition, villus morphology and the degree of villus reddening and scattered white spots differed between the jejunum and ileum. There have been previous reports of the use of capsule endoscopy to observe changes in the mucosa along the vertical axis of the small bowel[12,13]. New findings using magnified observation include the following: (1) irregular villus structure; (2) scattered white spots within the tips of villi; (3) small granular structure on the tips of villi; (4) irregular caliber of the loop-like capillaries; and (5) spotted reddening within villi. Histological analysis revealed that the scattered white spots reflect the pathological dilatation of the lymphatic ducts. This is consistent with the previous report by Asakura *et al*[18]. Furthermore, histology revealed that the spotted reddening within the villi reflects interstitial bleeding.

The magnified observation of the small intestine

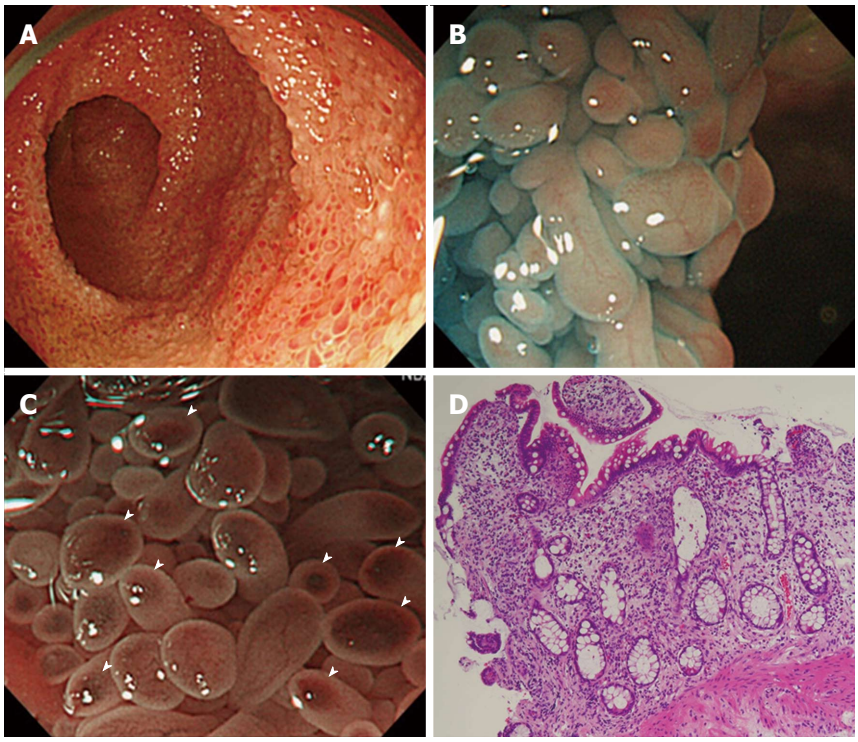

Figure 4 Trans-anal single balloon enteroscopy (ileum) and histopathological findings. A: White light observation demonstrated prominent reddening of villi. Scattered white spots were also observed; B: Magnifying enteroscopy with indigo carmine contrast demonstrated irregular villus structure; C: Narrow-band imaging magnified observation demonstrated spotted reddening within villi (white arrow heads) and irregular caliber of the loop-like capillaries; D: Histopathological findings demonstrated elongated crypts and atrophied villi as well as inflammatory cell infiltrates predominantly composed of eosinophils.

was firstly reported in 1980 by Tada *et al*[19]. However, the scope (SIF-M, Olympus) needed to be inserted using ropeway method. Therefore, the scope was not widely used. SIF-Y0007 provides a magnification of up to × 80, and has the outer diameter of 9.9 mm which is approximately the same contour as SIF-Q260 with the outer diameter of 9.2 mm. Although an increase in outer diameter of 0.7 mm compared to SIF-Q260, SIF-Y0007 is incorporated passive bending and high force transmission. Therefore, the ability of deep insertion is similar to SIF-Q260 and SIF-Y0007 can be used in a routine examination.

The magnified observation enables us to visualize the detailed morphology of the villus or the loop vessels. Therefore, we are actively using SIF-Y0007 for the patients with protein-losing enteropathy or with known polyps or aggregates of white villi detected by video capsule enteroscopy. Further analysis should be done to investigate the usefulness of magnified endoscopy as a diagnostic tool of intestinal pathogenesis.

