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EDITORIAL

Can a fibrotic liver afford epithelial-mesenchymal transition?

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Abstract

The question whether epithelial-mesenchymal transition (EMT) occurs during liver fibrogenesis is a controversial issue. *In vitro* studies confirm that hepatocytes or cholangiocytes undergo EMT upon transforming growth factor β (TGF-β) stimulation, whereas *in vivo* experiments based on genetic fate mapping of specific cell populations suggest that EMT does not occur in fibrotic animal models. In this review we present current data supporting or opposing EMT in chronic liver disease and discuss conditions for the occurrence of EMT in patients. Based on the available data and our clinical observations we hypothesize that EMT-like alterations in liver cirrhosis are a side effect of high levels of TGF-β and other pro-fibrotic mediators rather than a biological process converting functional parenchyma, *i.e.*, hepatocytes, into myofibroblasts at a time when essential liver functions are deteriorating.

Key words: Epithelial-mesenchymal transition; Liver fibrosis; Liver cirrhosis; Transforming growth factor-β

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Core tip: This review provides a personal notion about

whether a complete epithelial-mesenchymal transition (EMT) occurs in human fibrotic livers. We consider three aspects that might determine the occurrence of EMT: (1) capacity of parenchymal cells; (2) potential benefit for the liver and the whole body; and (3) microenvironment within a fibrotic liver. Clinical evidence suggests that in humans, EMT-like alterations occur mainly in advanced chronic liver disease, *i.e.*, cirrhosis. In such a severe disease state, the most urgent mission for a liver is to maintain a maximum number of functional hepatocytes, while hepatic stellate cells and portal fibroblasts provide an ample supply of myofibroblasts. It appears that there is no need for additional sources of myofibroblasts in a cirrhotic liver. EMT-like alterations in parenchymal cells are most likely a side effect of high levels of EMT-promoting factors such as TGF-β.

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INTRODUCTION

The progression of liver fibrosis is a dynamic process characterized by excessive deposition of extracellular matrix (ECM). Myofibroblasts (MFB) are the major ECM-producing cells[1,2]. MFB are derived from different cell types with sinusoidal hepatic stellate cells (HSC), portal fibroblasts and bone marrow-derived fibrocytes being the most prominent sources[3]. Whether hepatocytes and/or cholangiocytes differentiate into MFB by way of epithelial-to-mesenchymal transition (EMT) is still controversial[4-10]. In this review, we discuss actual data supporting or opposing the occurrence of EMT during liver fibrogenesis.

Why does EMT occur during embryogenesis?

A hypothetical biological process requires three preconditions: (1) the process has to provide a benefit to either the local organ or the system; (2) the cells must be capable of performing the process; and (3) the process must be supported by the surrounding microenvironment. EMT is classified into three subtypes[11]: Type 1 EMT, which is associated with implantation, embryo formation, and organ development; type 2 EMT, which is a repair-associated function that generates fibroblasts and other related cells in order to reconstruct tissues following trauma and inflammatory injury; and type 3 EMT in neoplastic cells that have previously undergone genetic and epigenetic changes, particularly in genes that favor clonal outgrowth and the dissemination of tumors. So far, type 1 EMT is the best-characterized subclass, occuring in the embryo at gastrulation[12,13]. A subset of cells from the epiblast moves to the midline to form the primitive streak. These cells undergo EMT and internalize to generate mesoderm and endoderm, while those remaining in the epiblast become ectoderm[12,13]. EMT and MET between endoderm and mesoderm are critical mechanisms for organogenesis, for example in the kidney[14-16]. However, EMT does not play an important role during liver organogenesis because hepatoblasts, from which hepatocytes and BEC are subsequently derived, arise from endoderm rather than mesoderm[17].

EVIDENCE SUPPORTING AND OPPOSING EMT IN LIVER FIBROSIS

According to a brief definition of EMT, that is, "epithelial cells changing their phenotype and acquiring mesenchymal properties" [11], two types of adult liver cells can undergo EMT under experimental conditions: hepatocytes and cholangiocytes[18]. Given that HSC are mesenchymal cell in the first place, regardless if quiescent or activated, the conversion of HSC into MFB is not considered EMT. Thus the term EMT refers to the process of hepatocytes or cholangiocytes obtaining phenotypes of mesenchymal cells and differentiating into MFB.

Parenchymal cells express mesenchymal markers in patients with advanced chronic liver disease

Evidence supporting the occurrence of EMT during liver fibrogenesis is based on immunohistochemistry and co-staining studies. Expression of multiple mesenchymal markers, including vimentin, S100A4 [fibroblast-specific protein (FSP-1)], heat shock protein 47 (HSP47), snail, and α-smooth muscle actin (α-SMA), has been reported in parenchymal cells of patients with different chronic liver disease[17,19,20]. Diehl AM's group showed that S100A4 is expressed in reactive ducts of patients with primary biliary cholangitis (PBC) and of cirrhotic patients with non-alcoholic steatohepatitis (NASH)[19,21]. Díaz *et al*[17] found that in pediatric patients with biliary atresia and adult patients with primary sclerosing cholangitis (PSC)/PBC, cholangiocytes and reactive ducts express FSP-1, the collagen chaperone HSP47, the intermediate filament protein vimentin, and the transcription factor snail. Dooley *et al*[22] showed that a portion of hepatocytes in patients with HBV-associated cirrhosis expressed Snail. These results suggest that parenchymal cells do indeed express mesenchymal markers in chronic liver disease. It should be noted that parenchymal cells expressing mesenchymal markers have only been found in patients with advanced chronic liver disease, *e.g.*, cirrhosis so far. There is no data showing that parenchymal cells of patients express mesenchymal markers at early stages of liver fibrosis.

In vitro studies confirm the occurrence of EMT in liver cells

Further evidence supporting the occurrence of EMT of liver parenchymal cells comes from *in vitro* studies.

Fetal rat hepatocytes treated with transforming growth factor β (TGF-β) underwent an EMT, presenting high levels of vimentin and Snail and lack of cytokeratin 18 and E-cadherin[23]. Murine primary hepatocytes cultured on monolayers of dry collagen undergo dedifferentiation and lose polarity and liver function within 3 d[24]. Changing culture conditions by seeding hepatocytes within a sandwich of two soft collagen gel layers preserves an epithelial phenotype for extended periods[24]. Upon TGF-β stimulation, primary hepatocytes on both dry collagen monolayer and soft collagen gel sandwich quickly exhibit myofibroblast-like morphological changes, lose tight junction proteins (*e.g.,* Occludin and E-cadherin), and express mesenchymal markers (vimentin, connective tissue growth factor, S100A4, *et al*)[4,24,25]. In contrast to hepatocytes of untreated mouse livers, hepatocytes derived from carbon tetrachloride (CCl_4)-induced cirrhotic mice express vimentin, a mesenchymal marker, *in vitro* and *in vivo*[25].

TGF-β induces hepatocytes' EMT through regulating the expression of transcription factors, in particular Snail, the master gene of EMT, and hepatocyte nuclear factor 4α (HNF4α), the master gene of hepatocyte differentiation[26,27]. The Snail family induces EMT in different epithelial cells, including hepatocytes. In fetal liver, TGF-β induces apoptosis of hepatocytes. Snail confers hepatocytes resistance to TGF-β-induced cell death[26,27]. In addition, Snail expression is sufficient to induce EMT in adult hepatocytes. HNF4α is an essential transcription factor maintaining the epithelial phenotype of hepatocytes[28]. During EMT of hepatocytes, expression of HNF4α is largely inhibited by TGF-β administration[27]. The inhibitory effects are performed by upregulating Snail, which represses transcription of the *HNF4α* gene through direct binding to its promoter[27]. The balance between these two transcription factors plays a pivotal role in regulating EMT/MET dynamics in hepatocytes[29].

Besides hepatocytes, primary cholangiocytes isolated from rats following one week of bile duct ligation (BDL) express S100A4 while showing reduced expression levels of epithelial markers such as cytokeratin 19 and 7[21]. When an immature cholangiocyte line was treated with conditioned medium from myofibroblastic HSC, these cholangiocytes underwent complete EMT[21]. Consistent with the findings in rat cholangiocytes, Rygiel *et al*[30] reported that administration of TGF-β induced expression of mesenchymal markers in cultured primary human cholangiocytes. These results show that (1) *in vitro* cell culture conditions (*e.g.,* putting cells on monolayer gel) induce hepatocytes' loss of epithelial feature; and (2) pro-EMT factors in cultured medium, such as TGF-β, induce rapid EMT of liver parenchymal cells.

Current fibrotic animal models deny the occurrence of EMT during liver fibrogenesis

Although a study of the CCl_4-induced fibrotic mouse model stated the occurrence of EMT during liver fibrosis[31], later studies based on genetic cell fate mapping provided convincing evidence that in contrast to liver parenchymal cells in primary culture, EMT does not occur in fibrotic animal models induced by BDL, CCl_4, and 3,5-diethoxycarbonyl-1,4-dihydrocollidine[4,5,10]. This issue has been discussed intensively[3,6,8].

TGF-β: BETWEEN FIBROSIS AND EMT

As mentioned above, one key finding supporting the occurrence of EMT in damaged liver is that parenchymal cells express mesenchymal markers. Why would they do that? One explanation might be that there are high levels of growth factors such as TGF-β surrounding these cells.

TGF-β is not only the most important pro-fibrotic cytokine[32], but also the most efficient growth factor promoting EMT[33]. It has been confirmed that liver parenchymal cells undergo EMT in culture medium with TGF-β stimulation[4,24,25]. During chronic liver diseases, TGF-β is produced by multiple systemic and local cells, including macrophages, monocytes, activated HSC and reactive ducts[34,35]. In addition, TGF-β treatment also induces BMOL cells, a murine liver progenitor cells (LPC) line, to undergo EMT-like phenotype change *in vitro* (unpublished data). There is a close correlation between phosphorylated Smad2 levels and fibrotic stages in HBV- and steatosis-associated chronic liver disease[36]. This means that parenchymal cells in cirrhotic livers often reside in an environment teeming with high levels of TGF-β. It is quite likely that such a microenvironment can force the expression of mesenchymal markers in parenchymal cells.

However, the occurrence of EMT should not be defined merely by parenchymal cells expressing mesenchymal markers. To accomplish a complete EMT in the liver, hepatocytes or cholangiocytes are required to finish at least the following steps: (1) expression of mesenchymal markers; (2) loss of anchoring proteins such as E-cadherin and Occludin; and (3) release from adjoined hepatocytes/cholangiocytes and conversion into an isolated MFB. To date, there is no conclusive evidence that hepatocytes or cholangiocytes expressing mesenchymal markers undergo the latter two steps and become real MFB.

It should be re-emphasized that parenchymal cells expressing mesenchymal markers are found only in advanced stages of chronic liver disease, particularly in cirrhosis. At this stage, survival of parenchymal cells for the maintenance of liver function is of prime importance. Therefore we surmise that the most likely scenario is that expression of mesenchymal markers by parenchymal cells represents a response to high levels of TGF-β rather than evidence for EMT in a liver with severely impaired functions.

CAN LIVERS EVEN AFFORD EMT IN CIRRHOTIC PATIENTS?

Based on current data, it is too early to conclude

that EMT of liver parenchymal cells contributes to the MFB pool *in vivo*. Given the vast difference between tissue culture and the human liver, observations of EMT following TGF-β incubation *in vitro* by no means provide convincing evidence that the same phenotypic alterations occur during progression of chronic liver disease *in vivo*. On the other hand, the fact that EMT does not occur in fibrotic animal models does not rule out the possibility of EMT in patients with chronic liver disease. The currently used fibrotic models have a maximum observation period of several months, whereas the history of a patient progressing to liver cirrhosis spans years and decades[37]. The fact that patients with chronic liver disease have such a long natural history bears witness to the huge capacity of the human liver for self-repair, even under continuous attack.

The liver is the largest gland in the body, and it supports nearly every other organ in some aspect. The majority of physiological functions of the liver are performed by hepatocytes, including metabolism of carbohydrates, proteins, amino acids, lipids and some important hormones, the production and excretion of bile, metabolism and excretion of toxic substances, and synthesis of coagulation factors[38]. In order to implement these copious complex physiological functions, the liver owns special blood systems and anatomic architecture. A hepatocyte has three boundaries: the sinusoidal, lateral and canalicular membranes[28]. The cell is highly polarized with transport directed from its sinusoidal surface to the canalicular surface[28]. The canalicular domains between two adjacent hepatocytes constitute the smallest bile lumen (diameter: 1 μm)[39]. The adjoining apical membranes of a bile lumen are sealed by tight junctions (zonula occludens), representing the only physical barrier between the blood and the canalicular lumen. These tight junctions determine "paracellular permeability" between blood and bile[39]. In normal liver, hepatocytes are arranged in one-cell thick cords[40]. Such arrangement makes hepatocyte-produced bile delivery easy. If a complete EMT should occur in these hepatocytes, one key issue would be that the loss of hepatocytes from these one-cell thick cords must not alter primary liver architecture. In a patient with chronic liver disease, the organ is under continuous insult and yet manages to maintain a normal function to support the body's physiological requirements for several decades. To achieve this feat, the liver has to avoid any response that is likely to disturb the above-mentioned hepatocyte arrangement.

Deposition of ECM by MFB is a key process in liver repair. In acute liver injury, particularly during acute liver failure, the severely damaged organ recruits enormous numbers of ECM-producing MFB in order to maintain a relatively intact liver architecture[41]. Furthermore, MFB and the ECM they produce are providing a niche for the activation of LPC, a major cell source for liver regeneration in acute liver failure[42,43]. Under these conditions, most mature hepatocytes have gone extinct[44,45]. Still these copious amounts of MFB do not cause fibrosis: Once the damaging etiology is removed, the damaged liver can recover its function and restore its architecture completely, although fibrotic septa produced by MFB persist for several months or years. This process is summarized as "wound healing".

In chronic liver disease, enduring damage induces excessive ECM deposition beyond the liver's capacity for degradation[46]. Such excessive ECM deposition combined with local hepatocyte death and regeneration finally results in distortion of the hepatic architecture and vascular structures[47]. The process is described as liver fibrosis and its end stage cirrhosis. Actually, the line between "wound healing" and "fibrosis" is a blurred one. Defining the two processes only according to disease time, for example acute or chronic, is artificial. It is impossible to claim that ECM deposition in the liver during chronic disease is completely "fibrogenesis", rather than "wound healing". During several decades of chronic liver disease progression, the human body is constantly trying to repair and restore the damaged liver. Before decompensated liver cirrhosis is established, withdrawal of etiology can still reverse liver fibrosis and even cirrhosis to some degree[48,49]. Regeneration and repair represent two aspects of host defence. When we discuss whether EMT occurs in chronic liver damage or not, it is important to consider whether there is actually any requirement for hepatocytes to transdifferentiate into MFB through EMT. In our view, it is highly doubtful if MFB derived from other cell sources, *e.g.,* HSC, should be insufficient to produce the amount of ECM required for healing and repair.

Morphologically, at least five fibrotic septa patterns are demonstrated in patients with liver fibrosis: portal to portal, portal to central, central to central, chicken-wire and portal pipestem[40,50]. Etiology, the topographic localization and nature of injury, and disease stage are critical factors that determine the pattern of liver fibrosis.

Patients with alcoholic steatohepatitis (ASH) or NASH usually have pericellular fibrosis, *i.e.,* the deposition of fibrillar matrix is concentrated around the sinusoids and groups of hepatocytes and displays a chicken-wire like shape[40,50]. It is well recognized that this "chicken-wire fibrosis" is dependent on sinusoidal HSC activation. Do hepatocytes undergo EMT and transdifferentiate into MFB in these circumstances? Most likely not: In patients with ASH or NASH, hepatocytes manifest with steatosis, ballooning degeneration, and containing Mallory-Denk bodies. These cells usually do not have an intact liver function. Severe ASH or NASH leads to lytic necrosis and apoptosis of hepatocytes. In the end-stage of these diseases, particularly in ASH, there may be large amounts of parenchymal extinction, suggesting secondary vascular events[40]. Under these circumstances, the most important mission

for surviving hepatocytes is maintaining liver function. It is difficult to fathom that such a liver would induce EMT in functionally impaired, or even in some of the few remaining functional hepatocytes. On the other hand, no data indicate that there might be insufficient HSC-derived MFBs to produce the amount of ECM required for tissue repair and/or fibrogenesis.

In contrast to ASH and NASH, ECM deposition in biliary disease is dependent on portal fibroblasts. In cholestatic diseases such as PBC and PSC, fibrosis initiates from portal tracts, induced by obstruction, loss, or inflammation of bile ducts[51]. Geographically, peribiliary fibroblast-derived MFB are primarily responsible for the deposition of portal tract collagen[52]. The biliary fibrosis due to activation of peribiliary and portal fibroblasts explains the lack of subdivision with parenchymal fibrotic septa until late stages of the disease[40]. Morphologically, the MFB of bridging septa in cholestatic livers strongly resemble the MFB of the portal field[53]. These cells can be distinguished from HSC-derived MFB using combined staining for fibrillin-1 and elastin[52]. Activated HSC generate fibrillin-1-positive but elastin-negative ECM, whereas MFB inside the portal tracts produce both fibrillin-1- and elastin-positive ECM[54]. In addition, activated portal tract fibroblasts express some different protein markers such as cellular retinol-binding protein-1[55].

Besides activation of portal fibroblasts, ductular reaction (DR), which is defined as "ductules accompanied by an inflammatory infiltrate and by fibrosis", is a critical histological feature in most cholestatic liver diseases[51,56-58]. It is mainly reactive ducts that have previously been reported to express mesenchymal markers[17,21]. Will these reactive ducts expressing mesenchymal markers differentiate into MFB? To date there is no evidence supporting this hypothesis. DR in cholestatic liver disease has several cell sources, including the small intralobular bile ducts, ductules, canals of Hering and from "ductular metaplasia" of periportal hepatocytes[58]. Cholestatic pathogenesis is initiated by bile leakage due to obstruction of extrahepatic bile ducts and loss of small intrahepatic bile ducts. DR accompanied by inflammation and fibrosis constitutes a protective response to the destruction of interlobular bile ducts. These reactive ducts provide abortive bypass mechanisms for the drainage of bile in the diseased liver, and thus protect hepatocytes from the deleterious effect of bile acid overload[59]. It has been well recognized that LPC residing in canals of Hering are the major source of DR in cholestatic diseases[58]. In advanced stages of PSC, severe destruction of small ductules including canals of Hering reduces the number and size of DR[60]. Thus, it is clear that DR is a key process of the liver in order to restore the architecture of a damaged biliary tree. LPC are activated and undergo differentiation to cholangiocytes to recover ruined bile ducts. On the other hand, DR and the accompanying inflammatory response indeed play an important role in portal and periportal fibrosis by producing and secreting a variety of biologically active fibrosis-associated mediators, including TGF-β2, connective tissue growth factor, platelet derived growth factor, tumor necrosis factor-α, interleukin (IL)-6, IL-8, monocyte chemotactic protein-1 and nitric oxide[61]. Thus, these data suggest that DR contributes to biliary fibrosis through producing critical pro-fibrogenesis factors rather than differentiating into mesenchymal cells.

HYPOTHESIS: HEPATOCYTES ARE NOT ALLOWED TO PERFORM EMT IN A CIRRHOTIC LIVER

Human liver cirrhosis develops over years or decades. Histologically it is characterized by diffuse nodular regeneration surrounded by dense fibrotic septa with subsequent parenchymal extinction and collapse of liver structures, causing pronounced distortion of hepatic vascular architecture[40,47,62,63]. Of all these histological features, parenchymal extinction is rarely found in animal models[46]. Parenchymal extinction denotes the loss of contiguous hepatocytes, producing lesions that remodel into septa that vary from 0.05 mm to several millimeters in thickness[60,62]. Only recently, an elegant study from Stueck and Wanless showed that repopulation of parenchymal extinction lesions in cirrhotic human liver is dependent on LPC activation[60]. This result suggests that without LPC-derived hepatocytes, the remaining mature hepatocytes in a cirrhotic liver are not sufficient to ensure liver function. The most urgent mission of a cirrhotic liver is to maintain a maximum number of functional mature hepatocytes, either by proliferation of the remaining hepatocytes, or from LPC. Proliferating cells cannot perform EMT in breast cancer[64,65]. Consistent with breast cancer cells, TGF-β administration or over-expression of Snail induce EMT as well as cell cycle arrest, which favors survival signals in hepatocytes[27]. Thus, a cirrhotic liver is unlikely to support or induce a biological process like EMT in the surviving parenchymal cells. On the other hand, the decision if EMT is required for hepatic fibrogenesis and tissue repair might also depend on whether MFB derived from other cell sources provide sufficient ECM. At the present time, there are no studies indicating that the activated HSC, portal fibroblasts and fibrocytes provide insufficient MFB.

CONCLUSION

It may be too early yet to exclude the occurrence of type 2 EMT in patients with chronic liver damage. However, current evidence indicates that EMT only occurs in the advanced stages of chronic liver disease. In this phase, *i.e.,* during cirrhosis, mature hepatocytes performing vital functions are decreasing in numbers. LPCs are activated to replenish hepatocytes in order

Table 1 Selected evidence supporting or opposing epithelial-mesenchymal transition during liver fibrogenesis

	Ref.
Supporting evidence	
In vitro	
Primary cultured hepatocytes or cholangiocytes with TGF-β stimulation undergo complete EMT	[4,21,23-27,29,30]
Patients	
Liver parenchymal cells in patients with advanced chronic liver disease express mesenchymal markers	[17,19-22,30]
Opposing evidence	
Techniques based on genetic cell fate mapping of specific cell populations provided convincing evidence that EMT does not occur in fibrotic animal models	[4,5,10]
Clinical observation	
There is no data showing that parenchymal cells of patients express mesenchymal markers during early stages of fibrosis	
Parenchymal cells expressing mesenchymal markers are found only in patients with advanced chronic liver disease, *e.g.*, cirrhosis.	
A cirrhotic liver is not likely to drive remaining parenchymal cells towards a non-essential biological process like EMT	
No studies indicate that activation of HSC, portal fibroblasts and fibrocytes produce insufficient MFB	
In the cirrhotic liver, parenchymal cells expressing mesenchymal markers might be caused by high levels of surrounding pro-EMT factors, *e.g.*, TGF-β	

TGF-β: Transforming growth factor β; EMT: Epithelial-mesenchymal transition; HSC: Hepatic stellate cells; MFB: Myofibroblasts.

to maintain crucial liver functions[60]. Under these conditions, it seems rather counterproductive for a severely damaged liver to induce conversion of hepatocytes into MFB. There are multiple alternative sources of MFB, including HSC, portal fibroblasts and fibrocytes. To date, there is no evidence suggesting that these cell sources produce insufficient MFB for liver repair or fibrogenesis. We propose that in the cirrhotic liver, parenchymal cells express mesenchymal markers in response to high levels of surrounding pro-EMT factors, *e.g.*, TGF-β.

The notions discussed in this paper are based on our observations only, and at present lack supporting experimental evidence. We hope that future studies and observations will provide clinical evidence to confirm, correct or refute our hypothesis. Table 1 summarizes current evidence supporting or opposing EMT during liver fibrogenesis.

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EDITORIAL

Impact of hepatitis C oral therapy in portal hypertension

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Abstract

Chronic hepatitis C is a leading cause of morbidity and mortality, mainly related to fibrosis/cirrhosis and portal hypertension. Direct antiviral agents are highly effective and safe and can now cure > 90% of the patients. Sustained viral response (SVR) after interferon-based regimens has been associated with improvement in liver function, fibrosis and portal hypertension in a significant proportion of patients, although a point of no return seems to exist from which viral elimination is no longer capable of preventing portal hypertension progression and liver decompensation. Indeed, although SVR is associated with improvement of hepatic venous pressure gradients and therefore a decreased risk of *de novo* esophageal varices, several studies show that viral clearance does not eliminate the risk of variceal progression, liver decompensation and death in patients with pre-established portal hypertension. Although evidence about the effects of direct antiviral agents (DAAs) on clinically significant outcomes is still scarce and with short follow-up, DAAs can decrease the burden of the disease if patients are timely treated before significant fibrosis and portal hypertension develops. Studies with longer follow-up are waited to establish the real magnitude of hepatitis C treatment on portal hypertension. Future studies should also focus on predictors of portal hypertension resolution since it can influence management and avoid unnecessary monitoring

Key words: Hepatitis C; Portal hypertension; Direct antiviral agents; Cirrhosis; Fibrosis; Interferon

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Core tip: Hepatitis C is associated with significant morbidity and mortality, mainly through portal hypertension complications. Hepatitis C treatment is associated with improvement of liver function and fibrosis, better quality of life and reduced mortality. The knowledge of the impact of viral clearance on portal hypertension is also relevant because it greatly influences clinical outcomes and can influence management after treatment. Several studies show

that the benefits on portal hypertension are higher if treatment is delivered before clinically significant portal hypertension is developed, encouraging timely and early treatment with the highly efficacious and safe direct antiviral agents.

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INTRODUCTION

Chronic hepatitis C is a leading cause of morbidity and mortality worldwide mainly due to complications of cirrhosis, portal hypertension and hepatocellular carcinoma. Hypertensive bleeding is the most significant complication of portal hypertension, being associated with a high early mortality (20% at 6 wk)[1]. Mortality attributed to viral hepatitis has been increasing in the last decades and viral hepatitis was the seventh leading cause of death worldwide in 2013[2].

Direct antiviral agents (DAAs) are highly effective and safe and are changing the prognosis and burden of the disease. Sustained virologic response (SVR) is now achieved in > 90% of the patients and is associated with improvements in liver function, fibrosis and overall survival. Portal hypertension is also expected to improve with virological response, paralleling the improvements in liver inflammation and liver fibrosis. Liver transplantation due to viral hepatitis C can also decrease in the next years[3]. The knowledge of the effects of hepatitis C virus (HCV) elimination on clinically significant outcomes like portal hypertension and its complications is thus of unremarkable importance since it can influence management after SVR and is the focus of this report.

IMPACT OF SVR IN PORTAL HYPERTENSION BEFORE DAAs

Prior to DAAs development HCV treatment was mainly recommended in patients with advanced fibrosis or cirrhosis, in order to balance treatment benefits with the risk of liver complications and treatment adverse events. SVR was achieved in 40%-60% of cases with interferon (INF) based therapies and was associated with improvements in liver fibrosis, portal hypertension, liver-related adverse events, liver-related mortality, overall mortality and decreased HCC incidence[4-7]. HCV therapy was also associated with quality of life improvement, namely reducing decompensation and hospitalization rates[8,9].

Liver fibrosis, a major determinant of portal hypertension, was shown to improve in several studies using paired liver biopsies[10-13] and non-invasive biomarkers[12,14]. However, a "point of no return" seems to exist for both liver fibrosis, liver function and portal hypertension. Indeed, in a large cohort of INF-treated patients, 740/1094 (68%) of the patients who achieved SVR maintained fibrosis stage 20 mo after treatment and fibrosis improved in only 277/1094 (25%)[10].

Concerning portal hypertension, SVR was associated with a statistically significant yet modest decrease in hepatic venous pressure gradient (HVPG) 6 mo after INF-based therapy[15]. In a small study including 8 patients who achieved SVR with antiviral triple therapy there was a significant decrease in both HVPG and liver stiffness 24 wk after therapy (10.3 mmHg *vs* 6.1 mmHg and 21.3 kPa *vs* 6.4 kPa, $P < 0.001$), with 5 patients (62.5%) achieving an HVPG < 6 mmHg[16]. Indirect markers of portal hypertension such as platelet count[17-19] and spleen size[17] were also shown to improve after HCV eradication in INF-treated cirrhotic patients.

Concerning clinical endpoints after HCV eradication, a prospective study with 12 years follow-up showed a lower incidence of esophageal varices in Child A cirrhotic patients with SVR (0% *vs* 32%-39% in the untreated/non-SVR group)[20]. A lower incidence of *de novo* esophageal varices was also reported in cirrhotic patients who achieved SVR, although the progression of variceal size was not statistically different in patients with and without SVR[21], supporting the concept of the point of no return. Another prospective study by Di Marco *et al*[22] also showed that SVR was associated with a lower incidence of *de novo* esophageal varices in cirrhotic patients treated with PEG-INF and ribavirin (HR = 0.23, 95%CI: 0.11-0.48), although it was not associated with a decrease in variceal progression or liver decompensation in those with pre-existing varices[22].

Petta *et al*[23] also reported a reduced incidence of *de novo* esophageal varices in patients with SVR (3.4% *vs* 37.4%) On the other hand, although SVR was associated with a decrease in liver decompensation and mortality at 10 years, patients with esophageal varices at baseline had an increased risk of decompensation and death. Further supporting these findings, Lens *et al*[24] recently reported that cirrhotic patients with clinically significant portal hypertension at baseline remain at risk for liver decompensation after 5 years, regardless of SVR. In these study, although SVR was associated with a non-statistically significant decrease in HVPG, a higher baseline HVPG was found as the only predictor of liver decompensation at multivariate analysis.

Besides the absence of improvement in HVPG, other factors may also influence the development of complications of portal hypertension after SVR. Indeed, Nagaoki *et al*[25] reported that portosystemic collateral shunts at baseline (assessed by CT) were associated

with exacerbation of esophageal varices and hepatic encephalopathy after SVR. Cofactors for liver disease such as obesity, alcohol consumption and hepatitis B may also contribute to minor improvement in portal hypertension after HCV eradication.

IMPACT OF SVR IN PORTAL HYPERTENSION WITH DAAs

In order to accomplish the goal of HCV elimination as an important health public threat by 2030[26], HCV treatment is nowadays recommended in almost all infected patients, even those without significant fibrosis[27]. This, together with the high SVR rates and the safety profile of DAAs, can change the history of HCV infection and improve clinical outcomes namely avoiding the development of portal hypertension and its improvement in patients with patients with established portal hypertension.

Liver fibrosis has also been shown to improve after INF-free DAA treatments, based on serum fibrosis biomarkers[28,29] and transient elastography[29,30]. SVR, liver function and fibrosis are undoubtedly important endpoints to assess the efficacy of HCV treatment, although portal hypertension and its complications (*i.e.,* liver decompensation and liver-related mortality) may be more adequate to assess treatment effectiveness. In fact, the knowledge of the impact of DAA treatments in portal hypertension and cirrhosis complications may influence patient management after achieving SVR. Due to the novelty of DAA INF-free therapies (and thus short follow up times) there are only few studies assessing the effects of HCV novel treatments on portal hypertension and clinical decompensation.

In patients successfully treated with DAAs, fibrosis and MELD score were shown to improve[31-33]. The decrease in necroinflammation along with fibrosis improvement can decrease intrahepatic resistance and thus portal pressure. In particular, improvement of liver inflammation, aminotransferases and liver function early during treatment can explain the rapid decreases in HVPG and liver stiffness that were found in some studies.

A well designed retrospective study conducted by the Austrian group evaluated the changes in HVPG and liver stiffness in 60 cirrhotic patients (84% Child A) treated with various combinations of DAAs[34]. SVR led to a reduction in HVPG in 80% of the patients (mean HVPG change -2.63 ± 0.38 mmHg, $P < 0.001$). Importantly, in the subgroup of patients with clinically significant portal hypertension (⩾ 12 mmHg) at baseline, 63% achieved a HVPG decrease ⩾ 10% and a decrease > 20% or to < 12 mmHg was found in 51%, at a median of 114 d after treatment. This beneficial effect was found in all strata of HVPG, although portal hypertension was less likely to improve in Child B patients. Liver stiffness and platelet counts improvements were also associated with SVR.

In another study including 33 cirrhotic patients treated with 48 wk sofosbuvir + ribavirin with clinically significant portal hypertension at baseline, 24% achieved a ⩾ 20% decrease in HVPG at the end of treatment, although the median HVPG change in the entire cohort was modest (-0.5 mmHg)[35]. Interestingly, higher baseline MELD score was associated with a higher HVPG response ($P = 0.04$). Longer follow-up results of this trial are waited since the full effects of SVR on architectural changes and fibrosis improvement may have their effects later on time. Indeed, a more pronounced liver stiffness improvement was found between baseline and end of treatment than between end of treatment and 6 mo after, suggesting an important role of necroinflammation on the early improvements in liver stiffness[36]. Deterding *et al*[37] suggested a two-phasic decline of portal hypertension consisting of a first rapid phase during treatment (associated with improved inflammation) followed by a slower second phase after 6-12 mo (associated with fibrosis regression). This hypothesis will surely be tested and hopefully confirmed when longer follow-up results become available.

The results of the few studies evaluating the changes in portal hypertension shortly after DAA treatment thus suggest that portal hypertension improves quickly during and after HCV eradication, which can lead to improvements in clinically significant outcomes such as variceal bleeding, ascites and encephalopathy. This theoretical concept can favor the treatment of patients with decompensated cirrhosis in whom INF-treatments were previously contraindicated. However, treatment in this setting is still a matter of debate. Indeed, although HCV eradication can decrease Child-Pugh and MELD scores in a subset of patients (decreasing the need of liver transplantation), it does not necessarily improve liver function and portal hypertension sufficiently to the point of a compensated patient with a functional live and the need for liver transplantation may persist but be delayed due to the MELD decrease (MELD purgatory).

CONCLUSION

The available evidence shows that HCV eradication with both INF-based and DAA INF-free therapies can improve liver fibrosis and portal hypertension. The evidence of portal hypertension improvement with DAAs is still scarce but consistent with a rapid and significant improvement, which can also improve clinically significant outcomes such as variceal bleeding. However, data suggest that a point of no return exist, encouraging early treatment before the development of significant fibrosis and portal hypertension. DAA therapy, with its extremely high efficacy and safety profile, have an undoubtedly important role since it allows the cure of almost all infected patients, preventing fibrosis and portal hypertension and improving clinical outcomes.

As we have seen, patients with established portal hypertension can improve although for now there are no data about the long term effects of DAAs. The available evidence is mainly based on retrospective studies with heterogeneous populations and endpoints definitions. As randomized controlled trials with active treatment and control groups are not ethically acceptable at this time point, the best studies to answer these unsolved questions are prospective studies with well-defined inclusion and exclusion criteria, well-defined clinically significant endpoints and with long follow-up. We suggest that further studies include patients along the spectrum of HCV infection (from asymptomatic with minimal liver damage to cirrhotic patients) with stratification according to the stage of liver disease (ideally evaluated by non-invasive methods validated in HCV infection such as elastography and non-invasive markers of fibrosis). Additionally, the assessed endpoints should be clinically significant and well defined (*e.g.*, variceal enlargement from small to large varices, *de novo* ascites, encephalopathy and hypersplenism) and follow-up should be longer than 5 years to evaluate the true impact of HCV treatment according to the stage of liver damage. Data collection should include an adequate characterization of disease stage at the beginning and at the end of follow-up (including aminotransferases, platelet count, ultrasound findings, liver stiffness, presence of ascites, varices and encephalopathy). These studies should then assess the treatment effects according to the stage of liver disease and should compare patients who achieve SVR with patients in whom these endpoint is not achieved.

Future studies should also focus on predictors of portal hypertension resolution since it can influence management and avoid unnecessary monitoring in the subset of patients with a very low probability of having clinically significant portal hypertension after treatment. Evaluation of molecular markers of extracellular matrix and hepatic stellate cell remodeling such as hyaluronic acid or alpha-2 macroglobulin may also have an investigational interest to assess if they can be a surrogate marker of the point of no return. The role of pre-existing significant porto-systemic shunts should also be evaluated.

Until the answers to these questions are available, screening for varices is still recommended in cirrhotic patients although recent Baveno VI consensus suggest that patients with Fibroscan® < 20 kPa and platelet count above 150000/μL can avoid screening endoscopy[38]. In patients who undergo screening endoscopy and no varices are found, a follow-up screening after 3 years is still recommended if SVR was achieved and there are no cofactors (a 2 year interval is advised if there is ongoing liver injury).

Concerning patients with established portal hypertension and varices before treatment, the effects of SVR on variceal progression and on bleeding rates are also still unknown and should be evaluated in future studies. For now, those with small varices who achieved SVR and without cofactors should undergo follow-up endoscopy in 2 years, while patients with large varices should undergo primary prophylaxis and adequate management[38]. It should be also noticed that HCC surveillance should be continued in patients with F3 fibrosis or greater[27].

In conclusion, the development of portal hypertension can be prevented and it can be improved in a significant proportion of patients as long as the treatment is delivered in a timely manner, before the point of no return. The long-term effects of DAAs on portal hypertension are not completely established and studies with longer follow-up are needed, but there is evidence from studies of the pre-DAA era that show significant benefits of SVR on portal hypertension, encouraging early treatment before significant fibrosis (F3/F4) and portal hypertension are established.

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REVIEW

Present and future of metastatic colorectal cancer treatment: A review of new candidate targets

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Abstract

In the last two decades, great efforts have been made in the treatment of metastatic colorectal cancer (mCRC) due to the approval of new target agents for cytotoxic drugs. Unfortunately, a large percentage of patients present with metastasis at the time of diagnosis or relapse after a few months. The complex molecular heterogeneity of this disease is not completely understood; to date, there is a lack of predictive biomarkers that can be used to select subsets of patients who may respond to target drugs. Only the *RAS*-mutation status is used to predict resistance to anti-epidermal growth factor receptor agents in patients with mCRC. In this review, we describe approved targeted therapies for the management of metastatic mCRC and discuss new candidate targets on the horizon.

Key words: Novel biomarkers; Monoclonal antibodies; Resistance; Mutation; RAS; Target therapy; Metastatic colorectal cancer

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Core tip: Colorectal cancer is a heterogeneous disease characterized by several molecular alterations that affect genes implicated in cancer development. The discovery of novel biomarkers, together with a better understand of the complex biology of the disease, is essential to identify patients who will most likely benefit from personalized treatment.

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INTRODUCTION

Colorectal cancer (CRC) is the third most-diagnosed cancer in Europe and the United States, and 30% of patients with CRC present with a metastatic disease[1,2]. In past decades, substantial progress has been made in the development of new treatment options, which have radically changed the median overall survival (OS) of these patients. The mainstay of metastatic CRC (mCRC) treatment remains the use of cytotoxic agents, as well as irinotecan or oxaliplatin, which results in an average survival of 18 mo when combined with 5-FU and leucovorin or capecitabine (FOLFIRI/FOLFOX or CAPIRI/CAPOX regimens). The addition of targeted therapy has markedly improved the OS of patients with mCRC, which ranges from 22 to 29 mo[3]. Despite the dramatic improvement in survival, after few months of therapy with anti-epidermal growth factor receptor (EGFR) and anti-vascular endothelial growth factor (VEGFR) antibodies, mCRC patients stop responding to treatment due to intrinsic and acquired resistance to the targeted agents[4]. Recent findings in molecular biology and the ability to collect information from large patient databases have improved our understanding of the genetic evolution of this disease. Specifically, CRC is a heterogeneous disease with different molecular landscapes that reflect histopathological and clinical information. Four different subgroups of CRC have been identified, and each subgroup is associated with different patient outcomes (Figure 1). In this review, we summarise the currently approved treatments for CRC and discuss new targets that are on the horizon.

ADJUVANT SETTING

The use of adjuvant chemotherapy with 5-FU-based regimens is considered the standard of care for stage Ⅲ and stage Ⅱ high-risk CRC and benefits these categories of patients[5]. Moreover, the recent CRC classification, based on distinct molecular phenotypes, has identified a new biomarker that can be used to select patients with high-risk stage Ⅱ colon cancer: mismatch repair (MMR) deficiency. The main function of the MMR system is to identify and repair the mismatches that occur during DNA replication, which ensures genomic conservation and stability. While microsatellite instable (MSI) sporadic CRC constitutes 3%-15% of all CRCs, hereditary CRCs with a high level of MSI (MSI-H) constitute approximately 3%-5% of CRCs and arise exclusively in patients with Lynch syndrome, often called hereditary non-polyposis CRC (HNPCC)[6]. Because MSI has been used to screen for HNPCC, it has garnered increasing interest in the setting of CRC. Moreover, patients with MSI-H stage Ⅱ CRC have a better prognosis but derive minimal benefit from 5-FU adjuvant treatment. However, the addition of targeted therapy to a cytotoxic agent in the adjuvant setting provides no benefit in terms of OS and progression-free survival (PFS)[7,8] due to the low level of neo-angiogenesis and a phenotypical difference in these tumours, which leads to an epithelial to mesenchymal transition that could explain the absence of efficacy with the use of anti-EGFR antibodies.

Despite the good prognosis of early-stage CRC, many patients relapse during or a few months after the completion of treatment. Thus, better tools for molecular selection and new biomarkers are undoubtedly needed.

METASTATIC SETTING

In recent decades, the approval of targeted therapy in association with cytotoxic drugs has significantly improved the OS of patients with mCRC[9-13]. Specifically, vascular endothelial growth factor (VEGF) - and epidermal growth factor receptor (EGFR) - targeting monoclonal antibodies (mAbs) have become integral components of the first-line treatment strategies for mCRC. Moreover, the Food and Drug Administration (FDA) and the European Medicine Agency (EMA) have approved targeted therapies for mCRC in recent years such as the EGFR mAbs cetuximab and panitumumab for use in patients with RAS wild-type tumours; for RAS mutant disease, the VEGF mAb bevacizumab, the anti-VEGF receptor 2 (VEGFR2) mAb ramucirumab, the recombinant fusion protein ziva flibercept, and the oral multikinase inhibitor regorafenib have been approved and are discussed below.

Anti-EGFR antibodies such as aflibercept [a decoy receptor for VEGF-A, VEGF-B and placental growth factor (PIGF)] and ramucirumab (an antibody against VEGFR-2) are effective as monotherapy in previously treated patients and in combination with chemotherapy in the second-line setting, and regorafenib (a multikinase inhibitor) is effective as monotherapy in the refractory setting[14].

Anti-VEGFR drugs

In the field of targeted therapy, blocking angiogenesis

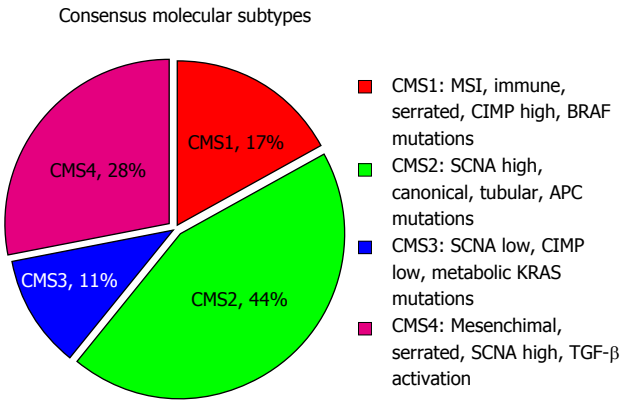

Figure 1 Colorectal cancer consensus gene expression-based subtypes[83]. CIMP: CpG island methylator phenotype; MSI: Microsatellite instability; SCNA: Somatic copy number alterations; BRAF: B-Raf proto-oncogene, serine/threonine kinase; KRAS: Kirsten rat sarcoma viral oncogene; TGF: Transforming growth factor; APC: Adenomatous polyposis coli.

has been considered a fundamental step in mCRC[14]. The deregulation of the VEGF receptor, its cognate cytokines and receptors as well as platelet-derived growth factor receptor has been established to be associated with tumour progression and metastatic spread *in vitro* and *in vivo*[14,15]. To date, the United States FDA and the EMA have approved 3 anti-VEGF agents for the treatment of mCRC.

Bevacizumab is an IgG-1 mAb with a high affinity for soluble VEGF-A that has been tested in early phase Ⅰ and Ⅱ trials[16] and subsequently investigated in phase Ⅲ randomised trials. As a first-line treatment for mCRC in combination with 5-FU/LV/irinotecan or oxaliplatin, bevacizumab has been shown to increase PFS and the response rate (RR)[17].

A recent trial that reported one of the longest survival periods to date investigated the use of the combination of FOLFOXIRI and bevacizumab as a first-line strategy, which resulted in better PFS and RR than FOLFIRI plus bevacizumab[18].

An Eastern Cooperative Oncology Group study (E3200) showed that the median survival for patients treated with FOLFOX4 and bevacizumab was 12.9 mo, whereas that of patients treated with FOLFOX4 or bevacizumab was 10.8 (HR = 0.75, $P < 0.001$) and 10.2 mo, respectively, in the second-line setting[19]. The use of bevacizumab as a maintenance treatment in patients who responded to treatment or present with stable disease upon induction therapy is controversial; however, in the AIO0207 trial, although non-inferiority for bevacizumab alone was demonstrated, the association of bevacizumab with capecitabine, compared to bevacizumab alone, may be the preferable option[20]. Furthermore, the CAIRO3 study showed that initial treatment with capecitabine, oxaliplatin, and bevacizumab (CAPOX-B) and continued with capecitabine and bevacizumab maintenance therapy provided a PFS benefit without compromising quality of life in patients compared with observation alone[21]. Bevacizumab is associated with specific class-related side effects, *e.g.,* hypertension, proteinuria, arterial thrombosis, mucosal bleeding, gastrointestinal perforation and wound-healing problems but does not increase chemotherapy-related side effects[2].

Ziv-aflibercept is a fusion protein that consists of the human extracellular VEGFR domains fused to the Fc portion of human immunoglobulin G1 and work as a trap VEGF-A, VEGF-B and PIGF.

A large phase Ⅲ trial investigating the activity of aflibercept in combination with FOLFIRI found a significant improvement in OS over FOLFIRI combined with placebo in patients with mCRC previously treated with an oxaliplatin-based regimen[22] (HR = 0.817, 95%CI: 0.713-0.937, $P = 0.0032$), with median survival times of 13.50 and 12.06 mo, respectively. Efficacy was maintained with a similar safety profile. Therefore, aflibercept was approved by the EMA after oxaliplatin-based therapy in combination with FOLFIRI.

Ramucirumab (IMC-1121B) is a fully humanized IgG-1 mAb that binds with high affinity to the extracellular VEGF-binding domain of VEGFR-2 and blocks VEGF ligands from binding this site and activating the receptor. The inhibition of VEGF-stimulated VEGFR-2 activation endows ramucirumab significant antitumour activity in a range of malignancies in *in vivo* models as a single agent or in combination with other drugs. Based on the RAISE trial, which enrolled 1072 patients (536 in each group) and randomized them to receive either ramucirumab or placebo, the EMA and FDA approved ramucirumab in the second-line setting for patients whose disease has progressed on first-line bevacizumab-, oxaliplatin- and fluoropyrimidine-containing regimens[23]. The median OS, *i.e.,* the primary endpoint, was 13.3 mo (95%CI: 12.4-14.5) for patients in the ramucirumab group *vs* 11.7 mo (10.8-12.7) for the placebo group (HR = 0.844, 95%CI: 0.730-0.976; log-rank $P = 0.0219$). PFS was significantly improved in patients who received the combination compared to placebo (median PFS 5.7 *vs* 4.5 mo, HR = 0.79, 95%CI: 0.70-0.90, $P < 0.001$).

Anti-EGFR drugs

The EGFR signalling pathway has been identified as a major driver of the development and progression of CRC[24,25].

Several ligands, such as EGF, amphiregulin, or epiregulin, bind specific extracellular domains of the EGFR, which activates an intracellular signalling cascade *via* different signalling pathways. The mitogen-activated protein kinase (MAPK) pathway, comprising RAS-RAF-MEK-ERK and the PI3K-AKT- (PTEN)-mTOR pathway are the main downstream effectors of EGFR implicated in different processes, such as cancer initiation, invasion, angiogenesis, inhibition of apoptosis and metastasis[24,25]. Therefore, EGFR is considered one of the most important targets in CRC treatment.

The anti-EGFR antibodies cetuximab (an IgG1 recombinant human/mouse chimeric anti-EGFR mAb) and panitumumab (an IgG2κ recombinant, fully human anti-EGFR mAb) have been investigated in several phase Ⅲ clinical trials and showed efficacy in terms of PFS, OS, RR, and quality of life among different lines of treatment[26,27]. These antibodies have been shown to prolong survival in patients with mCRC when introduced as monotherapy or in combination with irinotecan in a refractory population[10].

Despite the demonstrated strong benefit, cetuximab and panitumumab achieved a RR of only 10% when used in unselected patients[17]. This result is in concordance with the presence of genetic alterations in EGFR, in the downstream proteins of the EGFR pathway or in other receptor tyrosine kinases (RTKs) that cause resistance to these anti-EGFR antibodies, a phenomenon called primary or intrinsic resistance[28].

Moreover, genetic alterations induced by blocking EGFR cause the positive selection of independent clones or treatment-induced mutagenesis and result in tumour-intrinsic genomic instability that is related to the development of an acquired or secondary resistance to anti-EGFR therapy, emerging at treatment failure[29]. Furthermore, the overall scenario is complicated by the coexistence of different molecular alterations in distinct tumour lesions (inter-metastases heterogeneity) or within different regions of the same lesion (intratumour heterogeneity)[30].

In the era of "personalized treatment" both clinical and molecular data have shown that patients with metastatic CRC have a heterogeneous prognosis and response to treatment. Unfortunately, the complex molecular landscape of the tumour remains incompletely understood, and predictive biomarkers to select patients who may benefit from target drugs are lacking.

Predictive value of RAS

The *RAS* gene is often mutated in mCRC, and the most common of these mutations is Kirsten rat sarcoma viral oncogene (*KRAS*). The *KRAS* gene is mutated in approximately 40% of CRCs; specifically, somatic single-nucleotide point mutations occur in codons 12 and 13 of exon 2 of the *KRAS* gene and in a small percentage in codons 61 and leading to a constitutively activation of the MAPK pathway[31]. Because cetuximab and panitumumab demonstrated a lack of benefit when used as monotherapies for patients with chemorefractory mCRC, researchers investigate the negative impacts of these drugs. Retrospective analyses from randomized controlled trials established that these mutations can predict resistance to anti-EGFR mAb treatment in mCRC. Therefore, the EMA and FDA initially only approved cetuximab and panitumumab for the treatment of patients with *KRAS* exon 2 wild-type tumours[32].

In recent years, several biomarkers in addition to *KRAS* exon 2 mutations were identified to be involved in resistance to anti-EGFR therapy and help to determine a more appropriate patients' selection. Specifically, the presence of other mutations in *KRAS* (exon 3, codons 59/61 and exon 4, codons 117/146) and *NRAS* (exon 2, 3 and 4) correlates with a loss of efficacy of anti-EGFR antibodies, and retrospective and prospective trials have underlined the importance of a selection of patients based on RAS status. Notably, a retrospective analysis of the PRIME trial assessed the "expanded RAS" (*KRAS* and *NRAS*) status and demonstrated the efficacy of the panitumumab plus FOLFOX4 regimen in terms of the objective RR (ORR), PFS and OS compared with chemotherapy alone as a first-line treatment for *RAS WT* mCRC[13]. In other phase Ⅱ and phase Ⅲ trial analyses, the range of mutated patients changed from almost 15% (exon 2 *KRAS* mutation) to 53% (all RAS)[33-36], showing that this population is refractory to anti-EGFR therapy.