There have been few reports of small bowel lesions in cases of CCS. We reported a case in which SBE allowed observation of the entire small bowel affected by CCS.

COMMENTS

Case characteristics

A 72-year-old woman presented to our hospital because of diarrhea, dysgeusia and loss of appetite.

Clinical diagnosis

Physical examination revealed superficial ulcers on the tongue, chromatosis on the dorsum of the hands, nail abnormalities, bilateral leg edema, loss of head hair, eyebrows and eyelashes, indicating ectodermal abnormalities.

Differential diagnosis

Polyposis of the gastrointestinal tract, such as familial adenomatous polyposis, Peutz-Jeghers syndrome, juvenile polyposis.

Laboratory diagnosis

The laboratory results indicated anemia and hypoalbuminemia.

Imaging diagnosis

In addition to the diffuse polyposis in the stomach and colon, a magnifying single-balloon enteroscopy enables us to clearly observe an irregular villous structure, scattered white spots within the villi, fine granular structures at the tips of villi, irregular caliber of the loop-like capillaries, and spotted villous erythema in the jejunum and the ileum.

Pathological diagnosis

Histopathological findings of the inter-polypoid mucosa at gastric body indicated an edematous and myxomatous lesion, which suggest the diagnosis of Cronkhite-Canada syndrome.

Treatment

The patient responded to oral prednisolone of 30 mg/d and the dose was gradually tapered.

Related reports

There have been a few reports of the use of capsule endoscopy or conventional balloon-assisted enteroscopy to observe small intestinal lesion of Cronkhite-Canada syndrome.

Term explanation

Cronkhite-Canada syndrome is characterized by gastrointestinal polyposis accompanied by hair loss, nail abnormalities, chromatosis, dysgeusia, and other ectodermal abnormalities.

Experiences and lessons

Authors have visualized the detailed observation of small intestinal lesion of Cronkhite-Canada syndrome.

Peer-review

The endoscopic pictures and a supplementary video may interact your attention.

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CASE REPORT

Synchronous triple occurrence of MALT lymphoma, schwannoma, and adenocarcinoma of the stomach

Kyeong W Choi, Mee Joo, Han S Kim, Woo Y Lee

Kyeong W Choi, Woo Y Lee, Department of Surgery, Seoul Paik Hospital, Inje University College of Medicine, Seoul 100-032, South Korea

Mee Joo, Han S Kim, Department of Pathology, Ilsan Paik Hospital, Inje University College of Medicine, Goyang-si Gyeonggi-do 411-706, South Korea

Author contributions: Choi KW and Lee WY designed the report; Joo M and Kim HS performed histologic analysis; Choi KW and Lee WY wrote the paper.

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Informed consent statement: The patient presented in this case report gave his written informed consent authorizing use and disclosure of his protected health information.

Conflict-of-interest statement: There is no conflict of interest to declare.

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Correspondence to: Woo Y Lee, MD, Department of Surgery, Seoul Paik Hospital, Inje University College of Medicine, 9 Mareunnae-ro, Jung-gu, Seoul 100-032, South Korea. yongaaa5972@naver.com
Telephone: +82-2-22700247
Fax: +82-2-22700250

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Abstract

We present a case of a 56-year-old man with 3 synchronous gastric tumors. The patient presented with melena, and 3 gastric abnormalities were detected on gastroduodenoscopic examination, including a small ulcerative lesion in the gastric antrum, a submucosal mass in the gastric body, and severe erosion in the fundus. Histological examination of biopsy samples yielded respective diagnoses of gastric adenocarcinoma, gastritis, and mucosa-associated lymphoid tissue (MALT) lymphoma. The patient first received medication to eradicate any underlying *Helicobacter pylori* infection, which might have been a cause of the MALT lymphoma. Four weeks later, after examination of repeat biopsy samples revealed that the MALT lymphoma had resolved, the patient underwent subtotal gastrectomy. Further histological examination of resected tissue confirmed the antrum lesion as adenocarcinoma and the body lesion as schwannoma. To our knowledge, this is the first reported case of synchronous triple primary gastric adenocarcinoma, MALT lymphoma, and schwannoma.

Key words: Gastric cancer; Synchronous; Mucosa-associated lymphoid tissue lymphoma; Schwannoma; Triple

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Core tip: Synchronous occurrence of two types of gastric tumor is relatively well described, but this is the first report of synchronous triple gastric adenocarcinoma, mucosa-associated lymphoid tissue

(MALT) lymphoma, and schwannoma. Consideration of the possible underlying involvement of *Helicobacter pylori* in MALT lymphoma and preoperative treatment with appropriate medication allowed performance of subtotal gastrectomy and a successful outcome.

Choi KW, Joo M, Kim HS, Lee WY. Synchronous triple occurrence of MALT lymphoma, schwannoma, and adenocarcinoma of the stomach. *World J Gastroenterol* 2017; 23(22): 4127-4131 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i22/4127.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i22.4127

INTRODUCTION

The most frequent diagnosis of tumor of the stomach is adenocarcinoma (90%-95%), followed by lymphoma, gastrointestinal stromal tumor (GIST), and carcinoid tumor. Synchronous occurrence of two types of tumor in the stomach is relatively well known. Here, we present an extremely rare case of synchronous gastric adenocarcinoma, mucosa-associated lymphoid tissue (MALT) lymphoma, and schwannoma in a 56-year-old man in Korea.

CASE REPORT

A 56-year-old male visited to our clinic with melena. Apart from the presence of diabetes, he had no other remarkable symptom or past medical history. There were no specific findings in physical examination except for obesity (body mass index, 32). All laboratory results, including tumor markers, were in normal range. Gastroduodenoscopy showed slightly raised ulcer on the antral anterior wall. Additional findings included a positive rolling sign in the mid-body suggestive of a submucosal lesion and a diffuse erythematous lesion in the fundus area, and these areas were also biopsied (Figure 1). A computed tomography scan of the abdomen showed an ovoid homogeneous 2 cm × 2 cm mass at the greater curvature of the mid-body and mildly enlarged perigastric lymph nodes. Positron emission tomography - computed tomography showed no evidence of any other lymphoid involvement. Histological examination of the antral and fundic biopsy samples yielded respective diagnoses of moderately differentiated adenocarcinoma and low-grade MALT lymphoma with plasmacytic differentiation (Figure 2).

The patient was first prescribed medication to eradicate any underlying *Helicobacter pylori* (*H. pylori*) infection, which might have been a causative factor in the MALT lymphoma, and examination of repeat biopsy obtained at the fundus after 4 wk medication confirmed that the MALT lymphoma had resolved. The patient was then referred for surgical intervention. At laparotomy, the patient underwent radical subtotal gastrectomy with gastrojejunostomy. During gastrectomy, a well-defined nodular lesion measuring 2 cm × 2 cm was palpated in the greater curvature 5 cm proximal to the adenocarcinoma.

Histological examination of the partial stomach showed the presence of an early gastric cancer consisting of a moderately differentiated intestinal-type adenocarcinoma located in the antrum and measuring 3.2 cm × 2.5 cm. The tumor was infiltrating into the deep submucosa of the antrum. There was no lymph node metastasis in 17 retrieved nodes. The final pathologic stage for gastric adenocarcinoma was pT1bN0M0, p-Stage IA (American Joint Committee on Cancer 7th edition). Histological examination of hematoxylin and eosin-stained sections of the other submucosal tumor revealed that it consisted of spindle cells covered by a smooth muscle layer, lymphoid cuffs, and fundic-type glands. On immunohistochemical analysis, this submucosal tumor showed strong positivity for S-100 and negative expression for c-Kit. This immunostaining pattern differentiates gastrointestinal schwannoma from GIST (Figure 3).

Electron microscopic examination of ultrathin sections of the submucosal tumor revealed sheets of elongated cells with numerous complex cytoplasmic processes. The cytoplasmic membrane was completely covered with external lamina. The nucleus had irregular margins and a heterochromatic chromatin pattern, and the perikaryal cytoplasm contained several mitochondria, rough endoplasmic reticulum, ribosomes, and many lysosomes. Hence, a final diagnosis of schwannoma was made (Figure 4).

The patient's postoperative period was uneventful and he was discharged in good health. Follow-up visits, including endoscopy every 6 mo, for up to 2 yr after operation were unremarkable.