Results from a study published by our group, the phase Ⅱ CAPRI trial, demonstrated that patients with mCRC continued to benefit from cetuximab, even after they became refractory to FOLFIRI backbone chemotherapy[37]. After progression on a first-line treatment consisting of FOLFIRI plus cetuximab, patients were randomized to receive FOLFOX alone or in combination with cetuximab. The addition of cetuximab improved PFS when patients were appropriately selected for extended RAS assessment as well as two other potential biomarkers, B-Raf proto-oncogene, serine/threonine kinase (BRAF) and Phosphatidylinositol-4,5-Biphosphate-3-Kinase Catalytic Subunit Alpha (PIK3CA). The results from this trial confirmed the lack of efficacy of cetuximab in the subgroup of patients with *KRAS* and *NRAS* mutations[37] and suggest that continuing cetuximab treatment in combination with chemotherapy is effective in patients who have been molecularly selected. However, these results should be validated in randomized phase Ⅲ trials.

The results emerging from the FIRE 3 trial underscore the importance of expanded *RAS* mutational analysis in the selection of patients. Previously, untreated patients with *KRAS* exon 2 wild-type mCRC were randomized to receive FOLFIRI with either cetuximab or bevacizumab. The trial showed EGFR molecular antibodies were superior in the *RAS WT* population in terms of OS, RR, depth of response and early tumour shrinkage, whereas the initial results of this study did not demonstrate a statistical significant difference in terms of PFS or ORR[38].

Furthermore, a systematic review and meta-analysis of nine randomized, controlled trials evaluating EGFR antibody therapy in all lines of mCRC treatment confirmed these observations[31]. Specifically, the analysis showed that patients with tumours without *RAS* mutations had a significantly better treatment outcome with EGFR mAb therapy than patients whose tumours harboured *RAS* mutations.

Taken together, these results highlight the important role of the RAS status as a predictive biomarker in

the management of CRC. Therefore, the EMA and FDA restricted the indication of cetuximab and panitumumab to "all RAS WT" CRC patients in 2013[34,35].

Not all KRAS mutations are considered equal in giving resistance to anti-EGFR therapies[39]. For instance, retrospective analyses from a phase Ⅲ trial and preclinical data demonstrated that the presence of a *KRAS* G13D mutation allows mCRC patients to respond to cetuximab in both first-line and advanced settings[40,41]. Two phase Ⅱ trials investigated the prospective role of KRAS G13D mutation in response to cetuximab. Neither the first one, conducted from Schirripa *et al*[42] and Segelov *et al*[43] or the ICE-CREAM trial observed any response among the treated patients, with cetuximab therapy.

Finally, *KRAS* is amplified in a small percentage of tumours, and this amplification is considered to be responsible for both primary and acquired resistance[44].

New drugs targeting RAS

One of the most common approaches to inhibiting RAS has been the identification of downstream effectors, as well as MEK and PIK3CA. MAPK-ERK pathway is a convergence point where several upstream signalling pathways can be blocked. Specifically, the combination of trametinib (anti-MEK) and palbociclib (anti-CDK4/6) was investigated as a novel treatment approach in a xenograft model derived from patients with *KRAS*-mutant CRC, and the resulting data showed that this treatment was well tolerated and highly efficacious. Nevertheless, a clinical evaluation is necessary to confirm these preclinical data[45,46].

Reovirus Serotype 3 - Dearing Strain (Reolysin®, Oncolytics Biotech Inc., Calgary, AB, Canada) is a naturally occurring, ubiquitous, non-enveloped human reovirus that can replicate in RAS-transformed cells to cause cell lysis, and its role has been investigated in targeting *KRAS* in mCRC. Specifically, a multicentre phase Ⅰ study testing Reolysin in combination with FOLFIRI and bevacizumab in FOLFIRI-naive patients with *KRAS* mCRC is on-going (ClinicalTrials.gov Identifier: NCT01274624)[47].

Other biomarkers of resistance

The identification of genetic determinants of primary resistance to anti-EGFR therapies in CRC, in particular the activation of an alternative pathway, which can bypass EGFR blockade, is important to identify patients who should not be treated with EGFR mAbs[48]. Beyond *RAS*, additional mechanisms of intrinsic resistance have been identified.

BRAF

Despite the recognition of *KRAS/NRAS* mutations as predictors of a lack of response to anti-EGFR antibodies, a considerable percentage of WT *RAS* CRC tumours do not respond to the appropriately selected targeted therapy, which may be due to a downstream effector of the KRAS/NRAS pathway. Such effector is represented by *BRAF*, a serine/threonine protein kinase that is mutated in 12% -15% of patients with mCRC[49]. A $BRAF^{V600E}$ point mutation is the most common alteration and believed to be mutually exclusive with KRAS exon 2 mutations. Nevertheless, data from the CAPRI trial show concurrent other molecular alterations, such as *TP53, KRAS* and *PI3KCA* exon 9 and exon 20 alterations, in 12 of 15 BRAF-mutated samples[50,51]. The $BRAF^{V600E}$ encodes a constitutively active protein, which would account for the lack of blocking EGFR with cetuximab or panitumumab. Accordingly, several clinical trials have highlighted the poor prognostic role of the *BRAF* mutation in patients with mCRC. For example, Prahallad *et al*[52] reported a median OS for patients with *BRAF*-mutant mCRC of 10.4 mo, compared with 34.7 mo for patients with *BRAF* WT tumours. Furthermore, a retrospective analysis showed that two-thirds of *BRAF*-mutant tumours are located on the right side of the colon and associated with a major incidence of peritoneal disease and distant lymph node involvement. Moreover, a sizeable body of literature established the poor prognostic role of the $BRAF^{V600E}$ mutation, which is associated with increased colon cancer mortality[53,54], but its value as a predictive biomarker remains uncertain due to the absence of prospective trials. In a subset analysis of the PRIME trial, the $BRAF^{V600E}$ mutation indicates any prediction of benefit for the addition of panitumumab to FOLFOX in the first-line setting of mCRC. In addition, data from the MRC COIN trial showed that cetuximab was detrimental in patients with the $BRAF^{V600E}$ mutation[13]. A recent meta-analysis of phase Ⅲ trials confirmed this lack of benefit of mAbs in addition to doublet chemotherapy in terms of OS, PFS and ORR[55]. However, standard therapeutic options for this subgroup of patients are limited. Results derived from a subgroup analysis of the TRIBE trial of 28 patients with the $BRAF^{V600E}$ mutation indicated that patients are more likely to respond to an aggressive initial treatment that combines FOLFOXIRI (fluorouracil, leucovorin, oxaliplatin and irinotecan) and bevacizumab (median OS 19.1 mo *vs* 10.8 mo for the FOLFIRI and bevacizumab group), with a hazard ratio for progression of 0.55 in favour of the combination. Given the impressive results obtained in metastatic melanoma, vemurafenib and dabrafenib have been investigated in $BRAF^{V600E}$-mutated mCRC[56,57]. In a phase Ⅱ trial, vemurafenib was tested in previously treated patients with mCRC; unfortunately, the benefit in terms of RR was only 5% compared with the strong clinical activity demonstrated in melanoma tumours[58]. Moreover, *in vitro* experiments showed that mCRC cells do not respond to vemurafenib due to the persistent activation of the EGFR and the consequent dimerization of BRAF, which suggests that the EGFR signal should be blocked downstream. Current studies are focusing on the dual blockade of BRAF and EGFR or the downstream pathway[59]. According to initial

results, combining the BRAF inhibitor vemurafenib with the EGFR inhibitor panitumumab has been safe, but response has been modest. Nevertheless, ERK inhibitors, which are thought to suppress MAPK activity and overcome resistance to RAF inhibitors, may constitute a treatment strategy.

In this regard, the combination of anti-EGFR antibodies, BRAF inhibitors and MEK inhibitors has recently been investigated and is producing very interesting results[57].

Patients with metastatic CRC and tumours harbouring the BRAF^{V600E} mutation who received triple therapy with dabrafenib (Tafinlar), trametinib (Mekinist), and panitumumab (Vectibix) showed an improved best overall response and prolonged progression-free survival compared to patients who received panitumumab plus either dabrafenib or trametinib, according to results reported by Van Cutsem *et al*[60] at the 2016 European Society for Medical Oncology (ESMO) Congress in Copenhagen (Abstract 455O). Based on preclinical evidence showing that the addition of irinotecan to vemurafenib and cetuximab reduced tumour size, improved response rate and prolonged OS in xenograft models of BRAF^{V600E} metastatic CRC (Yang *et al*[61]), a phase Ⅱ study of irinotecan and cetuximab with or without vemurafenib in *BRAF* mCRC is currently recruiting patients[62]. Specifically, the trial investigates the activity of vemurafenib plus cetuximab and irinotecan compared to cetuximab plus irinotecan in patients with the BRAF^{V600E} mutation. The triplet had an acceptable toxicity profile and may be effective for patients with the BRAF^{V600E} mutation, but the need for a novel therapeutic agent remains.

PI3KCA

In addition to *NRAS/KRAS* and *BRAF* mutations, other predictive biomarkers also indicate resistance to cetuximab/panitumumab[63]. For example, *PIK3CA/AKT/mTOR* signalling pathway is associated with several RTKs, including EGFR. Approximately 10%-20% of CRCs harbour activating mutations of *PIK3CA*, which primarily occur in exons 9 and 20 and are responsible of lack of response to anti-EGFR therapy[64,65]. Accordingly, a retrospective analysis of 110 patients with mCRC treated with mAbs demonstrated the correlation between *PI3KCA* mutations and resistance to treatment with cetuximab or panitumumab in the subset of *KRAS WT* tumours[66].

The precise predictive role of *PI3KCA* mutations is not clear due to the concomitant presence of *KRAS* or *BRAF* mutations and their low incidence, especially exon 20 mutations. However, a large retrospective analysis of 1022 tumour samples of patients treated with cetuximab yielded two main results: only *PIK3CA* exon 20 mutations predict of a lack of response to cetuximab in the *KRAS WT* subpopulation; *PIK3CA* exon 9 mutations and *KRAS* mutations were associated, suggesting a secondary role of *PIK3CA* exon 9 mutations in cetuximab resistance[32]. *PIK3CA* mutations have also been identified as mechanisms of secondary resistance in samples from patients who relapse after treatment with EGFR-targeting mAbs[67]. With respect to the role of *PIK3CA* mutations as a prognostic biomarker, colon cancerspecific mortality is increased in patients with *PIK3CA*mutated tumours compared with patients with WT tumours, even if the worse prognosis in WT tumours is associated with both the presence of exon 9 and exon 20 mutations[68].

The PIK3CA signalling pathway may also be activated by the loss of *PTEN*, which is found in 30% of CRCs and associated with a lack of objective tumour response and worse OS in patients with *KRAS WT* tumours treated with a cetuximabbased regimen[69]. Nevertheless, the *PTEN* expression status does not affect clinical practice since its role as a predictive biomarker remains under investigation.

Several studies have investigated the predictive and prognostic roles of PTEN loss; *PTEN* encodes a phosphatase that is involved in the regulation of the intra-cellular levels of phosphatidylinositol-3, 4, 5-trisphosphate and acts as a tumour suppressor by negatively regulating the AKT/PKB signalling pathway[70]. *PTEN* loss in CRC can occur *via* several genetic and epigenetic mechanisms, such as mutations, promoter hypermethylation or 10q23 LOH and promoter hypermethylation, which leads to subsequent AKT hyperphosphorylation and inhibits apoptosis. Several studies have investigated the predictive and prognostic role of *PTEN* loss; however, data on the concordance rate of *PTEN* expression on primary tumours and matched metastases are controversial.

Mao *et al*[71] conducted a meta-analysis of eight studies to investigate the role of PTEN expression in CRC. In all studies, *PTEN* status was detected using immunohistochemistry (IHC) due to the multiple genetic and epigenetic mechanisms leading to a lack of protein function. In one study included in the meta-analysis[72], *PTEN* expression was analysed in 45 pairs of primary tumours and related metastases. The level of concordance reported was 60%, suggesting that *PTEN* loss contributes to tumour heterogeneity by anti-EGFR treatment pressure. Conversely, a more recent study conducted on 70 matched specimens found a high concordance rate of PTEN expression between primary tumours and liver metastases (98%)[73]. However, a large prospective trial should be conducted to confirm the emerging predictive value of PTEN loss using a validated scoring system for IHC.

New potential treatments that were recently investigated include the combination of the mTOR inhibitor everolimus with panitumumab and irinotecan as a first-line regimen for mCRC[74]. Notably, preliminary results derived from the use of lowdose aspirin in patients with *PIK3CA*mutant tumours indicated a benefit in survival due aspirin mediated COX2 inhibition. However, this observation requires further clinical evaluation[75].

Human epidermal growth factor receptor 2/human epidermal growth factor receptor 3

Human epidermal growth factor receptor 2 (HER2) is an oncogenic driver and member of the ERBbB family, which is targeted by trastuzumab antibody in breast and gastric cancer treatment[4]. The activation of this receptor requires heterodimerisation with other ligand-bound receptors of the same family because of the absence of known HER2 ligands. The heterodimer HER2-HER3 represents a powerful activator of intracellular signalling[76].

HER2 has been proposed as a target in CRC due to studies of *RAS/BRAF* wild-type and cetuximab-resistant CRC xenograft models. In the study conducted by Bertotti *et al*[77] the amplification of the *HER2* gene was recognised as a potential mechanism of primary resistance to cetuximab in a quadruple WT population (*KRAS*, *NRAS*, *BRAF*, and *PIK3CA* wild-type).

The authors only observed *HER2* amplification in a small percentage (2%-3%) of genetically unselected patients with CRC. This proportion increased when considering *KRAS WT* patients who are resistant to cetuximab, ranging from 13.6%-36% in the quadruple WT population. To examine the value of HER2 as a positive predictive biomarker, they performed a multi-arm xenotrial using lapatinib, a dual EGFR/HER2 small-molecule inhibitor, and cetuximab or pertuzumab, a mAb directed against the EGFR/HER2 heterodimer. The association resulted active in the subset of cetuximab resistant, quadruple WT HER2-amplified metastatic CRC xenopatients, with achievable implications in the clinical setting. Based on these preclinical results, Siena and colleagues conducted an Italian, phase Ⅱ, proof-of-concept clinical trial assessing the RR of trastuzumab combined with either lapatinib (cohort A) or pertuzumab (cohort B) in *KRAS* exon 2 (codons 12 and 13) WT and HER2 amplified mCRC patients resistant to standard therapies, including anti-EGFRs[78]. The results from cohort A have been recently published, and approximately 5% (48 of 914 patients screened) tumours were found to be HER2 positive. Of the 27 patients enrolled, eight (30%, 95%CI: 14-50) achieved an overall objective response, and the median duration of the response was 38 wk. The median PFS was 21 wk (95%CI: 16-32), whereas the median OS calculated post hoc was 46 wk (95%CI: 33-68). Notably, responses were significantly more common in tumours with a high *HER2* gene copy number, and the PFS was longer in this population. The combination exhibited a good safety profile, with most toxic effects being grade 1 or 2. To date, HER2 is the first druggable target in mCRC that is a good predictor of response to targeted treatments[79]. However, further investigations are needed in earlier lines of therapy, combining treatment with the inhibition of EGFR and HER2-4.

The amplification of *HER2* is not the only molecular alteration that can hyperactivate the HER2 receptor. The overproduction of Heregulin, a HER3 ligand, may also confer resistance to anti-EGFR treatment. Furthermore, a collection of tumour samples and plasma from patients with acquired resistance to cetuximab demonstrated an increased percentage of *HER2* amplification accompanied by higher levels of heregulin in treated patients compared with pretreatment tumour cells[79]. This result corroborates the assumption that a specific driver of primary resistance to anti-EGFR drugs may be implicated in secondary resistance, leading to the constitutive activation of the ERK-MEK pathway. Furthermore, these results underscore that CRC is a complex heterogeneous disease in which the evolution of single clones present at the beginning of treatment confers resistance in more advanced settings of therapy.

HER3, which is mutated in 11% of patients with CRC, may also be a marker of resistance and may limit the responsiveness to EGFR inhibitors, even if *HER2* is not amplified[80]. Moreover, the overexpression of HER3 was associated with a shorter PFS and OS in a subset of patients with metastatic CRC treated with irinotecan and cetuximab as second- or third-line therapy[81].

Moreover, MEHD7945A, a humanized IgG1 mAb with dual anti-HER3/EGFR activity, had a superior activity to monoclonal EGFR targeting agents in multiple xenograft models[82].

Despite the promising results derived from a phase Ⅰ study of patients with pretreated mCRC, a phase Ⅱ randomized trial of MEHD7945A + FOLFIRI *vs* cetuximab + FOLFIRI did not demonstrate the superiority of the experimental arm in patients with KRAS WT mCRC refractory to oxaliplatin[83].

Regarding secondary resistance, more than a molecular driver resulted implicated and *RAS* mutations are the most frequent, with a range of 50%-80% of patients. For instance, mutations that sustain the mechanism of primary resistance can also be validated as mechanisms of acquired resistance, as described above[29]. Genetic alterations were found in the EGFR receptor, preventing the mAb binding, in the downstream effector as well as *BRAF, PI3KCA*, loss of *PTEN* expression and in the activation of parallel pathways such as amplification of *HER2, MET*; all of these are components of EGFR signalling transduction pathway or interact with.

S492R and other EGFR mutations

Mutations in the extracellular domain of EGFR contribute to secondary resistance to cetuximab. Specifically, Montagut *et al*[84] identified a missense mutation in codon 492 (*S492R*) that appeared to hinder cetuximab binding. This allele has never been identified in previously treated tumour samples, which suggests that this alteration is an exclusive marker of secondary resistance. S492R clones continue to respond to panitumumab, which binds a different epitope, and this finding may be translated to the clinic. Specifically, the researchers reported that one patient with the *EGFR S492R* mutation, whose disease progressed after an initial response to cetuximab, achieved an

initial objective response of five months when treated with panitumumab. However, no further analyses were conducted. Furthermore, new mutations in the EGFR extracellular domain (ECD) were identified in two patients with acquired resistance to cetuximab: *R451C* and *K467T*. Tumour samples of 37 patients with mCRC treated or not with cetuximab were analysed, which revealed that these alterations allowed panitumumab binding to a different epitope of the EGFR ECD[67].

The development of new biological techniques has facilitated the identification of new targets in the setting of acquired resistance. For example, analyses of tumour ctDNA in plasma samples collected before and after treatment represent a complete picture of molecular changes in a patient's tumour. Notably, Bettegowda *et al*[85] described mutations in cell-free DNA, such as codons 714 and 794 of the EGFR kinase domain.

The development of new mAbs directed against different epitopes of the ECD of EGFR may be able to overcome resistance to EGFR blockade.

Sym004, which is a new drug composed by a mixture of two recombinant human mouse antibodies that bind non-overlapping epitopes of domain Ⅲ of the EGFR, induces rapid receptor internalization and degradation *via* EGFR cross-linking[67]. The binding region of Sym004 differs from cetuximab and allows the drug to also be used in the presence of mutations in the ECD of the EGFR. The efficacy of this new drug is under investigation in a phase Ⅱ trial as single agent in selected patients with *KRAS WT* CRC progressing on previous cetuximab- or panitumumabbased therapy within 6 mo of trial enrolment[86-88].

MM151 is a mixture of three fully human monoclonal IgG1 antibodies directed towards three different, non-overlapping epitopes of the EGFR, and the activity of MM151 has been demonstrated in preclinical models. Specifically, it improved EGFR pathway inhibition and downstream signalling and enhanced the downregulation of the EGFR and stimulation of the innate immune responses[89]. Notably, MM151 targets regions of the EGFR distinct from those affected by ECD mutations. Based on these preclinical studies, the efficacy of MM151 was explored in the clinical setting, and current phase Ⅰ results show an acceptable safety profile and objective clinical activity in refractory patients with cancer, including those failing cetuximab therapy[90].

FUTURE DIRECTIONS

Immunotherapy

In recent years, cancer immunology has been considered one of the most interesting fields, with substantial results obtained in the treatment of many tumours. For example, blocking the programmed death 1 (PD-1) pathway with antibodies to PD-1 or its ligands has led to remarkable clinical responses in patients with different types of cancer, including melanomas, non-small-cell lung cancer, renal-cell carcinoma, bladder cancer, and Hodgkin's lymphoma[91-93]. Moreover, the expression of PD-1 ligands (PD-L1 or PD-L2) on the surface of tumour cells or immune cells is an important predictive biomarker of response to PD-1 blockade. Unfortunately, CRC seems to present different molecular features, and the rate of response to PD-1 blockade is very low (1 of 33 patients treated), unlike in other malignancies[91].

Because MMR occurs in a small fraction of advanced CRCs and is associated with a prominent lymphocyte infiltrate and a large number of somatic mutations that can be recognized by the patient's own immune system, researchers hypothesized that mismatch repair-deficient tumours are more responsive to PD-1 blockade than mismatch repair-proficient tumours[94]. To this end, Le *et al*[94] conducted a phase Ⅱ study of Pembrolizumab (a humanized anti-PD-1 antibody) in a treatment-refractory stage Ⅳ CRC population. The immune-related objective response rate and immune-related PFS rate were 40% (4 of 10 patients) and 78% (7 of 9 patients) for MSI-H CRCs and 0% (0 of 18 patients) and 11% (2 of 18 patients) for microsatellite stable/proficient MSS CRCs, respectively. Only 1 of 10 patients with MSI-H CRC experienced disease progression, as compared to 11/18 MSS CRC patients. This study provides strong support for MSI testing in advanced CRC. Furthermore, the Checkmate-142 trial investigated the activity of nivolumab (anti-PD-1) as a single agent or in combination with ipilimumab (anti-cytotoxic T-Lymphocyte Antigen 4) in the same subset of patients with mCRC, MSI-H and non-MSI-H, and interim results were presented at the ESMO congress in 2016, which demonstrated an encouraging advantage and tolerable safety profile[95].

Further research is needed to enhance susceptibility of MSS CRCs to immune checkpoint inhibitors. To this end, a phase IB trial presented by Bendell *et al*[96] at the ASCO meeting in 2016 attempted to identify treatments for this subset of patients with MSS disease. Considering the low activity of atezolizumab monotherapy (an engineered antibody that inhibits PD-L1 from binding with its receptors PD-1 and B7.1) in mCRC, MEK-blocking agents have been associated to immune checkpoint inhibitors because they can induce intratumoural T-cell infiltration and enhance PD-L1 activity, as confirmed in a preclinical setting. Cobimetinib plus atezolizumab was well tolerated at the maximum-administered dose in patients with chemorefractory KRAS-mutant mCRC. The combination resulted in a higher clinical response rate in patients with MSS disease than that expected from either cobimetinib or atezolizumab alone. Furthermore, the use of the combination guaranteed an ORR of 17% and a 6-mo OS of 72%, leading to an expansion of the phase IB trial. A phase Ⅲ trial testing the combination of cobimetinib plus atezolizumab *vs* atezolizumab alone or regorafenib alone in patients with unresectable locally advanced or metastatic CRC is

Table 1 Baseline cancer biomarkers shown in preliminary analysis of the Screening Patients for Efficient Clinical Trial Access in advanced colorectal cancer's molecular screening platform

KRAS WT	KRAS exon 2 mutated	KRAS exon 3 and exon 4 mutated
151 of 284 patients (53%) NRAS mutated (KRAS WT) 14 patients (4.9%); 6 patients in exon 2 and 8 patients in exon 3	114 patients in exon 2 (40%) PI3KCA 41 patients (15%), 13 in exon 20 and 28 in exon 9	8 patients in exon 3 (3%), 11 patients in exon 4 (4%) BRAF 18 patients in exon 15 (7%)

KRAS: Kirsten rat sarcoma viral oncogene; BRAF: B-Raf proto-oncogene, serine/threonine kinase.

under investigation[97].

Furthermore, a study by Ahn *et al*[97] presented at the ESMO congress in 2016 defines a subset of patients with stage Ⅱ/Ⅲ CRC who harbour a mutation in the DNA polymerase epsilon (*POLE)* gene and have a better prognosis. These results may be explained by increased immune activity in *POLE*-mutant tumours, including increased $CD8^+$ lymphocyte infiltration, the expression of cytotoxic T cell markers, and effector cytokines, which is similar to that observed MSI cancers.

Although uncommon and found in only 66 of 6448 (1.0%) CRC samples, *POLE* mutations were significantly associated with several patient and tumour factors, including young age, male sex, right-sided location, early disease stage, and the absence of mismatch repair deficiency ($P \leqslant 0.003$ for all associations)[97].

Notably, a multivariable analysis revealed a statistically significant association between the *POLE* mutation and a greatly reduced risk of disease recurrence: HR = 0.34, 95%CI: 0.11-0.76 ($P = 0.006$). This reduced risk was particularly strong in stage II disease and when associated with MSI-H, an accepted biomarker of favourable prognosis in this setting[98].

Entrectinib

Entrectinib is a novel, orally available, selective tyrosine kinase inhibitor targeting tumours that harbour activating alterations in *NTRK1/2/3* (encodingTrkA/TrkB/TrkC), *ROS1* or *ALK*. Entrectinib is the most potent Trk inhibitor in the clinic and free of undesirable off-target activity. This product candidate is in a Phase 2 clinical trial called STARTRK-2, which is the second of the "Studies of Tumour Alterations Responsive to Targeting Receptor Kinases"[99]. The trial is a global, multicentre, open label, potentially registration-enabling Phase 2 clinical trial of entrectinib that utilises a basket design with the screening of patient tumour samples for the relevant targets. Such a basket design takes full advantage of entrectinib, whose preliminary clinical activity is demonstrated across a range of different tumour types and molecular targets.

SPECTAcolor platform

Treatments for patients with cancer are becoming increasingly tailored to the molecular characteristics of the particular patient and disease. Consequently, molecularly characterizing a patient's tumour is now a prerequisite for them to access the appropriate clinical trial for their particular cancer type. Efficient, GCP-conforming and quality-assured molecular screening to identify potential study patients is one of the major challenges for targeted drug development.

The European Organisation for Research and Treatment of Cancer built a collaborative molecular screening platform, Screening Patients for Efficient Clinical Trial Access in advanced CRC's (SPECTAcolor), which provides the necessary infrastructure to screen adult patients with advanced-stage CRC for mutations in CRC biomarkers. SPECTAcolor's successful start has demonstrated its ability to facilitate next-generation cancer clinical trials across 19 clinical centres by recruiting over 500 patients, and results have been presented by SPECTAcolor's coordinator, Dr. Gunnar Folprecht, at the ESMO congress in 2016[100].

The observed frequency of mutations is similar to that observed in previous CRC clinical trials. New therapeutic targets have been identified by gene panel sequencing and allow patients access to specific clinical trials (Table 1).

CONCLUSION

The treatment of CRC has markedly changed in recent years due to the development of new predictive biomarkers that facilitate optimized, tailored therapy. The discovery of new biologic techniques, such as the liquid biopsy approach, elucidate the increasingly complex heterogeneity of this disease and can be used to monitor minimal residual disease, track tumour clonal evolution and design novel therapeutic strategies to overcome the emergence of drug resistance. Despite this exceptional progress, a large subset of patients continues to be unresponsiveness. In the immediate future, further clinical investigations, such as clinical trials, are needed to guarantee to all patients a genetically determined treatment strategy.

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REVIEW

Diarrhea after bariatric procedures: Diagnosis and therapy

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Abstract

Diarrhea after bariatric procedures, mainly those with malabsorptive elements including Roux-Y Gastric Bypass and Biliopancreatic Diversion, is common and an essential determinant of quality of life and micro- and macronutrient deficiencies. Bariatric surgery is the only sustainably successful method to address morbid obesity and its comorbidities, particularly gaining more and more importance in the specific treatment of diabetic patients. Approximately half a million procedures are annually performed around the world, with numbers expected to rise drastically in the near future. A multitude of factors exert their influence on bowel habits; preoperative comorbidities and procedure-related aspects are intertwined with postoperative nutritional habits. Diagnosis may be challenging owing to the characteristics of post-bariatric surgery anatomy with hindered accessibility of excluded segments of the small bowel and restriction at the gastric level. Conventional testing measures, if available, generally yield low accuracy and are usually not validated in this specific population. Limited trials of empiric treatment are a practical alternative and oftentimes an indispensable part of the diagnostic process. This review provides an overview of causes for chronic post-bariatric surgery diarrhea and details the particularities of its diagnosis and treatment in this specific patient population. Topics of current interest such as the impact of gut microbiota and the influence of bile acids on morbid obesity and especially their role in diarrhea are highlighted in order to provide a better understanding of the specific problems and chances of future treatment in post-bariatric surgery patients.

Key words: Bariatric surgery; Diarrhea; Malnutrition; Malabsorption; Steatorrhea; Dumping syndrome; Bile acids and salts; Gut microbiota; Blind loop syndrome

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Core tip: Bariatric surgery is the only sustainable therapy for morbid obesity and its comorbidities. Postoperative diarrhea is common and an essential determinant of quality of life and micro- and macro-

nutrient deficiencies. The distinctive anatomic changes after bariatric procedures with exclusion of various length of small bowel have a severe impact not only on diagnostic but also puts limits on therapeutic means. This review provides an overview of causes for chronic diarrhea in the particular context of post-bariatric patients, and details specific problems in diagnosis and treatment of this challenging patient population.

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INTRODUCTION

Bariatric surgery was demonstrated to be the most efficacious method to achieve sustainable weight loss and resolution of co-morbidities among the morbidly obese. Roux-en-Y gastric bypass (RYGB), sleeve gastrectomy (SG) and to a lesser extent bilio-pancreatic diversion with duodenal switch (BPD-DS) are the most commonly applied procedures (Figure 1). Around 470000 bariatric procedures have been performed worldwide in 2013, with numbers expected to rise in the future as the threshold, currently at a body mass index of 35 kg/m^2, will be lowered to 30 kg/m^2 in diabetic patients[1-4]. Weight loss is achieved by different mechanisms, all having various effects on bowel habits. The implied alterations of the anatomy not only affect the sensitivity and specificity of diag–nostic procedures but also the pharmacodynamics and bioavailability of the used medication, potentially resulting in treatment failure[5].

Bariatric surgery, especially BPD-DS and distal RYGB, leads to a significant change of bowel habits with enhanced frequency of malodorous flatus and diarrhea[6]. Diarrhea exposes patients at risk for fecal incontinence - up to 50% of patients after BPD-DS are affected - and has a major impact on Quality of Life as well as on nutrient and vitamin absorption[7-9]. In addition, morbid obesity per se, and in particular its associated comorbidities, broadens the spectrum of possible causes for diarrhea.

The goal of this review is to detail causes for chronic diarrhea in the specific post-bariatric surgery context, especially after RYGB, and to give a focused overview of its diagnosis and treatment, with the intention to depict the differences and difficulties compared to an evaluation for diarrhea in non-operated patients[10-12]. Reasons nonspecific for post-bariatric surgery patients, such as cancer-related diarrhea, are not covered, even though the incidence of cancer is higher in the bariatric than in the non-obese population[13].

EATING HABITS AND LIFESTYLE CHANGES

Bariatric surgery results in a radical change of life with lifestyle modifications eventually resulting in change of food preference, meal size and frequency. The imposed restriction, especially in the earlier postoperative period, leads to a more liquid diet with decreased fiber intake[14]. While adaptive processes towards a stable metabolism and energy intake take at least 1 year[15], patients rarely benefit from an extensive evaluation prior to 6 mo postoperatively.

There are inconsistent data about postoperative food consumption, mainly because of self-reporting, incongruent assessment time points, changing food selection over time, and influence of nutritional counselling[16]. Overall energy intake is reduced after surgery; however, the proportion of fat, proteins and carbohydrates seems to remain constant[17]. In our practice, we observe an increased consumption of dietary and fat-reduced products in a purpose to eat "healthier". However, the use of non-absorbable sweeteners such as sorbitol in these products can lead to similar effects as carbohydrate malabsorption[18].

BARIATRIC PROCEDURES

SG

Sleeve gastrectomy has become the most popular bariatric procedure in the last years. The greater curvature of the stomach is resected alongside a bougie preserving around 5 cm of antrum (Figure 1).

Proximal and distal RYGB

RYGB was the most popular procedure for years. A small gastric pouch is created followed by a pouch-jejunostomy, thereby forming an alimentary Roux-Y limb of up to 150 cm and a biliopancreatic limb of around 50 cm resulting in a common channel of various lengths depending on the length of whole small bowel. In distal RYGB, the length of the common channel, mostly around 100 cm, is the determined factor and the alimentary limb is of variable length (Figures 2 and 3).

BPD-DS

The gastric volume is reduced akin to a LSG but with a generally bigger bougie size. The duodenum is transected near the pylorus and a duodeno-jejunostomy is created to form a common channel of around 80 cm.

Adjustable gastric banding

Adjustable gastric banding (AGB) experienced a massive decline in the past decade due to a failure of long-term weight loss. Currently it is used almost exclusively concomitant to or after RYGB to reduce pouch ex-tensibility. Apart from vagotomy as intraoperative

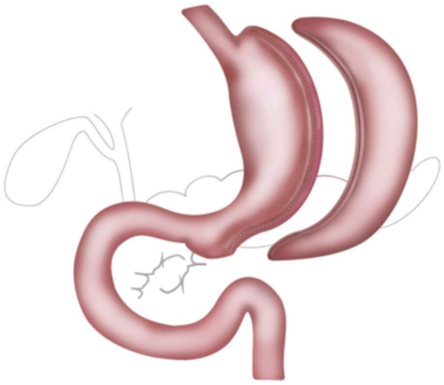

Figure 1 Laparoscopic sleeve gastrectomy. The greater curve of the stomach is resected alongside a bougie.

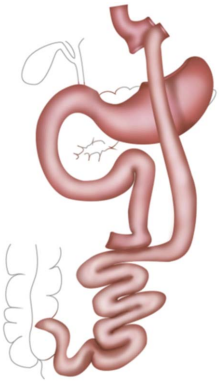

Figure 2 Proximal Roux-Y gastric bypass. The stomach is divided to form a small gastric pouch. Alimentary Roux-Y limbs of up to 150 cm and a biliopancreatic limbs of around 50 cm are formed; the resulting common channel is of various length depending on length of the whole small bowel.

complication, it has little influence on stool consistency, and when, patients rather tend to constipation[14].

BOWEL HABIT CHANGES AFTER BARIATRIC SURGERY

There is a consistent relationship between obesity and diarrhea[19]. The incidence of diarrhea in a preoperative bariatric population is around 8%[20], being twice as high as in lean people. A possible reason for this might be a higher intake of poorly absorbed sugars[21]. Indeed, digestive symptoms in general, including diarrhea, are frequent among obese patients, both before and after bariatric surgery[22,23].

There is a change of bowel habits after every bariatric procedure, even though the severity of symptoms differs between the individual techniques. Up to 75% of patients suffer from alterations of bowel habits and faecal transit time after RYGB[20]. Diarrhea is a common symptom after RYGB[6,20,24], and usual after BPD[14,25]. Length of the common channel, *i.e.*, the amount of absorptive surface, seems to play a role, given the higher frequency of diarrhea in long limb/distal RYGB patients than after short limb/proximal

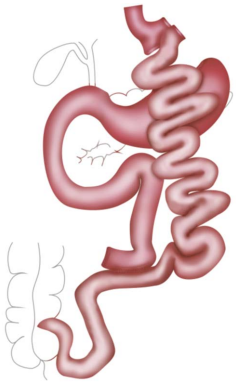

Figure 3 Distal Roux-Y gastric bypass. The same principles as in proximal RYGB are applied; however, the length of the common channel, mostly around 100 cm, is the determined factor and the alimentary limb is of variable length. RYGB: Distal Roux-Y gastric bypass.

RYGB procedures[26], in BPD compared to RYGB[25], and in BPD patients with shorter common channels[27].

SHORT BOWEL SYNDROME

Short bowel syndrome, defined by lack of absorptive surface, occurs in around 4% of patients after bariatric surgery[28]. The reported average small bowel length in obese patients ranges from 300 to 900 cm, with considerable variability between 230 cm and 1510 cm[29-31]. However, there is a remarkable intra- and interoperator variability when it comes to determination of bowel length, both in open and laparoscopic procedures[32,33]. In the most commonly performed technique, the proximal RYGB, only the lengths of the alimentary (AL) and the biliopancreatic limbs (BPL) are defined and counted through, whereas the common channel remains of variable, unknown size. While AL of 100-150 cm and BPL of 45-85 cm are commonly used[30,34,35], there is ongoing debate about the delineation of optimal limb lengths. Nonetheless, this debate does not respect absorptive capacity of small bowel and even less its adaptation over time. Even though there is considerable progress in assessing intestinal malabsorption, no direct test with sufficient sensitivity and specificity is currently available, not to mention the diagnostic issues due to the altered anatomy after RYGB[36]. Initial treatment consists of supportive measures; surgical options are lengthening of the common channel, enteral nutrition via gastrostomy into the gastric remnant, and restitution of normal anatomy if still possible[37]. In the United States, 6.3% of home parenteral nutrition patients have a history of bariatric surgery and over two-thirds of them underwent RYGB[38].

MALABSORPTION OF CARBOHYDRATES (LACTOSE, FRUCTOSE)

Carbohydrates were propagated in a low-fat diet for

years; they have a good palatability and are readily available in a high-caloric liquid form[39]. Liquids transit rapidly through the intestine and produce lesser satiety than the solid form. Sugar-sweetened beverages have a considerable impact on body weight, and there is clear association between their consumption and weight gain[40,41].

Even though a diet with a moderate amount of carbohydrates is recommended after bariatric surgery[42], the proportion of ingested macronutrients - lipids, proteins and carbohydrates - remains constant, whereas the overall energy intake that is reduced[18]. Lack of the enzyme lactase in the intestinal mucosa leads to lactose malabsorption and intolerance with diarrhea. While symptoms are dependent on small bowel transit time, there is a poor correlation between lactose malabsorption and intolerance[18,43]. Lactase activity is diminished progressively in adulthood[44], and the influence of bariatric surgery on this process in unclear[6]. Furthermore, there is no data on symptoms after bariatric surgery in a population of non-Western European heritage, *i.e.,* with a higher prevalence of lactase deficiency. On the other hand, a Scandinavian study found a lactose intolerance rate of 30% after jejuno-ileal bypass[45].

Fructose as monosaccharide is widely used as a sweetener in fat-reduced, low-caloric food. In contrast to the disaccharide form, its glucose-independent absorption capacity is limited[46]. Hydrogen breath tests can be used in the diagnosis of carbohydrate malabsorption. However, 18% of patients of European descent are hydrogen non-excretors resulting in a possible false-negative result. Furthermore, the effect of excluded small bowel segments on the test accuracy has not yet been elucidated. Abstaining from or at least reducing lactose and fructose in meals might be most productive for both diagnosis and therapy, even though enzyme replacement therapies, as referred to as lactase and xylose isomerase, are available[18].

PROTEIN-LOSING ENTEROPATHY

Micronutrients are absorbed in the mid to distal jejunum[47]. BPD-DS and to a lesser extent distal RYGB exclude these segments and are associated with macro- and micronutrient deficiencies[9,48]. Hypoalbuminemia occurs in up to 18% of BPD-DS patients[49], further aggravated by a protein intake of half of the recommended amount of 60-120 g protein daily in bariatric patients[47,50]. Hypoalbuminemia is associated with severe diarrhea in every fourth patient ending up with the need for parenteral nutrition[51]. The associated pathogenesis resembles the Kwashiorkor-type malabsorption of severely undernourished children resulting in a reduced production of gastric acid, pancreatic atrophy, small intestinal bowel overgrowth (SIBO) and alterations of the gut microbiota[52]. Treatment is usually initiated by employing supportive measures, rehydration and volume replacement, together with parenteral feeding, akin to World Health Organization Treatment guidelines for children[53]. Alternatively, surgical measures, such as limb length reshaping and reversal of RYGB, may be considered.

ENDOCRINE DISORDERS

Endocrine causes for diarrhea are rather related to morbid obesity and its comorbidities than to bariatric surgery. Ninety percent of patients with type 2 diabetes mellitus (T2DM) are overweight or obese[54]. Metabolic/bariatric surgery, mainly RYGB, is gaining importance in the treatment of T2DM[4,55]. However, minimally symptomatic patients with T2DM may become symptomatic after RYGB. Furthermore, depending on preoperative duration and severity of T2DM, the relapse rate after a disease-free postoperative interval is reported to be up to 11%[56]. Several factors have an influence on diarrhea in T2DM-patients: dietetic, sugar-free food, association of T2DM to celiac disease[57], and T2DM-induced disturbance of the enteric nerve system leading to altered gut motility, again resulting in SIBO and exocrine pancreatic insufficiency (EPI).

Loss of weight and fat volume after bariatric surgery[58] may require adaptation of drug apportioning otherwise leading to postoperative overdosage, *e.g.,* of thyroid hormones. Thus, switch from LSG to LRYGB for gastroesophageal reflux disease requires monitoring despite absent weight change, as L-thyroxine is absorbed in the (partly excluded) small bowel[59].

MICROBIOTA AND SIBO

In human adults, the gut microbiota is a complex and dynamic ecosystem that coevolves with its host[60], and remains remarkably constant slightly fluctuating around an individual core of stable colonisers. Low diversity of an individual's fecal bacteria is associated with a more pronounced overall obesity and dyslipidemia, impaired glucose homeostasis, and considerable low-grade inflammation[61]. Dietary changes, use of proton pump inhibitors or (recurrent as well as short- and long-term) antibiotic treatments may result in transient alterations of the gut microbiota composition[62,63]. It is still a matter of debate whether dietary intake or host genetics exert the stronger influence on microbial composition[64]. Not only development of obesity but also body weight reduction following bariatric surgery is, at least partly, attributed to alterations in gut microbiota. RYGB, as against SG, was demonstrated to substantially diminish the diversity of gut microbiota[65] paralleled by an increase in the proportion of Gammaproteobacteria and a decrease in Clostridia[66,67]. SG, however, was shown to cause a change in the Bacteroidetes/Firmicutes ratio, indeed, with a distinct increase in Bacteroidetes and a decline in the abundance of Firmicutes[68]. While the exact mechanisms remain unclear, change of the individual's microbiota composition is considered to be

a key factor of postoperative body weight reduction and may be one of the potential contributors to a stable weight loss after bariatric surgery[65,69]. Even though one would assume the importance of the microbiome regarding occurrence and/or resolution of diarrhea after bariatric surgery, this has not yet been elucidated. Bacteroides, normally increased after SG, was found to be substantially decreased in patients with idiopathic chronic diarrhea[70]. These results were confirmed by another group reporting an enrichment of Bacteroides, among other phyla, in controls when compared to diarrhea cases, irrespective of whether they were or not Clostridium difficile-associated[71]. These results cast a possible relationship between the normally observed post-bariatric shift of Bacteroides within the microbiota composition and diarrhea into doubt. Further studies addressing this question are warranted.

SIBO, defined as an excessive amount of bacteria in the small bowel[72], has a prevalence of 2.5% in healthy subjects[73], and up to 41% in obese patients, probably due to an impaired small intestinal motility[74]. In fact, side-side anastomoses with longer blind ends such as in candy cane syndrome are susceptible to SIBO[75,76]. A Brazilian group described a frequent occurrence of bacterial overgrowth in both the gastric pouch and the gastric remnant after RYGB in morbidly obese subjects, when assessed after a mean follow-up of 7.3 years[77]. These patients, however, did not complain of consistent or prolonged symptoms suggestive of SIBO, namely diarrhea, malabsorption, abdominal pain, intestinal obstruction, or extradigestive complaints (polyarthritis, dermatologic abnormalities, progressive liver insufficiency), after a mean follow-up of almost 15 years[78]. Another group reported a more than two-fold rise of SIBO after RYGB. Yet, weight loss itself does not seem to favor SIBO, given the comparable rate of bacterial overgrowth before and after exclusively restrictive surgery, such as AGB[23]. The influence of proton pump inhibitors, caloric intake and dietary composition is another topic of debate[23,79]. Diagnostic insecurities and a high prevalence of SIBO complicate the exact determination of its effects. In fact, it was shown that two thirds of patients after RYGB had digestive symptoms, but none of those were more frequent in patients with SIBO[23]. Consequences of SIBO after bariatric surgery are unclear. The nutrients escaping digestion in the small bowel due to SIBO might yield elevated levels of short- and medium-chain fatty acids through metabolization in the large bowel, implying a higher caloric uptake[80,81]. However, data on expected resulting reduced weight loss is conflicting[23,82]. The altered anatomy with excluded small bowel segments severely inflicts diagnostic measures; aspiration and culture might be impossible despite advanced endoscopic techniques, and breath testing underlies the same restrictions[83].

CLOSTRIDIUM DIFFICILE

Alteration of gastrointestinal climate caused by obesity, antibiotic therapy or surgery is a risk factor for clostridium-associated colitis[84], even though it may occur in absence of the aforementioned factors[23]. Furthermore, it may present as a protein-losing enteropathy with hypoalbuminemia without fulminant inflammation[85]. Individual types of stool tests can yield the diagnosis. However, the high rate of asymptomatic carriers demands for a combination of symptoms and positive test results[84]. Treatment aims at reestablishing a diverse microbial flora. Long-standing medical wisdom suggested treatment with only oral antibiotics, metronidazole rather than vancomycin, and avoiding antimotility agents such as loperamid. The latter lacks substantive data[86]; the former must be questioned, at least in post-RYGB and -BPD-patients. In view of the anatomic alterations after bariatric surgery and given the pharmacokinetics of metronidazole - it is almost completely absorbed in the small bowel - intravenous vancomycine might the preferred primary treatment option[87,88]. Fecal microbiota transplantation is a novel method to treat recurrent infections, and has also gained interest in the bariatric community due to its effects on weight loss[89].

ADDICTIVE DISORDERS

An "addiction-transfer" away from food may be an explanation for the increased number of impulse control disorders after bariatric surgery[90]; a quarter of the bariatric population has an eating disorder that impedes weight loss[91]. Amongst other substances, alcohol and nicotine both lead to diarrhea when consumed in excessive amounts[92]. Several cohort studies showed increased risk for alcohol abuse after bariatric surgery[93], further complicated by a faster rise of blood alcohol concentration[94]. Preoperative history of substance misusage is associated with postoperative abuse, as for alcohol ranging up to 12%[95,96]. Furthermore, consumption of excessive amounts of (sugar-free) drops, sometimes with the purpose of covering halitosis, must be kept in mind.

VAGOTOMY

Vagotomy during RYGB can be performed either intentionally to enhance weight loss *via* earlier satiety and lessened food intake[97,98], as esophageal lengthening procedure concomitant to hiatal hernia repair[99] or inadvertently as intraoperative complication due to the proximity of the vagal nerves to the gastric pouch[100]. Diarrhea occurs in around 10% of patients after truncal and to a lesser extent after more distal vagotomy; severe, debilitating diarrhea occurs in 2%-3%[101]. Controversially, intentional vagotomy to

gain esophageal length in hiatal hernia repair did not lead to higher rates of side effects[98,99]. Reasons for postvagotomy diarrhea are not completely elucidated, a change of microbial climate by altered intestinal motility and gastric hypoacidity certainly plays a role[102]. Beneath the usual dietary modifications proposed for RYGB, bulking agents to decrease the water content of the stool should be introduced. A short-term trial with octreoid can be attempted, however, isolated postvagotomy diarrhea is less responsive than dumping syndrome. In severe cases, surgical options such as alteration of limb lengths or even reversal of RYGB should be discussed.

BILE ACID MALABSORPTION

The role of bile acids in morbid obesity, its comorbidity and weight loss in post-bariatric patients experiences increased interest, as they seem to be profoundly involved in the postoperative metabolic improvement. The target of interest are FX- and TGR5-receptors and their influence on bile acid metabolism with subsequently increased hormonal - in particular incretine - answer and change of gut microbiota[103,104].

Bile acid malabsorption results from a disturbed enterohepatic cycle and bile acid production. About 95% of bile acids are reabsorbed in the ileum, an additional small percentage is absorbed in the colon with bowel motility, medication, microbiota and food composition as influencing factors[105-107]. FXR is involved in the regulation of bile acid production, in entero- and hepatocytes[108]. BAM is categorized as either idiopathic, secondary to ileal dysfunction, such as after resection, or unrelated to ileal dysfunction, mainly due to SIBO or cholecystectomy. A disturbed synthesis in idiopathic BAM might also play role in irritable bowel syndrome (IBS)-D patients, same as in post-cholecystectomy patients[109]. Cholecystectomy concomitant to a bariatric procedure was a standard of care in the last decades[110]. BAM might further be a cause for post-vagotomy diarrhea[102]. The short common channel after BPD and distal RYGB predisposes to BAM due to reduced reabsorption rate and diminished time for bile acids to exert its effects on digestion. So far, there is no data about the rate of BAM stratified for subtypes of bariatric procedures. Bile acid malabsorption can be detected by fecal bile acid quantification, radio-labelled Selenium homotaurocholic acid testing or determination of serum-C4-concentration[111]; however, they all are expensive, rather difficult to apply in clinical practice or not standardized. Cholestyramine, a bile-acid binder, is an effective treatment with an efficacy up to 96%[112]. Due to the downsides of the above-mentioned testing procedures, it is used in diagnosis in an empirical trial[12].

EXOCRINE PANCREATIC INSUFFICIENCY

Gastrectomy and RYGB both can lead to secondary EPI resulting in steatorrhea[34,113] with subsequent deficiencies of fat-soluble vitamins. Depending on type of RYGB, proximal or distal, the prevalence of EPI is up to 19%-48% respectively[34]. Changes in caloric content, composition and physical properties of meals after RYGB lead to a diminished, uncoordinated pancreatic response to nutrient stimuli[114]. The altered anatomy after RYGB leads to a shorter amount of contact time of enzymes with chyme. In addition, the degradation of pancreatic enzymes is accelerated in absence of food in the biliopancreatic limb[114]. In rare cases, left pancreatic resection for dumping syndrome or nesidioblastosis is performed, leading to primary endocrine and EPI[115]. Testing for EPI proves difficult due to altered anatomy. Direct stimulation tests require the the intubation of the duodenum either in a transgastric way or *via* a double-balloon technique and measure the exocrine pancreatic response after stimulation with CCK or secretin. Indirect stimulation test, such as measurement of fecal fat or elastase-1 are cheaper, more readily available but pose other problems. The former requires the ingestion of a defined amount of fat, which proves difficult after RYGB-induced, altered perception of food. In the latter, a normal result does not preclude EPI, as only the released amount of enzymes, but not its effect on chyme is measured; the shortened contact time within the common channel is not reflected. The recommended high-fat diet, based on lipids as strongest stimulators of exocrine pancreatic response, is hard to apply in post-RYGB patients[34]. Pancreatic enzyme replacement therapy is the mainstay of EPI treatment. Substitution is adapted to symptoms[114]; if using pancrealipase, removing the acid-resistant coating is imperative as the amount of produced acid in the gastric pouch is only minimal[34].