DISCUSSION

Occurrence of two types of primary gastric neoplasm is relatively well known, but no reports have been published regarding the simultaneous presence of gastric adenocarcinoma, MALT lymphoma, and schwannoma.

Gastric adenocarcinoma accounts for more than 90% of all malignant gastric tumors and may co-exist with synchronous tumors of a different histologic type, most commonly lymphoma.

Primary gastric lymphoma occurs in 5% of gastric malignancies and their worldwide incidence is increasing. Primary gastric lymphoma is mostly non-Hodgkin lymphoma. Diffuse large B cell and marginal zone B cell type are the most common subtypes. The pathogenesis is often related to *H. pylori* infection[1]. Since Rabinovitch *et al*[2] published the first case in 1952, 56 cases of simultaneous occurrence of gastric adenocarcinoma and gastric lymphoma have been reported. In that series, gastric lymphoma was mainly MALT type (69.6%) or low grade (87.2%), the

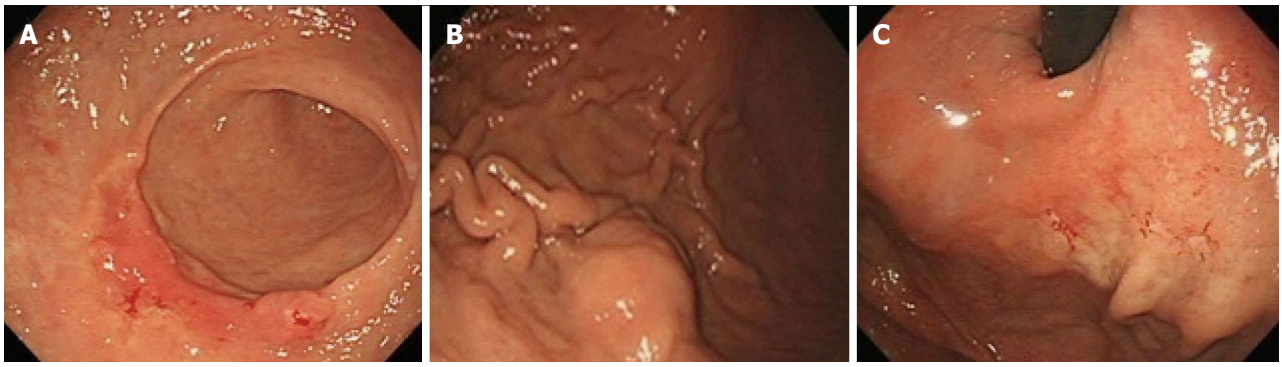

Figure 1 Images obtained during endoscopic examination. A: There is a slightly raised ulcerated area on the anterior wall of the antrum; B: A positive rolling sign is present in the mid-body, suggestive of a submucosal lesion; C: A diffuse erythematous lesion is seen in the fundic area. These three lesions underwent biopsy sampling.

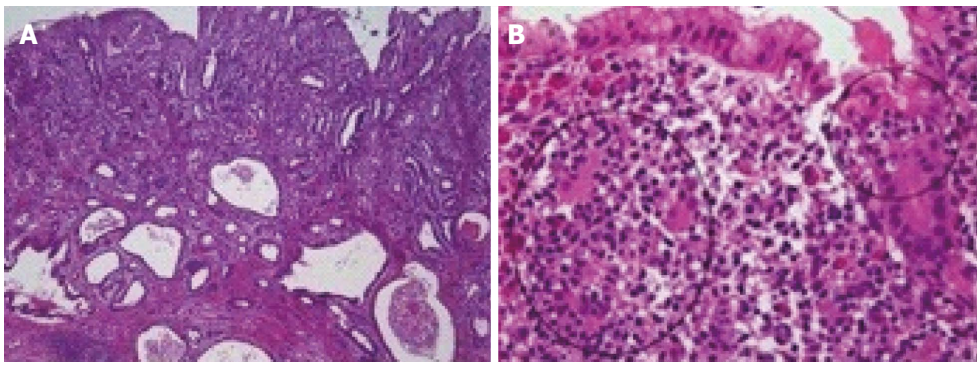

Figure 2 Histologic images. A: The tumor in the antrum is a moderately differentiated adenocarcinoma [× 40, hematoxylin and eosin (HE)]; B: In the fundus section, lymphoepithelial lesions (circled in black), typical for mucosa-associated lymphoid tissue (MALT) lymphoma, are seen, which are formed by infiltration of centrocyte-like cells into the gastric glandular epithelium (× 400, HE).