DUMPING SYNDROME

Diarrhea is one of many abdominal and systemic symptoms of mostly early dumping syndrome (DS), caused by a rapid exposure of the small bowel with undigested nutrients. Prevalence of DS after RYGB is reported to be up to 75%, and after SG up to 45%. Even though bothersome at least, DS is seen not seen as complication but rather as desirable feature by a few surgeons[116], it is thought to be an essential component of postoperative weight loss. Diagnosis relies mainly on symptom-based questionnaires like Sigstad's scoring system, together with an oral glucose tolerance test or a mixed-meal test. However, those tests have a high sensitivity, but low specificity and are complicated by the small gastric pouch[117]. First-line treatment of DS is directed at a change of diet towards a more fibre- and protein-rich regimen with a low proportion of rapid-absorbable carbohydrates. Dietary supplements such as pectin or glucomannan have a poor tolerability and may interfere with a post-bariatric diet[117]. Acarbose affects mainly late

DS, whilst diarrhea is associated rather with early DS. Somatostatin analogues are effective treatment options for both early and late DS, however, the have diarrhea as common side effect. Total parenteral nutrition is less practical, but sometimes inevitable option, other surgical possibilities consist in either remnant gastrostomy, reversal of RYGB or measure to enhance restriction of the pouch, such as AGB or pouch reshapings, only in very rare cases pancreatic resection have to be performed[118,119].

UNMASKED UNDERLYING INTESTINAL DISEASES

Inflammatory bowel disease

The cardinal symptom of both, Crohn's disease and ulcerative colitis is diarrhea. It is unclear whether obesity and the subsequent proinflammatory state have a role in the pathogenesis of Crohn's disease[120]. Even though bariatric surgery leads to a normalization of this state, bacterial overgrowth might lead to a local activation of innate immune factors favoring inflammation[121]. Whether bariatric surgery is of benefit remains to be elucidated, case series show a favorable outcome with LSG as procedure of choice[122-126]. Even though in most instances inflammatory bowel disease will be known prior to surgery, it might be missed in pre-operative evaluation as it might occur later or is not captured, as morbid obesity seems to be associated with more colonic disease[127]. Of note, fecal calprotectin as measure for tissue inflammation is elevated after bariatric surgery[128].

Celiac disease

Approximately 40% of patients suffering from celiac disease (CD), traditionally associated with malabsorption and insufficient body weight, are overweight or obese at diagnosis[129,130]. They, therefore, could be potential candidates for bariatric surgery. Diarrhea is classically the hallmark of symptomatic coeliac disease. However, a trend toward silent or atypical forms has been observed[131]. Triggers including pregnancy, traveller's diarrhea, gastroenteritis, and some type of gastrointestinal surgery were reported[132]. Indeed, a recent publication described the rapid onset of CD after bariatric surgery, *i.e.*, a duodenal switch procedure[133]. After all, CD should be considered as a differential diagnosis in patients presenting with persistent diarrhea after bariatric surgery.

A group of Italian surgeons suggested a preoperative work-up of specific CD tests (anti-endomysial and antitransglutaminase antibodies and total Immunoglobulin A) before bariatric procedures. Diagnosis of CD, consisting of harvesting duodenal biopsies is hindered after RGB due to the anatomical changes involved. In these cases, exlusively serologic testing remains an option. Indeed, a negative CD specific serology does not completely exclude the diagnosis of CD though it does make it much less likely[134]. The standard treatment of CD implementing a gluten-free diet (GFD) was shown to be successful in bariatric patients, either. In this line, a complete restoration of the intestinal muscosa within 12 mo after starting GFD was observed in a young adult with an incidentally diagnosed silent form of CD after bariatric surgery (vertical banded gastroplasty) 5 years earlier[135]. However, apart form a few case reports, little is known about onset, course, diagnosis and management of CD following bariatric surgery, particularly sleeve gastrectomy and RGB.

IBS

A prevalence of up to 30% of IBS fulfilling the ROME Ⅲ criteria among morbidly obese patients was reported. These patients are more likely to suffer from profound alterations to quality of life and severe psychological disturbances than those without IBS[136,137]. On the other hand, a recent study of a large cohort showed that obesity is protective of a diagnosis or worsening of IBS[138]. Visceral adiposity, rather than general obesity, has been associated with an increased risk of diarrhea dominant IBS[139]. However, the association between the two entities remains unclear[140]. Increase of intra-abdominal pressure owing to excess of visceral fat, local tissue as well as systemic inflammation mediated by adipokines and cytokines originating from abdominal adipocytes[141-143], and altered gut microbiota[144] have been suggested as possible mechanisms that link obesity and IBS. Bariatric surgery, *i.e.*, RGB, may improve the IBS symptoms[137]. Yet, the impact of bariatric surgery on visceral hypersensitivity and outcome of IBS is still unknown. Nevertheless, it might be advisable to systematically screen IBS and other functional bowel disorders in patients eligible for bariatric surgery.

CONCLUSION

There are a multitude of reasons for diarrhea in post-bariatric patients. Diagnosis can be challenging, as they are often intertwined and the influence of inconsistent, mood-dependent elements, must not be underestimated. The special anatomy after RYGB and BPD with excluded bowel segments complicates testing and the interpretation of results. Thus, empiric therapy of limited time will help in diagnosis and treatment.

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ORIGINAL ARTICLE

Basic Study

Fibrinogen deficiency suppresses the development of early and delayed radiation enteropathy

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Abstract

AIM

To determine the mechanistic role of fibrinogen, a key regulator of inflammation and fibrosis, in early and delayed radiation enteropathy.

METHODS

Fibrinogen wild-type ($Fib^{+/+}$), fibrinogen heterozygous ($Fib^{+/-}$), and fibrinogen knockout ($Fib^{-/-}$) mice were exposed to localized intestinal irradiation and assessed for early and delayed structural changes in the intestinal tissue. A 5-cm segment of ileum of mice was exteriorized and exposed to 18.5 Gy of x-irradiation. Intestinal tissue injury was assessed by quantitative histology, morphometry, and immunohistochemistry at 2 wk and 26 wk after radiation. Plasma fibrinogen level was measured by enzyme-linked immunosorbent assay.

RESULTS

There was no difference between sham-irradiated $Fib^{+/+}$ and $Fib^{+/-}$ mice in terms of fibrinogen concentration in plasma and intestinal tissue, intestinal histology, morphometry, intestinal smooth muscle cell proliferation, and neutrophil infiltration. Therefore, $Fib^{+/-}$ mice were used as littermate controls. Unlike sham-irradiated $Fib^{+/+}$ and $Fib^{+/-}$ mice, no fibrinogen

was detected in the plasma and intestinal tissue of sham-irradiated $Fib^{-/-}$ mice. Moreover, fibrinogen level was not elevated after irradiation in the intestinal tissue of $Fib^{-/-}$ mice, while significant increase in intestinal fibrinogen level was noticed in irradiated $Fib^{+/+}$ and $Fib^{+/-}$ mice. Importantly, irradiated $Fib^{-/-}$ mice exhibited substantially less overall intestinal structural injury (RIS, P = 0.000002), intestinal wall thickness (P = 0.003), intestinal serosal thickness (P = 0.009), collagen deposition (P = 0.01), TGF-β immunoreactivity (P = 0.03), intestinal smooth muscle proliferation (P = 0.046), neutrophil infiltration (P = 0.01), and intestinal mucosal injury (P = 0.0003), compared to irradiated $Fib^{+/+}$ and $Fib^{+/-}$ mice at both 2 wk and 26 wk.

CONCLUSION

These data demonstrate that fibrinogen deficiency directly attenuates development of early and delayed radiation enteropathy. Fibrinogen could be a novel target in treating intestinal damage.

Key words: Radiation enteropathy; Knockout mouse model; Fibrinogen; Inflammation; Fibrosis; Ionizing radiation

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Core tip: Fibrinogen, a plasma protein, and fibrin (breakdown product of fibrinogen) induce inflammation, and fibrosis. Suppression of coagulation, inflammation, and fibrosis attenuate intestinal radiation injury. While fibrinogen has been presumed to be involved in intestinal fibrosis development, a direct role has only been supported by indirect evidence. We investigated the direct role of fibrinogen deficiency in early and delayed intestinal radiation injury. Radiation caused less intestinal injury in mice deficient in the fibrinogen gene, than in mice bearing two or one wild type fibrinogen alleles. We conclude that fibrinogen is essential for full-blown intestinal radiation fibrosis to occur.

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INTRODUCTION

The intestine is an important dose-limiting organ during radiation therapy of abdominal and pelvic tumors. Despite advances in radiation delivery techniques having substantially reduced intestinal exposure and clinical bowel toxicity, early intestinal radiation toxicity (early radiation enteropathy) remains a significant clinical problem resulting in treatment delays, increased patient hospitalization rates and short term morbidity. Also, delayed radiation enteropathy continues to adversely affect the quality of life of a large number of long-term cancer survivors[1,2].

Radiation enteropathy is classified as early or delayed, depending on when it presents relative to radiation therapy. Early radiation enteropathy occurs during or within weeks of completion of radiation therapy. The cause is clonogenic crypt cell death, disruption of the epithelial barrier, and mucosal inflammation. Delayed radiation enteropathy is a chronic condition, characterized mainly by vascular sclerosis and progressive intestine wall fibrosis[1,2]. Clinical and experimental data from our laboratory show that intestinal radiation toxicity is associated with marked endothelial dysfunction, interstitial accumulation of enzymatically active thrombin, and interstitial fibrin deposition[3-5].

Fibrinogen, a plasma protein, and fibrin (breakdown product of fibrinogen), in addition to its known role in blood clotting, is considered a key modulator of tissue injury and inflammation[6-8], as well as in fibrosis development[9-13]. However, the putative significance of fibrinogen in radiation-induced inflammation and fibrosis is mostly based on correlative observations, and the results with pharmacological modulators of coagulation have been inconclusive. The availability of fibrinogen-deficient mice provides the means to directly evaluate the contribution of fibrinogen to radiation-induced intestinal injury. We used fibrinogen knockout ($Fib^{-/-}$), heterozygous ($Fib^{+/-}$), and homozygous ($Fib^{+/+}$) mice to determine the role of fibrinogen in radiation-induced early and delayed intestinal injuries. The results showed that the lack of fibrinogen was associated with a significant protection from radiation-induced early and delayed intestinal injuries, thus confirming the role of fibrinogen.

MATERIALS AND METHODS

Mice

A α-chain fibrinogen-deficient ($Fib^{-/-}$) male and fibrinogen-heterozygous ($Fib^{+/-}$) female breeder mice were a gift from Dr. Jay L Degen (Cincinnati Children's Hospital, Cincinnati, OH, United States). Breeding was performed at Department of Laboratory Animal Medicine, University of Arkansas for Medical Sciences. The animals were housed in conventional cages with free access to tap drinking water and standard mouse chow (TD8640, Harlan Teklad, Madison, WI, United States). A pathogen-free environment with controlled humidity, temperature, and 12-12 h light-dark cycle was maintained. All experimental protocols were approved by the University of Arkansas for Medical Sciences Institutional Animal Care and Use Committee.

$Fib^{-/-}$ mice lack all components of fibrinogen in the circulation[14]. Because A α-chain of fibrinogen is not the rate-limiting step for fibrinogen biosynthesis in normal mice, heterozygous ($Fib^{+/-}$) mice maintain

plasma fibrinogen concentrations that are 75%-100% of that in wild-type ($Fib^{+/+}$) mice[15]. Both male and female mice were included in the study. Genotype analysis of mice was performed by mouse tail biopsy and PCR as described elsewhere[14]. Briefly, approximately 2-3 mm mouse tail was incubated with direct PCR Lysis Reagent (Viagen Biotech, Los Angeles, CA, United States) including freshly prepared proteinase K at 55 ℃ overnight and 2 μL of lysate was used per PCR reaction. The sequences of three primers were used in PCR reaction (kindly provided by Dr. Jay L Degen, Cincinnati Children's Hospital, Cincinnati, OH, United States). 1). 5'-TAT TAC CAG TGA ATC TTT GTC AGC AG-3', 2) 5'- TGC TGG ATC AAT CCC CAG CAA CCG TGA GAG-3', and 3) 5'- GCT TCA GCT CCA GTT CTC CTC ATG AGC CAT-3'.

Experimental model, irradiation, and tissue procurement

Mice were between 10 and 15 wk of age at the initiation of experiments. Mice were anesthetized with 60 mg/kg sodium pentobarbital administered intraperitoneally (Abbott Laboratories, Chicago, IL, United States). A 5 cm segment of ileum, located 10 cm from the ileocecal junction, was exteriorized through a midline abdominal incision and marked for future identification with a tantalum clip on the mesentery. The mice were placed on a heating pad (maintained at 38 ℃). The exteriorized ileum was covered with saline-moistened gauze and exposed to 18.5 Gy localized single dose x-irradiation using a Seifert Isovovolt 320 X-ray machine (Seifert X-Ray Corp., Fairview Village, PA) operated at 250 kVp and 15 mA with 3 mm aluminum added filtration. The half-value layer was 0.85 mm Cu, and the dose rate was 4.49 Gy/min. Our previous experience with localized intestinal radiation model in mice suggests that 18.5 Gy radiation dose causes consistent structural, cellular, and molecular changes. After irradiation, the ileum was replaced into the abdomen and the incision was closed with 5-0 polypropylene. Antimicrobial prophylaxis was not used. Each strain of mice ($Fib^{+/+}$, $Fib^{+/-}$, $Fib^{-/-}$) was randomly assigned into 2 groups (2 wk and 26 wk), which represent, respectively, early (acute) and delayed (chronic) radiation enteropathy in our model system[16]. This model allows for accurate delineation and dosimetry to the exposed intestinal segment, whereas, the rest of the intestine is not exposed to radiation.

Groups of mice were euthanized 2 wk and 26 wk after irradiation. Specimens of irradiated and un-irradiated intestine were procured and fixed in methanol-Carnoy's solution for histological and immunohistochemical studies and snap-frozen for fibrinogen enzyme-linked immunosorbent assay (ELISA) analysis.

Quantitative histopathology and morphometry

Radiation injury score: The overall severity of structural radiation injury was assessed using the radiation injury score (RIS) system. The RIS is a composite histopathological scoring system that provides a global measure of the severity of structural radiation injury. It has been extensively used and validated in our laboratory[16,17]. Briefly, we assessed and graded (from 0-3) seven histopathologic parameters of radiation injury (mucosal ulcerations, epithelial atypia, subserosal thickening, vascular sclerosis, intestinal wall fibrosis, ileitis cystica profunda, and lymph congestion). The sum of the scores for the individual alterations constitutes the RIS. All specimens were evaluated in a blinded fashion by two separate researchers.

Mucosal surface area: Mucosal surface area was measured in vertical sections using a stereologic projection/cycloid method as described by Baddeley *et al*[18] and adapted by us for use in our model system[19]. The advantage of this technique is that it does not require assumptions about the shape or orientation distribution of the specimens and thus circumvents problems associated with most other procedures for surface area measurement.

Thickness of the intestinal wall and subserosa: Intestinal wall thickening is a measure of both reactive intestinal wall fibrosis and intestinal smooth muscle cell hyperplasia. In contrast, subserosal thickening reflects mainly reactive fibrosis. Intestinal wall thickness (encompassing submucosa, muscularis externa, and subserosa) and subserosal thickness were measured with computer-assisted image analysis (Image-Pro Plus, Media Cybernetics, Silver Spring, MD). All measurements were done with a 10 × objective lens. A total of 5 areas, 500 μm apart, were chosen for the measurement, with 3 measurements taken per area. The average of all 5 areas was used as a single value for statistical calculations[20].

Quantitative immunohistochemistry, histochemistry and image analysis

Immunohistochemical staining was performed with standard technique using avidin-biotin complex, diaminobenzidine chromogen, and hematoxylin counterstaining. Appropriate positive and negative controls were included. The primary antibodies, incubation times, dilutions, and sources were as follows: polyclonal anti-myeloperoxidase antibody (MPO), 2 h, 1:100 dilution, Dako (Carpinteria, CA); monoclonal antibody against proliferating cell nuclear antigen (PCNA), 2 h, 1:100 dilution, Calbiochem (Cambridge, MA), and polyclonal antibody against transforming growth factor-β (TGF-β), 2 h, 1:300 dilution, R&D (Minneapolis, MN).

Computer-assisted immunohistochemistry and histochemistry image analysis (Image-Pro Plus, Media Cybernetics, Silver Spring, MD) were used to assess the following established indicators of intestinal radiation injury: (1) neutrophil infiltration;

(2) proliferation of intestinal smooth muscle cells; (3) deposition of collagen in the intestinal wall; and (4) expression of extracellular matrix-associated TGF-β as described in detail and validated previously[20].

Neutrophil infiltration: The number of myeloperoxidase-positive cells was determined by color thresholding and counting in 20 fields at 40 × magnification, selected according to a predetermined grid pattern. Smooth muscle cell proliferation: Intestinal smooth muscle cell proliferation was assessed in the smooth muscle layer of intestine (muscularis propria). The numbers of total smooth muscle cells and PCNA-positive smooth muscle cells were determined in 20 fields at 40 × magnification using color thresholding and normalizing PCNA-positive smooth muscle cells per thousand smooth muscle cells. Collagen deposition: Masson's trichrome 2000 stain kit (American Master Tech, Lodi, CA) was used for collagen staining to evaluate collagen deposition. The percentages of areas (relative to the total intestinal wall area) positive for collagen were determined in 20 fields (under 40 × magnification), according to the procedure established by Raviv *et al*[21] and adapted to our model system[22].

TGF-β immunoreactivity: Areas relatively positive for TGF-β were determined in 20 fields (under 40 × magnification) according to the method described by Raviv *et al*[21] and adapted to our model system[22].

Fibrinogen analysis by ELISA and localization by immunohistochemical staining

Quantitative determination of fibrinogen in mouse plasma and small intestinal samples was done by double antibody sandwich ELISA method following manufacturer's instructions (Kamiya Biomedical Company, Seattle, WA). For plasma preparation, whole blood was collected and centrifuged at 2000 × *g* for 15 min at 4 ℃. Following the centrifugation, the supernatant (plasma) was collected and stored at -20 ℃ until further use. For intestinal tissue sample preparation, frozen tissues were homogenized in ice-cold PBS containing protease inhibitors and sonicated using cell disrupter. The samples were centrifuged at 2000 x *g* for 15 min at 4 ℃ and the supernatants were taken for ELISA procedure. The total protein concentration was first quantified using Bicinchoninic Acid protein assay kit following the manufacture's protocol (Pierce Biotechnology, Rockford, IL, United States).

Samples and standards were added to appropriate polystyrene microtiter wells pre-coated with anti-fibrinogen antibodies. After incubation and following extensive washing steps to remove any unbound proteins, Horseradish Peroxidase conjugated anti-fibrinogen antibody solution was added to the wells and incubated. After another extensive washing step, the enzyme bound to the immunosorbent was assayed by the addition of chromogenic substrate 3,3′,5,5′-Tetramethylbenzidine and was measured at a wavelength of 450 nm. The quantity of bound fibrinogen was proportional to the magnitude of the absorbance at 450 nm and thus, was a measure of concentration of fibrinogen in the test samples.

The localization of fibrinogen in the intestinal tissue was performed by immunohistochemical staining. Rabbit anti-mouse fibrinogen antibody (kindly gifted by Dr. Jay L Degen) was incubated for 2 h at 1:100 dilution.

Statistical analysis

Sample size calculation was performed with PASS 2000 for Windows (NCSS, Kaysville, UT). Differences between experimental groups and variability for the early and delayed endpoints was derived from similar experiments conducted in our laboratory and used for calculations, making sure statistical power was at least 0.8. Statistical analyses were performed with the software package NCSS2007 for Windows (NCSS, Kaysville, UT). Differences in endpoints between two groups were assessed with Equal-Variance T-Test of two samples *t*-Test. Differences in endpoints among the groups Fixed factor analysis of variance (different times and different mice such as $Fib^{+/+}$, $Fib^{+/-}$, or $Fib^{-/-}$ mice) was performed with ANOVA General Linear Models analysis. A *P* value less than 0.05 was considered statistically significant.

RESULTS

Determination of genotype from tail biopsy and fibrinogen level by ELISA in plasma and intestinal tissue of sham-irradiated mice

Genotype of the offspring were determined by identifying the number and position of DNA bands in agarose gel. Single band of about 283 bp region indicates that the alleles are truncated, which means homozygous null ($Fib^{-/-}$) genotype, single band at about 376 bp region signifies endogenous allele that means wild-type ($Fib^{+/+}$) genotype, and double bands, one at 283 bp region and the other at 376 bp region indicate heterozygous ($Fib^{+/-}$) genotype (Figure 1A).

Quantitative determination of fibrinogen levels was performed by ELISA. In plasma, fibrinogen was not detected in $Fib^{-/-}$ mice, while fibrinogen was easily detected in the plasma of $Fib^{+/+}$ and $Fib^{+/-}$ mice. There was no significant difference in plasma fibrinogen levels between $Fib^{+/+}$ and $Fib^{+/-}$ mice (Figure 1B). Similarly, in intestinal tissue, fibrinogen was not detected in $Fib^{-/-}$ mice, whereas, fibrinogen was detected in $Fib^{+/+}$ and $Fib^{+/-}$ mice. Again, there was no significant difference between in $Fib^{+/+}$ and $Fib^{+/-}$ mice in terms of intestinal tissue fibrinogen levels (Figure 1C).

Effects of radiation on intestinal fibrinogen level in $Fib^{+/+}$, $Fib^{+/-}$ and $Fib^{-/-}$ mice

Intestinal fibrinogen level was measured by immuno-

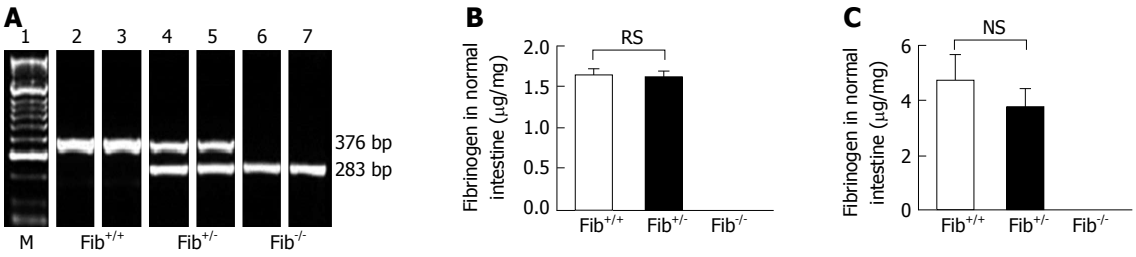

Figure 1 PCR analysis of mouse genotype and fibrinogen levels in plasma and intestinal tissue of Fib$^{+/+}$, Fib$^{+/-}$ and Fib$^{-/-}$ mice. A: Representative agarose gel image showing DNA marker (Lane 1), single band at around 376 base pair (Lane 2 and 3) that denotes Fib$^{+/+}$ mice, double bands, one at around 376 bp and the other is at around 283 bp (Lane 4 and 5), which signify Fib$^{+/-}$ mice, and single band at around 283 bp (Lane 6 and 7) that represents Fib$^{-/-}$ mice; B: Plasma fibrinogen level in un-irradiated Fib$^{+/+}$ (n = 5), Fib$^{+/-}$ (n = 6) and Fib$^{-/-}$ (n = 5) mice as detected by enzyme-linked immunosorbent assay (ELISA) assay; C: Fibrinogen level in the intestinal tissue of un-irradiated Fib$^{+/+}$ (n = 6), Fib$^{+/-}$ (n = 10) and Fib$^{-/-}$ (n = 3) mice as detected by ELISA assay.

histochemical staining. Fibrinogen immunoreactivity recognizes both fibrinogen and fibrin mainly located in the intravascular space, endothelial surface, connective tissues of the lamina propria, submucosa, muscularis, and serosa in the intestinal sections. Fibrinogen was detected in the intestinal tissue of sham-irradiated Fib$^{+/+}$ and Fib$^{+/-}$ mice, however, no significant difference in fibrinogen immunoreactivity was observed. In un-irradiated intestine of Fib$^{-/-}$ mice, fibrinogen immunoreactivity was undetectable (Figure 2A-C).

Like previously published studies, we used Fib$^{+/-}$ mice as littermate controls for Fib$^{-/-}$ mice[11,23,24]. Irradiation significantly increased intestinal fibrinogen level in both Fib$^{+/+}$ (P = 0.0004) and Fib$^{+/-}$ mice (P < 0.0001) but no detectable increase in fibrinogen level was observed in Fib$^{-/-}$ mice after radiation exposure, compared to the respective sham-irradiated groups. (Figure 2D-G). However, the increase in intestinal fibrinogen level between irradiated Fib$^{+/+}$ and Fib$^{+/-}$ mice was not significantly different (Figure 2G).

Effects of fibrinogen deficiency in early and delayed radiation-induced intestinal structural injury

Similar to previous studies performed in our laboratory[19], irradiated intestine exhibited epithelial atypia, reactive thickening of the subserosa and mucosal ulceration at 2 wk, and chronic ulceration, vascular and intestinal wall fibrosis at 26 wk. Compared with Fib$^{+/-}$ mice, Fib$^{-/-}$ mice showed much less intestinal radiation structural injury (RIS) (F-Ratio: 27.95, P = 0.000002), less intestinal wall thickening (F-Ratio: 9.50, P = 0.003) and serosal thickening (F-Ratio: 7.30, P = 0.009) at 2 wk and 26 wk after radiation (Figure 3A-C).

Effects of fibrinogen deficiency on early and delayed radiation induced intestinal inflammation and intestinal mucosal injury

Enhanced myeloperoxidase activity is a well-documented inflammation marker. Myeloperoxidase enzymatic activity in leukocytes correlates directly with neutrophil number (r = 0.99) and myeloperoxidase activity in tissue extract correlates directly with cellular infiltration (r = 0.94)[25]. Un-irradiated intestine from Fib$^{+/-}$ and Fib$^{-/-}$ mice showed few MPO immunoreactive cells and there was no difference between Fib$^{+/-}$ and Fib$^{-/-}$ mice (P > 0.05) (supplemental Figure 1D). After irradiation, the number of MPO positive cells in the intestine of Fib$^{+/-}$ and Fib$^{-/-}$ mice increased significantly (P < 0.01) at 2 wk and 26 wk (data not shown). However, compared with Fib$^{+/-}$, Fib$^{-/-}$ mice showed significant decrease in the number of positive MPO cells at 2 wk and 26 wk (F-Ratio: 6.37, P = 0.01) as shown in Figure 4A-C.

A decrease in intestinal mucosal surface area is a sensitive parameter of small bowel radiation injury[19]. Compared to un-irradiated mice, irradiated mice showed significant decreased (P < 0.01) mucosal surface area in both Fib$^{+/-}$ and Fib$^{-/-}$ mice at 2 wk and 26 wk (data not shown). However, Fib$^{-/-}$ mice showed significant improvement (F-Ratio: 6.37, P = 0.01) in mucosal surface area at both 2 wk and 26 wk compared to Fib$^{+/-}$ mice (Figure 4D-F).

Effects of fibrinogen deficiency on early and delayed radiation-induced intestinal smooth muscle cell proliferation, collagen deposition, and TGF-β immunoreactivity

In the intestine, collagen is mainly produced by intestinal smooth muscle cells, rather than by fibroblasts. Intestinal smooth muscle cell proliferation rate is very low at baseline, but increases steeply after irradiation[26]. In the current study, PCNA was used as intestinal smooth muscle cell proliferation marker. Un-irradiated intestine of Fib$^{+/-}$ and Fib$^{-/-}$ mice showed low proliferation rate. After irradiation, the proliferation rate of intestinal smooth muscle cells increased significantly (P < 0.01) at 2 wk and 26 wk (data not shown). However, compared to Fib$^{+/-}$, Fib$^{-/-}$ mice showed a borderline significant decrease (F-Ratio: 4.19, P = 0.046) in the proliferation of intestinal smooth muscle cells at both 2 wk and 26 wk (Figure 5A-C).

Collagen is the major component of fibrous tissue. We previously showed that collagen accumulation is increased in the intestine after irradiation[27]. The current study is consistent with this observation. Radiation significantly increased (P < 0.01) collagen accumulation at both 2 wk and 26 wk in Fig$^{+/-}$ mice, as well as in Fib$^{-/-}$ mice compared to sham-irradiated

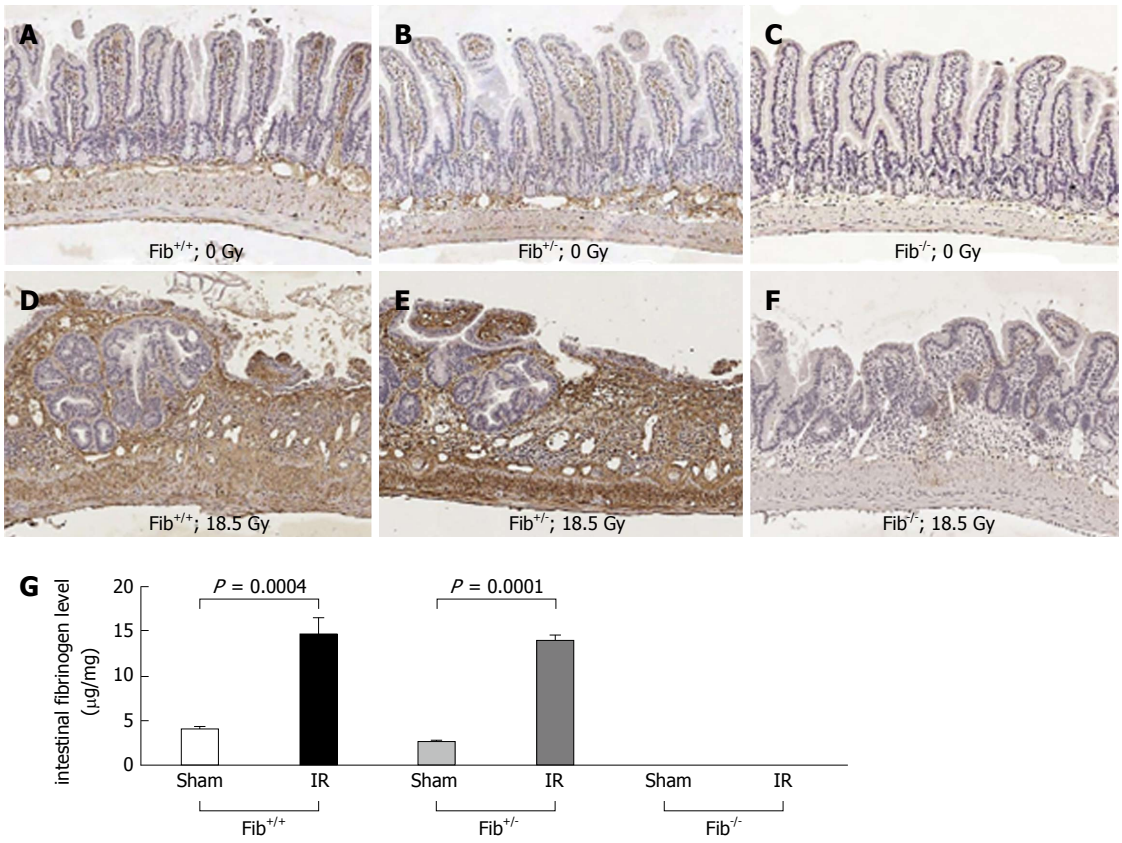

Figure 2 Fibrinogen immunoreactivity in the intestinal tissue of Fib$^{+/+}$, Fib$^{+/-}$ and Fib$^{-/-}$ mice before and after irradiation. A: Representative photomicrograph showing fibrinogen level in un-irradiated Fib$^{+/+}$ mice; B: Fibrinogen level in un-irradiated Fib$^{+/-}$ mice; C: Fibrinogen level in un-irradiated Fib$^{-/-}$ mice; D: Fibrinogen level in irradiated Fib$^{+/+}$ mice; E: Fibrinogen level in irradiated Fib$^{+/-}$ mice; F: Fibrinogen level in irradiated Fib$^{-/-}$ mice; and G: Column diagram showing comparison of intestinal fibrinogen levels among un-irradiated (n = 6) and irradiated (n = 6) Fib$^{+/+}$ mice, un-irradiated (n = 10) and irradiated (n = 10) Fib$^{+/-}$ mice, and un-irradiated (n = 3) and irradiated (n = 3) Fib$^{-/-}$ mice.

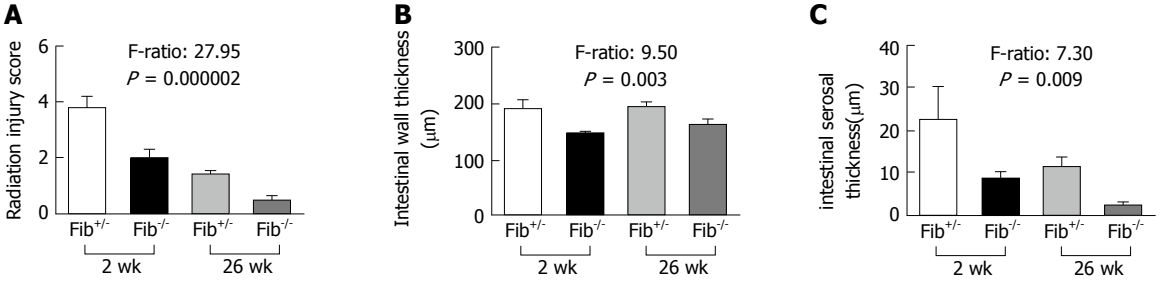

Figure 3 Morphological changes in the intestinal tissue of Fib$^{+/-}$ and Fib$^{-/-}$ mice observed at 2 wk and 26 wk after localized irradiation. A: Comparative assessment of structural radiation injury between Fib$^{+/-}$ (n = 15) and Fib$^{-/-}$ (n = 14) mice at 2 wk and Fib$^{+/-}$ (n = 28) and Fib$^{-/-}$ (n = 11) mice at 26 wk; B: Comparative assessment of intestinal wall thickness between Fib$^{+/-}$ (n = 14) and Fib$^{-/-}$ (n = 14) mice at 2 wk and Fib$^{+/-}$ (n = 24) and Fib$^{-/-}$ (n = 11) mice at 26 wk; C: Comparative assessment of intestinal serosal thickness between Fib$^{+/-}$ (n = 14) and Fib$^{-/-}$ (n = 14) mice at 2 wk and Fib$^{+/-}$ (n = 24) and Fib$^{-/-}$ (n = 11) mice at 26 wk.

groups (data not shown). However, in comparison to Fib$^{+/-}$, Fib$^{-/-}$ mice showed significant decrease (F-Ratio: 6.86, P = 0.01) in collagen accumulation at both 2 wk and 26 wk after radiation (Figure 5D-F).

TGF-β is overexpressed in many fibrotic conditions, including radiation fibrosis[28], and is mechanistically involved in radiation enteropathy[22]. Extracellular matrix-associated TGF-β staining was similar in the intestines of Fib$^{+/-}$ and Fib$^{-/-}$ mice before irradiation (data not shown). TGF-β immunoreactivity increased significantly (P < 0.01) in the intestine after radiation in both Fib$^{+/-}$ and Fib$^{-/-}$ mice compared to sham-irradiated mice at both 2 wk and 26 wk (data not shown). However, compared to Fib$^{+/-}$, Fib$^{-/-}$ mice showed a significant decrease (F-Ratio: 4.67, P = 0.03) in TGF-β immunoreactivity at both 2 wk and 26 wk after radiation (Figure 5G-I).

DISCUSSION

The current study demonstrated that there is no statistically significant difference with regard to

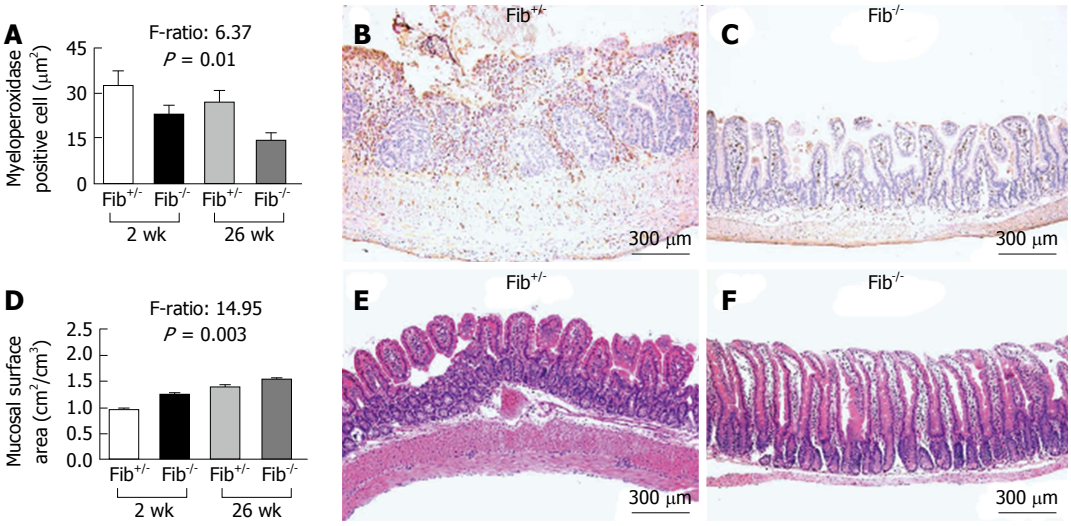

Figure 4 Intestinal inflammation and intestinal mucosal injury in Fib$^{+/-}$ and Fib$^{-/-}$ mice observed at 2 wk and 26 wk after localized irradiation. A: Comparative assessment of intestinal inflammation between Fib$^{+/-}$ (n = 13) and Fib$^{-/-}$ (n = 14) mice at 2 wk and Fib$^{+/-}$ (n = 28) and Fib$^{-/-}$ (n = 13) mice at 26 wk by measuring myeloperoxidase activity; B and C: Representative photomicrographs showing myeloperoxidase positive cells in Fib$^{+/-}$ and Fib$^{-/-}$, respectively, at 2 wk after exposure; D: Comparative assessment of intestinal mucosal injury between Fib$^{+/-}$ (n = 15) and Fib$^{-/-}$ (n = 14) mice at 2 wk and Fib$^{+/-}$ (n = 28) and Fib$^{-/-}$ (n = 11) mice at 26 wk; E and F: Representative photomicrographs showing difference in mucosal surface area by H&E staining in Fib$^{+/-}$ and Fib$^{-/-}$, respectively, at 2 wk after exposure.

intestinal wall thickness, mucosal surface area, smooth muscle cell proliferation, and neutrophil infiltration among three mouse strains (Fib$^{+/+}$, Fib$^{+/-}$ and Fib$^{+/-}$) before irradiation, both at 2 and 26 wk time points. Moreover, irradiated Fib$^{+/+}$ and Fib$^{+/-}$ mice show no significant difference in intestinal radiation injury score (F-Ratio: 0.19, P = 0.66), mucosal surface area (F-Ratio: 0.04, P = 0.84), and smooth muscle cell proliferation (F-Ratio: 0.00, P = 0.99), at both time intervals. In addition, ELISA results demonstrated that un-irradiated Fib$^{+/+}$ mice have similar plasma and intestinal fibrinogen level as un-irradiated Fib$^{+/-}$ mice. No significant difference in intestinal fibrinogen immunoreactivity was observed between Fib$^{+/+}$ and Fib$^{+/-}$ mice before and after irradiation, thus justifying the use of Fib$^{+/-}$ mice as littermate controls in the current study as in previous studies. Importantly, we demonstrated that fibrinogen deficiency profoundly attenuated early and delayed radiation-induced intestinal inflammation, mucosal injury and fibrosis, intestinal wall and serosal thickness, collagen deposition, intestinal smooth muscle proliferation, TGF-β immunoreactivity, and loss of intestinal mucosal surface area. This study thus confirms a significant role for fibrinogen in radiation enteropathy and suggests that fibrinogen is a potential target in suppressing radiation-induced gut injury.

Fibrinogen is a soluble glycoprotein synthesized by the liver with a molecular weight of 340 kDa. It circulates in the blood, where it is converted by thrombin into fibrin during coagulation and inflammation[29]. In addition, fibrinogen and their degradation products have been implicated in regulating fibrotic process[6-13], including radiation-induced enteropathy and nephropathy[5,30]. However, direct evidence supporting this notion has been lacking.

In pathological conditions, such as vascular wall injury, infection or inflammation, the blood concentration of fibrinogen increases significantly[31]. Even before extravasation into the perivascular space, increased fibrinogen and its derivative peptides in the blood induce blood mononuclear cells to synthesize pro-inflammatory cytokines, such as TNF-α, IL-6 and IL-1β[32,33], activate neutrophils[34], mediate leukocyte adhesion to the vascular endothelium[35,36], and increase leukocyte migration[36], notably leukocyte transendothelial migration[37]. Fibrinogen can bind to both endothelial cells and platelets increasing vascular permeability and causing their local aggregation, as well as inducing fibrin and thrombin production[6,38,39] permitting fibrinogen, fibrin, thrombin, and other plasma constituents to escape the vasculature[40]. Increased interstitial deposition of fibrinogen and/or fibrin has been observed in inflammatory conditions and is considered an early and persistent hallmark of inflammatory responses[8]. Our previous and current studies shows that radiation significantly increase the deposition of both intravascular and extravascular fibrinogen, as well as thrombin deposition in the intestinal tissue at both early and delayed stage[5].

Extravasated fibrinogen promotes leukocyte adhesion and stimulates macrophage production of a select set of chemokines such as macrophage inflammatory protein-1alpha (MIP-1α), MIP-1β, MIP-2, and monocyte chemoattractant protein-1, resulting in the attraction of inflammatory cells such as T cells, neutrophils and additional macrophages to the site of the insult[41]. Direct evidence of the proinflammatory role of fibrinogen comes from animal studies using genetic and pharmacologic approaches. Fibrinogen deficiency delays endotoxin induced inflammatory responses[42], decreases inflammation and delays the onset of demyelination in a

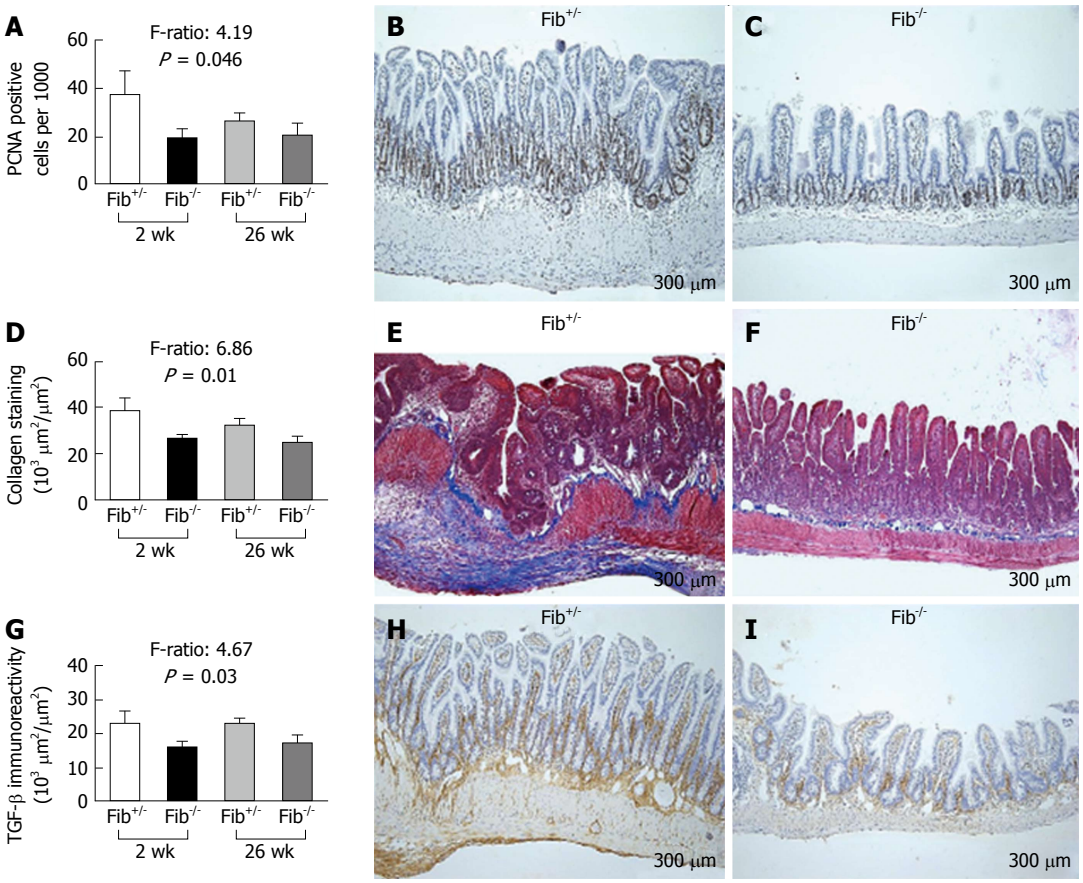

Figure 5 Intestinal smooth muscle cell proliferation, collagen deposition and TGF-β immunoreactivity in Fib$^{+/-}$ and Fib$^{-/-}$ mice observed at 2 wk and 26 wk after localized irradiation. A: Comparative assessment of intestinal smooth muscle cell proliferation between Fib$^{+/-}$ (n = 13) and Fib$^{-/-}$ (n = 13) mice at 2 wk and Fib$^{+/-}$ (n = 19) and Fib$^{-/-}$ (n = 12) mice at 26 wk by measuring PCNA immunoreactivity; B and C: Representative photomicrographs showing PCNA immunoreactivity in Fib$^{+/-}$ and Fib$^{-/-}$, respectively, at 2 wk after exposure; D: Comparative assessment of collagen deposition between Fib$^{+/-}$ (n = 11) and Fib$^{-/-}$ (n = 14) mice at 2 wk and Fib$^{+/-}$ (n = 17) and Fib$^{-/-}$ (n = 10) mice at 26 wk; E and F: Representative photomicrographs showing Collagen deposition by Trichrome staining in Fib$^{+/-}$ and Fib$^{-/-}$, respectively, at 2 wk after exposure; G: Comparative assessment of TGF-β immunoreactivity between Fib$^{+/-}$ (n = 13) and Fib$^{-/-}$ (n = 13) mice at 2 wk and Fib$^{+/-}$ (n = 21) and Fib$^{-/-}$ (n = 12) mice at 26 wk; H and I: Representative photomicrographs showing TGF-β immunoreactivity in Fib$^{+/-}$ and Fib$^{-/-}$, respectively, at 2 wk after exposure.

tumor necrosis factor transgenic mouse model[43]. Fibrinogen deficiency also diminishes macrophage infiltration and activation in a model of Duchenne muscular dystrophy[13] and in a model of crescentic glomerulonephritis[23]. Ancrod, a defibrinogenating agent, reduces macrophage infiltration and activation, and production of multiple cytokines in a model of Duchenne muscular dystrophy[13]. Reduction of fibrin formation by hirudin is accompanied by a diminution of inflammation and disease severity in experimental arthritis[6] and radiation-induced intestinal injury[5]. Current results are consistent with these and other previous studies, supporting that fibrinogen is a key regulator in inflammation and tissue injury.

Progressive vascular and intestinal wall fibrosis are characteristics of delayed radiation enteropathy, which involves excessive accumulation of collagen and other extracellular matrix (ECM) components. During inflammation and tissue injury, the extravasated fibrinogen is cleaved by the thrombin to form insoluble fibrin, which appears as an eosinophilic meshwork of threads and provides an initial scaffold along which the fibroblasts adhere and migrate. Fibrinogen and its degradation products act as a mitogen to stimulate fibroblast proliferation, resulting in collagen deposition and leading to tissue fibrosis[9-11]. Accordingly, genetic deletion of fibrinogen diminishes crescentic glomerulonephritis[23] and induced wound-healing defects[24]. Fibrin, once extravasated into the tissue, resists degradation[10]. The persistent fibrin is associated with enhanced collagen accumulation that may result in the development of fibrotic disorders[9-13]. Fibrinogen and/or fibrin deposition has been involved in the pathogenesis of arterial intimal thickening and atherosclerosis[44,45]. Studies show that fibrinogen and its degradation products stimulate vascular smooth muscle cells to produce inflammatory cytokines[46] and induce smooth muscle cell proliferations[47,48], resulting in collagen and other ECM production[47]. Fibrinogen and fibrin are also chemoattractant for smooth muscle cell[49,50]. This could be involved in the pathogenesis of vascular sclerosis, as well as intestinal wall fibrosis in radiation enteropathy, since collagen is produced largely by smooth muscle cells in the intestine. Moreover, because the intestinal fibrosis becomes more prominent with increasing radiation dose, the

protective effect of fibrinogen deficiency would likely increase with increasing radiation doses.

Fibrinogen and fibrin can bind multiple growth factors, including TGF-β[51]. TGF-β has been implicated in many fibrotic conditions, including radiation injury in skin, liver, heart, kidney, lung and intestine and plays important role in fibrotic condition. Fibrinogen in the blood stream serves as a carrier for latent TGF-β[52]. The fibrinogen-bound latent TGF-β leaks into the interstitial tissue after vascular injury and is activated, leading to the formation of active TGF-β and activation of downstream signaling pathways. Fibrin also triggers endogenous TGFβ1 expression[10,13]. Genetic or pharmacologic depletion of fibrinogen reduces active TGF-β and inhibits the fibrinogen-induced effects on glial scar formation[52]. The current study showed that fibrinogen deficiency significantly reduced TGF-β expression. These results thus suggest that TGF-β could be a molecular link between fibrinogen and intestinal fibrosis formation.

In conclusion, the current study shows that fibrinogen deficiency attenuated early and delayed radiation-induced intestinal inflammation, mucosal injury and fibrosis, suggesting that fibrinogen play a key role in early and delayed radiation enteropathy. Pharmacological inhibition of fibrinogen could be a novel strategy to reduce the risk of radiation enteropathy.

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COMMENTS

Background

One third of total cancer patients suffer from either abdominal or pelvic malignancies. About 14%-68% of patients with abdominal or pelvic malignancies undergo radiotherapy. The major limitation of abdominal or pelvic radiotherapy is the risk of development of radiation enteropathy, an adverse side effect.

Research frontiers

Radiation enteropathy is a well-known complication after abdominal or pelvic radiotherapy and causes substantial morbidity in patients. The underlying mechanisms involved in the development of intestinal injury from therapeutic radiation are largely unknown. A role for fibrinogen in intestinal fibrosis has been suggested, but not unequivocally confirmed.

Innovations and breakthroughs

To the best of our knowledge, this study for the first time show that deficiency of fibrinogen suppresses radiation-induced early and delayed intestinal injury using a fibrinogen knock-out mouse model.

Applications

Fibrinogen may represent a novel target to reduce intestinal damage in patients undergoing abdominopelvic radiotherapy or in case of accidental or intentional exposure to ionizing radiation.

Terminology

Radiation enteropathy, or intestinal damage caused by therapeutic radiation is a global problem and its successful mitigation has not been achieved. The mechanisms underlying the development of radiation enteropathy are highly complex and controversial. The present study demonstrated that deficiency of fibrinogen, a plasma protein that induces coagulation, inflammation, and fibrosis, substantially reduces radiation-induced intestinal damage.

Peer-review

Fibrinogen deficiency directly attenuates development of early and delayed radiation enteropathy. Fibrinogen could be a novel target in treating intestinal damage. The manuscript is an interesting piece of good work.

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ORIGINAL ARTICLE

Basic Study

Helicobacter pylori vacA genotype is a predominant determinant of immune response to *Helicobacter pylori* CagA

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Abstract

AIM

To evaluate the frequency of *Helicobacter pylori* (*H. pylori*) CagA antibodies in *H. pylori* infected subjects

and to identify potential histopathological and bacterial factors related to *H. pylori* CagA-immune response.