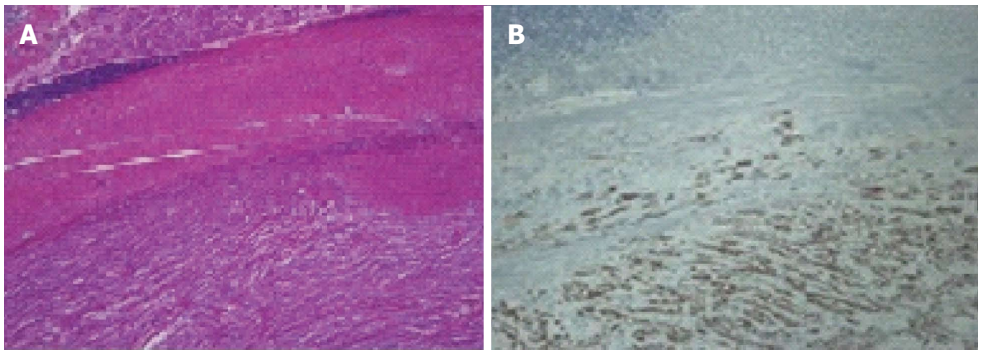

Figure 3 Histologic images. A: The tumor in the mid-body consists of spindle cells. Above the spindle cell tumor, a smooth muscle layer, lymphoid cuffs, and fundic-type glands are found (× 40, HE); B: On immunohistochemical analysis, the spindle tumor cells are positive for S-100, in contrast to the overlying smooth muscles fibers and lymphoid cells (× 100).

correlation between *H. pylori* and MALT lymphoma was 86%/72% in the Eastern and Western cases[3].

Gastric adenocarcinoma and gastric MALT lymphoma are considered to be one of the results of *H. pylori* infection. Gastric cancer can occur in about 1%-2% of *H. pylori* infection cases, and the risk is nine times higher than in patients without *H. pylori* infection. In addition, *H. pylori* infection is highly correlated with gastric MALT lymphoma and is found as a low-grade type MALT lymphoma in over 90% of cases[4]. At present, the most widely accepted initial therapy for localized low-grade MALT lymphoma is aimed at the eradication of *H. pylori* infection, with regimens combining antibiotics and proton-pump inhibitors. Many reports have confirmed the efficacy of antibiotic therapy and showed long-term remission in 60%-100% of patients with localized *H. pylori*-positive MALT lymphoma[5].

In our patient, we decided to treat the MALT lymphoma first by prescribing medication for the

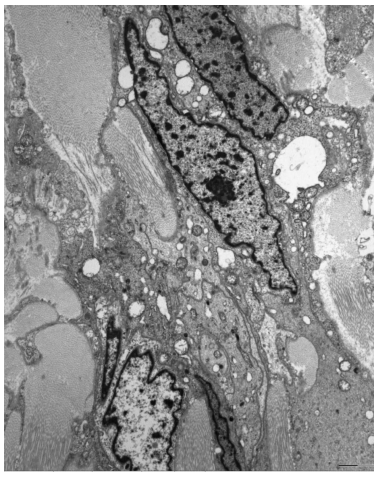

Figure 4 Electron microscopic findings of the submucosal tumor. Elongated neoplastic cells show with numerous complex interdigitating cytoplasmic processes. The cytoplasmic membrane was completely covered with external lamina. The nuclei reveal irregular margins and a heterochromatic chromatin pattern, and the perikaryal cytoplasm contained several mitochondria, rough endoplasmic reticulum, ribosomes, and many lysosomes.

eradication of *H. pylori* organisms to avoid total gastrectomy. After 4 wk, repeat biopsy was conducted at the fundic site and we confirmed that the MALT lymphoma had resolved. Subsequently, the patient underwent radical subtotal gastrectomy, which included removal of the submucosal tumor in the mid-body. The final pathologic findings were moderately differentiated intestinal-type adenocarcinoma (submucosal invasion without regional lymph node metastasis) and schwannoma, and follow-up visits up to 2 y after operation were unremarkable.