METHODS

Systematic data to *H. pylori* isolates, blood samples, gastric biopsies for histological and molecular analyses were available from 99 prospectively recruited subjects. Serological profile (anti-*H. pylori*, anti-CagA) was correlated with *H. pylori* isolates (*cagA*, EPIYA, *vacA s/m* genotype), histology (Sydney classification) and mucosal interleukin-8 (IL-8) mRNA and protein expression. Selected *H. pylori* strains were assessed for *H. pylori* CagA protein expression and IL-8 induction in co-cultivation model with AGS cells.

RESULTS

Thirty point three percent of microbiologically confirmed *H. pylori* infected patients were seropositive for CagA. Majority of *H. pylori* isolates were *cagA* gene positive (93.9%) with following *vacA* polymorphisms: 42.4% *vacA s1m1*, 23.2% *s1m2* and 34.3% *s2m2*. Anti-CagA-IgG seropositivity was strongly associated with atrophic gastritis, increased mucosal inflammation according to the Sydney score, IL-8 and *cagA* mRNA expression. *VacA s* and *m* polymorphisms were the major determinants for positive (*vacA* s1m1) or negative (*vacA* s2m2) anti-CagA serological immune response, which also correlated with the *in vitro* inflammatory potential in AGS cells. *In vitro* co-cultivation of representative *H. pylori* strains with AGS cells confirmed functional CagA translocation, which showed only partial correlation with CagA seropositivity in patients, supporting *vacA* as major co-determinant of the immune response.

CONCLUSION

Serological immune response to *H. pylori cagA*+ strain in *H. pylori* infected patients is strongly associated with vacA polymorphism, suggesting the crucial role of bacterial factors in immune and clinical phenotype of the infection.

Key words: *Helicobacter pylori*; Seropositivity; Virulence factors; CagA; VacA; Immune response

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Core tip: *Helicobacter pylori* (*H. pylori*) related diseases are commonly associated with *cagA+* strains, although seropositivity against CagA varies among different studies. In this prospective study, we evaluated potential factors related to the *H. pylori* CagA-immune response. We show that anti-CagA-IgG seropositivity was strongly associated with histopathological and inflammatory factors. Most importantly, we identified *H. pylori vacA* polymorphism as one of the main determinants of immune response to CagA and inflammatory potential of *H. pylori* strains *ex vivo* and *in vitro*. Our data support the crucial role of bacterial factors that co-determine the complex interaction with *H. pylori* and define the immune and clinical phenotypes of the infection.

Link A, Langner C, Schirrmeister W, Habendorf W, Weigt J, Venerito M, Tammer I, Schlüter D, Schlaermann P, Meyer TF, Wex T, Malfertheiner P. *Helicobacter pylori vacA* genotype is a predominant determinant of immune response to *Helicobacter pylori* CagA. *World J Gastroenterol* 2017; 23(26): 4712-4723 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i26/4712.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i26.4712

INTRODUCTION

Infection with *Helicobacter pylori* (*H. pylori*) causes chronic inflammation of the gastric mucosa with progression to severe complications in a subset of patients[1-3]. The determinants for the magnitude of inflammation and progression to complication include *H. pylori* with its bacterial virulence factors, host genetic background and environmental factors. *H. pylori* virulence factors facilitate colonization (urease, flagella and catalase) and induce inflammation (OipA, NapA, DupA, IceA, VacA and CagA) of the gastric mucosa[4,5]. CagA and VacA are the most relevant pro-inflammatory factors and are closely related to peptic ulcer disease (PUD) as well as gastric cancer (GC)[6-9].

CagA is the principal protein encoded in the complex of the *cytotoxin associated gene pathogenicity island* (*cag PAI*), which is shuttled from *H. pylori* into gastric epithelial cells through the type Ⅳ bacterial secretion system[7,8]. Intracellularly, CagA undergoes tyrosine phosphorylation by Src and Abl kinases to interact with several host proteins, influence their activity and subsequently alter morphological properties of the host cells[10-13]. CagA protein stimulates expression of inflammatory cytokine interleukin-8 (IL-8) in gastric epithelial cells by activating nuclear factor-κB and leads to increased inflammation of the gastric mucosa[14]. Overall, *H. pylori cagA*+ strains are associated with an increased risk of gastric cancer compared to *cagA*- strains[15]. The oncogenic role of CagA is further supported by *in vivo* experiments in mice, where transgenic *cagA* expression in stomach leads to gastric epithelial hyperplasia, adenocarcinoma, myeloid leukemia and B-cell lymphoma[16].

One of the interesting features related to CagA is the induction of a systemic immune response to CagA and this in fact led to the discovery of this protein[17]. Infection with *cagA*+ strains and serological detection of anti-CagA antibodies have been associated with increased risk for PUD as well as for GC[18,19]. A meta-analysis of 16 studies concludes that seropositivity for anti-CagA-IgG is associated with a 2.87-fold higher risk for gastric cancer development[20]. In earlier studies, Ando *et al*[21] found a significant correlation between anti-CagA-IgG and IL-8 expression in biopsy culture supernatant and described an association of

anti-CagA-IgG with increased neutrophil infiltration and *H. pylori* density. Therefore, it has been suggested that screening for the *cagA* status of *H. pylori* may provide an additional advantage for identifying patients at high risk for gastric cancer development[20]. However, low levels of anti-CagA-IgG in subjects infected with *cagA*+ strains have been reported[22,23]. *H. pylori* IgG seroprevalence in a large study in our center was 44.4%, and proportion anti-CagA-IgG positive was 43.3%[22]. In another prospective study on patients undergoing screening colonoscopy, we observed an even lower proportion (36.6%) of anti-CagA-IgG positivity[23]. In studies performed in various geographic regions of the world the CagA-seropositivity ranges from 35% to 80%[22-24]. The low number of CagA-seropositivity in spite of the high prevalence of *H. pylori cagA*+ strains has not been explained. At present only few studies addressed this observation, however, systematic data are not yet available[25-27]. In the present prospective study, we aimed to identify the factors related to serological reactivity or immune response to CagA.

MATERIALS AND METHODS

Study design

In a prospective study 413 patients were recruited between July 2011 and April 2014. Among those, 99 patients (98 patients of European descent) in total fulfilled the inclusion criteria such as microbiologically confirmed *H. pylori* infection with successful isolation and characterization of *H. pylori* strains and known *H. pylori* anti-CagA status (Figure S1). Patients, with current or past history of non-gastric cancers or stomach surgery, acute bleeding, oral anticoagulation, immunosuppressive or antibiotic therapy (within the last 2 wk before entering the study) were excluded. The study was conducted according to the "World Medical Association Declaration of Helsinki - Ethical Principles for Medical Research Involving Human Subjects" and approved by the ethical board of the Otto-von-Guericke University (Study Number 80/11). All patients provided written informed consent. Blood samples were drawn and systematic biopsy protocol was completed during upper GI endoscopy at the Department of Gastroenterology, Hepatology and Infectious Diseases at the Otto-von-Guericke University of Magdeburg, Germany.

Biopsy protocol and histopathological assessment

During upper GI endoscopy, biopsies from antrum and corpus were collected for microbiology cultivation of *H. pylori,* rapid urease test (CLOtest, Kimberly Clark, United States), histological assessment and further molecular analyses. Histological evaluation was performed according to the updated Sydney protocol from five biopsies (two from each antrum and corpus and one from incisura angularis)[28,29]. Following fixation, slides were stained with hematoxilin, eosin, PAS and modified Giemsa stain for *H. pylori* detection. Gastric cancer tumor tissues were characterized according to the International Classification of Diseases for Oncology and Lauren criteria.

Serological assessment of anti-H. pylori IgG and anti-CagA-IgG

Serological assessment for *H. pylori* was performed using *H. pylori* IgG ELISA Kit (Biohit, Helsinki, Finland) and CagA IgG ELISA Kit (GENESIS Diagnostics, Cambridgeshire, Great Britain). Both tests exhibited a high sensitivity for detection of *H. pylori* infection in our region and have been validated in multiple studies in the past[22,23]. All tests were performed according to manufacturer's instructions with internal and external validation. Cut-off values for positive testing were $\geqslant$ 30.0 EIU or $\geqslant$ 6.25 U/mL for *H. pylori* IgG ELISA and CagA IgG ELISA, respectively. To confirm the data on anti-CagA-IgG we performed immunoblot testing using Helicobacter ViraStripe® IgG immunoblot (Viramed Biotech AG, Planegg, Germany). The test result was considered positive if following criteria were fulfilled: quantitative evaluation of the blots using an automated scanning system provided by the manufacturer (positivity values $\geqslant$ 80% in comparison to control), and two researchers independently and blinded to results, confirmed the positivity.

H. pylori cultivation

Gastric biopsies were collected in 1.5 mL 0.9 vol% isotonic sodium chloride solution (Berlin-Chemie AG, Berlin, Germany) and immediately transported to the Institute of Medical Microbiology for further cultivation. Cultivation and identification of *H. pylori* was performed as described previously[30]. Positive cultures were harvested in 0.9 vol% isotonic sodium chloride solution, centrifuged at 13.000 rpm for 3 min and cell pellets were stored at -30 ℃ until further analysis.

Cell culturing with H. pylori

Six days before the experiment, frozen stocks of several *H. pylori* isolates from patients were inoculated on Columbia-agar-based medium that contained 10 vol% washed human erythrocytes and 10 vol% heat inactivated horse serum (purchased from the NRZ, Nationales Referenzzentrum Helicobacter Freiburg, Germany). Bacteria were cultivated under microaerophilic conditions at 37 ℃. The strain *H. pylori* ATCC® BAA-1606™ (BCM300) was cultivated on selective agar plates (bioMérieux, Marcy l'Etoile, France) under the same conditions. After 3 d bacteria were removed into PBS and cultivated on fresh agar plates for another three days under the same conditions. For the experiments, bacteria were re-suspended in PBS (with Ca^{2+} and Mg^{2+}) and concentration (bacteria/mL) was determined by measuring the optical density (λ = 580 nm). To check bacteria for viability, suspensions were

inspected microscopically for motility and shape.

AGS cells (CRL-1739; American Type Culture Collection-ATCC) were maintained in RPMI 1640 (Life Technologies, Carlsbad, CA, United States) with 10% Fetal Calf Serum, 100 U/mL Penicillin, 100 μg/mL streptomycin, and 100 μg/mL gentamycin (PAA, Cölbe, Germany) at 37 ℃ and 5% CO_2. Twenty-four hours prior to infection experiments, cells were seeded in 6 well plates at a concentration of 300000 cells/mL in the same medium as mentioned above. Four hours prior infection, medium was removed, cells were washed twice with PBS without Ca^{2+} and Mg^{2+} (Life Technologies, Carlsbad, CA, United States) and fresh antibiotic free medium was added. One well was harvested by trypsination (5 min, 37 ℃) and cell number was determined. Cells were infected with *H. pylori* at a "multiplicity of infection" of 100 for 24 h. Cell culture supernatant was removed, centrifuged at 13.000 rpm for 5 min and transferred into a new reaction tube. After cells were washed twice with PBS, cells were harvested, washed with PBS and cell pellet was stored at -80 ℃ until further analysis.

Genomic DNA extraction of H. pylori and PCR methods

DNA extraction of *H. pylori* was performed using DNA Mini Kit (Qiagen, Hilden, Germany) following manufacturer's recommendations. Amplification of DNA was done in a T3 Thermocycler machine (Biometra, Goettingen, Germany) with 15 μL HotStar Taq Plus DNA Polymerase Mix (Qiagen, Hilden, Germany), 11.6 μL RNase-free water, 0.2 μL of each forward and reverse primer (50 μmol/L) and 3 μL *H. pylori* DNA. Seven primer sets were used for the study: *cagA*, EPIYA, *vacA* s, *vacA* m, *glmM, cagE* and *virB11*. The primer sequences and size of product are shown in Table S1. The reactions were carried out as follows: enzyme activation at 95 ℃ for 15 min, 40 cycles of denaturation at 95 ℃ for 30 s, annealing for 30 s, extension at 72 ℃ for 1 min followed by final extension at 72 ℃ for 10 min PCR products were analyzed by agarose gel electrophoresis, ethidium bromide staining and Hyperladder IV (Bioline, Luckenwalde, Germany) as molecular weight marker. E.A.S.Y RH system (Herolab, Wiesloch, Germany) was used for gel imaging.

Extraction of total RNA and quantitative RT-PCR

H. pylori total RNA was extracted using RNeasy Protect Bacteria Reagent and RNeasy Mini Kit (QIAGEN, Hilden, Germany) following manufacturer's recommendations. Total RNA of gastric specimens and AGS cells was isolated using RNeasy Plus Universal Mini Kit (QIAGEN, Hilden, Germany) following the manufacturer's recommendations (without gDNA Eliminator Solution). RNA concentration was determined spectrophotometrically by measuring absorbance at 260/280 nm (Biophotometer, Eppendorf, Hamburg, Germany). cDNA synthesis was performed in a 40 μL reaction volume with 500 ng of total RNA of *H. pylori* or 1 μg RNA of antrum biopsies. *CagA* and *glmM* mRNA of *H. pylori* and β-actin with IL-8 of gastric tissue and AGS cells was determined with quantitative real-time PCR (qRT-PCR) using the CDX96-Cycler (BioRAD, Munich, Germany). A single 30 μL reaction contained 15 μL QuantiTect SYBR Green PCR Master Mix (QIAGEN, Hilden, Germany), 13.4 μL RNase-free water, 0.2 μL of each forward and reverse primer (50 μmol/L) and 1.2 μL *H. pylori* or antrum cDNA. For qRT-PCR programs see above (qualitative PCR program). Annealing temperature and primers are shown in supplementary data (Table S1). Quality of qRT-PCR products was verified by melt curve analysis and agarose gel electrophoresis (see above). Expression data were analyzed using the $2^{-\Delta Ct}$ method.

CagA expression in vitro using Western blotting

Cell pellets were mixed with 2x Laemmli buffer (4% SDS, 20% glycerol, 120 mmol/L Tris-Cl (pH 6.8) and 0.02% bromphenol blue) and boiled for 10 min at 95 ℃. Thereafter, samples were separated using 10% SDS-polyacrylamide gel and transferred to a polyvinylidene difluoride membrane. Membranes were blocked with TBS buffer and incubated with antibodies as previously described[31].

IL-8 quantification using ELISA

Interleukin 8 (IL-8) concentration in AGS co-culture supernatants was determined with quantitative sandwich enzyme-linked immunoassay (Quantikine® ELISA, R and D Systems, Abingdon, United Kingdom) according to manufacturer´s recommendations. Results are displayed in pg/mL.

Statistical analysis

Statistical analysis was performed using GraphPad Prism 6 (GraphPad Software Inc., La Jolla, CA, United States). All data are presented as mean ± SD. χ^2 test and Fisher´s exact test were used for contingency tables. The Mann-Whitney *U*-test and the Kruskal-Wallis analyses of variance were used to analyze the statistical significance for two unpaired groups or multiple groups, respectively. Post hoc analyses were performed using Dunn's multiple comparison tests. Correlation analyses were performed using Spearman's test. Two-sided *P*-values < 0.05 were considered as statistically significant.

RESULTS

Clinical characteristic of patients with and without CagA-IgG

From 99 patients with successful cultivation of *H. pylori* from the stomach, 30 (30.3%) patients had positive anti-CagA-IgG serology. First, we questioned if CagA-IgG seropositive and seronegative groups may have a difference in clinical phenotype. Clinical

Table 1 Characteristics of patients with active *Helicobacter pylori* infection regarding patient's CagA-IgG status *n* (%)

	Total	*H. pylori* + CagA-IgG-	*H. pylori* + CagA-IgG+	*P* value
Total	99	69 (69.7)	30 (30.3)	
Gender				NS
Female	72	51 (73.9)	21 (70)	
Male	27	18 (26.1)	9 (30)	
Age				
mean ± SD	54.1 ± 14.1	53.7 ± 13.7	55.1 ± 15.0	NS
H. pylori status				
Anti-*H.pylori*-IgG+	93	64 (92.8)	29 (96.7)	NS
Anti-*CagA*-IgG+	30	-	30 (100)	
mean *CagA*-IgG EIU		1.5 ± 1.7	39.6.5 ± 31.1	< 0.0001
Culture+	99	69 (100)	30 (100)	NS
Histology+	79	55 (79.7)	24 (80)	NS
Clinical phenotype				
Chronic active Gastritis (any severity)	92	63 (91.3)	29 (96.7)	NS
Chronic non-active Gastritis (grade > 2)[1]	7	6 (8.7)	1 (3.3)	NS
Corpus predominant gastritis	5	0 (0)	5 (16.7)	0.0020
Antrum-/pangastritis	87	63 (91.3)	24 (80)	NS
Chronic atrophic gastritis (any severity)	46	25 (36.2)	21 (70)	0.0023
Chronic atrophic gastritis (> 2/3)[1]	28	14 (20.3)	14 (46.7)	0.014
Intestinal metaplasia (any)	23	12 (17.4)	11 (36.7)	0.068
Gastric cancer	6	2 (2.9)	4 (13.3)	0.056
PUD or MALT-Lymphoma (any)	5	4 (5.8)	1 (3.3)	NS
Normal mucosa (no PMNs and ⩽ 1 chronicity)[1]	4	4 (5.8)	0 (0)	NS

[1]Score based on the Sydney classification. NS: Not significant, $P > 0.05$. PUD: Peptic ulcer disease; PMNs: Polymophonuclear neutrophils.

and demographical data are presented in Table 1. Among different histological conditions, corpus predominant gastritis and chronic atrophic gastritis were more frequently found in the group of patients with seropositivity for anti-CagA-IgG. More patients with chronic non-active gastritis or patients without any inflammation were found in the anti-CagA-IgG negative group, suggesting the weaker Inflammation related to *H. pylori* infection. With further focus on the clinical phenotype, we observed a slightly higher polymophonuclear neutrophil infiltration in corpus and a more severe atrophy with intestinal metaplasia in antrum of patients with anti-CagA-IgG based on the mean Sydney Scores for corpus and antrum separately (Figure 1). No difference in *H. pylori*-IgG antibody titer or *H. pylori* density was found histologically between those groups.

Characteristics of H. pylori strains in patients with and without anti-CagA-IgG

It has been previously suggested that immune response to CagA may be dependent on *H. pylori* strain characteristics and its virulence factors. As expected all six patients in CagA-IgG- group had *cagA*-strains. All strains from patients with CagA-IgG+ had *cagA*+ strains (Table 2). To evaluate if studied patients show immune response to *H. pylori* we compared CagA-IgG in both groups. We found that seropositivity against *H. pylori* was present in most of cases in 64 (92.8%) and 29 (96.7%) patients without and with anti-CagA-IgG, respectively, suggesting that the majority of patients are immunologically capable of showing the serological response to *H. pylori* or its virulence factors. Correlation analyses between the *H.pylori*-IgG and CagA-IgG titers did not reveal any correlation (Figure 2A).

Next, we speculated that CagA immune response may be further dependent on successful transcription of *cagA* mRNA. All strains in CagA-IgG+ group showed moderate or high *cagA* mRNA expression. At the same time 34 (56.7%) patients of the CagA-IgG-group had also positive *cagA* mRNA expression. We questioned if differences in EPIYA motifs or a missing Type Ⅳ secretion system could have an impact on production of CagA-IgG. A large proportion of the patients had an evidence for *H. pylori* with mixed EPIYA motifs and no specific differences were observed among CagA-IgG positive and negative groups (Table 2). As a surrogate for the presence of *cagA* pathogenicity island and type Ⅳ secretion system, we examined *cagE* (cagPAI marker) and *virB11* (T4SS marker) expression in 54 patients of the *cagA*+ and CagA IgG negative group. *CagE* was detectable in all tested *H. pylori* isolates, while only one strain was negative for virB11 (data not shown) further excluding the potentially missing T4SS.

It is well known that VacA and CagA are the main pro-inflammatory bacterial factors. It has been earlier hypothesized that *vacA* polymorphism may also be related to CagA seropositivity[25]. As shown in Table 2, all of the strains from patients with immune response had *H. pylori* with *vacA s1* subtype (with m1 76.7% and m^2 23.3%). None of the patients with *s2m2* showed CagA seropositivity. In support, the level of anti-CagA-IgG were higher and more frequent positive in *vacA s1m1* (50%) and *vacA s1m2* (36.8%) compared to *vacA s2m2* (0%), further suggesting the importance

Table 2 Characteristics of *Helicobacter pylori* strains in CagA IgG dependent status of the host *n* (%)

	Total	*H. pylori* + CagA-IgG-	*H. pylori* + CagA-IgG+	*P* value
Total	99	69 (69.7)	30 (30.3)	
cagA gene[1]				NS
Positive	93	63 (91.3)	30 (100)	
Negative	6	6 (8.7)	0	
cagA mRNA[2]	87	60	27	< 0.0001
Positive	61	34 (56.7)	27 (100)	
Negative	26	26 (43.3)	0 (0)	
EPIYA motifs				NS
Negative	5	5 (7.2)	0	
AB	4	2 (2.9)	2 (6.7)	
ABC	33	21 (30.4)	12 (40)	
ABCC	7	6 (8.7)	1 (3.3)	
ABCCC	2	1 (14.5)	1 (3.3)	
Mixed	48	34 (49.3)	14 (46.7)	
VacA-IgG[1,3]		8 (13.1)	5 (18.5)	NS
vacA subtype[1]				
s1	65	35 (50.7)	30 (100)	< 0.0001
s2	34	34 (49.3)	0	
m1	42	19 (27.5)	23 (76.7)	< 0.0001
m2	57	50 (72.5)	7 (23.3)	
s1m1	42	19 (27.5)	23 (76.7)	< 0.0001
s1m2	23	16 (23.2)	7 (23.3)	
s2m2	34	34 (49.3)	0	

[1]Six patients with evidence for different/mixed *cagA*$^+$/*cagA*$^-$ or *vacA* strains in corpus and antrum have been included to the potentially more pathogenic group for simplicity; [2]RNA analyses were possible only in 87 patients/strains; [3]Eighty-eight samples were available for VacA-IgG analyses. NS: Not significant, $P > 0.05$.

Table 3 Validation of CagA expression and cellular translocation in AGS cells

ID	Strain characterization				*In vitro*		
	cagA DNA	*cagA* RNA	CagA-IgG	*vacA*	*H.pylori* CagA	AGS + *H.pylori* CagA	AGS + *H.pylori* p-CagA
BCM300	+	+		s1m1	+	+	+
117	+	+	+	s1m1	+	+	+
6	+	+	-	s1m1	+	+	+
255	+	+	-	s1m1	+	+	+
13/1	+	+	+	s1m1	+	+	-
46	+	+	-	s1m1	+	+	-
89	+	+	-	s1m2	+	+	-
424	+	+	+	s1m2	-	-	-
21	+	-	-	s1m1	-	-	-
321	+	+	-	s2m2	-	-	-
374	+	+	-	s2m2	-	-	-
342	+	-	-	s2m2	-	-	-
314	-	-	-	s1m2	-	-	-
450	-	-	-	s1m2	-	-	-

AGS cells co-cultivated in similar conditions without *Helicobacter pylori* were considered as negative control. Strains were characterized based on *cagA* DNA/RNA/cagA-IgG seropositivity of the host and *vacA* polymorphism. "+": positive and "-": negative expression. p-CagA and CagA: Phosphorylated p-CagA and total CagA protein expression *in vitro*.

of *H. pylori vacA* virulence factor in immune response (Figure 2B).

H.pylori-induced inflammation in mucosa and in vitro model

CagA with functional TSS4 is known to induce IL-8 *in vitro* and *in vivo*. Having shown increased histological inflammation in subjects with anti-CagA-IgG, we questioned if this may correlate with *H. pylori*-related cytokine IL-8 in antrum mucosa. Independently of histological phenotype, IL-8 was significantly higher (about 2 fold) in patients with anti-CagA-IgG+ compared to anti-CagA-IgG- patients with CG (0.0082 ± 0.009 *vs* 0.0048 ± 0.004, $P = 0.026$) (Figure 3A). This, however, was not the case in mucosa from patients with GC and PUD, although the number of the patients was very small. We observed no correlation between IL-8 expression in mucosa and the level of anti-CagA-IgG. To confirm those strain-dependent observations, we performed *in vitro* analyses using *H. pylori* co-culture with AGS cell line. We randomly selected *H. pylori* strains with different strain characteristics including *cagA* mRNA expression, CagA-IgG and *vacA* polymorphisms (Table 3). As expected, *cagA*+ stains and strains with *cagA* mRNA expression induced slightly higher IL-8 mRNA expression compared to controls (AGS without *H. pylori*) and *cagA*- strains (Figure 3B and C). However, anti-CagA-IgG positivity did not correlate with IL-8 expression suggesting that host serological immunotype/phenotype does not correlate with *in vitro* potential of *H. pylori* to induce inflammation (Figure 3D). IL-8 mRNA expression in AGS cells correlated significantly with IL-8 expression in supernatant (Figure S2A), and we observed identical pattern for IL-8 release in supernatant of AGS cells in confirmation of the results (Figure 3B-D).

The inflammatory potential of *H. pylori cagA*+ strains showed relatively high distribution suggesting other bacterial factors potentially responsible for the observation. Therefore, we questioned whether *vacA* s and m polymorphisms may correlate with inflammatory potential of *H. pylori in vitro*. Strains with *vacA* s1 induced higher IL-8 mRNA (Figure 4) and IL-8 expression in supernatant; however, the highest difference was related to *vacA* m polymorphism with highest values for *vacA* m1 compared to *vacA* m2. This data further confirms the highest inflammatory potential defined by IL-8 expression of *vacA* s1m1 compared to *vacA* s1m2 or s2m2 (Figure S2B and C).

CagA expression in vitro

Having shown that multiple factors may be related to seropositivity to CagA, we questioned if the *H. pylori* strains indeed a capable of expression of functional CagA protein (including its phosphorylated form) in AGS cells. For this purpose, we performed CagA Western blotting using bacterial pellets and AGS cells co-cultivated with *H. pylori* (Table 3). As expected, we found that the majority of *H. pylori cagA*+ strains with *vacA* s1m1 polymorphism indeed were capable of CagA protein expression independently to anti-CagA-IgG positivity in host (Table 3). This provides

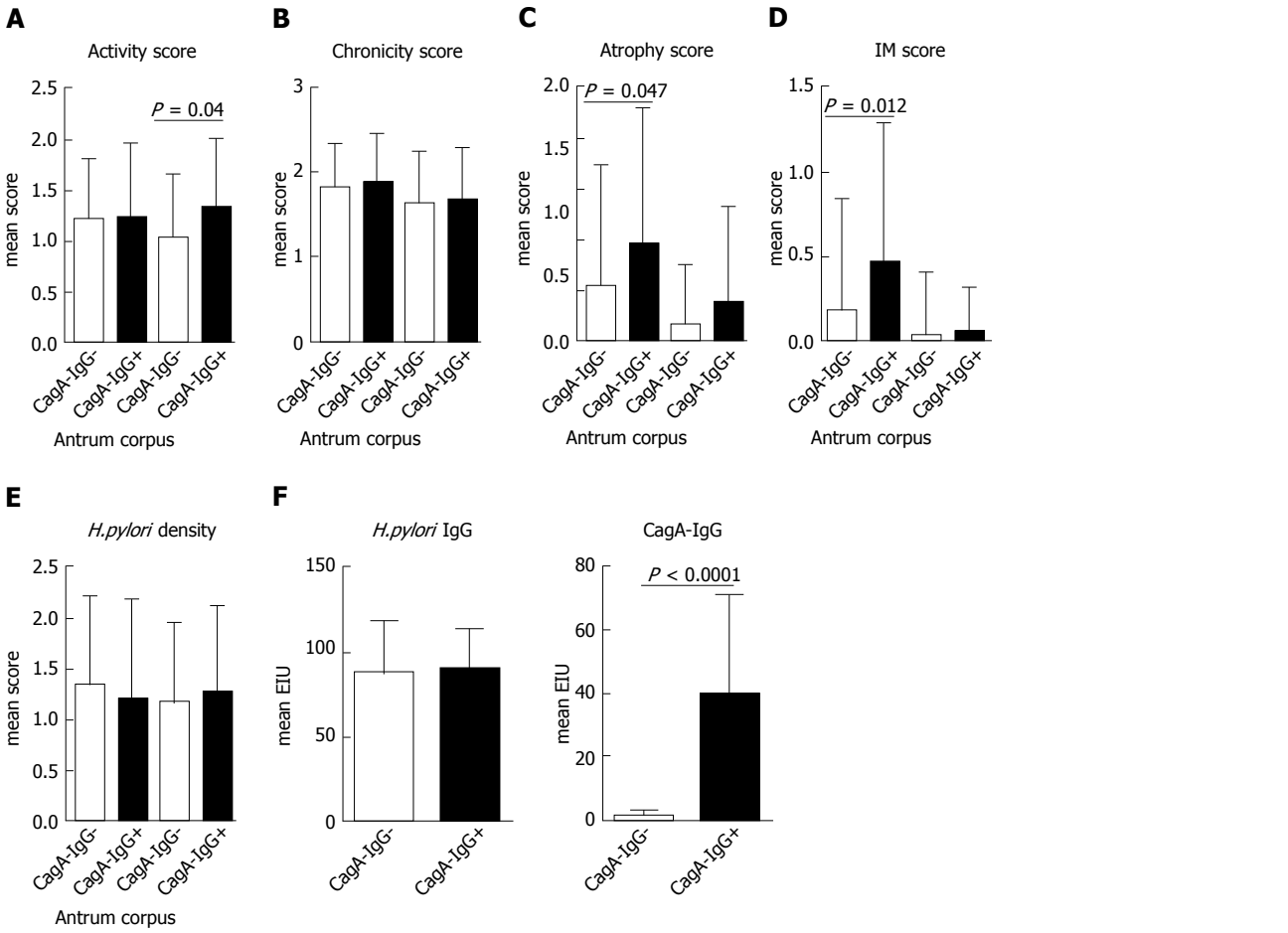

Figure 1 Difference in Sydney score and *Helicobacter pylori* seropositivity in patients with and without anti-CagA-IgG. Mean histological scores ± SD. A: Activity; B: Chronicity, C: Atrophy; D: Intestinal metaplasia are shown; E: Anti-*Helicobacter pylori* IgG; F: anti-CagA-IgG titer were evaluated using ELISA; Statistical analyses were performed using Mann-Whitney test.

an additional level of evidence that anti-CagA-IgG is dependent on various bacterial and probably host factors but may not be useful as a biomarker for lesser pathogenic *H. pylori* infection.

Validation of CagA-IgG data

For the analysis of IgG response against CagA, we used well established ELISA-based method[22,23]. To confirm these results and to further evaluate seropositivity, we performed an independent analysis using Immunoblot based method to evaluate the seropositivity. Helicobacter ViraStripe® IgG Kit includes, besides CagA, also various other Antigen-preparations such as VacA, p90, UreA, *etc.* Overall, there was a strong correlation between the two tests [r = 0.722 (95%CI: 0.6-0.81), P < 0.0001] (Figure S3A). All samples with positivity in anti-CagA-IgG ELISA test (Omega Genesis) showed very strong signal in immunoblot with values above 200 (Figure S3B). However, there were also several samples with positive signal in immunoblot and low values in ELISA, suggesting that certain samples with anti-CagA-IgG could be probably missed due to methodological issues (Figure S3C). However, the immunoblot-based method (ViraStripe CagA-IgG Blot) was positive in some patients without evidence for past or present *H. pylori* infection and the lower specificity could be at least in part be the explanation for the higher detection rate (data not shown).

DISCUSSION

A substantial number of patients infected with *H. pylori cagA*-positive strains do not develop systemic immune response to CagA. In this study, we performed prospective and systematic analysis of *H. pylori* and its virulence factors CagA and VacA to find the explanation for the missing CagA-seropositivity. We confirm that the seroprevalence of CagA in unselected population with microbiologically confirmed *H. pylori* infected patients is low despite the high prevalence of *H. pylori cagA*+ strains. Following multilevel analyses, we found that among various potential factors *vacA* polymorphism is the most important factor associated with anti-CagA-IgG seropositivity.

The anti-CagA-seropositivity varies between different regions with highest prevalence in Asian countries and lowest in Europe. While earlier data

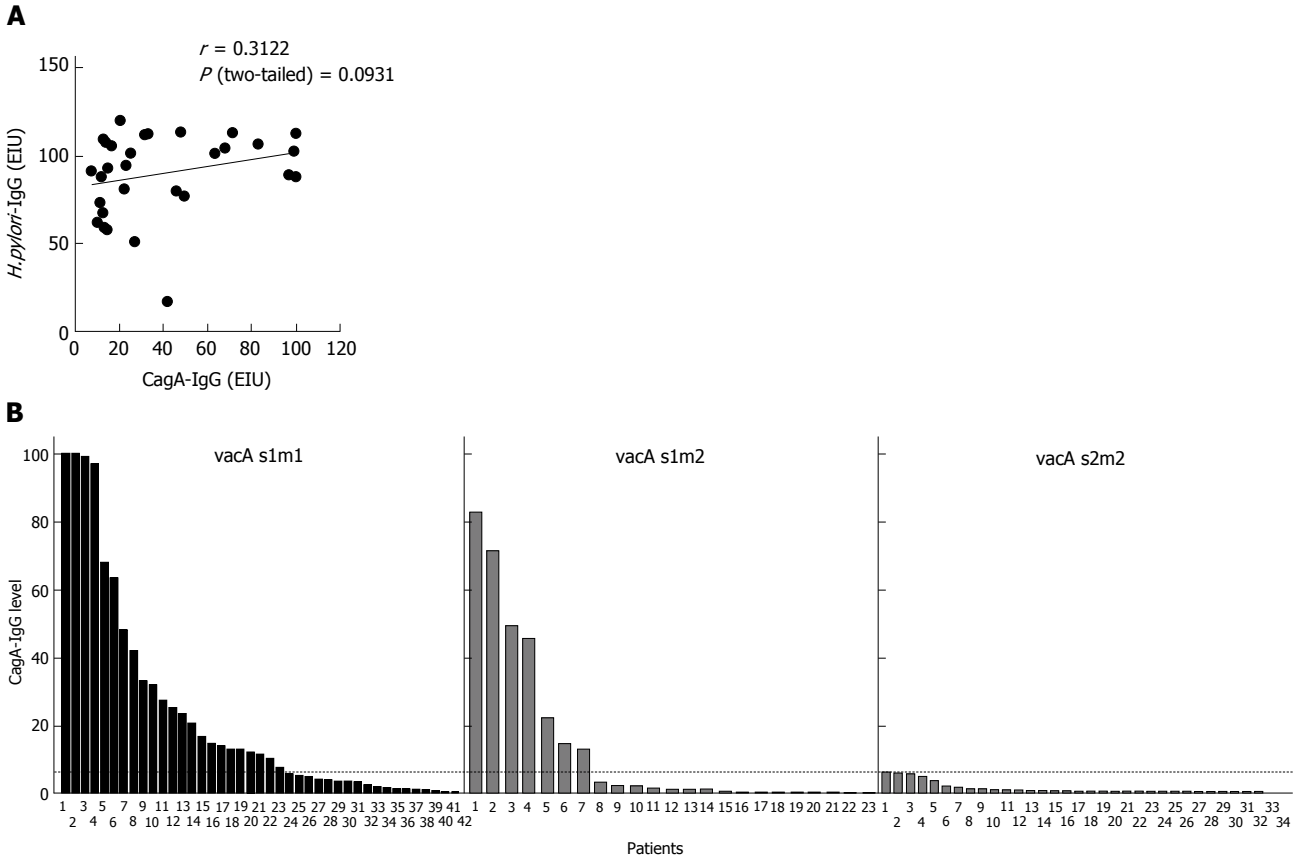

Figure 2 Correlation between anti-CagA-IgG and anti-*Helicobacter pylori* IgG and *vacA* s/m polymorphisms. A: Quantitative values for anti-*Helicobacter pylori* IgG and anti-CagA-IgG were correlated in patients with positive serology (n = 30) using Pearson's test; B: Patients with available data to *vacA* s1/2m1/2 polymorphisms were divided in subgroups dependent on s/m subtype. Anti-CagA-IgG values were sorted in increasing order. Dotted line shows the cut-off for seropositivity of the test (6.25 U/mL).

suggested correlation between *cagA* gene and seropositivity against CagA, our data showed that only 36%-43% patients had anti-CagA-IgG[22,23,32]. In a recent work from Blaser´s group, the prevalence of anti-CagA-IgG in a large cohort of children in Europe was 32%[33]. The data from those studies confirm the low seropositivity in a European population with seropositivity for anti-CagA-IgG of microbiologically confirmed *H. pylori cagA*+ infected subjects.

Experience from *H. pylori* vaccine trials suggests that an immune response to CagA is a common event. In the phase- Ⅰ vaccine trials, intramuscular application of CagA, VacA and NAP induced strong systemic immune reactions measured *via* anti-CagA-IgG[34]. So, basically any contact of inflammatory cells with CagA leads to antibody production in B-lymphocytes following antigen presentation. The failure in CagA presentation may happen during various steps of infection such as defective CagA expression, missing translocation due to T4SS system or missing or low cell death related to *H. pylori* infection and according low antigen presentation to immune cells. Indeed, the majority of *H. pylori* strains from subjects with anti-CagA-IgG exhibited mRNA expression *in vitro*, while a subgroup of bacteria showed no or very low *cagA* mRNA expression which further correlated with CagA protein expression the *in vitro* AGC cell model. However, multiple factors related to host and environment (for example low acidity, predisposition to inflammation) may play very important role[35]. Using *in vitro* model, we confirmed that the inflammatory potential of *cagA* positive strain was confirmed *in vitro* using the classical co-cultivation model of AGS cells and using CagA expression analysis in AGS cells (Table 3). To the first, direct analyses of strains with anti-CagA-IgG seropositivity did not reveal significant difference in inflammatory potential measured by IL-8 *in vitro*, suggesting that other bacterial factors could contribute to immune reaction. Second, co-cultivation analysis using AGC cells confirmed from mRNA expression showing that the multiple *H. pylori* strains from patients with negative anti-CagA-IgG have fully functional CagA and TSS4 (Table 3).

Increasing evidence highlights the role of *vacA* polymorphisms in gastric diseases[26]. Assessment of *H. pylori vacA* and *cagA* genotypes and serological host response earlier revealed the association with *vacA* s1[25]. Systematic analyses of *vacA* subtypes in background of anti-CagA-IgG have revealed crucial dependency of seropositivity on *H. pylori vacA* s1m1 polymorphism in our cohort. The *in vitro* data highlight the inflammatory potential of *H. pylori* strains with

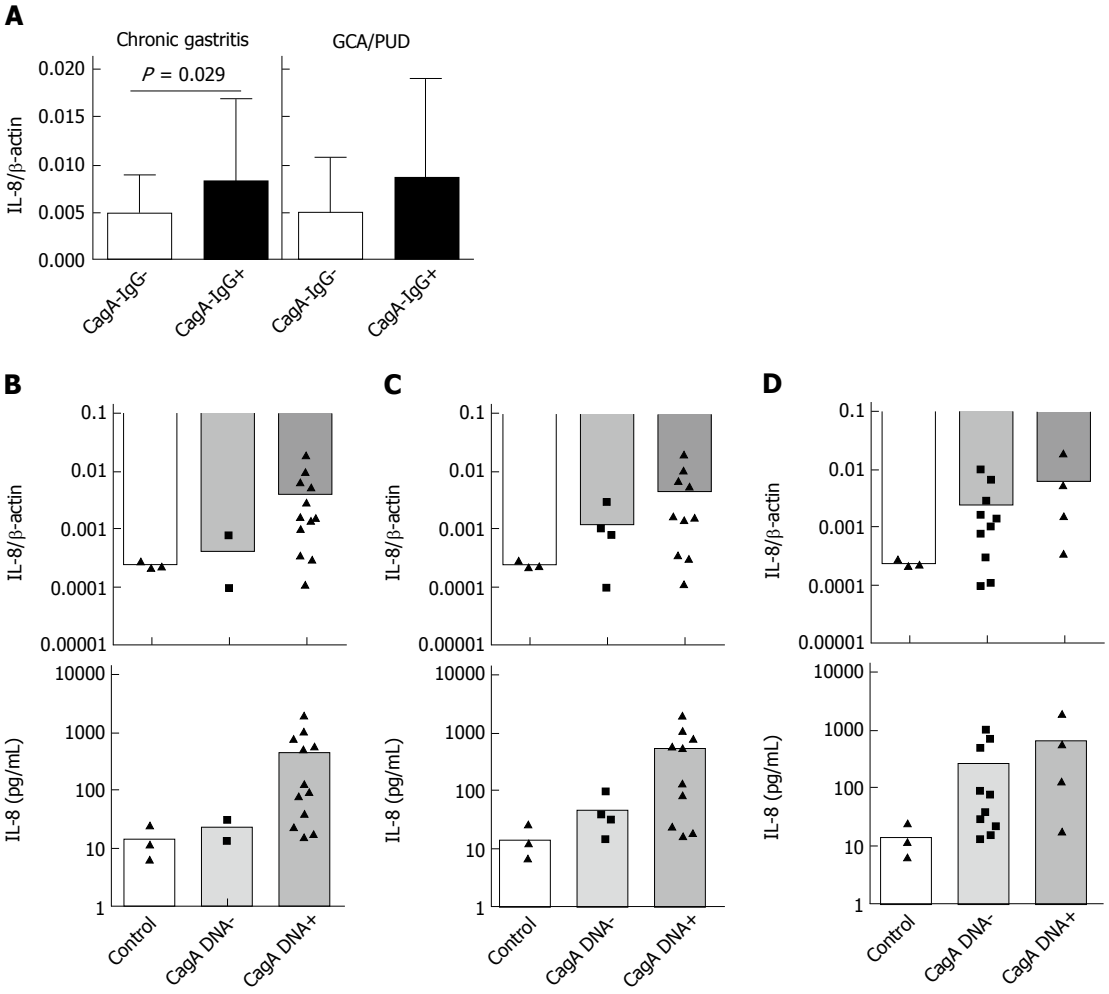

Figure 3 Inflammatory potential based on CagA-IgG in gastric mucosa *ex vivo* and *in vitro* using AGS cells. A: IL-8 mRNA expression was evaluated in antrum mucosa from patients with chronic gastritis [CG, AG, IM, without peptic ulcer disease (PUD) and gastric cancer (GC)] with (n = 23) and without CagA seropositivity (n = 48); B: IL-8 expression in antrum mucosa from patients with PUD and gastric cancer (n = 4 for CagA-IgG+ and n = 6 CagA-IgG-); C: IL-8 mRNA; D: IL-8 protein expression in supernatant are shown for subgroups dependent on *cagA*, *cagA* mRNA and anti-CagA-IgG status *in vitro* using co-culturing of *Helicobacter pylori* strains from patients with AGS cells.

vacA s1m1 polymorphism. This observation is further supported by data showing the dependency of apoptotic activity of *H. pylori* on *vacA*[36]. This led us to believe that the immune response to *cagA* may be at least in part triggered by the effect of VacA on the gastric mucosa. Therefore, the amount of inflammation related to cell toxicity and apoptosis through VacA may influence the interaction of cellular CagA with the immune system and ultimately determine the immune response. The interaction between VacA and CagA has been in focus of several recent studies providing evidence for complex interaction and showing that VacA and CagA can counter-regulate or antagonize each other and affect the host-bacteria interaction[37,38].

Whether a host will develop an immune response to an infection may be influenced by multiple factors. The seroprevalence may typically change during the course of infection, however, it only partially true for *H. pylori* infection that shows relatively similar pattern during the life-time starting with early infection to death. We have previously shown that anti-CagA-IgG seropositivity was similar in different age groups (above or below 30 years) from *H. pylori* positive subjects[22]. Recently, the similar serological pattern was shown in children, where anti-CagA-IgG was positive in 32% despite the very young age[33]. Based on this observation, we speculate that the initial Infection with *H. pylori* and according very first contact to CagA may determine the serological status of the host, which will then remain stable through the whole life until *H. pylori* treatment, disappearance or death. In this regard, the host factors and especially genetic predisposition may play the very important role. Certain host factors such as genetic polymorphism (exp. HLA) have previously been suggested to be associated with susceptibility or resistance to *H. pylori* infection[39]. Furthermore, nonfunctional TLR1 SNP 602S/S has been associated with a reduced risk of *H.pylori*-induced gastritis[40]. Also, a genome-wide association study identified an association between TLR1 and *H. pylori* seroprevalence that could potentially explain the variation[41]. However, TLR1 is not the only candidate gene, and IL1-beta

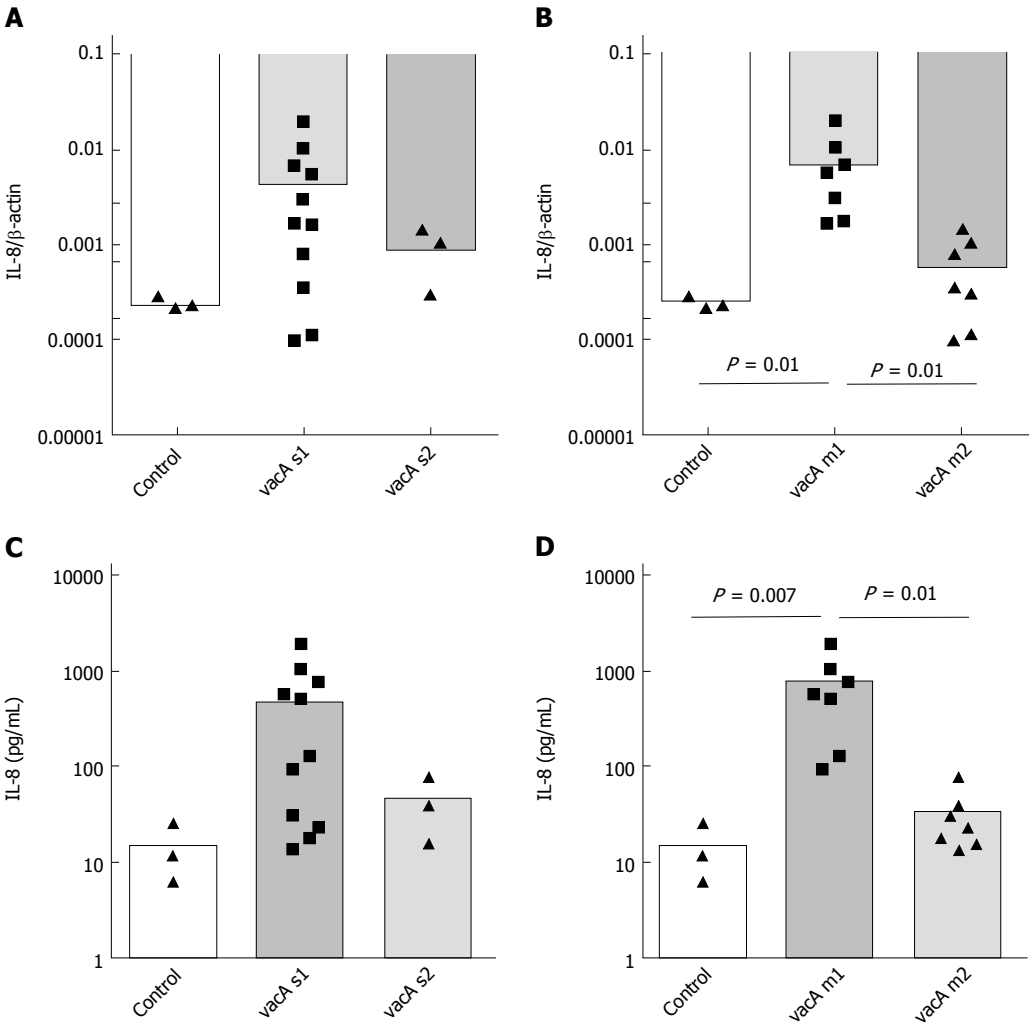

Figure 4 Inflammatory potential of *Helicobacter pylori* strains in relation to *vacA* polymorphism. *Helicobacter pylori* strains from patients were co-cultivated with AGS cell and A-B: IL-8 mRNA; C-D: IL-8 expression in supernatant were measured using qPCR and ELISA.

should be also considered as a potential determinant[42].

From the clinical perspective, our data support significant association of *H. pylori* CagA seropositivity and corpus predominant gastritis, atrophic alterations in gastric mucosa. However, the absence of anti-CagA-IgG does not preclude the infection of individual with the more virulent CagA positive *H. pylori* strain. Based on the current data, the knowledge of individual anti-CagA-IgG status does not allow any specific prognostic clinically-relevant management in support of existing recommendation[43,44].

One of the limitations of our study is that due to the low number of patients, we were unable to suitably address the host related genetic factors. In the present study, we focused on the systemic anti-CagA-IgG production and the locally produced IgA response may be an interesting target for evaluation. Even though we could correlate CagA-IgG data from two different tests, there still may be some difference related to different techniques[25]. Nevertheless, we observed the best specificity with the ELISA kit while immunoblot although had slightly higher sensitivity, it was also associated with high number of false positive results (data not shown). Furthermore, even though the *H. pylori* CagA-IgG positive and negative groups were well balanced, the higher number of subjects were female and potential gender specificity cannot be fully excluded.

In summary, we show that seropositivity for CagA in subjects with *H. pylori* infection is positive in one third of *H. pylori* infected European population despite the presence of CagA positive strain. The immune response to CagA was associated with various bacterial factors and most importantly with *H. pylori vacA* gene polymorphisms. Our data support a crucial role of bacterial and probably host-related factors that co-determine the complex interaction with *H. pylori* and define the immunologic and clinical phenotypes of the infection.

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COMMENTS

Background

Helicobacter pylori (*H. pylori*) -related peptic ulcer disease and gastric adenocarcinoma are commonly associated with cagA+ *H. pylori* strains. However, seropositivity against CagA varies among different studies with positivity below 50% in multiple studies from Europe.

Research frontiers

Infection with *H. pylori* induces strong and sustained inflammation in mucosa and triggers immune positivity against *H.pylori*. Although similar immune response to CagA is expected for *H. pylori cagA*+ strains, the positivity is substantially lower. The mechanism responsible for the seropositivity to CagA is not sufficiently understood. This knowledge may be helpful to identify the factors responsible for the differences in clinical phenotype of *H. pylori* infection. Furthermore, it may also facilitate the preventive and treatment strategies.

Innovations and breakthroughs

In this well-characterized cohort of patients, we demonstrated a low anti-CagA-IgG positivity in *H. pylori* infected patients, which was independent to the high rate of *H. pylori cagA* positive strains. Immune response to *H. pylori* CagA was strongly associated with atrophic gastritis, increased mucosal inflammation and IL-8 expression. Most importantly, we observed a strong association of anti-CagA positivity to *H. pylori vacA* s and *m* polymorphisms, which also correlated with the inflammatory potential *in vitro* in AGS cell lines. Altogether, our data suggest that *H. pylori vacA* polymorphism may determine the immune response to CagA through modulation of mucosal inflammation.

Applications

These data strengthens the role of *H. pylori vacA* polymorphisms and immune response to CagA and in *H. pylori* infection. Whether *H. pylori vacA* may become a clinical tool for risk stratification of *H. pylori*-related diseases needs further evaluation.

Peer-review

The results in this manuscript have demonstrated that seropositivity for CagA in subjects with CagA positive *H. pylori* status is present in one third of *H. pylori* infected European population. The immune response to CagA is associated with various bacterial factors and most importantly with vacA gene polymorphisms. The data supported that both bacterial and host-related factors determined the complex interaction of *H. pylori* with the immunologic system and clinical phenotypes of the infection.