According to Nakamura's study[6], the survival rate of patients with synchronous gastric adenocarcinoma and gastric lymphoma was similar to survival rate of gastric adenocarcinoma alone and was significantly lower than survival rate of gastric lymphoma alone.

Schwannoma is a rare gastrointestinal mesenchymal tumor. The most common site for schwannoma in the gastrointestinal tract is the stomach, followed by the colon. There are some histological differences between gastric schwannoma and soft-tissue schwannoma. Unlike soft tissue schwannoma, encapsulation, nuclear palisading and vascular hyalinization are rare in gastric schwannoma[7]. Gastric schwannoma seems to have a good prognosis without recurrence and metastasis. As for simultaneous occurrences of schwannoma with other types of gastric cancer, only three cases have been reported prior to 2015[8].

In conclusion, synchronous triple gastric adenocarcinoma, MALT lymphoma, and schwannoma has not been reported previously in the literature. *H. pylori* eradication and surgery are the mainstay treatment. Further biologic and genetic studies will be required to explain the simultaneous development of tumors of different histotypes.

COMMENTS

Case characteristics
A 56-year-old man presented to our hospital with melena and three gastric abnormalities were detected on gastroduodenoscopic examination.

Clinical diagnosis
Mucosa-associated lymphoid tissue (MALT) lymphoma, adenocarcinoma, and submucosal tumor in stomach.

Differential diagnosis
Carcinoid tumor, primary lymphoma, gastrointestinal stromal tumor.

Laboratory diagnosis
All laboratory results were within normal limits.

Imaging diagnosis
Gastroduodenoscopic examination showed a small ulcerative lesion in the gastric antrum, a submucosal mass in the gastric body, and severe erosion in the fundus. CT showed an ovoid homogeneous 2 cm × 2 cm mass at the greater curvature of the mid-body and mildly enlarged perigastric lymph nodes.

Pathological diagnosis
MALT lymphoma, adenocarcinoma, schwannoma.

Treatment
Antibiotics treatment and radical subtotal gastrectomy.

Related reports
Synchronous occurrence of two types of tumor in the stomach is relatively well known. This is rare case of synchronous triple tumors in stomach.

Term explanation
MALT lymphoma is a form of lymphoma involving the MALT, frequently of the stomach. Gastric schwannoma is a benign nerve sheath tumor composed of Schwann cells.

Experiences and lessons
Further biologic and genetic studies will be required to explain the simultaneous development of tumors of different histotypes.

Peer-review
It is a well written article, a case report about synchronous triple primary gastric adenocarcinoma, MALT lymphoma, and schwannoma.

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LETTERS TO THE EDITOR

Is tremor related to celiac disease?

Lucia Ameghino, Malco Damian Rossi, Daniel Cerquetti, Marcelo Merello

Lucia Ameghino, Malco Damian Rossi, Daniel Cerquetti, Marcelo Merello, Movement Disorders Section, Neuroscience Department, Raul Carrea Institute for Neurological Research, Buenos Aires C1428AQK, Argentina

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Correspondence to: Marcelo Merello, MD, PhD, Movement Disorders Section, Neuroscience Department, Raul Carrea Institute for Neurological Research, Montañeses 2325, Buenos Aires C1428AQK, Argentina. mmerello@fleni.org.ar
Telephone: +54-11-57773200

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Abstract

Neurological features in celiac disease (CD) are not rare (5%-36%), but tremor is scarcely described. Subjects with CD and healthy controls completed an online survey using WHIGET tremor rating scale. One thousand five hundred and twelve subjects completed the survey, finally 674 CD patients and 290 healthy subjects were included. A higher prevalence of tremor in CD patients was observed in comparison to controls (28% *vs* 14%, $P < 0.001$). Frequency of family history of tremor in CD patients with and without tremor was 25% and 20% ($P = 0.2$), while in the control group it was 41% and 10% ($P < 0.001$). Controls with tremor showed a higher frequency of family history of tremor when compared to CD patients with tremor (41.5% *vs* 24.6%, $P = 0.03$). The results suggested that tremor in CD might be more frequent and possibly related to the disease itself and not due to associated essential tremor.