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ORIGINAL ARTICLE

Basic Study

Jianpi Qingchang decoction regulates intestinal motility of dextran sulfate sodium-induced colitis through reducing autophagy of interstitial cells of Cajal

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Abstract

AIM

To investigate the underlying effect of Jianpi Qingchang decoction (JQD) regulating intestinal motility of dextran sulfate sodium (DSS)-induced colitis in mice.

METHODS

C57BL/6 mice were randomly divided into four groups: the control group, the DSS group, the JQD group, and the 5-aminosalicylic acid group. Except for the control group, colitis was induced in other groups by giving distilled water containing 5% DSS. Seven days after

modeling, the mice were administered corresponding drugs intragastrically. The mice were sacrificed on the 15th day. The disease activity index, macroscopic and histopathologic lesions, and ultrastructure of colon interstitial cells of Cajal (ICC) were observed. The levels of tumor necrosis factor-alpha (TNF-α), interleukin (IL)-1β, IL-10 and interferon gamma (IFN-γ), the expression of nuclear factor-kappa B (NF-κB) p65, c-kit, microtubule-associated protein 1 light chain 3 (LC3-Ⅱ) and Beclin-l mRNA, and the colonic smooth muscle tension were assessed.

RESULTS

Acute inflammation occurred in the mice administered DSS. Compared with the control group, the levels of IL-1β, TNF-α, IL-10 and IFN-γ, the expression of LC3-Ⅱ, Beclin-1 and NF-κB p65 mRNA, and the contractile frequency increased ($P < 0.05$), the expression of c-kit mRNA and the colonic smooth muscle contractile amplitude decreased in the DSS group ($P < 0.05$). Compared with the DSS group, the levels of IL-10 and IFN-γ, the expression of c-kit mRNA, and the colonic smooth muscle contractile amplitude increased ($P < 0.05$), the levels of TNF-α and IL-1β, the expression of LC3-Ⅱ, Beclin-1 and NF-κB p65 mRNA, and the contractile frequency decreased in the JQD group ($P < 0.05$).

CONCLUSION

JQD can regulate the intestinal motility of DSS-induced colitis in mice through suppressing intestinal inflammatory cascade reaction, reducing autophagy of ICC, and regulating the network path of ICC/smooth muscle cells.

Key words: Intestinal motility; Interstitial cells of Cajal; Autophagy; Ulcerative colitis; Jianpi Qingchang decoction

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Core tip: Interstitial cells of Cajal (ICC) lead to a variety of changes in the physiological properties of the neurons in the related circuitry, which then affects gastrointestinal motility. Therefore, ICC have been accepted as a therapeutic target for gastrointestinal motility disorders. It is demonstrated in the current study that Jianpi Qingchang decoction can regulate the intestinal motility of dextran sulfate sodium-induced colitis in mice through suppressing intestinal inflammatory cascade reaction, reducing autophagy of ICC, and regulating the network path of ICC/smooth muscle cells.

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INTRODUCTION

Ulcerative colitis (UC) is a chronic intestinal disease, and its clinical manifestations are associated with the pathophysiological aspects of intestinal motility disorders, such as diarrhea and tenesmus[1,2]. Intestinal motility disorders of UC can seriously impact the quality of life (QOL) of patients due to the long disease duration. At present, improving the QOL, inducing and maintaining clinical remission and mucosal healing, and preventing complications are together regarded as the targets of UC treatments[3].

Previous clinical and experimental studies have demonstrated that Traditional Chinese Medicine (TCM) is advantageous in treating UC; its curative effect is reliable, with less side effects and low recurrence rate[4,5]. Jianpi Qingchang decoction (JQD) was made on the basis of the theory in TCM to treat UC. Previous studies found that JQD could be used to treat patients with initial or mild UC, improve their intestinal symptoms, such as diarrhea, mucous bloody stool and tenesmus, and regulate their systemic functional state, such as fatigue, consequently improving their QOL[6,7]. Furthermore, JQD could up-regulate the expression of peripheral blood mononuclear cell glucocorticoid receptor-α to improve hormone-dependent status to treat steroid-dependent UC[8]. Besides, JQD could suppress activation of the nuclear factor-kappa B (NF-κB) signaling pathway and regulate the expression of cytokines in dextran sulfate sodium (DSS)-induced colitis in mice[9].

Recent investigations have demonstrated that inflammation influences the morphology and structure of interstitial cells of Cajal (ICC), leading to a variety of changes in the physiological properties of the neurons in the related circuitry, which then affects gastrointestinal motility[10]. Therefore, ICC have been accepted as a therapeutic target for gastrointestinal motility disorders[11]. Defective autophagy has been strongly linked to UC pathogenesis, with evidence showing that enhancing autophagy may be therapeutically beneficial by regulating inflammation and clearing intestinal pathogens[12,13].

The regulation of autophagy might be a potential strategy for UC, which can be achieved by multi-level and multi-path interference[14]. Therefore, it was essential to study relationships between intestinal motility disorder of UC and autophagy of ICC, and to explore upstream signaling pathway regulation to strengthen or advance the beneficial autophagy response, which might be beneficial in preventing and treating intestinal dysmotility of UC[15]. JQD could improve the clinical symptoms of intestinal dysmotility, such as diarrhea and tenesmus in patients with UC,

Table 1 Composition of Jianpi Qingchang decoction

Chinese name	Latin name	English name	Quantity in g
Zhi Huang Qi	*Radix astragali*	Milkvetch root	30
Huang Lian	*Coptis chinensis*	Coptis root	3
Dang Shen	*Codonopsis pilosula*	Pilose Asiabell root	15
Ma Chi Xian	*Portulaca oleracea L*	herba portulacae	30
Sheng Di Yu	*Radix sanguisorbae*	Garden Burnet root	15
San Qi	*Panax notoginseng*	Notopterygium root	6
Bai Ji	*Bletillae rhizoma*	Tuber Bletillae	3
Mu Xiang	*Radix aucklandiae*	Common Vladimiria root	6
Sheng Gan Cao	*Radix glycyrrhizae*	Licorice root	6

but the specific mechanisms remain unclear[6,7]. The aim of the present study was to investigate the effects of JQD on intestinal motility of DSS-induced colitis in mice.

MATERIALS AND METHODS

Materials

DSS (MW 36000-50000; MP Biomedicals, Santa Ana, CA, United States); the nine medicinal herbs of JQD raw powder, as shown in Table 1 (Pharmacy Department, Longhua Hospital affiliated to Shanghai University of TCM, Shanghai, China) dissolved in 0.5% carboxymethylcellulose sodium (CMC) solution; mesalazine [5-aminosalicylic acid (5-ASA); Sunflower Pharmaceutical Group, Jiamusi Lu Ling Pharmaceutical Co., Ltd., Liaoning, China; batch number: 111206], external coat removed, broken down, and dissolved in 0.5% CMC solution; fecal occult blood (FOB) (Huashengyuan Medical Science and Technology Co. Ltd., Beijing, China); tumor necrosis factor-alpha (TNF-α), interleukin (IL)-1β, IL-10 and interferon gamma (IFN-γ) ELISA kits (eBioscience, San Diego, CA, United States); NF-κB antibody (Cell Signaling, Danvers, MA, United States); primer synthesis kit (Yushen Bio-Technique Co. Ltd., Shanghai, China).

Colitis model construction and treatment

Specific pathogen-free C57BL/6 6- to 8-wk-old male mice (weight, 25 ± 3 g) were purchased from Shanghai Xierpu-Bikai Bio-Technique Co. Ltd. [Certificate No. SCXK(Hu)2013-0016]. All experiments were approved by the local ethics committee for Animal Research Studies at the Shanghai University of TCM (SZY20151002). Forty-six mice were randomly divided into four groups: the control group (n = 10), the DSS group (n = 12), the JQD group (n = 12), and the 5-ASA group (n = 12). Except for the control group, colitis was induced in the other groups by giving distilled water containing 5% (w/v) DSS[16].

Seven days after modeling, the mice were administered corresponding drugs intragastrically for 7 d. The intragastric administration dose of the mice in each group was calculated according to the conversion factor of experimental animals and clinical administration dose, which was 0.2 mL/(10 g•d) according to the body mass of the mice[17,18]. The control group and the DSS group mice were administered 0.5% CMC solution intragastrically. The JQD group mice were administered JQD solution (17.1 g/kg per day) and the 5-ASA group mice were administrated 5-ASA solution (100 mg/kg per day) intragastrically[19].

The color, activity, feces condition, weight, and eating volume of mice were observed daily during modeling and drug administration. FOB was tested, and the severity of colitis was assessed daily using the disease activity index (DAI), which was calculated by grading on a scale of 0 to 4 using the following parameters: loss of body weight (0: normal; 1: 0%-5%; 2: 5%-10%; 3: 10%-15%; 4: > 15%), stool consistency (0: normal; 2: loose stools; 4: watery diarrhea) and FOB (0: negative; 2: positive; 4: gross bleeding)[20].

Tissue collection

After the drug administration for 14 d, the mice in each group were sacrificed and colon tissues (from the ileocecal junction to the anal verge) were collected. The general form of colon tissues was observed, and the length was recorded. The colon tissues were cut into three sections. One part was tiled on the filter paper, fixed at the two ends by pins, and then fixed in 4% liquor formaldehyde for hematoxylin and eosin (H and E) staining and in 2.5% glutaraldehyde for transmission electron microscopic (TEM) observation[21]. One part was cut into smooth muscle strips (2 mm wide and 10 mm long) for determining tension. The other tissues were packaged using aluminized paper, put into liquid nitrogen, and stored at -80 ℃ before being used for ELISA, western blotting, and mRNA analyses.

TEM

Colon tissues were doubly fixed with glutaraldehyde and osmic acid, dehydrated with gradients of ethyl alcohol and acetone, embedded and saturated, sliced into ultrathin sections (thickness, 50-60 nm), and doubly stained with uranyl acetate and lead citrate. The structure of ICC and autophagosomes were observed by TEM (Philips, Eindhoven, Netherlands).

ELISA

Colons were weighed and homogenized in 1 mL of ice-

Table 2 Polymerase chain reaction primers' gene sequences

Target gene	Primer sequence	Product length in bp
c-kit	Forward: AGGCTATCCCTGTTGTGTCTG Reverse: ACATGGAGTTCACGGATGTAGA	111
LC-3II	Forward: TTATAGAGCGATACAAGGGGGAG Reverse: CGCCGTCTGATTATCTTGATGAG	109
Beclin-1	Forward: ATGGAGGGGTCTAAGGCGTC Reverse: TCCTCTCCTGAGTTAGCCTCT	197
GAPDH	Forward: AGGTCGGTGTGAACGGATTTG Reverse: TGTAGACCATGTAGTTGAGGTCA	123

cold RIPA lysis buffer containing 1% protease inhibitor cocktail and 1% phosphatase inhibitor cocktail. The lysate was centrifuged (15000 × *g*, 4 ℃) for 15 min, and the supernatant was used for TNF-α, IL-1β, IL-10 and IFN-γ analyses. The levels of TNF-α, IL-1β, IL-10 and IFN-γ in colons were measured by commercial ELISA kits following per manufacturer's instructions, respectively.

Western blotting analysis

The bicinchoninic acid method was utilized to quantify the protein concentrations of colon tissue extracts, which were subsequently separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The protein spots in the gels were transferred onto polyvinylidene fluoride membranes (Amersham Pharmacia Biotech, Piscataway, NJ, United States), which were then blocked by 5% skimmed milk, Tris-buffered saline and Tween 20 at ambient temperature for 2 h. The membranes were incubated with primary antibody against NF-κB p65 (rabbit anti-NF-κB polyclonal antibody, 1:500) overnight at 4 ℃ and then incubated with the appropriate horseradish peroxidase-conjugated secondary antibodies. Finally, the blots were visualized using an enhanced chemiluminescence detection kit (Millipore, Temecula, CA, United States), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Yushen Bio-Technique Co. Ltd., Shanghai, China) was used as a loading control. Three independent replicates were conducted for each experiment.

RNA preparation and reverse transcription-polymerase chain reaction analysis

Total RNA was isolated from mice colon tissues using TRIzol reagent (Invitrogen, Carlsbad, CA, United States) and reverse-transcribed into cDNA using a Reverse Transcription System (Promega, Madison, WI, United States). The thermal cycling conditions were as follows: 95 ℃ for 3 s, 95 ℃ for 5 s for 40 cycles, and 60 ℃ for 30-34 s. The mRNA expression levels of c-kit, microtubule-associated protein 1 light chain 3 (LC3-Ⅱ) and Beclin-1 were quantitatively analyzed and normalized to GAPDH levels. Three replicates were run for each assay. Table 2 shows the sequences of reverse and forward primers. The relative expression of the target genes was analyzed by the $^{\Delta\Delta}$Ct method.

PowerLab analysis

Colon smooth muscles from mice were perfused with Krebs (mmol/L: NaCl 8 g, KCl 0.2 g, $MgSO_4 \cdot 7H_2O$ 0.26 g, $NaH_2PO_4 \cdot 2H_2O$ 0.065 g, $NaHCO_3$ 1 g, $CaCl_2$ 0.2 g, glucose 1 g; 37.5 ± 0.5 ℃, pH 7.3-7.4) bubbled with 97% O_2-3% CO_2[22,23]. One end of the smooth muscle strip was fastened to the tension converter in the bath of the perfusion system of isolated tissue, and the other end was fastened to the hook at the bottom of the bath. The load was 1.0 g, and the balance time was 30 min. After the spontaneous contraction of the smooth muscle strips became steady, electrical signals were recorded with a PowerLab 8/30 (ADInstruments, Bella Vista, Australia) and the contractile amplitude and frequency were analyzed every 5 min.

Statistical analysis

One-way ANOVA was used to analyze the data expressed as mean ± SD for comparison between multiple groups and least significant difference *t*-test for internal group comparison. All statistical analyses were performed using SPSS version 18 (SPSS Inc., Chicago, IL, United States). The threshold of statistical significance was set to $P < 0.05$. GraphPad Prism version 5.0 (GraphPad Software Inc., La Jolla, CA, United States) was used to generate histograms.

RESULTS

JQD decreased DAI scores

On the 2nd day of modeling, the mice in the model groups began to lose weight. Their stools became sticky, and the FOB test was positive, although the color was yellow. On the 7th day of modeling, the body weight of the model group decreased obviously, and black stool or bloody stool was visible in the anus. During modeling, the DAI scores of mice in the model groups increased gradually. Because of weight loss and bloody stool, two mice died in each of the DSS, JQD and 5-ASA groups.

Following drug intervention for 7 d, two mice died in the DSS group due to obvious hematochezia. One mouse died in each of the JQD and 5-ASA groups due to severe weight loss. In the DSS group, the bloody stool gradually decreased. The stool took shape slightly with soft quality, and the FOB test was positive (6/8). The FOB test turned negative (8/9) in the JQD group and (7/9) in the 5-ASA group. During drug administration, the DAI scores decreased gradually (Figure 1).

JQD improved colonic macroscopic appearances

In the control group, the colon had good toughness. It was not easily breakable, and the colonic mucosa was smooth. In the DSS group, the length of the colon was significantly shortened compared with the control group ($P < 0.05$). The colon had poor toughness, with

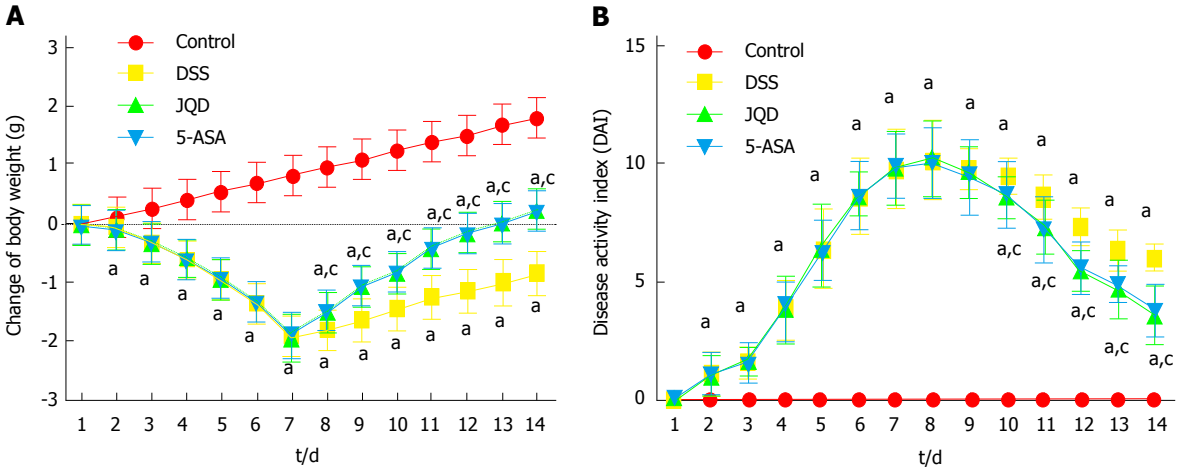

Figure 1 Body weight change and disease activity index of mice. A: Body weight change (the change of body weight in each group of mice was compared with the body weight in the same group of mice on the 1st day); B: Disease activity index. $^{a}P < 0.05$ *vs* control group, $^{c}P < 0.05$ *vs* DSS group. 5-ASA: 5-aminosalicylic acid; DSS: Dextran sulfate sodium; JQD: Jianpi Qingchang decoction.

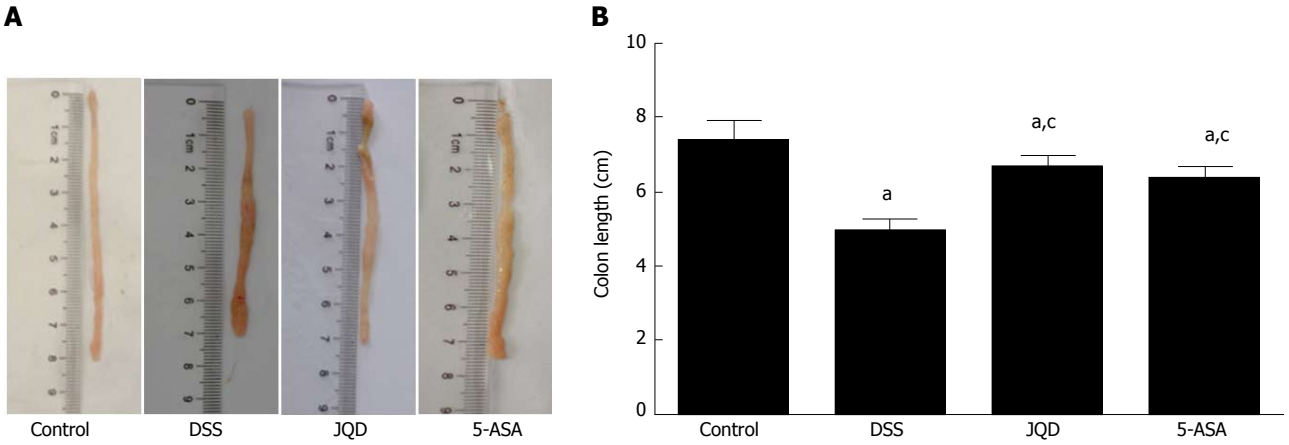

Figure 2 Representative image of colons. A: Macroscopic appearance; B: Length of colon (from the appendix to the anus). $^{a}P < 0.05$ *vs* control group, $^{c}P < 0.05$ *vs* DSS group. 5-ASA: 5-aminosalicylic acid; DSS: Dextran sulfate sodium; JQD: Jianpi Qingchang decoction.

congestion and edema. Partial colon had adhesion with surrounding tissue, irregular ulcer, and bleeding on the mucosa. In the JQD and 5-ASA groups, the colon had better toughness, with local congestion and edema. Partial colon had a small amount of punctate erosion (Figure 2).

JQD promoted repair of the colon

H and E staining showed that in the control group, the colons presented a normal morphology of crypts, abundant goblet cells, a small number of lamina propria mononuclear cells, no signs of mucosal thickening, and complete absence of ulcerations. However, in the DSS group, the colons presented severe epithelial damage with extensive cellular infiltration into the lamina propria and colon mucosa, mucosal thickening, glands arranged irregularly, ulcer and crypt abscess formation, and proliferating granulation tissue. In contrast, in the JQD and 5-ASA groups, the colons presented the migration and repair of epithelial cells on the erosive mucosal surface with infiltration of fewer inflammatory cells and recovery of the glandular structure (Figure 3).

JQD suppressed ICC excessive autophagy

In the control group, ICC was spindle-shaped with a huge ovate nucleolus, caveolae, and numerous free ribosomes. A mass of mitochondria, and smooth and rough endoplasmic reticulum were present in the cytoplasm. ICC was located around the nerve fibers and connected with neighboring smooth muscle cells (SMCs) by intermediate junctions. A few autophagic vacuoles could be observed in ICC. In the DSS group, huge vacuoles, reducing organelles, chromatin margination, cytoplasmic liquefaction and dissolution, and almost invisible autophagic vacuoles could be observed in ICC. In the JQD and 5-ASA groups, the configuration of ICC was normal, with intact connections between cells and the ridge of mitochondria, and a few autophagic vacuoles existed (Figure 4).

JQD inhibited the NF-κB/TNF-α pathway, regulated the cytokine expression of IL-1β, IL-10 and IFN-γ levels in colon tissue

Compared with the control group, the levels of IL-1β, IL-10, IFN-γ and TNF-α increased in the DSS group (*P*

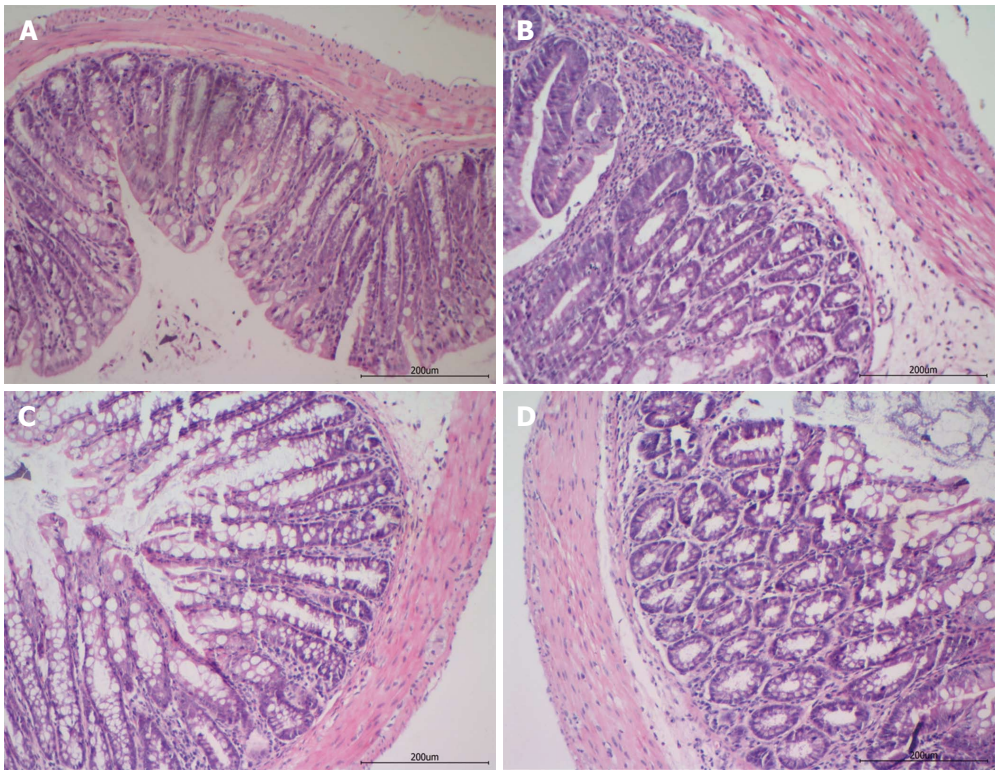

Figure 3 Histologic images of rat colon (H and E, magnification × 200). A: Control group; B: DSS group; C: JQD group; D: 5-ASA group. 5-ASA: 5-aminosalicylic acid; DSS: Dextran sulfate sodium; JQD: Jianpi Qingchang decoction.

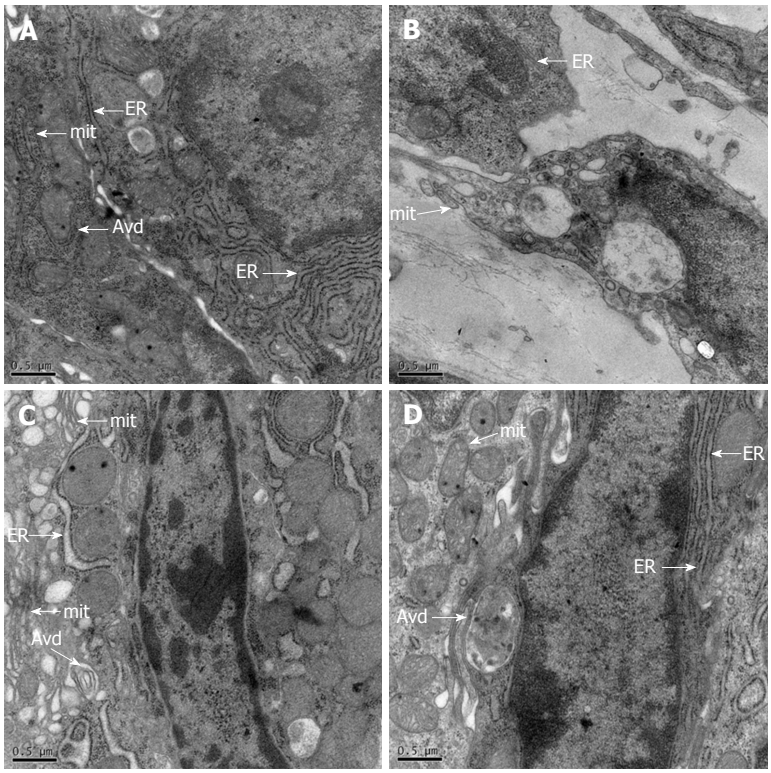

Figure 4 Transmission electron micrographs of interstitial cells of Cajal and autophagosomes in the region of rat proximal colon (magnification × 20000). A: Control group; B: DSS group; C: JQD group; D: 5-ASA group. 5-ASA: 5-aminosalicylic acid; AVd: Autophagic vacuoles; DSS: Dextran sulfate sodium; ER: Endoplasmic reticulum; JQD: Jianpi Qingchang decoction; mit: Mitochondria.

< 0.05). After intervention, compared with the DSS group, the levels of IL-1β and TNF-α decreased in the JQD and 5-ASA groups ($P < 0.05$). However, no statistically significant difference was found between

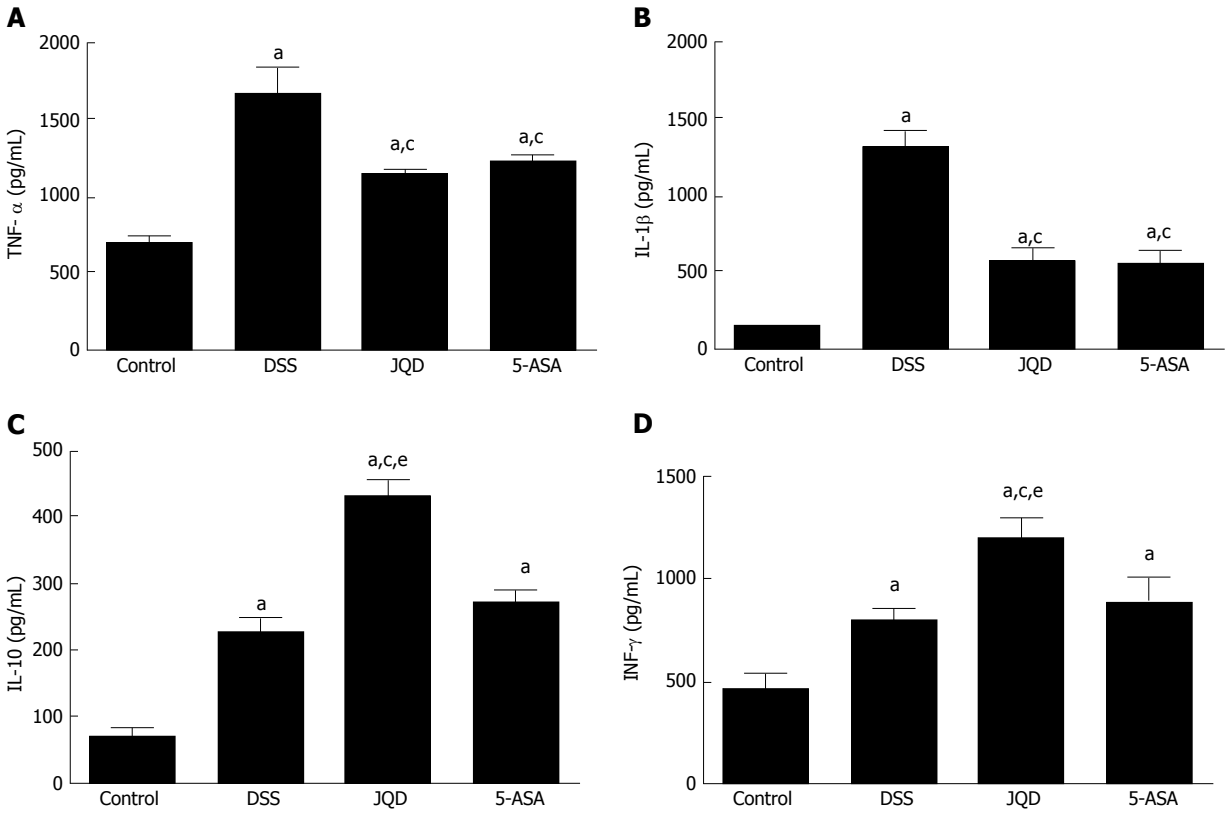

Figure 5 Enzyme linked immunosorbent assay analysis of tumor necrosis factor-alpha, interleukin-1β, interleukin-10 and interferon-gamma expression (*n* = 6). A: TNF-α expression in the colon; B: IL-1β expression in the colon; C: IL-10 expression in the colon; D: IFN-γ expression in the colon. $^{a}P < 0.05$ *vs* control group, $^{c}P < 0.05$ *vs* DSS group, $^{e}P < 0.05$ *vs* 5-ASA group. 5-ASA: 5-aminosalicylic acid; DSS: Dextran sulfate sodium; INF-γ: Interferon-gamma; IL: Interleukin; JQD: Jianpi Qingchang decoction; TNF-α: Tumor necrosis factor-alpha.

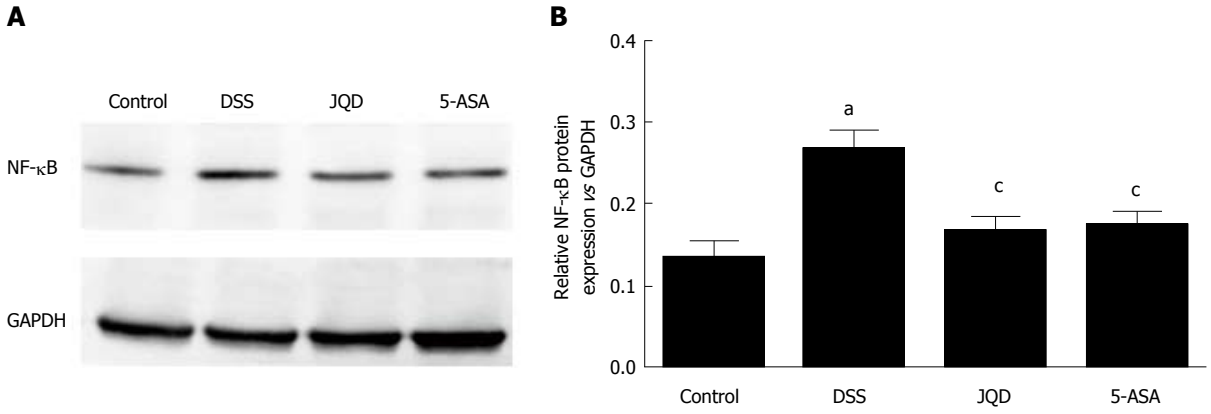

Figure 6 Western blot analysis of nuclear factor-kappa B p65 expression (*n* = 4). A: Representative western blots of NF-κB p65. B: Quantitative analysis of NF-κB p65 protein. $^{a}P < 0.05$ *vs* control group, $^{c}P < 0.05$ *vs* DSS group. 5-ASA: 5-aminosalicylic acid; DSS: Dextran sulfate sodium; JQD: Jianpi Qingchang decoction; NF-κB: Nuclear factor-kappa B; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.

the two groups ($P > 0.05$). Compared with the DSS group, the levels of IL-10 and IFN-γ increased in the JQD and 5-ASA groups. A statistically significant difference was found between the JQD and DSS groups ($P < 0.05$), but no difference was noted between the 5-ASA and DSS groups ($P > 0.05$) (Figure 5).

Compared with the DSS group, NF-κB p65 expression increased in the DSS group ($P < 0.05$). After intervention, compared with the DSS group, NF-κB p65 expression decreased in the JQD and 5-ASA groups ($P < 0.05$). However, no statistically significant difference was observed between the two groups ($P > 0.05$) (Figure 6).

JQD regulated c-kit, LC-3Ⅱ and Beclin-1 mRNA expression in colon tissue

Compared with the control group, c-kit mRNA expression decreased ($P < 0.05$) and LC-3Ⅱ mRNA and Beclin-1 mRNA expression increased in the DSS group ($P < 0.05$). After intervention, compared with the DSS group,

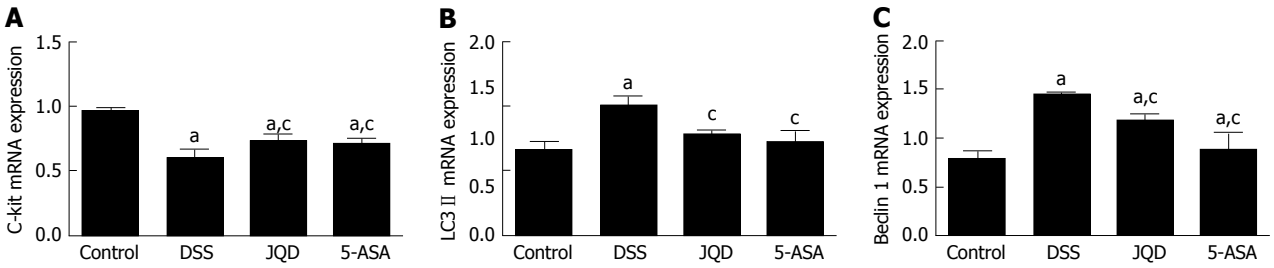

Figure 7 Real-time PCR analysis of c-kit mRNA, LC-3 II mRNA and Beclin-1 mRNA expression (*n* = 3). A: c-kit mRNA; B: LC-3 II mRNA; C: Beclin-1 mRNA. [a]*P* < 0.05 *vs* control group, [c]*P* < 0.05 *vs* DSS group. 5-ASA: 5-aminosalicylic acid; DSS: Dextran sulfate sodium; JQD: Jianpi Qingchang decoction; LC-3 II: Microtubule-associated protein 1 light chain 3.

c-kit mRNA expression increased in the JQD and 5-ASA groups ($P < 0.05$), LC-3II mRNA and Beclin-1 mRNA expression decreased in the JQD and 5-ASA groups ($P < 0.05$). However, no statistically significant difference was found between the two groups ($P > 0.05$) (Figure 7).

JQD regulated intestinal motility

Compared with the control group, the colonic smooth muscle contractile amplitude decreased, while the contractile frequency increased in the DSS group ($P < 0.05$). After intervention, compared with the DSS group, the colonic smooth muscle contractile amplitude increased, while the contractile frequency decreased in the JQD and 5-ASA groups ($P < 0.05$). There was statistically significant difference between the two groups ($P < 0.05$) (Figure 8).

DISCUSSION

The main clinical manifestations of UC include the intestinal motility disorder symptoms[24]. The abnormal expression of NF-κB/TNF-α pathway and cytokine expression is related to not only the intestinal mucosal inflammation of UC but also the intestinal motility. Research has revealed that the secretion of inflammatory factors increases in UC. TNF-α and IL-1β can inhibit L-type Ca^{2+} channels in the circular smooth muscle of colon and activate NF-κB. Then, NF-κB enter into the cell nucleus, which inhibits subunit description of L-type Ca^{2+} channel α1C. Subsequently, the number of Ca^{2+} channels in the cytomembrane of smooth muscle and Ca^{2+} entering into cells decrease. This inhibits the recovery of SMC contraction[25]. IL-1β can increase the levels of cytokine IL-6, IL-8 and TNF-α produced by macrophagocytes. Therefore, the neutrophils aggregate near the inflammatory site, which causes a series of pathological changes such as intestinal epithelial cell damage, crypt abscess and small-vessel vasculitis, leading to the abnormal proliferation of SMCs. The proliferation of abnormal SMCs makes them become thin in UC, resulting in motility disorder[26].

This study found that JQD could repair colonic tissues of DSS-induced colitis in mice, and that this effect involved inhibiting the NF-κB/TNF-α pathway, reducing the level of proinflammatory factor IL-1β, increasing the level of anti-inflammatory factor IL-10 and immunomodulatory factor IFN-γ, and suppressing the intestinal inflammatory cascade reaction. The aggravation of inflammatory reaction led to the filtration of a large number of immune cells, which damaged the colonic mucosa. The repair of colonic mucosa might be due to the decrease in immune inflammatory response after JQD treatment.

This study also found that compared with the control group, the levels of IL-10 and IFN-γ increased in the DSS group, which was in accordance with some previous studies[27,28]. This finding reflected that the bodies of mice had self-regulation and self-recovery effects on colitis induced by DSS, but the weak effect was not enough to cope with intestinal inflammation so that the lesions in intestinal mucosa still existed. However, the self-regulation and self-recovery effects were enhanced under the intervention of JQD, and the increasing levels of IL-10 and IFN-γ blocked the secretion of proinflammatory cytokines. Therefore, JQD promoted the recovery of intestinal mucosa.

The ICC/SMC network pathway is the basic functional unit of gastrointestinal motility. It causes effective transmission of nerve impulse to the surrounding smooth muscle, leading to gastrointestinal motility[29]. The abnormal ICC/SMC network pathway has a certain relationship with intestinal motility disorder of UC[30]. ICC often shows multiple secondary lysosomes, large confluent lipid bodies, and disrupted aggregates of vacuolated glycogen clusters. Intermediate filaments show margination and clumping in patients with UC[31].

The intestinal motility disorder in DSS-induced colitis is caused by the decreased expression of sarcoplasmic endoplasmic reticular calcium ATPase 2 and phospholamban in SMCs, and the increased activity of calmodulin kinase II and the level of histone deacetylases 4 in the cytoplasm. In addition, the increased expression of the contractile proteins associated with the actin filaments of SMCs, which inhibits the interaction between the myosin filaments, is also an important factor[32].

This study found that under physiological conditions, moderate autophagy maintained the growth,

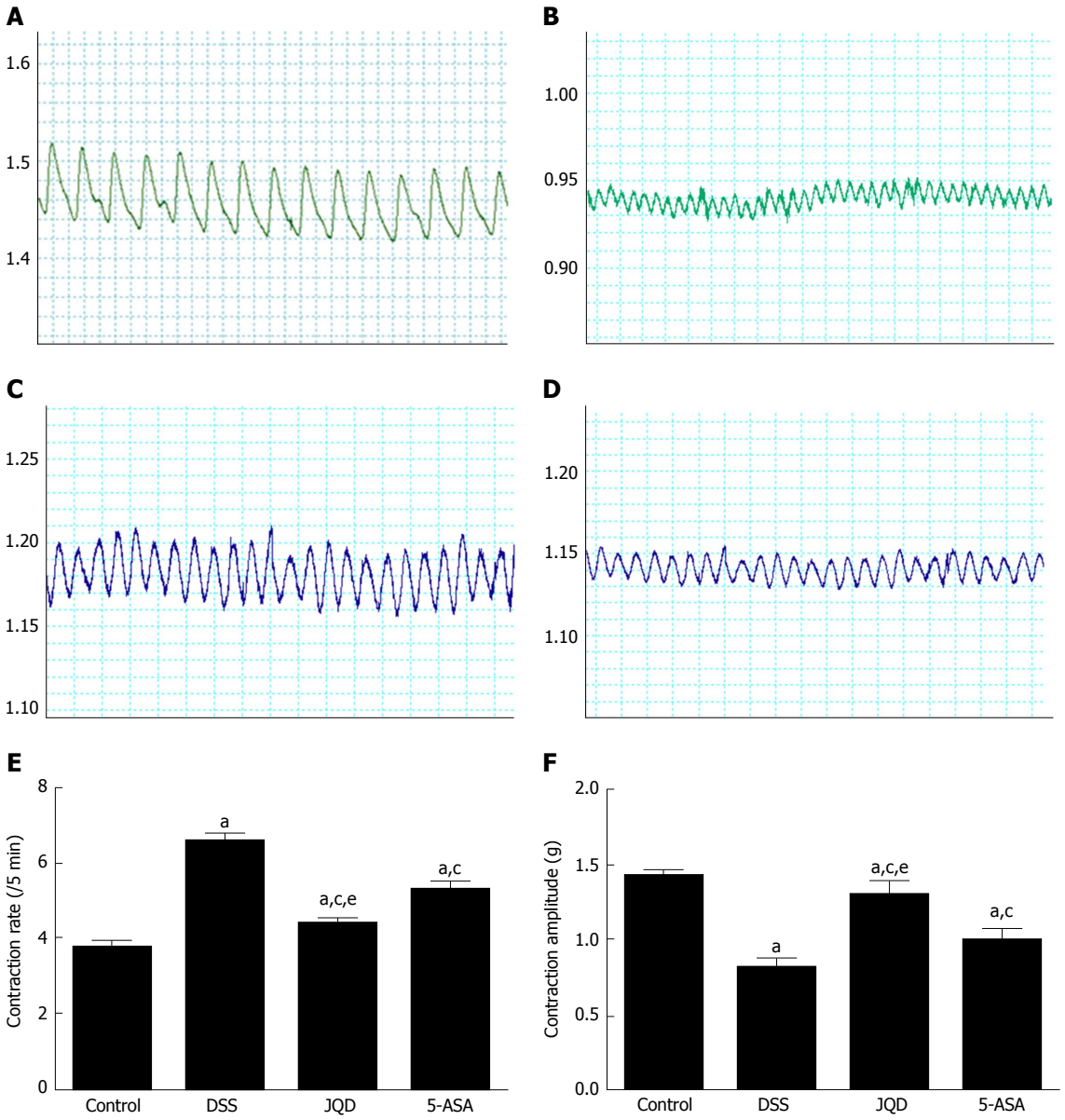

Figure 8 PowerLab analysis on the contraction of colonic smooth muscle in mice (*n* = 6). A: Control group; B: DSS group; C: JQD group; D: 5-ASA group; E: Contraction rate (/5 min); F: Contraction amplitude (g). $^{a}P < 0.05$ *vs* control group, $^{c}P < 0.05$ *vs* DSS group, $^{e}P < 0.05$ *vs* 5-ASA group. 5-ASA: 5-aminosalicylic acid; DSS: Dextran sulfate sodium; JQD: Jianpi Qingchang decoction.

differentiation, survival and homeostasis of colonic ICC. Under pathological conditions, in a model of DSS-induced colitis, excessive autophagy occurred in ICC, leading to programmed cell death. Pathological microstructure revealed reduced organelles, chromatin margination, cytoplasmic dissolution, changes in mitochondria and vacuoles, and reduced or absent autophagic vacuoles. Findings at the molecular level were reduced c-kit protein expression and increased LC3-Ⅱ and Beclin-1 protein expression. The colonic smooth muscle contractile amplitude decreased while contractile frequency increased in the model of DSS-induced colitis. The main abnormal manifestations of intestinal motility were reducing muscle tension and increasing unordered propulsion motility. This was similar to the intestinal dysmotility in patients with UC, finally causing diarrhea and tenesmus. JQD can inhibit excessive autophagy in ICC, regulate the ICC/SMC network pathway, increase the contractive amplitude, and decrease the contractive frequency of smooth muscle. Hence, the colonic smooth muscle tends to be normal and regulates intestinal tract dynamics.

JQD reflects the features of TCM treatment for UC on the basis of syndrome differentiation[33]. As a complete decoction, *Codonopsis pilosula* and *Radix astragali* could nourish qi, *Portulaca oleracea L* and *Radix sanguisorbae* could clear heat and dampness, *Panax notoginseng* and *Bletillae rhizoma* could promote blood circulation by removing blood stasis. Modern studies have found that *R. astragali* containing *Astragalus polysaccharide* can effectively ameliorate 2, 4, 6-trinitrobenzene sulfonic acid-induced experimental

colitis in rats, probably through restoring the number of regulatory T cells, inhibiting IL-17 levels in Peyer's patches, and regulating the expression of TNF-α and IL-1β[34]. *Berberine*, the main component of *Coptis chinensis*, can down-regulate the level of IFN-γ and IL-12, up-regulate the levels of IL-4 and IL-10 in DSS-induced colitis, and relieve inflammatory reaction in intestinal epithelial cells[35,36]. *Ginsenoside-Rg1*, the main component of *P. notoginseng*, can prolong the bleeding and clotting time, down-regulate the thromboxane B2 level, and up-regulate the 6-keto-prostaglandin F1a level to improve the hypercoagulable state in DSS-induced colitis in mice[37].

This study found that the NF-κB/TNF-α pathway was activated in DSS-induced colitis in mice. Abnormal expression of cytokines, excessive autophagy of ICC finally resulted in abnormal motility in UC. JQD can repair the colonic tissues in DSS-induced colitis in mice and regulate the intestinal motility through suppressing intestinal inflammatory cascade reaction, reducing excessive autophagy of ICC, and regulating the network path of ICC/SMCs.

ACKNOWLEDGMENTS

We would like to thank our colleagues in the Institute of Digestive Disease affiliated to Shanghai University of Traditional Chinese Medicine for their help and support in this research.

COMMENTS

Background

The main clinical manifestations of ulcerative colitis (UC) include the intestinal motility disorder symptoms of abdominal pain, diarrhea and tenesmus, besides bloody stool with mucus and pus. Intestinal motility disorder of UC can seriously impact the quality of life (QOL) of patients.

Research frontiers

Interstitial cells of Cajal (ICC) have been accepted as a therapeutic target for gastrointestinal motility disorders. The regulation of autophagy might be a potential strategy for UC, which can be achieved by multi-level and multi-path interference. Therefore, it was essential to study relationships between intestinal motility disorder of UC and autophagy of ICC, and to explore upstream signaling pathway regulation to strengthen or advance the beneficial autophagy response, which might be beneficial in preventing and treating intestinal dysmotility of UC.

Innovations and breakthroughs

Previous studies found that Jianpi Qingchang decoction (JQD) could be used to treat patients with initial or mild UC, improve their intestinal symptoms, such as diarrhea, mucous bloody stool and tenesmus, regulate their systemic functional state, such as fatigue, and consequently improve their QOL. This study provided evidence that JQD can repair colonic tissues of dextran sulfate sodium-induced colitis in mice and regulate the intestinal motility through suppressing intestinal inflammatory cascade reaction, reducing excessive autophagy of ICC, and regulating the network path of ICC/smooth muscle cells.

Applications

The present study provides evidence that JQD, a traditional Chinese medicine recipe, regulates intestinal motility in UC.

Terminology

ICCs serve as electrical pacemakers, active propagation pathways for slow waves, and mediators of enteric motor neurotransmission and are involved in abnormality of intestinal motility. Recent investigations have demonstrated that inflammation influences the morphology and structure of ICC, leads to a variety of changes in the physiological properties of the neurons in this circuitry, and then affects the gastrointestinal motility.

Peer-review

This work focuses on the application of an herbal remedy in an animal model of inflammation.

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ORIGINAL ARTICLE

Basic Study

Lactobacillus acidophilus alleviates pouchitis after ileal pouch-anal anastomosis in rats

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Author contributions: Liu G conceived and designed the study; Xu YY and Zhang YY collected the data; He AQ and Li KY contributed to data analysis; Xu YY, Gao SY and Zhang YY contributed to interpretation of the data; Liu G participated in streamlining the study protocol; Zhang YY, Xu YY and He AQ proofread the study protocol; Li KY and Gao SY supervised the data collection process; Li KY, Xu YY, Gao SY, Zhang YY and He AQ contributed to drafting the manuscript; all authors contributed to the revision of the manuscript and approved the final version.

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Abstract

AIM

To assess the therapeutic potential of *Lactobacillus acidophilus* (LA) for the treatment of pouchitis in a rat model.

METHODS

Sprague Dawley rats underwent proctocolectomy and ileal pouch-anal anastomosis followed by administration of dextran sulfate sodium (DSS) to induce pouchitis. Rats with pouchitis were randomly divided into three groups: no intervention (NI), normal saline (NS, 3 mL/d normal saline for 7 d), and LA (3 mL/d LA at 1 × 10^{10} colony-forming units for 7 d). General body condition was recorded and pouch specimens were obtained for histological examination. mRNA expression levels of interleukin (IL)-1β, IL-6, IL-10, and tumor necrosis factor-α were determined by RT-PCR. Zonula occludens protein 1 (ZO-1) levels were measured by immunohistochemistry.

RESULTS

LA reduced weight loss associated with pouchitis (P < 0.05) and improved the symptoms of pouchitis in rats. Compared with the NI and NS groups, rats in the LA

group showed earlier disappearance of hematochezia (6.17 ± 0.75, 6.50 ± 0.55, 3.17 ± 0.75, $P < 0.05$) and higher fecal scores (2.67 ± 0.48, 2.50 ± 0.51, 4.42 ± 0.50, respectively, $P < 0.05$). Histological scores were also lower in the LA group compared with the other two groups (7.17 ± 0.98, 8.00 ± 0.89, 4.00 ± 0.89, respectively, $P < 0.05$). mRNA expression levels of IL-1β, IL-6, and tumor necrosis factor-α were significantly reduced, while IL-10 mRNA levels were significantly increased in the LA group ($P < 0.05$, respectively). ZO-1 protein levels were also significantly increased after administration of LA ($P < 0.05$).

CONCLUSION

LA alleviates pouchitis induced by DSS after ileal pouch-anal anastomosis by decreasing pro-inflammatory factors and increasing anti-inflammatory factors, and restoring ZO-1 expression in the mucosa.

Key words: *Lactobacillus acidophilus*; Pouchitis; Ileal pouch-anal anastomosis; Dextran sulfate sodium; Rats

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Core tip: This study aimed to assess the therapeutic potential of *Lactobacillus acidophilus* (LA) for the treatment of pouchitis in a rat model. Rats with pouchitis were randomly divided into three groups: no intervention, normal saline (NS, 3 mL/d normal saline for 7 d), and LA (3 mL/d LA at 1×10^{10} colony-forming units for 7 d). General body condition was recorded and pouch specimens were obtained for histological examination. mRNA expression levels of interleukin (IL)-1β, IL-6, IL-10, and tumor necrosis factor-α were determined by RT-PCR. Zonula occludens protein 1 levels were measured by immunohistochemistry.

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INTRODUCTION

Ulcerative colitis (UC) is defined as a chronic nonspecific inflammatory disorder with recurrent symptoms, involving the mucosa and submucosa of the colon and rectum[1]. Ileal pouch-anal anastomosis (IPAA) is an ideal surgical treatment for UC, allowing complete removal of the colorectal lesion while retaining the anus and avoiding the need for a permanent ileostomy. Ileal pouchitis is a common complication after IPAA in patients with UC, and occurs in approximately 50% of patients[2]. However, the pathogenesis of pouchitis is unclear and basic studies regarding this complication are lacking.

Lactobacillus acidophilus (LA) is a gram-positive bacterium that can form a protein crystal layer on the surface of intestinal cells[3], thus conferring a protective effect on the intestinal barrier. The intestinal microbiota are considered to play a vital role in the development of UC[4] and pouchitis. Gionchetti *et al*[5] demonstrated the efficacy of probiotics such as VSL#3 for the prophylaxis and treatment of pouchitis, and our results are consistent with the results of this study. However, although various studies have shown beneficial effects of probiotics on the prevention and treatment of pouchitis, the specific mechanism remains unclear. Furthermore, the ability of specific bacteria and their combination to improve pouch inflammation is unknown[6].

Interleukin (IL)-1β is a multi-protein complex, which can play an important role in the maintenance of intestinal immune balance through the identification of bacteria and injury-related molecules[7]. IL-6 is an important inflammatory factor secreted by endothelial cells, macrophages, and mast cells, and participates in the activation of lymphocytes[8]. Tumor necrosis factor (TNF)-α plays a key role in intestinal inflammation, involving multiple immune responses, affecting the expression of endothelial cell adhesion molecules and maintaining intestinal permeability[9]. IL-10 is a regulatory cytokine secreted by mononuclear macrophages and plays an anti-inflammatory role[10]. Intestinal inflammation is often accompanied by abnormalities in inflammatory factors such as IL-1β, IL-6, TNF-α and IL-10, which may reflect the severity of inflammation in pouchitis[11]. Furthermore, damage to the intestinal mucosal barrier is often accompanied by destruction of tight junction proteins, especially the loss of zonula occludens protein-1 (ZO-1), leading to increased intestinal permeability and further increasing the intestinal inflammatory response[12]. Rats subjected to IPAA provide an effective model for the study of pouchitis[13]. Shebani *et al*[14] established a rat model of dextran sulfate sodium (DSS)-induced pouchitis suitable for further studies of the pathogenesis of the disease.

We investigated the therapeutic effect of probiotic LA in a DSS rat model of ileal pouchitis, including determination of the expression of inflammatory markers (IL-1β, IL-6, TNF-α and IL-10) and ZO-1 protein in the intestinal mucosa by immunohistochemistry.

MATERIALS AND METHODS

Animals

Male Sprague Dawley rats ($n = 18$) weighing 360-380 g (purchased from the Laboratory Animal Center of the Military Medical Science Academy of the Chinese People's Liberation Army) were housed individually in a specific pathogen-free animal laboratory at a temperature of 25 ℃ with a 12 h light/dark cycle, and provided with standard rat chow and running water *ad libitum*. Animal care and experiments were conducted

Table 1 Primers used for reverse transcription-polymerase chain reaction

	Oligonucleotide sequence (5'-3')	Product length (bp)
IL-1β		190
sense	5'-AATGCCTCGTGCTGTCTGACC-3'	
antisense	5'-GTGGGTGTGCCGTCTTTCATCA-3'	
IL-6		111
sense	5'-GACTTCCAGCCAGTTGCCTTCT-3'	
antisense	5'-TGGTCTGTTGTGGGTGGTATCC-3'	
IL-10		
sense	5'-GGGTTGCCAAGCCTTGTCAGAA-3'	196
antisense	5'-CTTCACCTGCTCCACTGCCTTG-3'	
TNF-α		
sense	5'-GGGCTCCCTCTCATCAGTTCCA-3'	113
antisense	5'-TGCTCCTCCGCTTGGTGGTT-3'	
R-GAPDH		
sense	5'-TACCCACGGCAAGTTCAACG -3'	122
antisense	5'-CACCAGCATCACCCCATTTG -3'	

IL: Interleukin; TNF-α: Tumor necrosis factor-α.

according to the international guidelines on animal research and ethics.

Ileal pouchitis model

IPAA was performed by microsurgery and pouchitis was induced by administration of 4% DSS for 4 successive days after postoperative day 31. Rats with pouchitis were divided randomly into three groups: no intervention (NI) group, normal saline (NS) group, and LA group (n = 6 per group). Rats in the NS group received 3 mL/d normal saline by lavage, and rats in the LA group received 3 mL/d LA at a concentration of 1×10^{10} colony-forming units for 7 d by lavage.

General body condition and sample collection

Body weight changes, hematochezia, and fecal scores were observed and recorded in all rats before sacrifice under anesthesia on day 7 after LA or normal saline intervention. Fecal scores were evaluated on a 5-point scale according to the method described by Drzymala-Czyż *et al*[15]: (1) lack of stool; (2) diarrhea; (3) blob of stool; (4) textured stool; and (5) normal stool. The ileal pouch was harvested after sacrifice and washed with normal saline. Half of each sample was fixed in 10% neutral formalin solution for histological examination and immunohistochemistry, and the remaining portion was immediately frozen in liquid nitrogen and stored at -80 ℃ for RT-PCR analysis.

Histological assessment of pouchitis tissue

Tissues were paraffin-embedded, stained with hematoxylin and eosin, and examined under a microscope. Pouch specimens were assessed according to the criteria described by Atila *et al*[16]. Erosion was evaluated as: 0, negative; 1, focal erosion; 2, erosion in many regions; or 3, extensive erosion. Ulceration was evaluated as: 0, none; 1, focal ulceration of the mucosa in half the superficial regions; 2, total mucosal ulceration at multiple foci; or 3, extensive mucosal ulceration extending to the muscularis mucosa or beyond. Intra-epithelial inflammation was evaluated by counting the number of lymphocytes in 100 epithelial cells at the tips of the villi. Villous atrophy was evaluated as: 0, none; 1, mild; 2, moderate; or 3, severe with villous flattening. Edema at the lamina propria was evaluated as: 0, none or 1, positive. Abscess formation and submucosal inflammation were also evaluated.

RT-PCR detection of IL-1β, IL-6, IL-10 and TNF-α mRNA

IL-1β, IL-6, IL-10 and TNF-α mRNA were detected using a RT-PCR kit (MJ-RESEARCH, United States) according to the manufacturer's instructions. Total RNA was extracted from pouch tissues using an animal tissue total RNA Extraction Kit (Tiangen, Beijing, China). The primer pairs are shown in Table 1.

Immunohistochemistry

Samples were subjected to immunohistochemistry to assess the expression of ZO-1 protein, using rabbit anti-ZO-1 (PB0072) (Boster, WuHan, China). Immunohistochemical images were analyzed using Image-Pro Plus6.0 software to assess the optical density.

Statistical analysis

All statistical analyses were carried out using SPSS 19.0. The data were expressed as the mean ± SD. Data analysis was performed using independent-samples t-tests or one-way ANOVA, and comparisons of two among the three groups were made using Students-Newman-Keuls tests. A difference of $P < 0.05$ was considered statistically significant.

RESULTS

Changes in physiological condition

Rat body weight decreased linearly after the generation of pouchitis (72.92 ± 6.60). Following intervention for 7 d, body weight in the NI (63.50 ± 5.99) and NS groups (64.67 ± 11.93) continued to decline (Figure 1A), with no significant difference between the two groups ($P > 0.05$). However, the weight of rats in the LA group which initially decreased started to increase again on day 4 of lavage with LA (20.17 ± 3.25). The difference in body weights among the three groups was significant ($F = 61.34$, $P < 0.05$) (Figure 1B).

Bloody stools were observed in all groups at the end of the DSS intervention. Hematochezia disappeared in the LA group at 3.17 d ± 0.75 d, and in the NS and NI groups at 6.50 d ± 0.55 d and 6.17 d ± 0.75 d, respectively. The recovery time in the LA group was significantly shorter than in the NS and NI groups ($F = 108.70$, $P < 0.05$) (Figure 2).

Stools in the NI and NS groups had a similar, loose-paste appearance (Figure 3A) and there was no significant difference in fecal scores between the

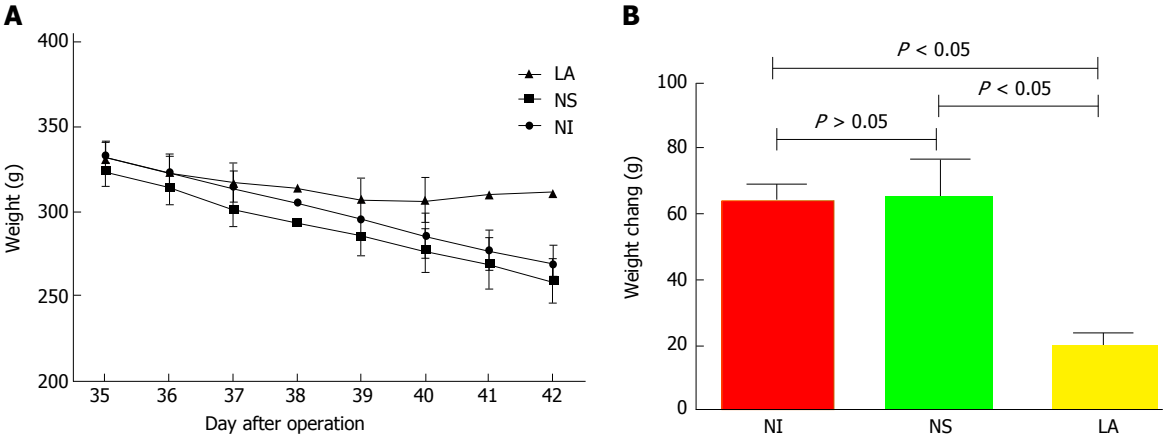

Figure 1 Changes in rat body weight during the experiment. A: Body weight changes in rats following pouchitis. Weight in the NI (63.50 ± 5.99) and NS groups (64.67 ± 11.93) continued to decline, while weight in the LA group initially decreased and then increased on day 4 of LA lavage (20.17 ± 3.25); B: The differences in body weight among the three groups were statistically significant (F = 61.34, P < 0.05), but there was no significant difference between the NI and NS groups (P > 0.05). NI: No Intervention group; NS: Normal Saline group; LA: *Lactobacillus acidophilus* group.

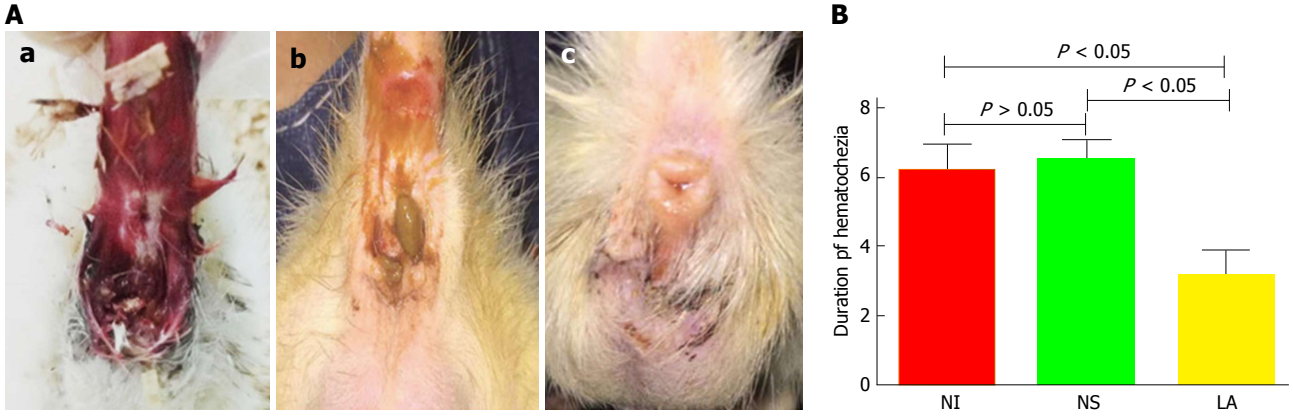

Figure 2 Hematochezia in rats. A: Anal condition of rats in each group at 4 d post-intervention. The NI (a) and NS groups (b) still had hematochezia, but this had disappeared in the LA group (c); B: Recovery was significantly faster in the LA group compared with the NS and NI groups (F = 108.70, P < 0.05). NI: No Intervention group; NS: Normal Saline group; LA: *Lactobacillus acidophilus* group.

NI (2.67 ± 0.48) and NS groups (2.50 ± 0.51) (t = 1.16, P > 0.05). However, feces in the LA group (4.42 ± 0.50) were more normal, and the fecal score was significantly higher than in the NI (t = 12.30, P < 0.05) and NS groups (t = 13.09, P < 0.05) (Figure 3B).

Histopathological changes and pouch scores

In terms of gross morphology, mucosal surface congestion and edema, toughness, visible multiple ulcers, and scattered bleeding were observed in the NI and NS groups, but mucosal congestion and edema were less evident in the LA group (Figure 4A).

On microscopic examination, the structure of the mucosal villi in the pouch tissue was irregular and disordered, the passivation of the villi, with extensive inflammatory cell infiltration in the central matrix of the villi in the NI and NS groups, while less severe lesions were observed in the LA group (Figure 4B). There was no significant difference in histological scores between the NI (7.17 ± 0.98) and NS groups (8.00 ± 0.89) (t = 1.536, P > 0.05), but the histological score was significantly lower in the LA group (4.00 ± 0.89) compared with the other two groups (F = 5.84, P < 0.05) (Figure 4C).

Expression of IL-1β, IL-6, IL-10, and TNF-α mRNA

mRNA levels of IL-1β, IL-6, and TNF-α in the LA group were significantly lower than in the NS and NI groups (F = 373.60, P < 0.05; F = 285.50, P < 0.05; F = 132.90, P < 0.05, respectively). In contrast, IL-10 mRNA levels were significantly higher in the LA group compared with the other two groups (F = 61.05, P < 0.05). There was no significant difference in expression levels of inflammatory factors between the NS and NI groups (P > 0.05) (Figure 5).

Expression levels of ZO-1 protein

ZO-1 is a tight junction protein, indicated by yellow staining in the cell membrane (Figure 6A). ZO-1 protein expression levels were significantly lower in the NI (0.27 ± 0.03) and NS groups (0.22 ± 0.08) compared with the LA group (0.35 ± 0.02) (F = 8.23, P < 0.05)

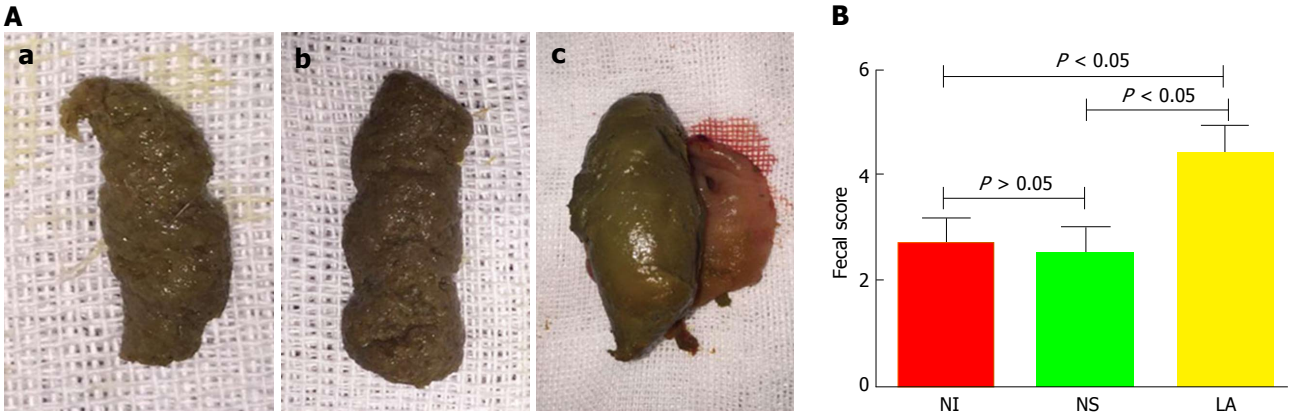

Figure 3 Feces of rats. A: Stools appeared as loose paste in the NI (a) and NS (b) groups, but were more normal in the LA group (c); B: The fecal score was significantly higher in the LA group compared with the NI (t = 12.30, P < 0.05) and NS groups (t = 13.09, P < 0.05). NI: No Intervention group; NS: Normal Saline group; LA: *Lactobacillus acidophilus* group.

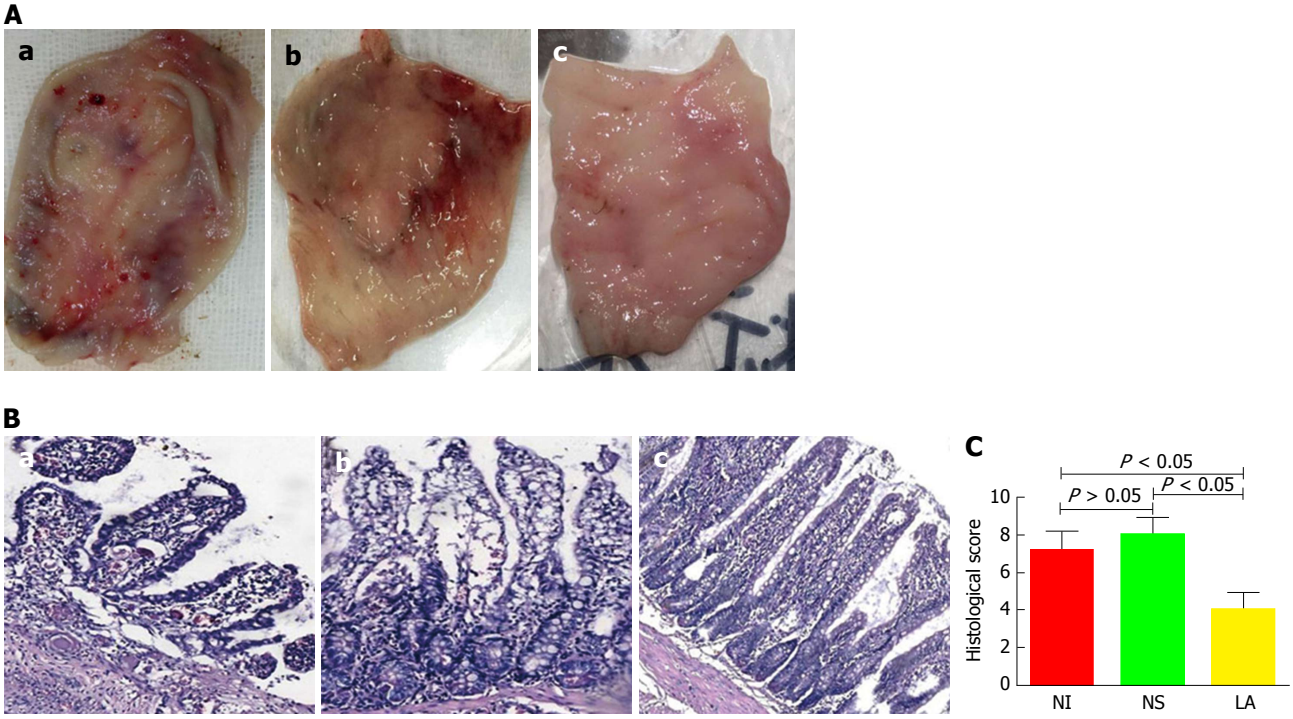

Figure 4 Gross and microscopic histological observations. A: Mucosal surface congestion and edema, toughness, visible multiple ulcers, and scattered bleeding were evident in the NI (a) and NS (b) groups, but mucosal congestion and edema had disappeared in the LA (c) group; B: Microscopic examination of the pouch revealed irregular and disordered mucosal villi, passivation of the villi, and extensive inflammatory cell infiltration in the central matrix of the villi in the NI (a) and NS (b) groups, but less severe lesions in the LA (c) group; C: There was no significant difference in histological scores between the NI (7.17 ± 0.98) and NS groups (8.00 ± 0.89) (t = 1.536, P > 0.05), but the histological score was significantly lower in the LA group (4.00 ± 0.89) than in the other two groups (F = 5.84, P < 0.05). NI: No Intervention group; NS: Normal Saline group; LA: *Lactobacillus acidophilus* group.

(Figure 6B).

DISCUSSION

The intestinal microflora is generally thought to be involved in the pathogenesis of inflammatory bowel disease (IBD)[17]. The numbers of intestinal *Bifidobacterium* and *Lactobacillus* were decreased and *Clostridium perfringens* was significantly increased in patients with pouchitis[18]. LA is a component of VSL#3, and VSL#3, which is beneficial for maintaining remission in patients with pouchitis[19,20]. Probiotics containing LA can reduce expression of the inflammatory cytokine IL-1β and inhibit inflammatory damage caused by infiltration of polymorphonuclear cells in the tissues. Lammers *et al*[11] suggested that probiotics could be used to prevent and treat pouchitis, and LA has also shown immunomodulatory effects in *in vitro* experiments[21-24].

NaCl absorption involves coupling of the Cl^-/HCO^{3-} exchanger(s) primarily with the Na^+/H^+ exchanger 3 at

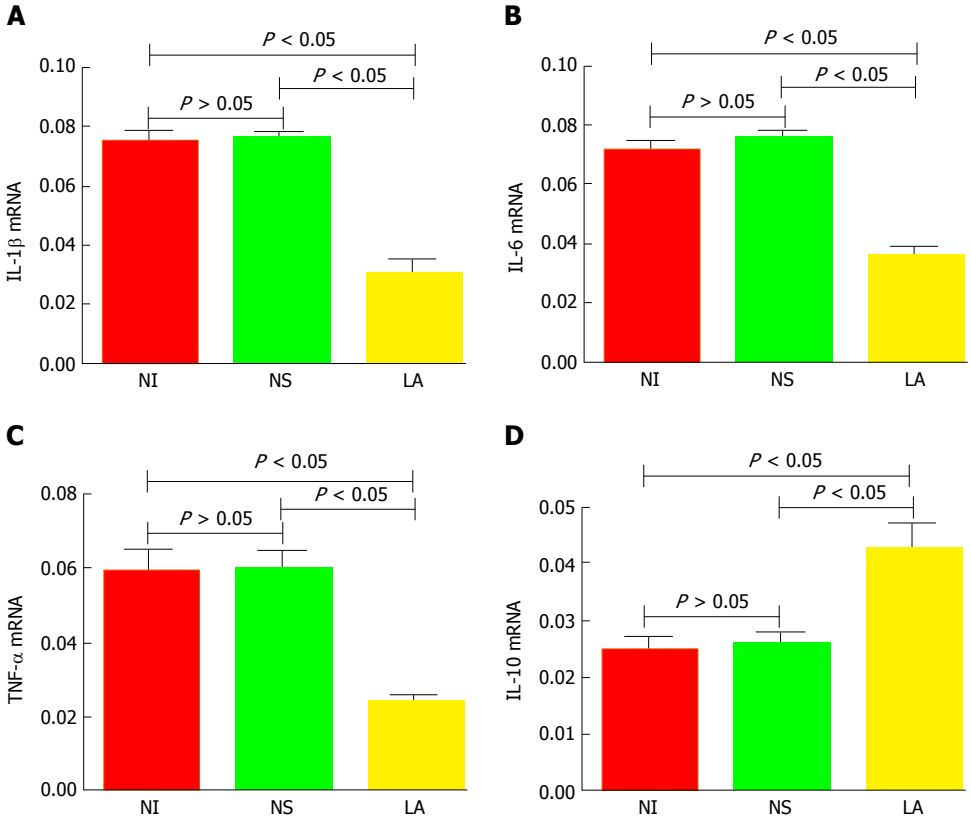

Figure 5 mRNA expression levels. mRNA levels of IL-1β (A), IL-6 (B), and TNF-α (C) were significantly lower in the LA group compared with the NS and NI groups (F = 373.60, P < 0.05; F = 285.50, P < 0.05; F = 132.90, P < 0.05, respectively), while IL-10 (D) levels were significantly higher (F = 61.05, P < 0.05). IL: Interleukin; TNF-α: Tumor necrosis factor-α; NI: No Intervention group; NS: Normal Saline group; LA: *Lactobacillus acidophilus* group.

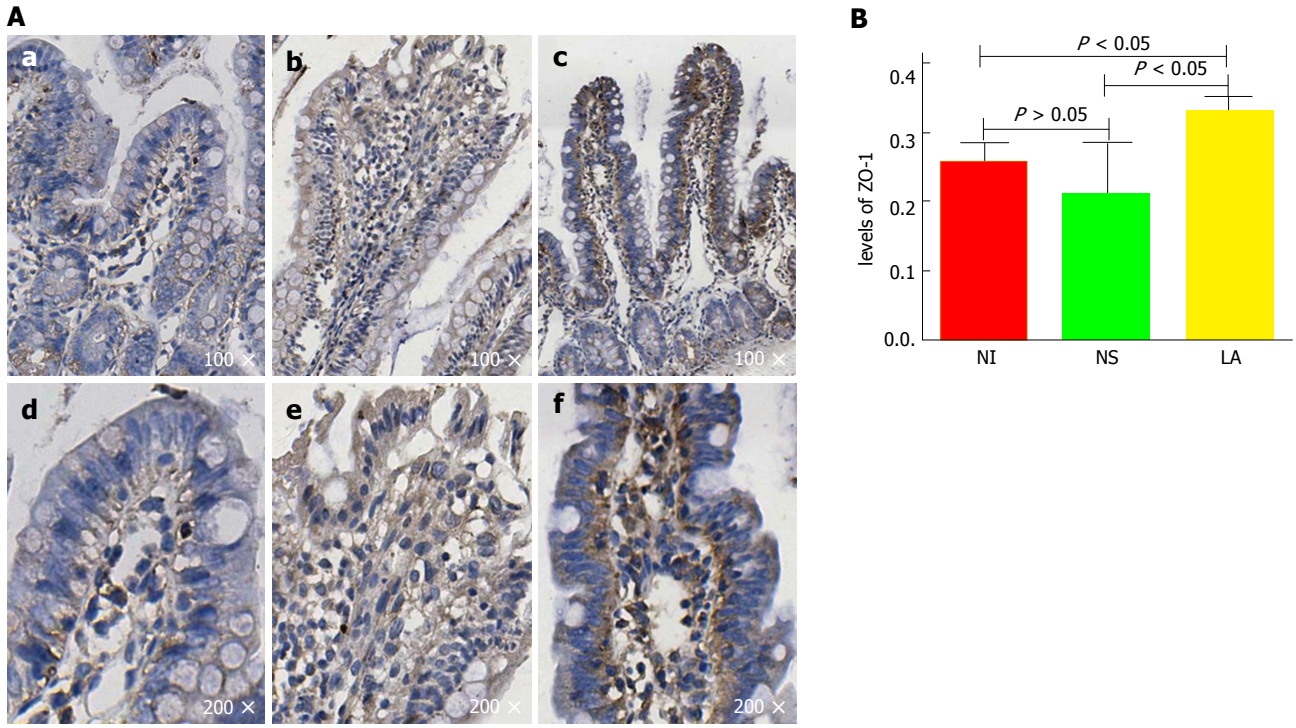

Figure 6 Zonula occludens protein 1 protein expression levels. A: ZO-1 protein expression levels in the NI (a, d) and NS groups (b, e) were significantly lower than in the LA group (c, f); B: The differences between the three groups were statistically significant (F = 8.23, P < 0.05). ZO-1: Zonula occludens protein 1; NI: No Intervention group; NS: Normal Saline group; LA: *Lactobacillus acidophilus* group.

the apical membrane of intestinal epithelia. Disturbances to this process occur in diarrheal diseases[25], and may also be involved in the formation of mucus stools in pouchitis. LA-conditioned medium stimulated intestinal cells to absorb NaCl by different mechanisms in *in vitro* experiments, and this mechanism may be an important factor in the improvement and treatment of IBD-associated diarrhea symptoms[26,27]. Borthakur *et al*[26] found that LA increased the exchange effect by increasing cell surface phosphoinositide 3-kinase dependent $Cl^-/HCO3^-$ channels. Singh *et al*[27] showed that LA could promote the expression of Na^+/H^+ exchanger 3 (SLC9A3), which is widespread in epithelial cells of the digestive tract, resulting in improved intestinal absorption of electrolytes and an antidiarrheal effect. Chen *et al*[28] showed that LA was able to prevent bacterial colitis and activate the immune response with a protective effect on the intestinal mucosa.

Changes in intestinal inflammation are often accompanied by abnormalities in a range of inflammatory cytokines, including IL-1β, IL-6, TNF-α and IL-10, and the level of inflammation can be determined by detecting changes in these inflammatory factors in the pouch mucosa[11]. The results showed that the expression levels of IL-1β, IL-6 and TNF-α were significantly higher in patients with pouchitis than in those with active UC and non-pouchitis. Intestinal tract damage can lead to activation of the inflammatory response resulting in activation of caspase-1 and release of IL-1β[29]. Our study found that the expression of IL-1β in pouch tissues was significantly increased after pouchitis was induced by DSS, and compared with the control group, LA can significantly reduce the expression of IL-1β.

IL-6 expression levels were significantly increased in intestinal tissues of rats with inflammation. The interaction of antigen presenting cells with bacteria in IBD patients was shown to result in abnormal activation of $CD4^+$ T cells, causing continuous release of pro-inflammatory cytokines and increased levels of IL-6 and TNF-α[30]. Our results showed that the expression levels of IL-6 and TNF-α in the mucosa of DSS-induced pouchitis were significantly higher than those in the control group, while the expression was decreased following LA gavage and the pathological score of the pouch was significantly decreased. Sang *et al*[31] treated rats with colitis using VSL#3 and showed that probiotics could significantly reduce IL-6 expression in intestinal tissues. Chen *et al*[32] also found that LA could significantly inhibit the expression of TNF-α in the intestinal mucosa in a rat colitis model.

IL-10 has previously been shown to play an anti-inflammatory role in pouchitis[33], and LA significantly increased IL-10 expression in human peripheral blood cells[34]. The results of the current study showed that DSS caused an intestinal inflammatory response in rats, associated with significantly increased levels of IL-1β, IL-6, and TNF-α, and significantly decreased levels of IL-10. This is consistent with the results of Chen *et al*[35], who used probiotics in a DSS-induced pouchitis model in rats. In addition, IL-6 can increase the amount of myeloperoxidase (MPO) by activating neutrophils, the increase in MPO protein not only reflects the degree of inflammation, but can produce a large number of oxygen free radicals in the intestinal barrier and further aggravate intestinal inflammation.

ZO-1 is a member of the membrane-associated guanylate kinases family[36]. Shen *et al*[37] showed that abnormal tight junctions resulted in increased permeability of the intestinal barrier, often accompanied by decreased expression of ZO-1 protein. Changes in the intestinal barrier caused by reduced ZO-1 expression in the intestinal tract will further promote the development of intestinal inflammation[12]. ZO-1 decreased following DSS administration in a rat colitis model, and the severity of colitis increased with time[12]. Our results also showed that ZO-1 expression levels were significantly higher in the LA group compared with the NI and NS groups, accompanied by decreased expression of inflammatory factors and improved pathological changes.

In summary, DSS-induced destruction of the intestinal barrier in the pouch manifested as increased levels of pro-inflammatory IL-1β, IL-6 and TNF-α, and decreased levels of anti-inflammatory IL-10, together with decreased expression of the intestinal barrier tight junction protein ZO-1. These factors reflect a complex network with the potential to aggravate mucosal inflammation in the pouch. LA may reduce the expression of pro-inflammatory factors and increase anti-inflammatory factors through a variety of mechanisms, increase expression of the tight junction protein ZO-1 in the intestinal mucosa thus promoting recovery of intestinal mucosal barrier function, and block interactions among various pro-inflammatory factors. Further studies are needed to clarify the potential role of LA in the prevention and treatment of pouchitis.

COMMENTS

Background

Ileal pouchitis is a common complication after ileal pouch-anal anastomosis (IPAA) in patients with ulcerative colitis (UC), and occurs in approximately 50% of patients. However, the pathogenesis of pouchitis is unclear and basic studies regarding this complication are lacking. *Lactobacillus acidophilus* (LA) confers a protective effect on the intestinal barrier, but the therapeutic potential of LA on pouchitis is uncertain.

Research frontiers

Gastrointestinal microbiota in the field of inflammatory bowel disease (IBD) and pouchitis is a hot research topic.

Innovations and breakthroughs

Flora imbalance is involved in the pathogenesis of IBD, and has a similar role in the genesis and development of pouchitis. The authors confirmed that LA can be effective in the treatment of pouchitis, which provides a new treatment option

for pouchitis.

Applications

This study provides a new treatment option for pouchitis. The successful application of LA in the treatment of pouchitis in rat models has indicated that further research on microbial treatment in humans should be carried out.

Terminology

Pouchitis: An intestinal pouch complication of the IPAA procedure in patients with UC. Symptoms of pouchitis include diarrhea, hematochezia, increased stool frequency and abdominal cramps. LA: LA is a gram-positive bacterium that can form a protein crystal layer on the surface of intestinal cells, thus conferring a protective effect on the intestinal barrier and plays a vital role in the development of UC and pouchitis.

Peer-review

This manuscript is an interesting and well written paper regarding possible facilitatory effects of LA pouchitis in a rat model of IPAA.

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ORIGINAL ARTICLE

Basic Study

Effect of EPEC endotoxin and bifidobacteria on intestinal barrier function through modulation of toll-like receptor 2 and toll-like receptor 4 expression in intestinal epithelial cell-18

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Author contributions: Yang X and Gao XC contributed equally as co-first authors; Ren HY conceived and designed the experiments; Yang X and Gao XC performed the majority of experiments, analyzed the data and wrote the paper; Ren HY and Liu J provided reagents, materials and analytical tools; all authors read and approved the final manuscript.

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Abstract

AIM

To investigate toll-like receptor 2 (TLR2) and TLR4 expression, following bifidobacteria and low-dose EPEC endotoxin treatment, and intestinal barrier function in rat intestinal epithelial cell-18 (IEC-18).

METHODS

Six experimental groups were established - normal control, EPEC, *Bifidobacteria infantis (B. infantis)*, *B. longum*, *B. bifidum*, and *B. youth* groups. Optimal EPEC endotoxin concentration, bifidobacteria fold dilution, and treatment duration were determined. Quantitative real-time polymerase chain reaction and western blot, respectively, were conducted to detect TLR2 and TLR4 mRNA and protein expression in IEC-18 cells. Transepithelial electrical resistance (TEER) was measured by the EVOM chopstick voltohmmeter in each group. All experiments were conducted in triplicate and data were analyzed on SPSS 16.

RESULTS

TLR2 and TLR4 mRNA and protein expression in the

EPEC group were significantly higher than in the control group ($P < 0.05$). TLR2 mRNA and protein expression in the *B. infantis*, *B. longum* and *B. youth* groups were significantly lower than in the normal control group ($P < 0.05$). TLR4 mRNA and protein expression in the *B. bifidum* and *B. youth* groups were significantly lower than in normal controls ($P < 0.05$). In addition, the TEER in *B. infantis*, *B. longum*, *B. bifidum*, and *B. youth* groups were decreased by 19%, 18%, 23% and 23%, respectively, after 120 min of intervention, as compared to the control group. However, the TEER in the EPEC group was significantly decreased by 67% in comparison to the normal control group ($P < 0.05$).

CONCLUSION

Bifidobacteria protect IEC-18 cells against injury by down-regulating TLR2 and TLR4 expression and enhance intestinal barrier function to protect the intestinal epithelial cells from pathogenic invasion.

Key words: Bifidobacteria; Intestinal barrier function; Intestinal epithelial cells

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Core tip: Toll-like receptors (TLRs) are key components of the innate immune system that trigger antimicrobial host defense responses. EPEC promoted the expression of toll-like receptor 2 (TLR2) and TLR4 and increased cell membrane permeability, where as bifidobacteria inhibited the expression of TLR2 and TLR4 and prevented TLR-mediated inflammation. Therefore, bifidobacteria can potentially play a protective role by inhibiting inflammation and preventing penetration of pathogenic bacteria in patients with inflammatory bowel disease.

Yang X, Gao XC, Liu J, Ren HY. Effect of EPEC endotoxin and bifidobacteria on intestinal barrier function through modulation of toll-like receptor 2 and toll-like receptor 4 expression in intestinal epithelial cell-18. *World J Gastroenterol* 2017; 23(26): 4744-4751 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i26/4744.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i26.4744

INTRODUCTION

The incidence of inflammatory bowel disease (IBD) is high in North America and Nordic countries, but lower in Asia. However, increasing incidence of IBD has been observed over the past few decades in parallel with better living standards and advances in diagnostics for IBD[1]. Current knowledge on the specific etiopathogenesis of IBD is still poorly understood. In general, however, IBD is considered to have several risk factors, including environmental, genetic, infectious and immune contributors[2]. The increasing incidence of IBD in developing countries indicates that progression of IBD epidemiology is associated with westernization and industrialization[3]. IBD is not only a polygenic disease, but also displays genetic heterogeneity. A recent study found that intraintestinal interaction of symbiotic gut bacteria is genetically determined and associated with epithelial dysfunction, which further induces IBD[4]. Thus far, no specific pathogens have been found to be associated with IBD. However, in an immunodeficient animal model of IBD, researchers found that intestinal inflammation did not occur in a sterile intestinal environment[5], suggesting that infective factors may play a key role in IBD pathogenesis.

More than 200 species in approximately one hundred trillion numbers constitute the characteristic distribution of commensal bacteria in a healthy human; of these, 99.9% of the bacteria grow on the intestinal mucosal surface, and along with those in feces, comprise three main species-bifidobacteria, *Escherichia coli*, and *Bacteroidesfragilis*[6]. Low-grade chronic inflammation in the intestinal lamina propria of healthy patients confers immune tolerance through various antibodies. However, such intestinal immune tolerance may be absent in patients with IBD, with resultant activation of intestinal mucosal immune response in the absence of a self-limiting mucosal immune response. Deficits in intestinal immune tolerance may majorly be attributed to intestinal flora imbalance[7]. Gut bacteria compete for nutrients and space with foreign pathogens in the intestinal environment, thereby effecting biological antagonism through inhibiting foreign pathogens from adhering to the intestinal mucosa[8]. Moreover, normal flora can promote the development of immune pathways and activation of macrophages to resist damage caused by foreign pathogens[9].

The intestinal epithelial barrier forms the first line of defense against invading pathogens. Several types of *pattern-recognition receptors* (PRRs), including a group of signaling toll-like receptors (TLRs) found in epithelial cells, play an important role in the intestinal mucosal immune system[10]. They facilitate recognition of microorganisms; for instance, TLR2 recognizes Gram-positive bacterial peptidoglycan, TLR4 recognizes lipopolysaccharide of Gram-negative bacteria, and TLR9 recognizes CpG DNA sequences of microorganisms[11]. Pathogen-associated molecular patterns (PAMPs) are recognized by TLRs and other PRRs[12]. Distinguishing between intestinal commensal bacteria and pathogens by intestinal epithelial cells (IECs) is a prerequisite for immune surveillance, although their mechanism of action is unclear.

Bifidobacterium, a bacterial commensal, reduces release of endotoxins through reduction of superfluous Gram-negative bacilli to normal levels, thereby functioning as a detoxifying agent[13]. Acidic products produced by protective bifidobacterium are essential to maintain normal intestinal conditions and improve mediation of intestinal defense by epithelial cells[14]. Furthermore, cholesterol in the intestine is converted

to cholestane and fecal alkyls by acidic products generated by bifidobacterium[15]. In recent years, increasing attention has been paid to bifidobacterium-based therapy due to its stable efficacy and fewer side effects. Bifidobacterium has been used to treat diarrhea, IBD and other disorders of digestion[16-19].

This study was conducted to investigate the role of intestinal commensal bacteria in inducing immune tolerance and IBD pathogenesis. To evaluate intestinal barrier function, four different subtypes of bifidobacteria and the EPEC endotoxin were used to induce effects in rat normal small IEC-18, and their transepithelial electrical resistance (TEER) was detected after intervention. Changes of TLR2 and TLR4 expression levels were investigated to evaluate the effect of bifidobacteria on immune signal recognition and transduction in IECs.

MATERIALS AND METHODS

Materials and groups

Rat IEC-18 cells [Catalog No. CRL-1589; American Tissue Culture Collection (ATCC), Manassas, VA, United States] were purchased from the ATCC and cultured in 95% Dulbecco's modified Eagle's medium supplemented with 5% fetal bovine serum and 0.1 U/mL bovine insulin (Gibco, Carlsbad, CA, United States). *Bifidobacteria infantis (B. infantis)*, *B. longum*, *B. bifidum* and *B. youth* were provided by the Institute of Light Industry (Wuhan, China). EPEC (serotype O127: B8) endotoxin was purchased from Sigma-Aldrich (St Louis, MO, United States) and constituted for use at a concentration of 1 mg/mL.

Six groups were established in this experiment, including normal control, EPEC, *B. infantis*, *B. longum*, *B. bifidum*, and *B. youth*. The EPEC group received intervention with the EPEC endotoxin, whereas the bifidobacteria groups underwent treatment with *B. infantis*, *B. longum*, *B. bifidum* and *B. youth* separately. Normal controls did not undergo any intervention.

Quantitative real-time polymerase chain reaction(qRT-PCR) assays for detection of mRNA expression levels of TLR2 and TLR4 in rat IEC-18

In order to obtain optimal conditions for the intervention, IEC-18 cells were cultured in 6-well plates and treated with EPEC endotoxin at three different concentrations (0.5, 1 and 5 mg/mL) and with four different kinds of bifidobacteria (*B. infantis*, *B. longum*, *B. bifidum and B. youth*), with each subspecies diluted 100-fold and 300-fold, respectively. Then the cells were cultivated in a 37 ℃ humidified atmosphere, consisting of 95% air and 5% CO_2. After intervention for 4, 8, 12 and 16 h, levels of TLR2 and TLR4 mRNA were detected in each intervention group by using qRT-PCR. Briefly, total mRNA was extracted from each intervention group using TRIzol reagent (Invitrogen Corp, Carlsbad, CA, United States) according to the manufacturer's instruction. One microgram mRNA was used for cDNA synthesis in the PrimeScrip RT Reagent Kit (Takara Biotechnology, Dalian, China) under the following conditions: reverse transcription at 37 ℃ for 25 min, followed by incubation at 85 ℃ for 5 s in 20 μL reaction volume. Then, real-time PCR based on SYBR premix EX TAQ was conducted to quantify mRNA levels on the ABI StepOnePlus Real-Time PCR System (Applied Biosystems Inc, Foster City, CA, United States) under the following conditions: denaturation at 95 ℃ for 30 s, followed by 40 cycles of amplification (95 ℃ for 5 s, and 60 ℃ for 30 s). Primers were designed using NCBI Primer-BLAST as follows: TLR2 (forward, 5'-TGGAGGTCTCCAGGTCAAATC-3'; reverse, 5'-ACAGAGATGCCTGGGCAGAAT-3'); TLR4 (forward, 5'-TCCCTGCATAGAGGTACTTC-3'; reverse, 5'-CACACCTGGATAAATCCAGC-3'), GAPDH (forward, 5'-TCTTCCAGGAGCGAGATCCC-3'; reverse, 5'-TTCAGGTGAGCCCCAGCCTT-3'). Cycle threshold (CT) values were standardized to CT values of GAPDH, and $2^{-\Delta\Delta CT}$ was used to determine fold-change differences between intervention groups.

Western blot for detection of TLR2 and TLR4 protein expression in rat IEC-18

Based on the results obtained from qRT-PCR of TLR2 and TLR4 mRNA expression post-intervention in all the study groups, we obtained the optimal values of EPEC endotoxin concentration, bifidobacteria dilution concentrations, and duration of action. Thereafter, we conducted tests with these optimal concentrations of EPEC endotoxin and four different strains of bifidobacteria at the optimal duration of action, and expression levels of TLR2 and TLR4 proteins were detected in each group using western blotting.

Briefly, total protein was extracted from each group using RIPA buffer (Sigma-Aldrich) in accordance with the manufacturer's instructions. Protein concentration was determined with the BCA Protein Assay Kit (BosterBio, Wuhan, China). For this, protein samples were resolved on a 10% SDS-PAGE (Bio-Rad, Hercules, CA, United States) and transferred to polyvinylene difluoride membranes (Millipore Corporation, Billerica, MA, United States). These blotted membranes were blocked with TBST buffer supplemented with 5% fat-free milk for 2 h at room temperature, and incubated with primary rabbit polyclonal antibody TLR2 and TLR4 (1:2000 dilution; Abcam Inc, Cambridge, MA, United States) overnight. GAPDH (BosterBio, Wuhan, China) was used as a loading control. Thereafter, these membranes were washed and incubated with peroxidase-conjugated secondary antibodies (BosterBio, Wuhan, China) for 2 h at room temperature according to the manufacturer's instructions. Then the membranes were washed thrice with TBST for 10 min each at room temperature to terminate the reaction. Immunoreactive bands were visualized by enhanced chemiluminescence

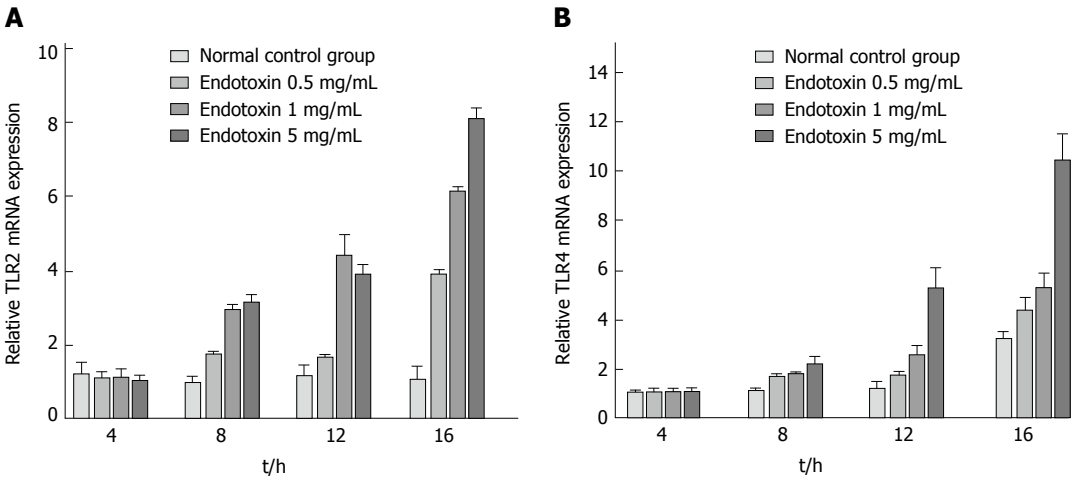

Figure 1 Toll-like receptor 2 and toll-like receptor 4 mRNA expression in intestinal epithelial cell-18 cells post-treatment with EPEC endotoxin at different time points. A: TLR2 mRNA expression in the EPEC groups was significantly higher at 12 h and 16 h, compared to the control group ($P < 0.05$); B: TLR4 mRNA expression was highest in the EPEC group at 16 h. TLRs: Toll-like receptors.

using SuperSignal West Pico Trail Kit (ThermoFisher, Rockford, IL, United States), and the intensity of the detected bands was analyzed using Image J software.

Measurement of epithelial cell membrane

IEC-18 cells were cultured in Transwell chambers with 3.0-mm polycarbonate filters (Corning Costar, Corning, NY,United States) at 37 ℃ in a humidified atmosphere of 95% air and 5% CO_2. Optimal concentrations of the EPEC endotoxin and four different types of bifidobacteria were added to the upper compartment of the Transwell plates in five intervention groups.An epithelial cell transmembrane resistance meter was used to test TEER changes in IEC-18 cells at 30, 60, 90 and 120 min in all of the five intervention groups and the normal control group. The TEER was calculated as: TEER (1 cm^2) = Measured value of the resistance (Ω) × Effective membrane area (cm^2).

Statistical analysis

All data were analyzed using SPSS 16.0 for Windows (SPSS Inc, Chicago, IL, United States). Between-group comparisons were conducted by two-way ANOVA. Three replicate wells were tested per assay, and each experiment was performed in triplicate. All statistical tests were two-tailed, and statistical significance was assumed for values with $P < 0.05$.

RESULTS

mRNA expression levels of TLR2 and TLR4

TLR2 mRNA expression was significantly increased after intervention with EPEC endotoxin at 12 h and 16 h, as compared with the control group ($P < 0.05$; Figure 1A). However, TLR2 mRNA expression did not change markedly in the intervention groups treated for 4 h and 8 h ($P > 0.05$; Figure 1A). Moreover, TLR4 mRNA expression was highest in the 16-h intervention groups ($P < 0.05$; Figure 1B). After treatment for 16 h, TLR2 mRNA expression was significantly higher in the 1 mg/mL and 5 mg/mL EPEC endotoxin groups, as compared to the 0.5 mg/mL EPEC and control groups ($P < 0.05$; Figure 1A). However, TLR2 mRNA expression did not differ considerably between the 1 mg/mL and 5 mg/mL EPEC groups ($P > 0.05$; Figure 1A). After treatment for 8 h and 16 h, TLR4 mRNA expression was significantly up-regulated in the 5 mg/mL EPEC group, as compared with that in the 0.5 mg/mL and 1 mg/mL EPEC groups and normal controls ($P < 0.05$; Figure 1B). However, there were no remarkable between-group differences in TLR4 mRNA expression among the different EPEC groups following intervention for 4 h and 12 h ($P > 0.05$; Figure 1B). We determined that the optimal EPEC endotoxin concentration was 5 mg/mL and optimal treatment duration was 16 h. qRT-PCR of IEC-18 cells post-treatment with EPEC endotoxin (0.5, 1 and 5 mg/mL) and with four different types of bifidobacteria diluted 100-fold and 300-fold at different time points, revealed that the optimal bifidobacteria dilution concentration was 300-fold.

Next, we applied these optimal parameters (EPEC endotoxin 5 mg/mL, four different strains of bifidobacteria diluted 300-fold, for 16 h) and evaluated TLR2 and TLR4 protein expression (Figure 2). TLR2 and TLR4 mRNA expression was significantly up-regulated in the 5 mg/mL EPEC group, as compared with the control group ($P < 0.001$; Figure 2). TLR2 mRNA expression in the *B. infant*, *B. longum* and *B. youth* groups were significantly lower than in the control group ($P < 0.05$), although reduction in expression levels in the bifidum group did not reach significance (Figure 2A). Moreover, TLR4 mRNA expression in the *B. bifidum* and *B. youth* groups were significantly lower than in the control group ($P < 0.05$; Figure 2B).