Key words: Tremor; Celiac disease; Gluten; Gluten-free diet; Movement disorders

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Core tip: We performed an online inquest, completed by 1512 subjects, 674 had celiac disease and 290 controls. We observed a higher (double) prevalence of tremor in celiac patients in comparison to healthy controls (28% *vs* 14%, $P < 0.001$). The frequency of a family history of tremor was higher in controls with tremor, but not in Celiac disease (CD) patients with tremor, suggesting that tremor in CD might be more frequent than controls and possibly related to the disease process itself and not due to essential tremor.

Ameghino L, Rossi MD, Cerquetti D, Merello M. Is tremor related to celiac disease? *World J Gastroenterol* 2017; 23(22): 4132-4134 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i22/4132.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i22.4132

TO THE EDITOR

Celiac disease (CD) is a systemic autoimmune disease that mainly affects small intestine. Neurological

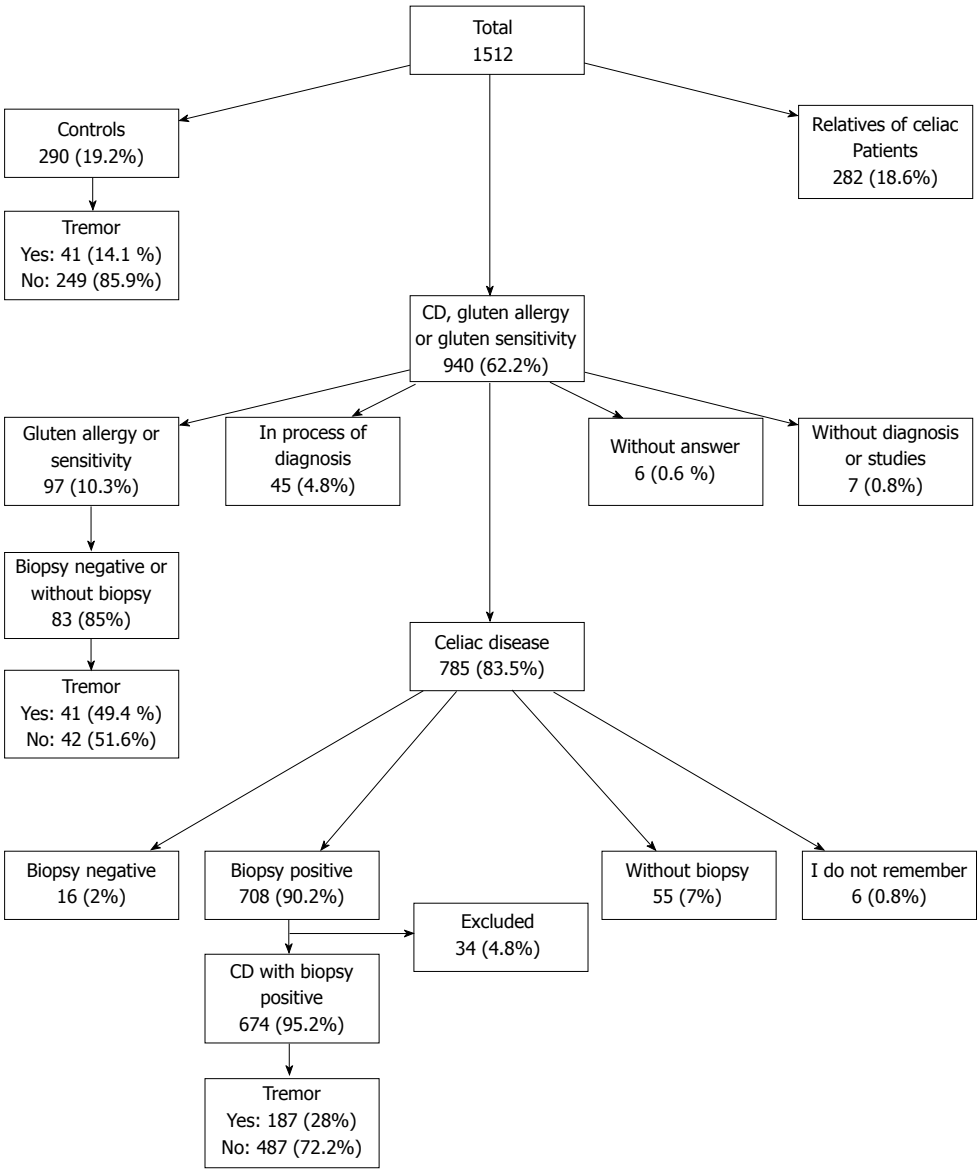

Figure 1 Flow chart of the study selection process.