TLR2 and TLR4 protein expressionin the intervention and control groups

After treatment with EPEC endotoxin (5 mg/mL) and

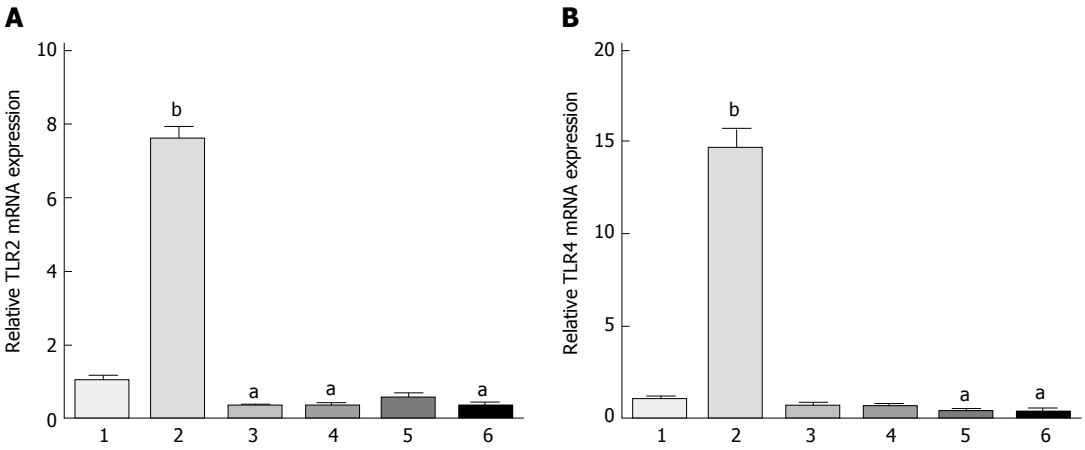

Figure 2 Toll-like receptor 2 and toll-like receptor 4 mRNA expression in intestinal epithelial cell-18 cells after treatment with EPEC endotoxin and bifidobacteria for 16 h. TLR2 and TLR4 mRNA expression was significantly upregulated in the EPEC group. A: TLR2 mRNA expression in *B.infantis*, *B.longum* and *B.youth*groups were significantly lower than in the control groups; B: TLR4 mRNA expression in *B.bifidum* and *B.youth* groups were significantly lower than in the normal control group.1, 2, 3, 4, 5 and 6 represent normal control, EPEC, *B.infantis*, *B.longum*, *B.bifidum* and *B. youth* groups, respectively. $^{a}P < 0.05$, $^{b}P < 0.001$ *vs* the control group. TLRs: Toll-like receptors.

four different bifidobacteria strains (diluted 300-fold) for 16 h, TLR2 and TLR4 protein expression in the EPEC groups were significantly higher than in controls ($P < 0.05$; Figure 3). TLR2 protein expression in the *B. infant*, *B. longum* and *B. youth* groups were lower than those in the control group ($P < 0.05$; Figure 3). TLR4 protein expression in the *B. bifidum* and *B. youth* groups were down-regulated in comparison with that in the control groups ($P < 0.05$; Figure 3).

Changes in IEC membrane resistance

The TEER in the *B. infantis*, *B. longum*, *B. bifidum* and *B. youth* groups decreased by only 19%, 18%, 23% and 23%, respectively, post-intervention for 120 min compared with the control group. However, the TEER in the EPEC endotoxin intervention groups reduced significantly by 67%, as compared with the control group ($P < 0.05$; Figure 4).

DISCUSSION

Recent studies have shown that infective factors may play a key role in the pathogenesis of IBD, and that changes in the composition of intestinal microbiota are closely correlated with IBD progression[17]. In addition, it has been suggested that probiotics can potentially influence equilibrium of commensal and pathogenic bacteria, and thereby destroy homeostasis in inflammatory conditions[13]. Moreover, probiotics modulate intestinal mucosal immune function, which plays a protective role in maintaining the intestinal barrier function[20]. Therefore, we focus on understanding the role of probiotics in the induction and maintenance of IBD remission and discuss their influence on gut microbiota.

Besides bifidobacteria, lactobacilli, and other probiotic organisms, a large number of opportunistic pathogens exist as intestinal commensals. Immune stimulation from microbial antigens, food antigens, and allergens occur in intestinal epithelial tissue all the time, leading to different immune responses. Although there are various pathogenic bacteria in the intestinal epithelial tissue of the healthy population, these people seldom fall sick, which is attributed to immune tolerance and homeostasis. Moreover, macrobiotic imbalance will induce loss of intestinal immune tolerance and immune homeostasis and induce a series of immune responses, which eventually damages normal tissue. To investigate the effect of bacteria and probiotic interventions on intestinal immune barrier function and the signal transduction pathway,we established an *in vitro* normal intestinal immune system experimental model using the probiotic bacterial species bifidobacterium and toxins from EPEC.

Probiotics are defined as "living microorganisms which, when administered in adequate amounts, confer a health benefit on the host"[17]. Probiotics, as a food additive, are being adopted extensively and can help improve imbalances of intestinal flora induced by antibiotic use or by pathologic conditions, particularly IBD[2,20]. Probiotics can prevent or improve diarrheal symptoms, alleviate symptoms of lactose intolerance, enhance the immune system, and promote intestinal digestion and absorption of nutrients[7]. Therefore, research on the mechanism of action of probiotics will be beneficial to understand the etiopathogenesis and treatment of IBD, and could potentially be applied as a preventive measure against IBD. In addition to important applications in the treatment of gastrointestinal diseases, probiotics may have beneficial therapeutic effects on other systemic diseases, such as diabetes and disorders of the reproductive system.

Bifidobacterium is an anaerobic Gram-positive bacteria, with 32 subspecies, of which 28 subspecies have been detected in the human gut[13]. Intestinal bifidobacteria appear within 3 to 4 d postnatally, and

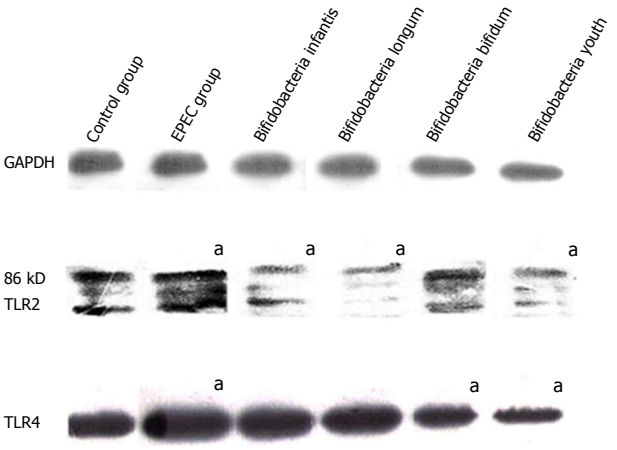

Figure 3 Toll-like receptor 2 and toll-like receptor 4 protein expression in intestinal epithelial cell-18 cells after treatment with EPEC endotoxin and bifidobacteria intervention for 16 h. TLR2 and TLR4 protein expression levels in the EPEC group were significantly higher than in the control group. TLR2 protein expression levels in the *B.infantis*, *B.longum* and *B. youth* groups were significantly lower than in the control group. TLR4 protein expression was down-regulated in the *B.bifidum* and *B. youth* groups. $^{a}P < 0.05$ *vs* the control group. TLR: Toll-like receptor.

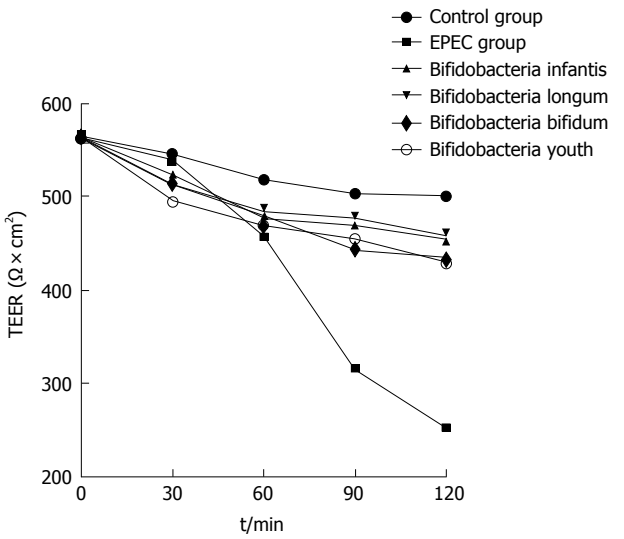

Figure 4 Changes in intestinal epithelial cell-18 cell membrane resistance after treatment with EPEC endotoxin and bifidobacteria for 16 h. The transepithelial electrical resistance in the *B.infantis*, *B.longum*, *B.bifidum* and *B. youth* groups decreased by only 19%, 18%, 23% and 23% respectively after 120 min of intervention,as compared with the control group. However, the transepithelial electrical resistance in the EPEC intervention group reduced significantly, by 67% compared to the normal control group ($P < 0.05$).

bifidobacteria comprise approximately 25% of total intestinal bacteria[20]. The number of bifidobacteria reduce gradually with increasing age, and decrease to 7.9% in individuals over age 65[20]. Reduction in the number of bifidobacteria is suggested to be an indicator of lack of health[21].

EPEC was first discovered as a causative agent of diarrhea in epidemiological studies. Considerable EPEC proliferation occurs on mucosal surfaces of the duodenum, jejunum and ileum. The adhesion of EPEC to microvilli can lead to brush border damage, atrophy of microvilli, epithelial cell disorder and dysfunction, and, eventually, diarrhea[12]. EPEC and opportunistic pathogens grow in the normal intestine; however, when immune function is impaired, these bacteria can cause disease.

Due to a complicated interaction between the pathogen and the host immune system in microbial infection, microorganisms express a large amount of special molecules, such as the viral nucleic acid sequences known as the PAMPs[10]. PAMPs are recognized by a number of receptors, including PRRs. Following PAMP recognition by PRRs, downstream signaling pathways activate innate immunity and cell functions such as phagocytosis and release of inflammatory cytokines, which are important for a balanced adaptive immune response against pathogens[22,23]. TLRs appear to be the most important members of the PRR family; furthermore, TLR2 and TLR4, two main subtypes of the TLR family, play a key role in the maintenance of intestinal epithelial homeostasis[24]. Previous studies have shown that high expression of TLR2 and TLR4 is significantly associated with increased risk of IBD[25,26]. Activation of TLR4 by pathogenic bacteria damages the mucosal barrier function, facilitating pathogen translocation from the gut and release of proinflammatory factors, such as interleukin (IL)-1, IL-6, and tumor necrosis factor-alpha[15,24,27]. Therefore, TLR2 and TLR4 were selected in this research to validate the effect of intervention on intestinal immune barrier function and the signal transduction pathway, as well as to further study the mechanism of immune responses in the gut.

EPEC is highly pathogenic, and culturing IEC cells with live EPEC induces cell death. EPEC endotoxin, the main PAMP, is present in the cell wall of Gram-negative bacteria, whereas exopolysaccharide, a secretory product expressed by bifidobacterium during proliferation and death, is found in Gram-positive bacteria. Therefore, EPEC endotoxin and live bifidobacterium were selected as interventional agents for use in IEC-18 cells. Due to the uncertain effects of EPEC endotoxin and bifidobacteria in IEC-18 cells, it was necessary to treat the IEC-18 cells with different concentrations of EPEC endotoxin (0.5, 1 and 5 mg/mL) and four different kinds of bifidobacteria, diluted 100-fold and 300-fold, at different time points to identify the optimal EPEC endotoxin treatment concentration, bifidobacteria diluted concentration, and duration of action.

Following intervention with the optimal concentration of EPEC endotoxin (5 mg/mL) and four different strains of bifidobacteria(diluted 300-fold) for 16 h, TLR2 and TLR4 mRNA and protein expressions were up-regulated in the EPEC group but down-regulated in some bifidobacterium groups, as compared with those in the normal control group. From these results, we infer that after IECs are stimulated by pathogenic bacteria, bifidobacteria can induce immunologic

tolerance to those bacteria through down-regulation of TLR2 and TLR4 expression or compete with pathogens for binding TLRs to prevent TLR-mediated inflammation. Therefore, bifidobacteria can provide a protective role or benefit through inhibition of inflammation in patients with IBD.

In our study, the TEERs in the *B. longum*, *B. infantis*, *B. bifidum* and *B. youth* groups were decreased only by 18%, 19%, 23% and 23%, respectively, after 120 min of intervention, as compared to the normal control group. However, the TEER in the EPEC group was reduced significantly by 67%. The TEER is inversely proportional to cell membrane permeability, and decreased TEER would lead to gap junction damage, increase cell osmotic pressure, and increase cell permeability; this could further result in pathogen penetration of the cell barrier and consequent inflammation[28,29]. These results indicate that EPEC may increase cell membrane permeability, thus playing a pathogenic role in patients with IBD. However, bifidobacteria exert a protective role in preventing penetration of pathogenic bacteria by the natural barrier function of the IEC. The lack of animal experimental model could be considered a major limitation of this study. Therefore, additional *in vivo* experiments should be conducted to further explore the mechanism of IBD in future research endeavors.

In conclusion, the EPEC endotoxin can promote TLR2 and TLR4 expression and increase cell membrane permeability in the IEC. In contrast, bifidobacteria can inhibit TLR2 and TLR4 expression and prevent TLR-mediated inflammation. Individualized use of a suitable probiotic may be helpful to design effective therapeutic strategies for symptom improvement in IBD.

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COMMENTS

Background

The incidence of inflammatory bowel disease (IBD) has increased in the past few decades, making it a global healthcare problem and an interesting research area. Current knowledge about the specific mechanism of IBD is deficient.

Research frontiers

IBD has been considered to be caused by a variety of risk factors, including environmental, genetic, infectious and immune triggers. Toll-like receptors (TLRs) are key components of the innate immune system that trigger antimicrobial host defense responses. This study aimed to investigate alterations of intestinal barrier function and the TLR2 and TLR4 expression in intestinal epithelial cell (IEC)-18 cells and to further elucidate the mechanism of IEC immune defense function following induction by low-dose EPEC endotoxin and bifidobacteria.

Innovations and breakthroughs

This study analyzed the potential protective role of bifidobacteria on IECs through regulation of TLR2 and TLR4 expression and intestinal cell membrane permeability. The findings revealed that the EPEC endotoxin promoted TLR2 and TLR4 expression and increased intestinal cell membrane permeability. In contrast, bifidobacteria inhibited TLR2 and TLR4 expression and prevented TLR-mediated inflammation.

Applications

Bifidobacteria can provide a protective role through inhibiting inflammation and preventing penetration of pathogenic bacteria across the intestinal cell barrier in patients with IBD. Individualized use of a suitable probiotic may be helpful to design an effective and specific therapy to improve disease symptoms in IBD.

Peer-review

This is a very well designed, performed and written experimental study for investigation of the effect of enteropathogenic *Escherichia coli* endotoxin and the effect of bifidobacteria on the expression of TLR2 and TLR4 mRNA and protein in IECs and possible influence of intestinal barrier function of cells and its role in pathogenesis of IBD.

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ORIGINAL ARTICLE

Retrospective Cohort Study

Hospital costs, length of stay and prevalence of hip and knee arthroplasty in patients with inflammatory bowel disease

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Author contributions: Ehrenpreis ED was involved in the following portions of the study: Study design, data analysis, manuscript writing, manuscript revisions; Zhou Y was involved in the following portions of the study: Study design, data analysis, manuscript writing, manuscript revisions.

Institutional review board statement: The study was reviewed by North Shore University Health System Institutional Review Board and deemed appropriate for exempt status of Institutional Review Board oversight due to the de-identified nature of HCUP-NIS data.

Informed consent statement: The clinical data utilized in this study is de-identified and was obtained from a publicly available database. Therefore, informed consent was not required for this study.

Conflict-of-interest statement: The authors have no conflicts of interest related to this study to report.

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Abstract

AIM

To examined the prevalence of hip and knee arthroplasty in patients with inflammatory bowel disease (IBD) by comparing the diagnostic codes for these procedures in patients with IBD and a control group of patients.

METHODS

The National Inpatient Sample database (NIS) is part of the Healthcare Cost and Utilization Project (HCUP), the largest publicly available inpatient healthcare database in the United States. The NIS samples about 20% of discharges from all community hospitals participating in HCUP, representative of more than 95% of the United States population, with approximately 7000000 hospitalizations reported annually. NIS contains data on diagnoses, procedures, demographics, length of stay (LOS), co-morbidities and outcomes. ICD-9-CM diagnostic codes for primary hospitalizations for arthroplasty of the hip or knee with a co-diagnosis of IBD [combining both Crohn's disease (CD) and ulcerative colitis (UC)] were used to identify study subjects for cost and LOS analysis for NIS from 1999-2012. Statistical analysis: 1:2 propensity score matching between IBD *vs* a control

group based on following factors: Patient age, gender, race, total co-morbidities, # of procedures, admission type, insurance, income quartiles, and hospital bed size, location and hospital teaching status. Categorical variables were reported as frequency and compared by χ^2 tests or Fisher's exact tests. Individual 1:3 matching was also performed for patients carrying diagnostic codes for CD and for patients with the diagnostic code for UC. After matching, continuous variables were rcompared with Wilcoxon signed rank or Paired T-tests. Binary outcomes were compared with the McNemar's test. This process was performed for the diagnosis of hip or knee arthroplasty and IBD (CD and UC combined). Prevalence of the primary or secondary diagnostic codes for these procedures in patients with IBD was determined from NIS 2007.

RESULTS

Costs and mortality were similar for patients with IBD and controls, but LOS was significantly longer for hip arthroplasties patients with IBD, (3.85 +/-2.59 d *vs* 3.68 +/-2.54 d, respectively, P = 0.009). Costs, LOS and survival from the procedures was similar in patients with CD and UC compared to matched controls. These results are shown in Tables 1-10. The prevalence of hip arthroplasty in patients with IBD was 0.5% in 2007, (170/33783 total patients with diagnostic codes for IBD) and was 0.66% in matched controls (P = 0.0012). The prevalence of knee arthroplasty in patients with IBD was 1.36, (292/21202 IBD patients) and was 2.22% in matched controls (P < 0.0001).

CONCLUSION

Costs and mortality rates for hip and knee arthroplasties are the same in patients with IBD and the general population, while a statistical but non-relevant increase in LOS is seen for hip arthroplasties in patients with IBD. Compared to the general population, arthroplasties of the hip and knee are less prevalent in hospitalized patients with IBD.

Key words: Ulcerative colitis; Outcomes; Inflammatory bowel disease; Hip arthroplasty; Knee arthroplasty; Hospital length of stay; Mortality; Crohn's disease

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Core tip: Patients with inflammatory bowel disease (IBD) have predisposing risk factors for arthroplasty of the hip and possibly the knee. IBD patients are also at higher risk for thromboembolic events, and longer and more complex hospitalizations for non-intestinal surgeries. Despite these considerations, this study of the National Inpatient Survey, the largest publicly available inpatient healthcare database in the United States, demonstrates the unexpected findings that patients with IBD have similar costs, lengths of stay and mortality when hospitalized for hip and knee arthroplasties. In addition, these surgeries are significantly less prevalent in patients with IBD than the general population.

Ehrenpreis ED, Zhou Y. Hospital costs, length of stay and prevalence of hip and knee arthroplasty in patients with inflammatory bowel disease. *World J Gastroenterol* 2017; 23(26): 4752-4758 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i26/4752.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i26.4752

INTRODUCTION

Rheumatologic complications of inflammatory bowel disease (IBD) are common. Patients with inflammatory bowel disease are at risk for spondyloarthropathies, osteoporosis, bone fractures and avascular necrosis of the hip[1-8]. Previous studies have also indicated that patients with inflammatory bowel disease are likely to have longer and more complicated hospitalizations[9], especially when undergoing non-intestinal surgeries. This may be especially true in light of the increased risk of thromboembolic events that occurs in patients with inflammatory bowel disease[10]. Despite these considerations, there has been little attention paid to hospital outcomes in patients with IBD that undergo orthopedic procedures. In addition, although it may be assumed that patients with IBD are at higher risk of undergoing arthroplasties of the hip (and possibly the knee), the prevalence of these procedures in the IBD patient population has not been performed. In the current study, we utilized a large national database of hospitalized patients we examined of the effect of a diagnosis of IBD on hospital cost, LOS and survival during primary hospitalizations for hip and knee arthroplasty. We also examined the prevalence of hip and knee arthroplasty in patients with IBD by comparing the diagnostic codes for these procedures in patients with IBD and a control group of patients.

MATERIALS AND METHODS

Data source

A review of the 2005-2011 National Inpatient Sample database (NIS) was conducted for this analysis. The NIS is the largest publicly available inpatient healthcare database in the United States. It is part of the Healthcare Cost and Utilization Project (HCUP) sponsored by the Agency for Healthcare Research and Quality. The NIS contains approximately a 20% sample of discharges from all community hospitals participating in HCUP, representative of more than 95% of the United States population. An estimated seven million hospital admissions per year are reported containing data on primary and secondary diagnoses and procedures, patient demographics, hospital characteristics, length of stay, insurance status, median income by zip code and co-morbidity measures[11,12].

Table 1 Comparison of length of stay, hospitalization costs, and mortality for hip arthroplasty between patients with inflammatory bowel disease and matched controls

	IBD (n = 1484)	No IBD (n = 4452)	P value
Mean length of stay (d)	3.85 ± 2.59	3.68 ± 2.54	0.009
Mean cost of hospitalization ($)	50074.72	33161.78	0.732
Deaths	0	5 (0.11)	0.340

IBD: Inflammatory bowel disease.

Table 2 Comparison of length of stay, hospitalization costs, and mortality for knee arthroplasty between patients inflammatory bowel disease and matched controls

	IBD (n = 1484)	No IBD (n = 7560)	P value
Mean length of stay (d)	3.53 ± 1.96	3.54 ± 1.76	0.864
Mean cost of hospitalization ($)	45319.13	25714.03	0.441
Deaths	4 (0.16)	16 (0.21)	0.605

IBD: Inflammatory bowel disease.

Sample selection

The International Classification of Diseases, 9th Revision Clinical Modification (ICD-9-CM) diagnostic codes was used to identify the study population of interest. The ICD-9-CM is used to code and classify morbidity data from hospitals, physicians' offices and National Center for Health Statistics surveys[13]. The dataset was created by searching NIS for all patients presenting with ICD-9-CM codes for a primary diagnosis of arthroplasty of the hip or knee with a co-diagnosis of IBD [combining both Crohn's disease (CD) and ulcerative colitis (UC)] and individually for patients with a diagnosis of CD or UC.

Statistical analysis

Continuous variables were reported as mean ± standard deviation and median (range). The normality assumption for continuous variables was assessed using the Shapiro-Wilk test. Continuous variables were compared between IBD and controls by two-sample T-test or Wilcoxon rank-sum test as appropriate. Categorical variables were reported as frequency (percentage) and were analyzed by χ^2 tests or Fisher's exact test for co-diagnosed disease *vs* control comparisons. Propensity score matching method is widely used in observational studies to reduce selection bias. To fairly compare the outcomes of interests between for co-diagnosed disease group and controls, we conducted the 1:2 for IBD *vs* controls or 1:3 for UC and CD *vs* controls propensity score matching between patients with co-diagnosed disease *vs* controls using greedy 8-to-1 match algorithm[14]. Propensity scores were computed by modeling the probability of having the co-diagnosed disease through multivariable logistic regression with following factors: age, gender, race, total numbers of co-morbidities in records, total numbers of procedures in records, admission type, insurance, income quartiles, hospital beds, hospital control, hospital location, hospital region and hospital teaching status. Prevalence of hip and knee arthroplasty were determined by selecting the group of patients from the 2007 HCUP database with of diagnostic codes for Crohn's disease or ulcerative colitis and also having either primary or secondary diagnostic codes for either hip or knee arthroplasty. A control group for each IBD group having hip or knee arthroplasty was then created from the 2007 database by 3:1 matching using the categorical variables mentioned above. Statistical analyses were performed on SAS 9.3 (Cary, NC) Windows platform. A $P < 0.05$ was considered as statistically significant.

The study was reviewed by NorthShore University HealthSystem Institutional Review Board (IRB) and deemed appropriate for exempt status of IRB oversight due to the de-identified nature of HCUP-NIS data.

RESULTS

Procedure costs for hip and knee arthroplasties were similar for patients with IBD and for controls. However, LOS was statistically but not clinically longer for hip arthroplasties patients with IBD compared to controls, (3.85 +/-2.59 d *vs* 3.68 +/-2.54 d, respectively, $P = 0.009$). Survival from the procedures was similar in both groups. These results are shown in Tables 1 and 2. Procedure costs, LOS and survival for hip and knee arthroplasties were also similar for patients with CD and UC when compared to controls. Results for patients with CD and UC undergoing hip arthroplasties are seen in Tables 3, 4, 5 and 6. Results for patients with CD and UC undergoing knee arthroplasties are seen in Tables 7, 8, 9 and 10.

The prevalence of hip arthroplasty in patients with IBD was 0.5% in 2007, (170/33783 total patients with diagnostic codes for IBD) and was 0.66% in matched controls ($P = 0.0012$). The prevalence of knee arthroplasty in patients with IBD was 1.36% in 2007, (292/21202 IBD patients) and was 2.22% in matched controls ($P < 0.0001$).

DISCUSSION

Spondyloarthropathies are the most common extra-intestinal manifestations of IBD. IBD-associated spondyloarthropathies have a variety of forms including pauciarticular peripheral arthritis, polyarticular peripheral arthritis, sacroiliitis and spondylitis[1]. These and other musculoskeletal abnormalities may occur in up to 50% of patients with IBD[2-8,15-17]. Although generally treated with medical therapy, the occasional requirement for surgery as a specific treatment of

Table 3 Comparison of length of stay, hospitalization costs hip arthroplasty between patients with Crohn's disease and matched controls

		n	mean ± SD	Median	Min	Max	*P* value
Leght of stay (d)	Controls	2601	3.70 ± 2.95	3	0	101	0.082
	CD	867	3.84 ± 2.25	3	1	25	
Total charges ($)	Controls	2539	48596 ± 27763.6	41393	7428	342434	0.630
	CD	847	48941.9 ± 28236.1	41187	2025	372931	

CD: Crohn's disease.

Table 4 Comparison of Mortality for Hip Arthroplasty between Patients with Crohn's disease and Matched Controls *n* (%)

	Controls	CD	*P* value
Alive	2598 (99.96)	867 (100)	1.00
Dead	1 (0.04)	0 (0)	

CD: Crohn's disease.

Table 5 Comparison of length of stay, hospitalization costs for hip arthroplasty between patients ulcerative colitis and matched controls

		n	mean ± SD	Median	Min	Max	*P* value
Leght of stay (d)	Controls	1863	3.72 ± 2.27	3	0	34	0.5157
	UC	621	3.87 ± 3.01	3	1	49	
Total charges ($)	Controls	1799	50966.2 ± 29412.4	43153	2703	291387	0.7605
	UC	596	52620.7 ± 34203.1	43704.5	145	307530	

UC: Ulcerative colitis.

Table 6 Comparison of mortality for hip arthroplasty between patients ulcerative colitis and matched controls *n* (%)

		UC	*P* vaule
Alive	1859 (99.89)	620 (100)	*P* = 1.00
Dead	2 (0.11)	0 (0)	

UC: Ulcerative colitis.

IBD- related spondyloarthropathies could increase the likelihood that patients with IBD will undergo orthopedic surgery. Patients with IBD also have up to a 40% greater risk of bone fractures and a higher prevalence of osteoporosis compared to the general population[1,4-6,8]. Avascular necrosis of the hip may also occur in patients with IBD receiving corticosteroid treatment[2,7,8].

Hospital LOS, complications, costs, in-hospital adverse events, mortality and readmission rates

Table 7 Comparison of Length of stay, hospitalization costs for knee arthroplasty between patients with crohn's disease and matched controls[1]

		n	mean ± SD	Median	Min	Max	*P* value
Leght of stay (d)	Controls	4032	3.59 ± 2.22	3	0	82	0.5894
	CD	1344	3.57 ± 2.19	3	0	55	
Total charges ($)	Controls	3965	45161 ± 27971.7	38408	1002	552569	0.5738
	CD	1317	44975.6 ± 26145.8	38353	8598	234950	

[1]Cleaned data. CD: Crohn's disease.

Table 8 Comparison of mortality for knee arthroplasty between patients with Crohn's Disease and matched controls[1] *n* (%)

	Controls	CD	*P* vaule
Alive	4022 (99.83)	1341 (99.78)	0.7183
Dead	7 (0.17)	3 (0.22)	

[1]Cleaned data. CD: Crohn's disease.

associated with a variety of conditions, including hip and knee replacements are subject to public reporting[18-22]. These data are a useful means to ensure accountability for individual hospitals and to determine areas of discrepancy in the medical system. However, the impact of a pre-existing IBD on patient outcomes for medical and surgical hospitalizations has received little attention. Our group has recently published an analysis of the NIS database that demonstrated the surprising finding that patients with IBD have improved survival when hospitalized for acute myocardial infarction. We hypothesized that this might reflect an unexpected effect of anti-inflammatory agents, particularly TNF-alpha inhibitors[23].

Intestinal and non-intestinal surgery in patients with IBD has been associated with a higher risk of thromboembolic events, post-operative complications and prolonged hospital LOS[9,10,24-26]. It would therefore be anticipated that patients with IBD undergoing hip or knee arthroplasties are likely to have more complications, with higher costs, LOS and mortality with these procedures. Because of these considerations, patients with IBD and their healthcare providers should benefit from a better understanding of the risks and costs associated with undergoing these procedures.

We chose the NIS database from HCUP as it has been previously used to investigate the epidemiology and cost factors occurring during hospitalizations for a variety of diseases[27-32]. Our study demonstrates that the economics of hip and knee arthroscopies, including costs, LOS and mortality, were similar for patients with IBD and controls. The only statistical (but not clinical) difference found was that the LOS was significantly

Table 9 Comparison of length of stay, hospitalization costs for knee arthroplasty between patients with ulcerative colitis and matched controls

		n	mean ± SD	Median	Min	Max	*P* value
Leght of stay (d)	Controls	3546	3.59 ± 2.24	3	1	74	0.364
	UC	1182	3.50 ± 1.67	3	1	30	
Total charges ($)	Controls	3465	46545.8 ± 28108	39769	2025	502498	0.727
	UC	1145	46681.3 ± 27892.7	39813	4550	428244	

Table 10 Comparison of mortality for knee arthroplasty between patients with ulcerative colitis and matched controls[1] *n* (%)

	Controls	UC	*P* value
Alive	3535 (99.77)	1180 (99.92)	0.336
Dead	8 (0.23)	1 (0.08)	

[1]Cleaned data.

longer for hip arthroplasties patients with IBD, (3.85 +/-2.59 d *vs* 3.68 +/-2.54 d, respectively, $P = 0.009$). This finding is not likely to be clinically relevant. There were also no differences in costs, LOS and mortality between patients carrying the specific diagnostic codes for CD or UC and matched controls. Our study also demonstrates the unexpected finding that in the group of patients undergoing hospitalization, having a diagnosis of IBD is associated with a lower prevalence of hip and knee arthroplasties. The prevalence of hip arthroplasty in patients with IBD was 0.5% in 2007 compared to 0.66% in 3:1 matched controls ($P = 0.0012$). The prevalence of knee arthroplasty in patients with IBD in 2007 was 1.36% compared to 2.22% in matched controls (< 0.0001).

Similarities in the costs and mortality associated with hip and knee arthroplasties in patients with IBD, along with relatively comparable LOS as determined in this analysis should be reassuring to patients and their practitioners with IBD. These findings suggest that the immediate occurrence of complications in the IBD patient group undergoing hip and knee arthroplasties is probably not higher than patients in the general public. However, this methodology and data analysis does not determine whether late thromboembolic events are more likely to occur in patients with IBD. Patients with IBD are in general at higher risk for thromboembolic events and following these procedures, may have prolonged immobilization and casting. For these reasons, appropriate use of antithrombotic therapy and careful monitoring for thromboembolic events is required in patients with IBD subsequent to their hospitalizations for hip and knee arthroplasty. In addition, one study showed a higher rate of reoperation for hip arthroplasty ion patients with IBD[33].

The paradox of lower prevalence of these surgeries in hospitalized patients with IBD compared to the general population may be explained in several ways. It is possible that because of known risk, IBD patients may be undergoing more intensive screening and treatment for osteoporosis compared to the general population. As a consequence, precautionary measures to prevent hip fractures and other orthopedic injuries may occur more frequently. It is also possible that there is more hesitancy in performing these surgeries on an elective basis in patients with IBD. Because the NIS database reviews hospitalized patients only, patients having the diagnostic codes for CD or UC that are hospitalized could also represent a sicker group of patients in whom elective procedures are more limited. Nonetheless, the possibility that hip and knee arthroscopies are less commonly performed in patients with IBD should be encouraging to patients and their healthcare providers. Evaluation of outpatient databases and further analyses of large and patient databases such as NIS will be required in the future to fully understand the mechanisms associated with this finding.

Similar to other retrospective studies that utilize data derived from large databases, analysis of the NIS is subject to various limitations. These include the potential of inaccuracy of ICD-9-CM diagnostic coding in the database and that inpatient discharge data is only representative of hospitals participating in HCUP[10,11]. When a group of patients is studied using NIS, there is always a possibility that diagnostic codes for chronic illnesses such as IBD may not be included when patients are admitted to the hospital, particularly if suffering from life-threatening conditions[34]. Missing data is common in large databases such as NIS and is accounted for by statistical analysis.

In summary, this investigation of a large inpatient database shows that the costs, LOS and mortalities in patients undergoing arthroscopy of the hip or knee are essentially similar in patients with IBD compared to the general population. The study also revealed the unexpected finding that patients with IBD have a lower the prevalence of these procedures, possibly because of better screening for osteoporosis improved fracture prevention.

COMMENTS

Background

Comorbidity is defined as the existence of pre-existing conditions that enhance the likelihood of adverse outcomes. By definition, the presence of preexisting co-morbidities are expected to have a negative impact on outcomes in patients that are hospitalized for medical conditions, including surgeries. Some studies

have shown that patients with inflammatory bowel disease (IBD) have longer, more complex hospitalizations, especially for non-intestinal surgeries. Because IBD patients often develop spondyloarthropathies, osteoporosis and avascular necrosis of the hip, the economics of orthopedic surgeries in patients with IBD is a highly relevant topic. However, the prevalence, costs, hospital length of stays and mortality for hip and knee arthroplasties have not been investigated in the IBD population.

Research frontiers

The following are important areas of clinical research that are related to the information provided in this study; (1) screening and treatment for osteoporosis in patients with IBD; and (2) economics of medical care in patients with IBD.

Innovations and breakthroughs

The economics of hip and knee arthroplasty in patients with IBD have not been previously investigated in the medical literature. The authors' group has recently demonstrated that patients with IBD have improved survival when hospitalized for acute myocardial infarction, possibly because of effects of immunosuppressant medications[13]. In the current study, they found that patients with IBD have similar hospital length of stays, costs and in-hospital mortality when undergoing hip and knee arthroplasty. Compared to the general population, these surgeries are less common in patients with IBD.

Applications

The information obtained in this study will help clinicians understand the economics of hip and knee arthroplasty patients with IBD. Future studies may focus on determing the effect of screening for osteoporosis and other methods that may lower the likelihood tat patients with IBD will require these surgeries.

Peer-review

The study is well written and welcome. With practical importance, it shows no difference between the IBD patients and general population for hip and knee artroplasty in a large cohort of patients.

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ORIGINAL ARTICLE

Retrospective Study

Eight-week ledipasvir/sofosbuvir in non-cirrhotic, treatment-naïve hepatitis C genotype-1 patients with hepatitis C virus-RNA < 6 million: Single center, real world effectiveness and safety

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Author contributions: Latt NL and Sahota AK designed the research; Latt NL and Yanny BT performed the data abstraction, contributed to the analysis; Latt NL wrote the paper; Gharibian D and Sahota AK provided clinical advice and supervised the report; Gevorkyan R helped with the data abstraction and provided clinical advice.

Institutional review board statement: This study was reviewed and approved by Kaiser Permanente Southern California Institutional Review Board.

Informed consent statement: Patients were not required to give informed consent to the study. The study was qualified for an 'exempt status' by the Institutional Review Board due to its retrospective nature. We collected only the existing data from electronic medical records, the date were stored without any patient identifiers.

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Abstract

AIM

To evaluate sustained viral response (SVR) of 8-wk ledipasvir/sofosbuvir therapy among non-cirrhotic, genotype-1 hepatitis C virus (HCV) patients with RNA < 6 million IU/mL.

METHODS

We performed a retrospective cohort study to examine SVR rates, predictors of treatment failure and safety analysis of 8-wk ledipasvir/sofosbuvir (LDV/SOF) therapy

among non-cirrhotic, genotype 1 HCV patients with viral load < 6 million IU/mL. Primary outcome was an achievement of SVR at 12 wk after treatment. Secondary outcomes were identifying predictors of treatment failure and adverse events during treatment.

RESULTS

Total 736 patients: 55% males, 51% Caucasians and 65% were genotype 1a. Non-cirrhotic state of 53% was determined by clinical judgment (imaging, AST, platelet count) and 47% had documented liver fibrosis testing (biopsy, vibration-controlled transient elastography, serum biomarkers). Overall SVR12 was 96%. No difference in SVR12 was seen between patients whose non-cirrhotic state was determined by clinical judgment and patients who had fibrosis testing. Age groups, gender, ethnicity and genotype 1 subtype did not predict SVR. Non-cirrhotic state determined by clinical judgment based on simple, non-invasive tests were not associated with lower SVR [OR = 1.02, 95%CI: 0.48-2.17, P = 0.962]. The AUROC for hepatitis C RNA viral load was 0.734 (P < 0.001, 95%CI: 0.66-0.82). HCV RNA 2.2 million IU/mL was identified as the cutoff value with sensitivity 73% and specificity 64%. HCV RNA < 2.2 million IU/mL was associated with significantly higher SVR 98% with OR = 0.22 (95%CI: 0.1-0.49, P < 0.001) compared to SVR 92% in HCV RNA ⩾ 2.2 million IU/mL. No death or morbidities were reported.

CONCLUSION

Our outcomes validate safety and effectiveness of 8-wk LDV/SOF therapy in non-cirrhotic, untreated HCV genotype 1 patients with HCV RNA < 6 million IU/mL.

Key words: Hepatitis C; Sustained viral response; Ledipasvir; Cirrhosis; Sofosbuvir

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Core tip: We highlight that sustained viral response (SVR) outcomes in patients with their non-cirrhotic status determined by clinical judgment using simple, cheap, non-invasive tests such as platelet count, sonographic finding of spleen size and hepatic morphology, are comparable with those who had specialized tests such as liver biopsy, vibration-controlled transient elastography or specialized serum biomarker test. We also validate the fact that hepatitis C virus (HCV) RNA plays a role in predicting SVR (AUROC = 0.743, 95%CI: 0.66-0.82) with a cutoff value of 2.2 million IU/mL. Significantly higher 98% SVR was observed among HCV RNA < 2.2 million IU/mL, compared to 92% SVR with HCV RNA ⩾ 2.2 million IU/mL.

Latt NL, Yanny BT, Gharibian D, Gevorkyan R, Sahota AK. Eight-week ledipasvir/sofosbuvir in non-cirrhotic, treatment-naïve hepatitis C genotype-1 patients with hepatitis C virus-RNA < 6 million IU/mL: Single center, real world effectiveness and safety. *World J Gastroenterol* 2017; 23(26): 4759-4766 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i26/4759.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i26.4759

INTRODUCTION

Hepatitis C virus (HCV) infection is a major cause of cirrhosis, hepatocellular carcinoma and liver-related mortality[1]. Recent studies estimate the prevalence of HCV to be between 1.2%-1.5% in the United States, which is approximately 4.5-5 million. This population is composed of 1 million incarcerated/homeless individuals, hospitalized patients, and people living on Indian reservations, and also includes 3.6 million from the 2003-2010 National Health and Nutrition Examination Survey[2,3]. The prevalence of HCV is declining, but HCV-related cirrhosis is still expected to peak in the year 2020[4]. Therefore, effective, safe and well-tolerated treatment regimens with shorter duration are urgently needed.

Hepatitis C treatment has evolved from a 78-wk interferon monotherapy to 48-wk pegylated interferon plus ribavirin therapy and now 12-wk therapy with newer all-oral direct-acting antiviral (DAA) agents. DAA regimens have revolutionized the treatment of hepatitis C with their excellent sustained virologic response (SVR), tolerable side effect profiles and shorter duration of therapy. Despite the high cost of newer DAAs, a cost-effective analysis has demonstrated that ledipasvir/sofosbuvir (LDV/SOF)-based regimens will reduce long-term HCV-related complications and are cost-effective in the majority of chronic HCV patients[5].

ION-3 trial demonstrated that an 8-wk LDV/SOF therapy in non-cirrhotic, treatment naïve genotype 1 HCV patients with HCV RNA < 6 million IU/mL is non-inferior to 12-wk LDV/SOF therapy (SVR: 94% *vs* 95%)[6]. The shorter duration of treatment can remarkably increase patient compliance and substantially reduce treatment cost. Real-world studies have reported that SVR rates are comparable to those observed in ION-3 trial[7-10]. However, conflicting data has been reported by a large real-world cohort from Veteran's Affairs in which researchers found that non-cirrhotic patients with HCV-RNA < 6 million IU/mL were less likely to achieve SVR with 8-wk LDV/SOF treatment compared to 12-wk treatment[9].

Eight-week LDV/SOF therapy for non-cirrhotic, genotype 1 HCV patients is not included in HCV guidelines by American Association for the Study of Liver Diseases and Infectious Diseases Society of America due to lack of real-world validation for comparable SVR with 12-wk therapy[11]. European Association for the Study of the Liver and United States Food and Drug Administration recommend considering 8-wk therapy with caution in treatment-naïve genotype-1 HCV patients without cirrhosis who have pre-treatment viral load < 6 million IU/mL[12,13]. We contemplated that

shorter duration of treatment could provide the lower cost, the higher patient compliance and adherence as long as the shorter duration therapy can provide the comparable outcomes. We performed a retrospective cohort study to examine the SVR rates, the predictors of treatment failure and the safety analysis of 8-wk LDV/SOF therapy among non-cirrhotic, previously untreated genotype-1 HCV patients with viral load < 6 million IU/mL.

MATERIALS AND METHODS

Study population

Kaiser Permanente Southern California (KPSC) is a large, integrated healthcare system with over 4 million members. Integrated healthcare is delivered to members at 14 medical centers throughout the region. All interactions with the healthcare system, such as clinic/emergency department/urgent care visits, hospital admissions and outpatient laboratory tests are captured in an integrated electronic medical record (EMR) system and the data is available for research purposes. Emergency care delivered at outside facilities is captured in a claims system that is also available. The KPSC Regional guidelines for 8-wk LDV/SOF therapy were developed and all providers were notified for eligibility criteria: genotype-1, non-cirrhotic, HCV-RNA < 6 million IU/mL and no prior treatment failure. Cirrhotic status of some patients was confirmed by liver biopsy or other non-invasive testing such as vibration-controlled transient elastography (VCTE) or FIBROSPECT Ⅱ test in some KPSC centers. In some patients, non-cirrhotic status of some patients was determined by clinical judgement of treating healthcare providers using sonographic morphology of the liver, the spleen size and the platelet count in other KPSC centers. All patients with platelet count less than 150 × 10^9/L underwent a form of hepatic fibrosis testing such as liver biopsy, VCTE or FIROSPEcT Ⅱ. Every patient had hepatic sonography and baseline laboratory testing prior to hepatitis C treatment. We developed a protocol for KPSC nurse practitioners, physician assistants and pharmacists, who specialized in hepatitis C treatment, to document intended treatment duration and rationales, pre-treatment testing and close monitoring of patients during treatment such as laboratory testing every 2 wk, calling/messaging to identify any barriers/adverse events and providing coping mechanisms/strategies if any event occurred.

Inclusion criteria: patients with age ⩾ 18 years, HCV viral load < 6 million IU/mL, no cirrhosis or prior treatment failure and who had received 8-wk LDV/SOF therapy for chronic HCV genotype-1 infection. Exclusion criteria: patients without SVR12 (SVR at 12 wk after end of treatment) data, patients who did not complete the intended therapy and patients who missed doses for more than seven consecutive days. Individuals who fulfilled above criteria were included in the final study analysis.

Study design

We conducted a retrospective cohort study from December 2015 to December 2016, of all patients who had completed 8-wk LDV/SOF therapy. Patient's clinical and demographic information was captured from KPSC-EMR system. We developed a standardized protocol with explicit criteria for data abstraction including pre- and post-treatment laboratory results, co-morbid medical conditions, liver biopsy (Metavir fibrosis staging), VCTE (kilopascal), FIBROSPECT Ⅱ test (serum biomarkers), adverse events, clinic/urgent care/emergency department visits and hospitalizations during treatment. Two data abstractors, who are familiar with the EMR system, collected the data according to the protocol criteria to maximize the inter-rater reliability of data abstraction.

Safety analysis

Patient reported side effects such as fatigue, headache, insomnia, arthralgia/myalgia, nausea, cough, rash, dizziness, diarrhea, pruritus, irritability and edema, were recorded. Serious adverse events were defined as any event requiring care at the emergency department or hospital admission.

Study outcome

Primary outcome of our study was achievement of SVR at 12 wk after treatment. Secondary outcomes were identifying predictors of treatment failure and adverse events during treatment. SVR was defined as non-detectable level of HCV-RNA test.

Statistical analysis

For the primary endpoint evaluating SVR12 and for the evaluation of adherence, the final analysis was restricted to *per-protocol* fashion of those patients who completed therapy and returned for follow-up HCV-RNA testing 3 mo after the end of treatment. The rationale to exclude patients who were lost to follow-up was to counter artificial lowering of the calculated SVR rates. Descriptive statistics were used to compare the baseline differences between those individuals who did or did not achieve SVR12. We used cross-tabulation with Pearson χ^2 test to determine the significant difference between categorical variables and 2-tailed Independent-samples *t*-test to determine the significant difference between 1 categorical variable and 1 quantitative variable. We used multivariate logistic regression to estimate OR and 95%CI to identify predictors of treatment failure while adjusting for confounding variables. All data were entered into and analyzed using IBM SPSS Statistics 20 (IBM, Armonk, NY, United States).

RESULTS

We identified total of 775 non-cirrhotic, genotype 1

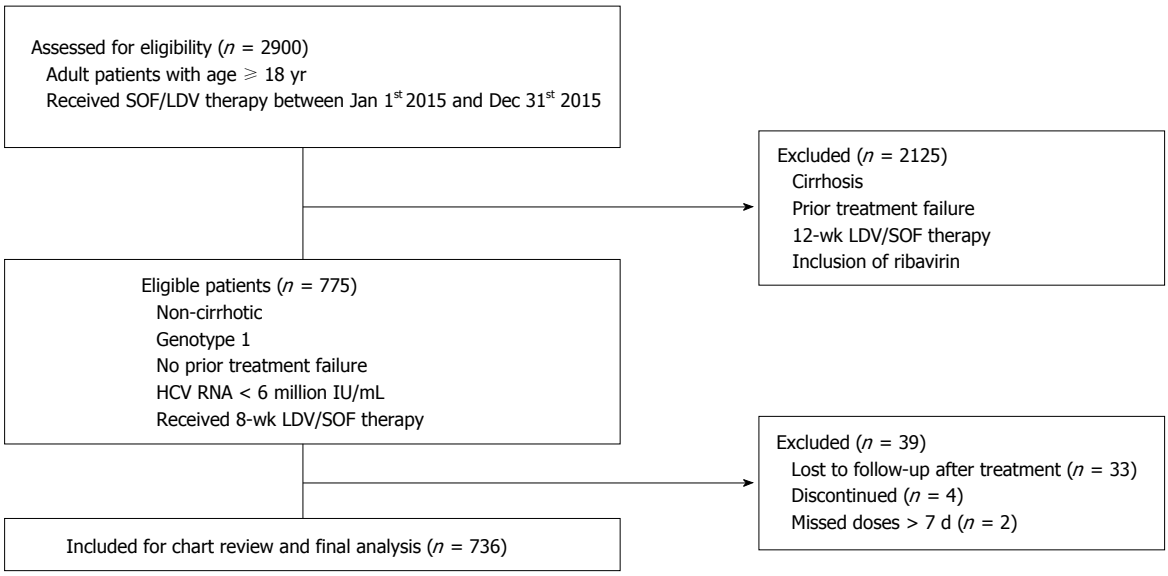

Figure 1 Flow chart of patient selection process. This flow chart summarizes patient identification for eligibility and inclusion/exclusion criteria.

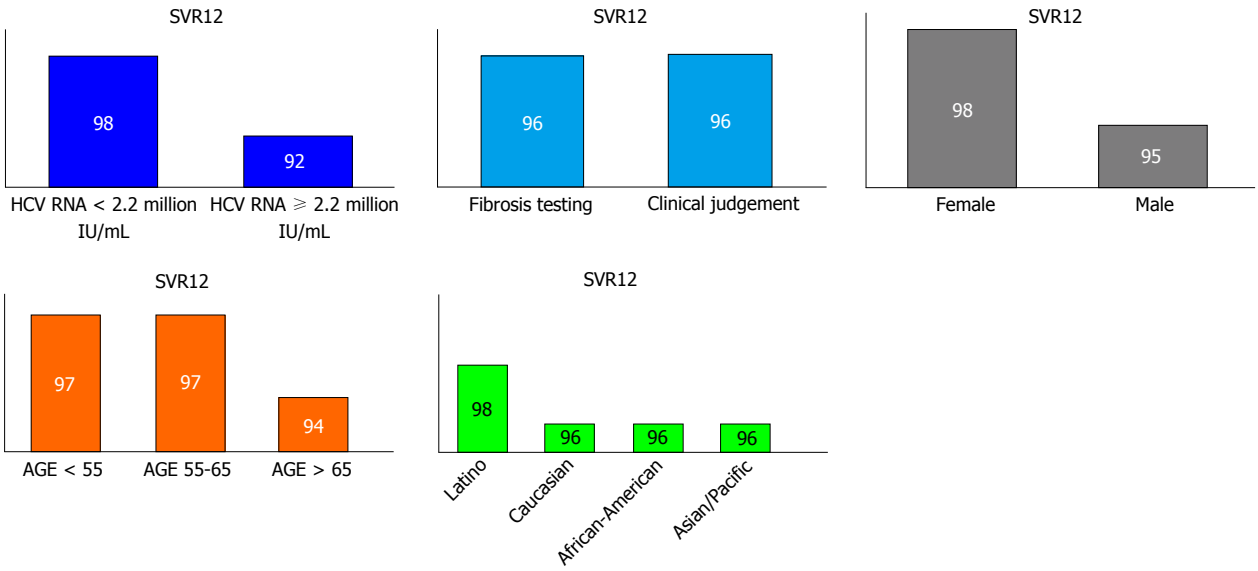

Figure 2 Sustained virologic response rates among patients with various clinical and demographic characteristics. SVR: Sustained viral response.

HCV patients with HCV-RNA < 6 million IU/mL who received 8-wk LDV/SOF treatment. Seven hundred and thirty-six patients were included in the final analysis after exclusion of patients who reported missing doses, discontinued treatment due to adverse events and patients who did not follow-up for SVR12. Figure 1 demonstrates the flow chart of patient selection process.

The demographic and clinical characteristics of patients are outlined in Table 1. The mean age was 58 years, 55% were males, 51% were Caucasian and 65% had genotype 1a infection. Fifty-three percent of patients considered to be non-cirrhotic were determined by healthcare providers based on clinical judgement (platelet count, spleen size, hepatic morphology on ultrasound) and 47% patients had documented liver fibrosis testing (43% liver biopsy, 3% VCTE and 0.4% FIBROSPECT II). Mean HCV-RNA $\log_{10}$ was 6.2.