features are not rare in CD and the physiopathology of the nervous system compromise is still controversial. However, it is believed that a cross antibodies reaction due to molecular mimicry occurs, mainly exerted by the celiac panel antibodies against the Purkinje cells in the cerebellum. With an overall frequency of 5%-36%, ataxia, myoclonus, chorea, peripheral neuropathy, headache and seizures are the most frequent neurological symptoms of CD[1,2]. Tremor was scarcely described[3,4] and its prevalence and clinical correlates were not established. Therefore, in this pilot study we aimed to prospectively evaluate tremor features in CD patients.

In a prospective way, an online inquest using the tremor scale Washington Heights-Inwood Genetic Study of Essential Tremor (WHIGET)[5] was published in the Argentinean Celiac Association website from June through July 2015. Subjects with CD that reported bowel biopsy confirmation of villous atrophy (Marsh score 3) were asked to complete the WHIGET scale. A case of tremor was considered when subjects rated one or more points. Demographic data, medications, CD antibodies and gluten-free diet (GFD) compliance were registered. Non-blood relatives served as age- and gender-matched controls. Individuals under 18 years or with known functional or structural bowel disorders were excluded. The local IRB approved the protocol and participants signed written informed consent in accordance to Helsinki Declaration principles. Descriptive data are shown as mean ± SE of the mean or proportions. χ^2 test was used to compare categorical data and analysis of variance (ANOVA) was employed for numerical variables.

A total of 1512 subjects [mean age, 38.5 ± 12.8 years; 1335 (88%) females] completed the survey, of which 940 (62.2%) met inclusion criteria (Figure 1). Among the included participants, 674 (70%) had CD [mean age, 37.9 ± 12.7 years; 622 (92%) females]

and 290 (30%) were healthy controls [mean age, 37.7 ± 12.4 years; 237 (82%) females]. No age differences were observed between both groups (P = 0.5). The overall prevalence of tremor was 28% in CD patients and 14% en controls (P < 0.001). A 25% of CD patients with tremor had a positive family history of tremor in comparison to a 20% of CD patients without tremor (P = 0.2). Frequency of family history of tremor in controls with tremor was 41% compared with 10% of controls without tremor (P < 0.001). A lower frequency of family history of tremor was found in CD patients with tremor in comparison to controls with tremor (25% *vs* 41%, P = 0.03). Comparison of CD patients with and without tremor revealed no statistically significant differences in family history of CD (29% *vs* 24%, respectively; P = 0.2), disease duration (5.3 ± 5.5 *vs* 6.5 ± 8.1, respectively; P = 0.07), positive CD antibodies (26.2% *vs* 26.4%, respectively; P = 0.9), GFD compliance (83% *vs* 82%, respectively; P = 0.7) and treatment (thyroid hormone and β2 adrenergic agonist).

In a large sample, a double prevalence of tremor in the CD group was found compared to healthy individuals. We consider that tremor in CD might be possibly secondary to the disease process itself and not due to essential tremor as the frequency of a family history of tremor was lower in CD patients with tremor than in controls presenting with tremor. Tremor in CD was independent from demographics and CD characteristics, like disease duration, CD antibodies or GFD compliance. However, we might have failed to find any relationship between tremor and CD antibodies or GFD compliance, as symptoms may improve with gluten-free diet along with the disappearance of the CD-specific antibodies. Bürk and colleagues reported neurological features in a series of 72 patients with CD, of which 3% (2 patients) of them had intention tremor. Tremor features or its association with CD characteristics were not described[3]. Kheder *et al*[4] reported a patient with positive antigliadin antibodies and progressive ataxia and palatal tremor that obtained improvement in his symptoms after two years of GFD.

Limitations of this study include the absence of a physical examination of CD patients with tremor, the absence of IgA deficiency exclusion, and that possible vitamin E, vitamin B12 or Mg deficiencies due to malabsorption, that may cause tremor secondary to a sensory neuropathy was not ruled out. Finally, a population bias may have occurred as an online inquest may have included mostly young subjects with Internet access. Further confirmatory studies are required to confirm the exact frequency of tremor in CD and the associated clinical correlates.

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