Table 2 demonstrates the study outcomes (SVR). Overall SVR12 was 96%. None of the patients who achieved SVR12 had viral relapse at 24-wk post treatment. Fifty-nine percent patients had SVR24 data at the time of analysis. We found no difference in SVR12 between patients whose non-cirrhotic state was determined by clinical judgment and patients who had fibrosis testing. No significant difference in SVR12 was seen among gender, genotype 1 subtype, ethnicity, type of fibrosis tests and fibrosis stages. Special populations; those co-infected with HIV and HBV achieved 100% SVR12. When reviewed by age groups, patients with age > 65 years had lower SVR compared to age groups 55-65 and < 55 years but no statistical significance was observed (Figure 2).

Table 1 Demographic and clinical characteristics of patients prior to hepatitis c treatment *n* (%)

Characteristics	*n* = 736
Age, mean ± SD (yr)	58 ± 10
Range	(23-85)
Male sex	403 (55)
Ethnicity	
Caucasian	374 (51)
African American	178 (24)
Hispanic	158 (21)
Asian/Pacific islanders	26 (4)
HCV genotype-subtype	
1a	475 (64)
1b	242 (33)
1 without confirmed subtype	19 (3)
Liver biopsy	317 (43)
Vibration-controlled transient elastography	25 (3)
FIBROSpect Ⅱ	3 (0.4)
Overall fibrosis score	
Stage 0	45 (13)
Stage 1	164 (48)
Stage 2	104 (30)
Stage 3	29 (8)
Stage 3-4 or 4	2 (1)
Non-cirrhotic state determined by clinical judgement	391 (53)
HCV RNA - $\log_{10}$ IU/mL, mean ± SD	6.2 ± 0.2
HCV RNA ≥ 2.2 million IU/mL	219 (30)
Pre-treatment laboratory values	
GFR, mean ± SD (Range)	79 ± 11 (40-89)
Platelet count (10^3/mm^3), mean ± SD (Range)	218 ± 55 (45-495)
INR, mean ± SD (Range)	0.99 ± 0.7 (0.8-1.3)
Albumin, mean ± SD (Range)	3.9 ± 0.4 (2.1-5.1)
Missing	101 (14.0)
Co-morbid conditions	
Psychiatric diagnoses	145 (20.0)
Chronic kidney disease	35 (5.0)
Psoriasis	16 (2.0)
HIV co-infection	5 (0.7)
Cryoglobulinemia	4 (0.5)
HCV-related glomerulonephritis	2 (0.3)
HBV co-infection	2 (0.3)
Hepatocellular carcinoma	1 (0.1)

HCV: Hepatitis C virus; HBV: Hepatitis B virus; GFR: Glomerular filtration rate; INR: International normalized ration; HIV: Human immunodeficiency virus.

We found that HCV RNA viral load plays a role in predicting SVR with high accuracy - the area under a receiver operating characteristic (AUROC) was 0.743 (95%CI: 0.66-0.82) with a cutoff value of 2.2 million IU/mL, as depicted in Figure 3. A significantly lower SVR was observed among patients with HCV-RNA more than 2.2 million IU/mL (91% *vs* 98%, $P < 0.001$). Table 3 exhibits the odds ratios for SVR12 in multivariate logistic regression. We found that patients with HCV-RNA less than 2.2 million IU/mL were more likely to achieve SVR compared to those with more than 2.2 million IU/mL (OR = 0.22, 95%CI: 0.1-0.49, $P < 0.001$). Age groups, gender, ethnicity and genotype 1 subtype did not predict SVR. Non-cirrhotic state determined by clinical judgment based on simple, non-invasive tests was not associated with lower SVR (OR = 1.02, 95%CI: 0.48-2.17, $P = 0.962$).

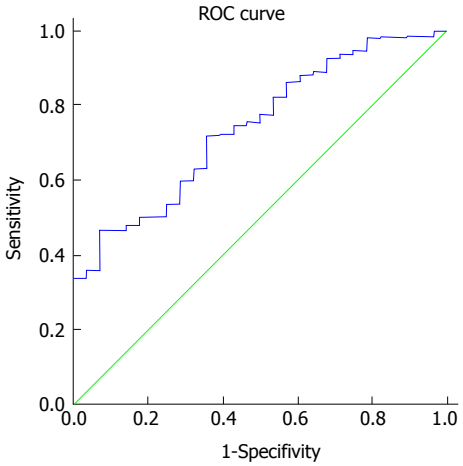

Figure 3 Area under a receiver operating characteristic curve for hepatitis C RNA viral load was 0.734 ($P < 0.001$, 95%CI: 0.66-0.82).

Table 4 reveals the safety analysis of the patients who received 8-wk LDV/SOF therapy. Three (0.5%) patients discontinued treatment due to intolerable adverse events: severe rheumatoid arthritis exacerbation, intractable nausea and declining renal function with glomerular filtration rate 22. One patient who developed drug-induced liver injury (DILI) from LED/SOF therapy with positive biopsy findings discontinued the treatment. Interestingly, one of two patients who were excluded from the study due to missing more than 7 d of therapy achieved SVR. This patient HCV-RNA was 625000 IU/mL. No death or significant morbidities were reported. Four (0.5%) patients experienced serious adverse events during therapy: 2 were hospitalized for observation to evaluate non-cardiac chest pain, 1 was hospitalized for DILI and 1 was due to emergency department admission for pneumonia. The most common minor adverse events were fatigue (14%), headache (13%), insomnia (5%), arthralgia/myalgia (4%) and nausea (4%).

DISCUSSION

Our findings have validated that SVR rate of 8-wk LDV/SOF therapy in treatment naïve, non-cirrhotic, genotype 1 HCV patients with RNA < 6 million IU/mL is comparable with clinical trials and preliminary outcomes from small real-world studies[7-9]. We demonstrated that there is no difference in SVR between patients whose cirrhosis state was determined by fibrosis testing or clinical judgment. All patients had at least baseline ultrasound of the liver and blood tests such as transaminases levels, platelet count and International normalized ration. We calculated overall fibrosis stages on biopsy, VCTE and FIBROSPECT Ⅱ and found no difference in SVR across fibrosis stages although very few patients had stage 0, 3 and 4. Our finding suggests that clinical judgment of non-cirrhotic state results in same outcome of SVR 96% compared to SVR of patients who had liver biopsy, VCTE or FIBROSPECT

Table 2 Sustained viral response 12 rate by various patient characteristics for non-cirrhotic patients with genotype 1 hepatitis C virus infection treated with 8-week ledipasvir/sofosbuvir therapy

Characteristics	SVR12 (%) (*n* = 736)	*P* value
Overall	96 (708/736)	
Non-cirrhotic state determined by clinical judgement	96 (376/391)	0.962
Non-cirrhotic state determined by biopsy, VCTE, FIBROSPECT Ⅱ	96 (332/345)	
HCV RNA ≥ 2.2 million IU/mL	92 (201/219)	< 0.001
HCV RNA < 2.2 million IU/mL	98 (507/270)	
HIV co-infection	100 (6/6)	
HBV co-infection	100 (2/2)	
Gender		0.071
Male	95 (383/403)	
Female	98 (325/333)	
HCV genotype-subtype		0.414
1a	96 (454/475)	
1b	98 (236/324)	
Undetermined	95 (18/19)	
Ethnicity		
Caucasian	96 (357/374)	
African American	96 (171/178)	
Hispanic	98 (155/158)	0.544
Asian/Pacific Islander	96 (25/26)	
Age groups		0.311
< 55 yr	97 (216/223)	
55-65 yr	97 (369/382)	
> 65 yr	94 (123/131)	
Fibrosis Tests		0.489
Liver biopsy	97 (306/317)	
Vibration-controlled transient elastography	92 (23/25)	
FIBROSPECT Ⅱ	100 (3/3)	
Overall fibrosis stage (cumulative: biopsy/VCTE/FIBROSPECT Ⅱ)		
Stage 0	98 (44/45)	
Stage 1	95 (155/164)	0.611
Stage 2	98 (102/104)	
Stage 3	97 (28/29)	
Stage 4	100 (2/2)	

HCV: Hepatitis C virus; VCTE: Vibration-controlled transient elastography; SVR: Sustained viral response.

Table 3 Odds ratios for sustained viral response at 12 week in multivariate logistic regression for non-cirrhotic, hepatitis c genotype 1 patients treated with 8-week ledipasvir/sofosbuvir therapy

Characteristics	OR (95%CI) for SVR12 (*n* = 736)	*P* value
Age 55-65 yr (ref. < 55)	0.92 (0.36-2.34)	0.861
Age > 65 yr (ref. < 55)	0.5 (0.18-1.41)	0.188
Male (ref. female)	0.47 (0.21-1.08)	0.077
African-American (ref. Caucasian)	0.84 (0.11-6.57)	0.868
Hispanic (ref. Caucasian)	2.07 (0.21-20.66)	0.537
Asian/Pacific Islander (ref. Caucasian)	0.98 (0.16-8.28)	0.983
Non-cirrhotic state determined by clinical judgement (ref. Fibrosis Test: biopsy/VCTE/FIBROSPECT)	1.02 (0.48-2.17)	0.962
HCV RNA ≥ 2200000 IU/mL (ref. < 2200000 IU/mL)	0.22 (0.1-0.49)	< 0.001
HCV genotype - subtype 1b (ref. 1a)	2.19 (0.25-19.15)	0.480

HCV: Hepatitis C virus; VCTE: Vibration-controlled transient elastography; SVR: Sustained viral response.

tests.

In our cohort, all patients had pre-treatment HCV-RNA < 6 million IU/mL. We divided to 2 subgroups containing RNA < 800000 IU/mL and > 800000 IU/mL. We found that patients with lower RNA < 800000 IU/mL achieved significantly higher SVR compared to patients with higher RNA in both univariate and multivariate analyses. This finding suggests that HCV viral load plays an important role in predicting SVR although the determination of the optimal cut-off value of HCV-RNA level to consider 8-wk therapy to achieve SVR is currently not available [14]. Our study highlights that HCV RNA 2.2 million IU/mL was associated significant impact on outcomes with AUROC 0.73. While female gender and Latino ethnicity achieved slightly higher SVRs, there is no statistical difference compared to male gender and other ethnicities. We found no difference in SVR rates between African-

Table 4 Adverse events, hospital admissions and discontinuation rates of patients with genotype 1 hepatitis C virus infection who received 8-wk ledipasvir/sofosbuvir therapy

Adverse events	*n* (%)
No. adverse event, mean ± SD (Range)	0.5 ± 0.7 (0-6)
Serious adverse events	4 (0.5)
Hospital admissions	
Non-cardiac chest pain	2
Drug-induced liver injury	1
Pneumonia	1
Minor adverse events	
Fatigue	104 (14)
Headache	98 (13)
Insomnia	35 (5)
Arthralgia/myalgia	29 (4)
Nausea	29 (4)
Cough	15 (2)
Rash	19 (3)
Dizziness	12 (2)
Diarrhea	14 (2)
Pruritus	11 (1)
Irritability/anxiety	10 (1)
Edema	2 (< 0.5)
Discontinuation	4 (0.5)
Drug-induced liver injury	1
Severe rheumatoid arthritis exacerbation	1
Intractable nausea	1
Decreased renal function during treatment (GFR < 30)	1
Death	0

GFR: Glomerular filtration rate.

Americans and Caucasians in contrast to other studies which demonstrated the decreased likelihood of SVR in African-American population[12].

The wholesale acquisition cost for LDV/SOF combination drug in the United States is $1125 per pill. Cost of 8-wk course of therapy is $63000 and cost of 12-wk course is $94500 - net cost saving of $31500 per patient when 8-wk treatment is administered. Healthcare expenses can substantially be reduced by selecting 8-wk LDV/SOF therapy in treatment-naïve, non-cirrhotic genotype 1 HCV patients.

The strengths of our study are its real-world experience and an integrated healthcare model involving all clinical services. We were able to abstract data regarding all clinic/emergency department/urgent care visits, hospitalizations, telephone/electronic-mail encounters and all laboratory tests from the integrated EMR system. All providers used KPSC-Regional HCV treatment guidelines which is readily available on the EMR system for review. Treatment duration, reasons for discontinuation and medication compliance were clearly documented. All patients in the final analysis had good post-treatment follow ups with available SVR12 data. The limitation of our study is its retrospective nature.

In conclusion, our outcomes from real-world cohort validate high SVR rates in non-cirrhotic, treatment naïve HCV genotype 1 patients with HCV RNA < 6 million IU/mL who received 8-wk LDV/SOF therapy. There was no difference in SVR between patients whose non-cirrhotic state was determined by clinical judgment and patients who had fibrosis testing. HCV RNA less than 2.2 million IU/mL was associated with significantly higher SVR. LDV/SOF therapy is safe and well-tolerated with high adherence rates. Therefore, 8-wk LDV/SOF therapy can be used in selected subset of patients with chronic HCV genotype 1 infection who meet aforementioned clinical criteria. Further studies are in need to evaluate and validate HCV RNA cutoff value to achieve the optimal more than 95% of SVR.

COMMENTS

Background

Hepatitis C treatment has evolved from a 78-wk interferon monotherapy to 48-wk pegylated interferon plus ribavirin therapy and now 12-wk therapy with newer all-oral direct-acting antiviral (DAA) agents. DAA regimens have revolutionized the treatment of hepatitis C with their excellent sustained virologic response (SVR), tolerable side effect profiles and shorter duration of therapy. Although ION-3 trials and other real-world studies have revealed that 8-wk ledipasvir/sofosbuvir therapy is effective and has comparable sustained viral response outcomes for non-cirrhotic patients who have untreated genotype-1 hepatitis C virus (HCV) infection and HCV RNA < 6 million IU/mL, the current AASLD guidelines recommend 12-wk therapy. Eight-week therapy may provide significantly lower cost, better patient compliance and adherence.

Research frontiers

The authors validated that SVR outcomes in 8-wk LED/SOF therapy was comparable with 12-wk therapy in this large, real-wold cohort.

Innovations and breakthroughs

This study highlights that HCV RNA 2.2 million IU/mL was associated significant impact on outcomes with AUROC 0.73. Patients with HCV RNA more than 2.2 millon IU/mL were observed to have significantly lower SVR (92% *vs* 98%, *P* < 0.001). They found no difference in SVR rates between African-Americans and Caucasians in contrast to other studies which demonstrated the decreased likelihood of SVR in African-American population.

Applications

This study validate other real-world studies which have shown that 8-wk therapy in selected subset of patients (non-cirrhotic, untreated, genotype-1 with HCV RNA < 6 million IU/mL) is effective and comparable to 12-wk therapy. We can apply these findings and amend changes in national guidelines regarding HCV treatment which can save significant amount of HCV treatment cost and boost patient compliance and adherence.

Peer-review

This study is good, and it's important knowledge for clinicians before treating HCV patients.

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ORIGINAL ARTICLE

Retrospective Study

Early radiological assessment of locally advanced pancreatic cancer treated with electrochemotherapy

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Author contributions: Granata V, Izzo F and Petrillo A designed research and supervised the research; Granata V, Fusco R, Setola SV, Piccirillo M, Leongito M, Lastoria S, Izzo F and Petrillo A performed research; Fusco R analyzed data; Granata V and Fusco R wrote the paper; all authors read and approved the final manuscript.

Institutional review board statement: The patients were enrolled in a clinical phase I/II study approved by the Ethical Committee of the National Cancer Institute "G. Pascale Foundation - IRCCS" of Naples (deliberation n. 482 of 02/07/2014).

Informed consent statement: All patients enrolled have signed the informed consensus.

Conflict-of-interest statement: The authors declare that they have no competing interests.

Data sharing statement: No additional data are available.

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Abstract

AIM

To report early imaging assessment of ablated area post electrochemotherapy (ECT) in patients with locally advanced pancreatic cancer (LAPC).

METHODS

ECT was performed in 19 LAPC patients enrolled in an approved ongoing clinical phase Ⅰ/Ⅱ study. Before and after ECT, 18 patients underwent computed tomography (CT) scan, 11 patients underwent morphological and functional magnetic resonance (MR) scan (dynamic contrast enhanced-MRI) calculating wash-in slope (WIS) and wash-out slope (WOS); diffusion weighted imaging calculating pseudo-diffusivity (Dp), perfusion fraction (fp) and tissue diffusivity (Dt); 10 patients underwent positron emission tomography (PET). Response evaluation criteria in solid tumour (RECIST) on MR and

CT were used to assess tumour therapy response. Choi on CT, PET response criteria in solid tumors (PERCIST) on PET and functional parameters on MR were used to evaluate treatment response.

RESULTS

For each patient no significant reduction was measurable by CT and MR using RECIST. According Choi criteria a partial response was obtained in 18/18 (100.0%) patients. According PERCIST criteria 6/10 (60.0%) patients showed a partial response, 3/10 (30.0%) stable disease and 1/10 (10.0%) progression disease. Moreover, using functional MR parameters, a significant reduction of viable tumour after ECT can be observed. According ΔWIS and ΔWOS 9/11 (81.8%) patients exhibited a partial response and 2/11 (18.2%) stable disease; 8/11 (72.7%) patients were considered in partial response by ΔDp evaluation and 3/11 (27.3%) in stable disease; according ΔDt 7/11 (63.6%) patients showed a partial response, 1/11 (9.1%) showed progression of disease and 3/11 (27.3%) were stable. Perfusion fraction fp showed a significant reduction after ECT only in four patients. No significant difference was observed after ECT in signal intensity of T1-weighted images and T2-weighted images, and in equilibrium-phase of contrast study, according to χ^2 test was observed. A good correlation was reported between ΔHounsfield unit and Δmaximum standardized uptake value and between Δfp and ΔWOS, with a significant statistically difference ($P < 0.05$) using Spearman correlation coefficient.

CONCLUSION

Perfusion and diffusion MR derived parameters, Choi, PERCIST criteria are more performant than morphological MR and CT criteria to assess ECT treatment response.

Key words: Reversible electroporation; Response assessment; Positron emission tomography/computed tomography; Pancreatic cancer; Magnetic resonance imaging

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Core tip: Aim of this study was to assess and to report early imaging assessment of ablated area post electrochemotherapy in patients with locally advanced pancreatic cancer emphasizing the role of new functional imaging tools in magnetic resonance imaging compared to standard morphological response evaluation criteria in solid tumour, Choi criteria and positron emission tomography response evaluation criteria in solid tumour.

Granata V, Fusco R, Setola SV, Piccirillo M, Leongito M, Palaia R, Granata F, Lastoria S, Izzo F, Petrillo A. Early radiological assessment of locally advanced pancreatic cancer treated with electrochemotherapy. *World J Gastroenterol* 2017; 23(26): 4767-4778 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i26/4767.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i26.4767

INTRODUCTION

Adenocarcinoma of pancreas is among the most aggressive forms of cancer. Surgical resection is the only potentially curative treatment for pancreatic cancer. Unfortunately, the majority of patients have grossly unresectable disease; over 80% of patients with pancreatic cancer have locally advanced or metastatic disease[1]. Current standard therapy is chemotherapy and/or radiotherapy. The most frequently used chemotherapy agent in LAPC was Gemcitabine; moreover some studies have shown the combination of Gemcitabine with other chemotherapy agents increases overall survival[1-4]. Because a limited group of patients responds to chemotherapy, additional therapies were explored in order to obtain tumor debulking or interstitial ablation[5-8]. A potential therapy was the electroporation that can be delivered in either an irreversible[9-11], as a direct ablation modality, or a reversible manner[12-15], as a physical delivery system, based on the strength and duration of the electrical field. Reversible electroporation has been performed to increase uptake of chemotherapy into tumor cells. Reversible electroporation combined with low doses of chemotherapeutic drugs was known as Electrochemotherapy (ECT)[15]. The delivering of an electrical field determines a transient increase of cell permeability with a consequent increase of intracellular dose of chemotherapeutic drugs, using low doses and reducing the chemotherapy cytotoxic effects[12-15]. Preclinical studies showed the effectiveness of ECT on pancreatic cancer[15,16]. Our previous studies investigated the safety and effectiveness of ECT in patients with locally advanced pancreatic cancer[17,18]. Recently, Tarantino *et al*[19] investigated the feasibility of percutaneous electrochemotherapy in the treatment of portal vein tumor thrombosis at hepatic hilum in patients with hepatocellular carcinoma in cirrhosis.

Until now oncologic therapy is principally based on different combinations of surgery, radiotherapy, and chemotherapy, however targeted therapies, hormonotherapy, immunotherapy, and interventional techniques, with the introduction a new promise ablation techniques to treat deeper tumors have emerged as alternative potential cancer treatments[17-23]. Currently, standard imaging techniques and morphologic response criteria do not provide the necessary information to evaluate tumor response. Magnetic resonance imaging (MRI) offers a combination of anatomic, physiologic, and molecular information, which may overcame these limitations, and is being increasingly used for therapy response assessment[23,24].

The purpose of our study is to report the early

Table 1 Characteristics of locally advanced pancreatic cancer patients treated with electrochemotherapy

Patients (n = 19)	
Histotype, %	
Adenocarcinoma	100 (19/19)
Location, %	
Head	57.9 (11/19)
Body/tail	42.1 (8/19)
Largest diameter lesion, cm (range)	5.2 (2.2-9.9)
Venus involvement (SMV or PV), %	
Yes	84.2 (16/19)
No	15.7 (3/19)
Arterial encasement, %	
Yes	57.9 (11/19)
No	42.1 (8/19)

imaging assessment of treated area with ECT in locally advanced pancreatic cancer, emphasizing the role of new functional imaging tools in MRI compared to standard morphological response evaluation criteria in solid tumour (RECIST), Choi[25] and PERCIST criteria[26].

MATERIALS AND METHODS

Study population

The patients were enrolled in a clinical phase Ⅰ/Ⅱ study approved by the Ethical Committee of the National Cancer Institute "G. Pascale Foundation - IRCCS" of Naples (deliberation n. 482 of 02/07/2014). The study endpoints were the feasibility and safety of ECT in the multimodal treatment of pancreatic cancer in patients with locally advanced disease and not suitable for radical surgery.

Nineteen patients (9 female and 10 male) from November 2011 to December 2016 were enrolled in this prospective study. Inclusion criteria were: age between 18-80 years; good mental health; life expectancy $\geqslant$ 3 mo; histologically confirmed diagnosis of pancreatic adenocarcinoma; locally advanced disease (stage Ⅲ) confirmed with preoperative radiological assessment, unfit for curative surgery. Exclusion criteria were: pregnant women, significant heart disease, coagulation disturbances, allergy to bleomycin, lung and kidney dysfunction, implanted defibrillator or pacemaker, concomitant presence of distant metastases. All patient enrolled have signed the informed consensus.

All patients enrolled with diagnosis of locally advanced pancreatic adenocarcinoma received systemic chemotherapy before ECT treatment. Patient characteristics were summarized in Table 1. Two chemotherapy regimens were adopted: Gemcitabine + Oxaliplatin (GEMOX) or 5-FU/Leucovorin, Irinotecan, and Oxaliplatin (FOLFIRINOX). Details of chemotherapy regimens were reported in our previous publication[27].

Fourteen (14/19, 73.9%) patients were subjected to GEMOX and five patients (5/19, 26.3%) were treated with FOLFIRINOX before ECT treatment (mean time between the start of chemotherapy treatment and ECT was 118 d, range 115-136). The patients with stable disease or partial response after chemotherapy, proven by clinical and radiological examination, were suitable to receive ECT treatment.

Treatment protocol

ECT was delivered in open laparotomy through an adequate midline incision to allow both appropriate staging of the disease and appropriate mobilization of the pancreatic malignancy based on its tumor location and infiltration. In the case of pancreatic head lesions, an extensive Kocher maneuver was performed in order to mobilize the duodenum and the head of the pancreas over the area of local invasion to allow easier caudal and cranial needle placement. Similarly, mobilization of the transverse colon were done inferiorly, depending on the degree of infiltration. In this way, the surgeon who performed the treatment was able to decide whether to use needle electrodes with fixed geometry (hexagonal or linear) or with variable configuration using multiple insertion of single-needle; through the transverse mesocolon or, if the mobilization of the transverse mesocolon was impossible, the needle electrodes were inserted superior to the base of the transverse mesocolon.

Bleomycin was administrated intravenously (15000 IU/m^2) before the application of electrical pulses to the target area. Electric pulses were applied by needle electrodes with linear, hexagonal configuration or variable geometry (IGEA S.p.A., Carpi, Italy) depending on the size and location of the tumors. Cliniporator™ (IGEA S.p.A., Italy) was used to deliver electric voltage with the following parameters: 8-96 pulses of 400-1000 V and 910-1000 V/cm, of 100 μs duration, at 1-5000 Hz repetition frequency in according to ESOPE (European Standard Operating procedure of Electrochemotherapy) protocol[28] or a single pulse for a single relived R-wave (ECG synchronization) for custom geometry. Electric impulses were synchronized with the ECG for a safe delivery of the electric impulses to pancreas. ECG synchronization was obtained with Accusync 42 (medical device provided by IGEA S.p.A., Carpi, Italy). Treatment was completed within the window from 8 to 28 min after the end of the bleomycin bolus.

Imaging techniques

According to the study protocol, the patients underwent baseline MRI and/or computer tomography (CT) and/or ^{18}F-FDG positron emission tomographic (PET)/CT study 5-7 d before ECT and post-treatment at 1, 3, 6 and 12 mo. Long-term follow-up was carried out with radiological imaging obtained every three months in the time thereafter.

We considered, in this paper, only the morphological and functional results obtained in CT, PET/CT and MR

Table 2 Parameters for each magnetic resonance sequence

Sequence	Orientation	TR/TE/FA (ms/ms/deg.)	FOV (mm^2)	Acquisition matrix	Slice thickness/gap (mm)
HASTE T2-W	Axial	1500/90/180	380 × 380	320 × 320	5/0
FLASH T1-W, in-out phase	Axial	160/4.87/70	285 × 380	192 × 256	5/0
FLASH T1-W, out phase	Axial	178/2.3/80	325 × 400	416 × 412	3/0
DWI	Axial	7500/91/90	340 × 340	192 × 192	3/0
VIBE T1-W	Axial	4.89/2.38/10	325 × 400	320 × 260	3/0
TWIST T1-W, Pre and post contrast agent injection	Axial	3.01/1.09/25	300 × 300	256 × 256	2/0

W: Weighted; TR: Repetition time; TE: Echo time; FA: Flip angle; AT: Acquisition time; HASTE: Half-Fourier acquisition single-shot turbo spin-echo; FLASH: Fast low angle shot; DWI: Diffusion-weighted imaging; VIBE: Volumetric interpolated breath hold examination; TWIST: Time-Resolved Angiography with Stochastic Trajectories.

imaging at one month as prognostic early indicator of therapy response. The gold standard to defining the assessment after therapy has been the consensus between two radiological modalities (Choi, PERCIST, Dynamic Contrast Enhanced-Magnetic Resonance Imaging and Diffusion Weighted Imaging; for two latter considering the consensus of response of two parameters).

MR and CT protocol: MR protocol consists of morphological and functional imaging including dynamic contrast enhanced-magnetic resonance imaging (DCE-MRI) and diffusion weighted imaging (DWI) sequences. Imaging was performed with a 1.5 T scanner (Magnetom Symphony, Siemens Medical System, Erlangen, Germany) equipped with a phased-array body coil. Patients were placed in a supine, head-first position.

Morphological pre-contrast axial T2-weigthed (T2-w) 2D Half-Fourier Acquisition Single-Shot Turbo Spin-Echo (HASTE), with and without fat suppression; morphological pre-contrast axial T1-weigthed (T1-w) Fast Low Angle Shot (FLASH) 2D in-out phase; morphological pre contrast axial T1-w fat suppression FLASH 2D out phase; morphological post contrast axial and coronal T1-w Volumetric Interpolated Breath-hold Examination (VIBE) fat suppression were acquired.

A free breathing axial single shot echo planar DWI pulse sequence parameters were obtained with multiple *b* value = 0, 50, 100, 150, 400, 800, 1000 s/mm^2.

After, DCE-MRI sequences, we obtained 1 sequence before and 120 sequences, without any delay, after intravenous injection of 2 mL/kg of a positive, gadolinium-based paramagnetic contrast medium (Gadobutrol Gd-DTPA, Bayer Pharma AG, Berlin, Germany). The contrast medium was injected using Spectris Solaris® EP MR (MEDRAD Inc., Indianola, PA, United States), with a flow rate of 2 mL/s, followed by a 10-mL saline flush at the same rate. DCE-MRI T1-w were acquired using Time-Resolved Angiography with Stochastic Trajectories 3-D axial images in order to reduce temporal resolution (3 s).

Parameters details for each MR sequence were provided in Table 2.

Non contrast-enhanced phase and triple-phase contrast-enhanced MDCT was performed with a 64-detector row scanner (Optima 660, GE Healthcare, United States). MDCT scanning parameters were 120 kVp, 100-470 mAs (NI 16.36), 2.5-mm slice thickness and table speed 0.984/1 mm/rotation. Scans were carried out including a region encompassing the liver from diaphragm to iliac crests. Phases were as follows; hepatic arterial phase 30-40 s after injection of 120 mL of a nonionic contrast medium (iomeprol, Iomeron 400, Bracco, Milan, Italy) with a bolus-triggered technique [120 kVp; 40-60 mA; trigger threshold, 180 Hounsfield units in descending aorta], portal and equilibrium phase 90 s and 120 s after contrast injection. The contrast medium was administered at a rate of 4 mL/s through antecubital vein with an automated injector system (Empower CTA, E-Z-EM Inc., New York, United States).

MR and CT image analysis: Four blinded observers with at least 10 years' experience in interpretation of MR and CT images of the pancreas independently and randomly reviewed the images acquired before and after ECT. The interval between reviews of the CT and MR images was at least 5 d. The response to ECT was evaluated according the RECIST 1.1 criteria[29]. Objective therapeutic responses according to RECIST 1.1 are as follows: complete remission (CR) is disappearance of target lesion for at least 4 wk; partial remission (PR) is a decline of at least 30% in tumor diameter; stable disease (SD) is neither PR nor progressive disease (PD); and PD is at least a 20% increase in tumor diameter and 5-mm absolute increase was required. Moreover for CT images, the response to ECT was evaluated according the Choi criteria[24]: CR is disappearance of target lesion; PR is a decrease in tumor size $\geqslant$ 10% or decrease in tumor density $\geqslant$ 15% on CT; SD is neither PR nor PD; and PD is an increase in tumor size $\geqslant$ 10% and does not meet PR criteria by tumor density.

For functional MR analysis tumor borders were manually segmented on transversal T1-W images VIBE fat suppression post contrast (equilibrium phase). In each slice a region of interest (ROI) was delineated according to the tumor geometry. The border of the ROI was placed in the tumor periphery close to the

tumor margin, so that the ROI encompassed almost the whole tumor area. DW-MRI analysis[30,31] and DCE-MRI semi-quantitative analysis[32] was performed on the ROIs previously described. For each pixel Time Intensity Curves (TICs) were obtained and per each TIC, 2 shape descriptors were computed: the WI slope (WIS), the WO slope (WOS) as described in[31]. DW-MRI analysis was performed using Variable Projection Curve-Fitting algorithm (VarPro), as reported by[33], to estimate the IVIM-related parameters of bi-exponential model: pseudo-diffusivity (Dp), perfusion fraction (fp) and tissue diffusivity (Dt). Per each descriptor (dimensional parameters, density on CT images and perfusion and diffusion coefficients) median value was obtained and the percentage changes between pre and post treatment [ΔX = (Xpre - Xpost)/Xpre; X is the generic shape descriptor] were calculated. No image registration was applied to our data acquired. We take care to exclude from the analysis the slices where was visible motion artifacts. Moreover, a volumetric analysis for functional parameters measurements was performed thus minimizing errors due to voxel misalignments.

This data analysis was performed using in-house software written in Matlab R2007a (The MathWorks, Inc., Natick, MA, United States). The following parameters were also evaluated for each single target area in MRI before and after treatment: signal intensity respectively in T1-weighted images, in T2-weighted sequences and in equilibrium phase of contrast study.

^{18}F-FDG PET Data acquisition and images analysis: ^{18}F-FDG PET/CT studies were acquired 60 min after the administration of 300-385 MBq of FDG either with a Siemens ECAT EXACT 47 or a General Electric DST 600 PET-CT scanner. All calibrations on the scanners to obtain accurate SUV readings were regularly performed. Patients fasted for at least 6 h, and blood glucose level was < 150 mg/dL. Each patient underwent the baseline and the pre-operative study on the same scanner.

Irregular volumes of interest (VOIs) were semi-automatically drawn by the expert investigator on orthogonal planes using a dedicated workstation and software using an arbitrary threshold, as reported previously[34]. For each patient both studies were analyzed at the same time in order to minimize discrepancies in VOI positioning. For each study maximum standardized uptake value (SUV_{max}) values of the pancreas lesion were recorded. The analysis of ^{18}F-FDG PET/CT results was performed by comparing measurements obtained in the pancreatic lesion at baseline (SUV1) and after treatment (SUV2). This change was expressed as the percentage of SUV reduction [ΔSUV = (SUV1−SUV2)/SUV1 × 100]. Objective therapeutic responses was defined according to PERCIST 1.0 are as follows[26]: complete metabolic response (CMR) is complete resolution of ^{18}F-FDG uptake within the measurable target lesion and in-distinguishable from surrounding background blood-pool levels with no new ^{18}F-FDG-avid lesions; partial metabolic response (PMR) is reduction of a minimum of 30% in the target tumor ^{18}F-FDG SUV_{max}; stable metabolic disease is disease other than CMR, PMR or progressive metabolic disease; and progressive metabolic disease is a 30% increase in ^{18}F-FDG PET/CT SUV_{max} or advent of new ^{18}F-FDG-avid lesions that are typical of cancer.

Statistical analysis

Data were expressed in terms of median value ± SD. Spearman correlation coefficient for non-parametric variables was used to assess the correlation between percentage changes of tissue density of CT, and of perfusion and diffusion parameters. A P value < 0.05 was considered statistically significant. Percentage of objective response was reported for each modality. χ^2 test was, also, used to compare pre- and post-ECT imaging findings. A P value < 0.05 was regarded as statistically significant.

All analyses were performed using Statistics Toolbox of Matlab R2007a (The Math-Works Inc., Natick, MA, United States).

RESULTS

Radiological response assessment

Basal imaging involved CT, PET and MR scans. Mean time between basal imaging assessment and ECT was 9 d (range 7-14). Mean time between ECT and first follow-up radiological assessment was 36 d (range 31-43).

CT was performed for eighteen patients before and after ECT; morphological and functional MR was obtained for 11 patients before and after ECT and 10 patients were subjected to ^{18}F-FDG PET/CT before and after treatment. One died to complication after treatment (24-48 h after ECT). Four patients rejected MR scan due to claustrophobia complications. Three patients were affected by allergy to Gadolinium chelates (MR contraindication). In 4 patients the patient clinical conditions did not allow to perform ^{18}F-FDG PET/CT study in the range that would make the data comparable, before and after treatment; in other 4 patients the PET study was performed in a different hospital with low quality of the images.

In Table 3 we reported the measure of largest diameters obtained by CT and MR for each patients, before and after one month of treatment.

In Table 4 we showed the percentage change, between before and after ECT, of largest diameter by CT and MR and the median value percentage change of tissue density in ΔHU, of perfusion and diffusion quantitative parameters derived by MR imaging (WIS, WOS, Dt, fp and Dp) and of maximum SUV value. Moreover, median values ± SD of percentage changes, before and after ECT were reported in Table 4.

Table 3 Tumor size before and after electrochemotherapy for individual patient evaluated by magnetic resonance and computed tomography

Patient No.	Age	Sex	Tumor size		Tumor response after ECT treatment	
			CT (mm)	MR (mm)	1st radiological evaluation after ECT (CT); size (mm)	1st radiological evaluation after ECT (MR); size (mm)
1	48	M	99	95	90	87
2	63	F	43	48	38	43
3	71	F	59	64	54	57
4	61	F	22	26	19	23
5	72	F	51	49	49	-
6	80	F	48	-	45	-
7	60	F	33	-	24	-
8	62	F	30	-	22	-
9	67	M	99	-	-	-
10	57	M	56	-	46	-
11	74	M	56	58	59	51
12	67	M	63	68	55	55
13	59	M	28	30	28	24
14	79	M	50	41	46	38
15	71	M	35	34	56	-
16	80	M	53	-	49	-
17	80	M	64	55	49	46
18	59	F	51	51	66	65
19	62	F	53	53	50	49

ECT: Electrochemotherapy; CT: Computed tomography; MR: Magnetic resonance.

Table 4 Treatment response assessment based on response evaluation criteria in solid tumour 1.1 criteria evaluated by magnetic resonance and computed tomography, based on Choi criteria evaluated by computed tomography, based on perfusion and diffusion parameters evaluated by dynamic contrast enhanced-magnetic resonance imaging and diffusion weighted imaging data and based on positron emission tomography response criteria in solid tumors criteria evaluated by positron emission tomography/computed tomography

No.	ΔCT largest diameter	ΔHU	ΔMR largest diameter	ΔWIS	ΔWOS	ΔDt	Δfp	ΔDp	ΔSUV_{max}	Response assessment
1	11.6%	22.7%	8.4%	35.4%	40.0%	-78.1%	12.1%	61.9%	-177.8%	PR
2	9.1%	40.4%	10.4%	84.7%	85.0%	-32.7%	27.3%	78.7%		PR
3	8.5%	34.0%	11.5%	94.0%	74.4%	-64.4%	28.5%	50.3%	38.5%	PR
4	13.6%	7.8%	2.0%	88.0%	76.0%	-16.8%	32.7%	36.6%		PR
5	3.9%	48.7%								
6	6.3%	18.9%								
7	27.3%	49.5%								
8	26.7%	51.6%								
9										
10	17.9%	42.6%							100.0%	PR
11	-5.4%	49.1%	12.1%	18.4%	9.7%	-20.4%	-33.3%	5.4%	66.5%	SD
12	12.7%	6.8%	19.1%	57.9%	98.0%	-34.2%	11.7%	12.0%	-17.9%	SD
13	6.7%	44.4%	20.0%	7.9%	-17.4%	32.5	2.2%	36.9%	46.8%	PR
14	8.0%	44.8%	7.3%	67.6%	110.0%	-32.9%	44.3%	92.0%	44.4%	PR
15	-60.0%	83.3%								
16	7.5%	23.4%								
17	23.4%	35.5%	16.4%	55.7%	307.1%	-18.0%	62.6%	32.2%	18.8%	PR
18	-29.4%	40.0%	-9.8%	34.9%	58.7%	-16.3%	46.7%	66.4%	17.0%	PR
19	5.7%	44.0%	7.5%	67.3%	-24.6%	-30.5%	-22.6%	-90.7%	32.3%	PR

CT: Computed tomography; HU: Hounsfield unit; MR: Magnetic resonance; WIS: Wash-in slope; WOS: Wash-out slope; Dt: Diffusivity; fp: Perfusion fraction; SUVmax: Maximum standardized uptake value.

For each patient no significant reduction of largest diameter percentage change by CT and MR was observed. According to RECIST criteria, all patients had stable disease using MR imaging while using CT imaging one patient showed progression disease (Figure 1 for MRI and 2, 6 for CT imaging). According to Choi criteria 18/18 (100.0%) patients were considered in partial response (Figure 2). According to PERCIST criteria 6/10 (60.0%) exhibited partial response (Figure 3), 3/10 (30.0%) stable disease and 1/10 (10.0%) progression disease. Moreover, using functional MR derived parameters, significant reduction of viable tumor tissue were observed: a reduction of 30% of ΔWIS, ΔWOS, Δfp, ΔDp and an increase of

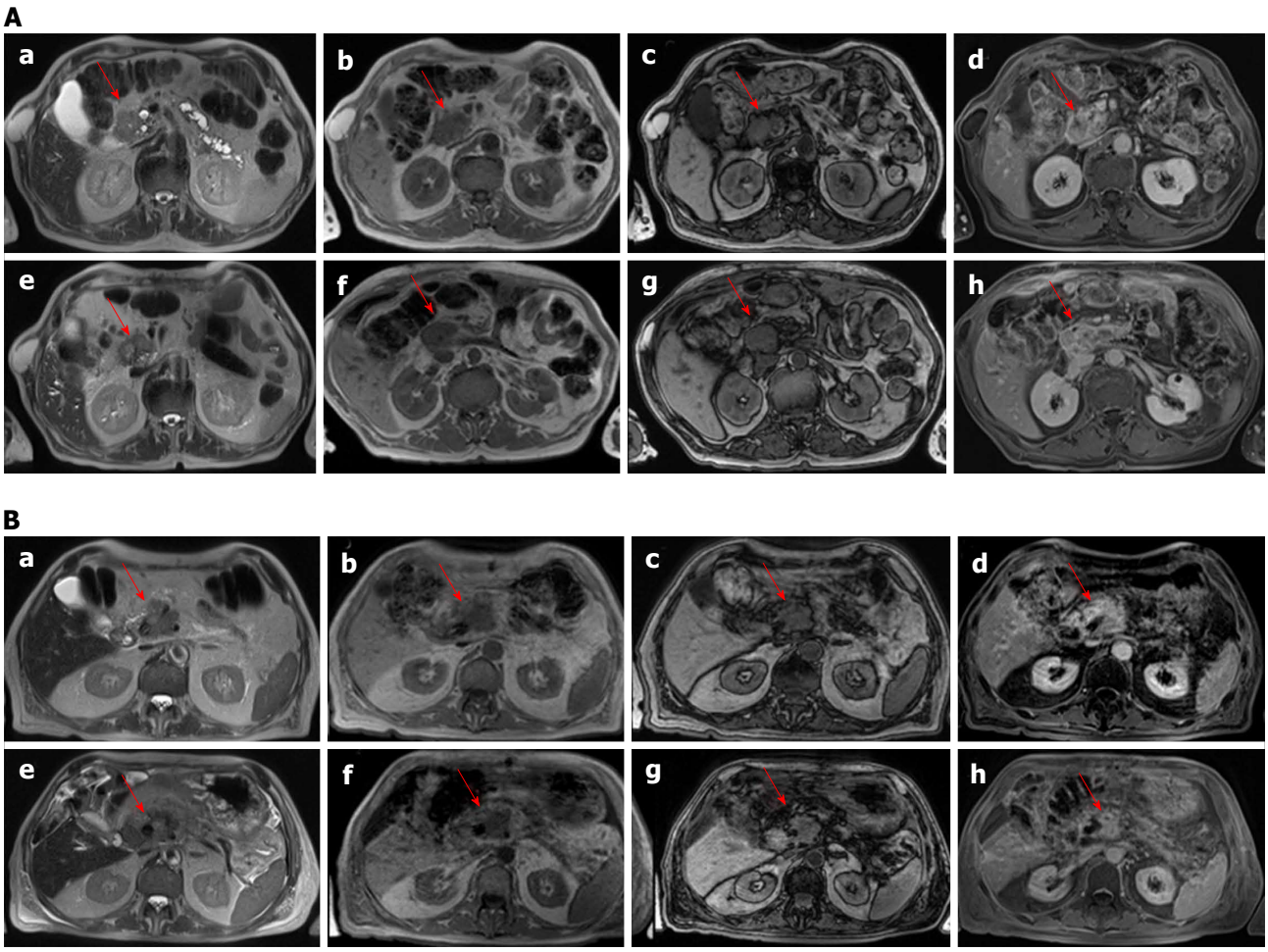

Figure 1 Magnetic resonance imaging assessment using morphological criteria for two patients (in A man 79 years old and in B man 74 years old). Before treatment in HASTE T2-W sequence (a), the lesion (arrow) appears hyperintense, in in-phase T1-W sequence (b) and out-phase T1-W sequence (c) appears hypointense and hypovascular in VIBE T1-W in equilibrium phase (d). After the treatment the lesion in HASTE T2-W sequence (e), in-phase T1-W sequence (f), out-phase T1-W sequence (g) and VIBE T1-W in equilibrium phase (h): there were not significant differences in signal compared to the similar before the treatment. HASTE: Half-Fourier Acquisition Single-Shot Turbo Spin-Echo; VIBE: Volumetric Interpolated Breath-hold Examination.

30% of ΔDt was considered as significant variation after treatment and was defined as partial response. For both ΔWIS and ΔWOS 9/11 (81.8%) patients showed partial response and 2/11 (18.2%) were considered stable. Eight elevenths (72.7%) patients were considered in partial response by ΔDp evaluation and 3/11 (27.3%) was considered in stable disease. According to ΔDt 7/11 (63.6%) patients showed partial response, 1/11 (9.1%) progression disease and 3/11 (27.3%) stable disease (Figure 4). Perfusion fraction fp showed a significant reduction after ECT only for four patients. Final decision on treatment response was taken considering the accordance with at least two imaging technique (see last column of Table 4).

We found no statistically significant difference of target area signal intensity obtained by T1-weighted images, T2-weighted images and equilibrium-phase of contrast study between before and after treatment, according to Chi-square test.

Spearman correlation coefficient was performed for each couple of parameters and was reported in Table 5. A good correlation was reported between ΔHU and ΔSUVmax and between Δfp and ΔWOS, with a significant statistically difference ($P < 0.05$).

DISCUSSION

Although it has been shown that the ECT is a promising technique for cancer treatment[15-18,21,28], there is still the problem of how to assess treated tumor response. In our preliminary experience, we demonstrated that RECIST 1.1, using the variation of largest diameter, both on CT and MR images, do not provide a appropriate patients stratification in responders or non-responders after ECT. In fact, according to RECIST criteria, all patients were classified with stable disease by MR imaging while using CT scan one patient showed progression disease. The RECIST criteria restrictions are well known, as also reported by Lencioni *et al*[35] in the assessment of residual viable tumor of treated HCC and by Choi[36] in Gastrointestinal Stromal Tumor. ECT potentiates the cytotoxic effect of chemotherapy and, therefore, the CHOI or PERCIST criteria would appear to be more suitable for early

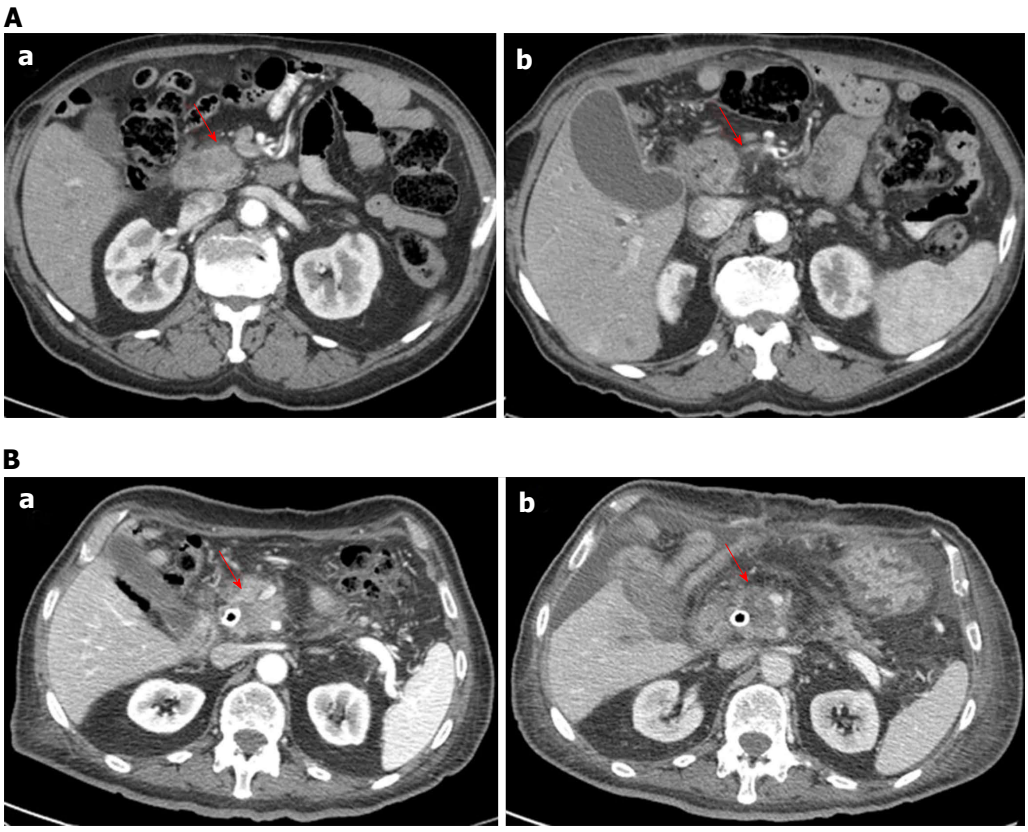

Figure 2 Computed tomography imaging assessment using morphological criteria for two patients (in A man 79 years old and in B man 74 years old). In pancreatic phase on CT study (a) the lesion appears hypodense (arrow). After the treatment in pancreatic phase on CT study (b) the lesion appears similar than in (a) but there was a significant variation in CT density value. CT: Computed tomography.

treatment evaluation[25,26,36,37]. In fact, according to our results, using Choi criteria (tissue density percentage change) 18/18 (100%) patients were considered in partial response. For PERCIST criteria 6/10 (60.0%) showed partial response, 3/10 (30.0%) stable disease and 1/10 (10.0%) progression disease. A good correlation was reported between ΔHU and ΔSUV_{max}. The accuracy of Choi criteria is known in the evaluation of target therapies[38]; during imatinib treatment therapy, Choi criteria have proved to be very useful to differentiate responders by non-responders and offer an potent prognostic indicator in terms of progression-free survival[36]. A recent study of van der Veldt *et al*[38] found that the Choi criteria may be helpful in assessing early metastatic renal cell carcinoma treated with sunitinib while Stacchiotti *et al*[39] showed that the Choi criteria were superior to the RECIST criteria to evaluate soft-tissue sarcoma response after chemotherapy and radiation therapy. Because many newer cancer therapies may be more cytostatic than cytocidal, appreciable tumor response may be associated to a decrease in metabolism, without a reduction in tumor size. Then, metabolic response can be a hopeful early indicator of tumor response and may be even more predictive of outcome than morphologic criteria[37]. So, the PERCIST criteria were proposed in 2009 to define and validate quantitative approaches to evaluating PET tumor response[26]. In a study on evaluation of response to chemotherapy in non-small cell lung cancer, PERCIST is more sensitive in detecting complete remission and progression, and these criteria might be the significant predictor of outcomes[40]. Avallone *et al*[34] demonstrated that, after preoperative radio-chemotherapy in locally advanced rectal cancer, FDG-PET is both an early predictor of pathologic response and a valuable prognostic tool. So, according to literature, on the value of the functional data obtained in CT and PET, compared with only morphological data in CT and MRI, to evaluate the response to ECT, it appears more appropriate to use Choi or PERCIST Criteria, although it would be better to link the two data[22-26,34-40]. García-Figueiras *et al*[24] demonstrated that standard imaging modalities and current morphologic response criteria do not always offer the adequate information for early therapy assessment, especially when target or ablated therapy were considered. According to García-Figueiras *et al*[24], MRI is able to predict treatment success before size changes become evident, thanks its capability to integrate anatomic, physiologic, and functional tissue information, which may overcome these limitations. In our study the morphologic information obtained by MRI examination did not show a dimensional change of treated pancreas lesion neither no statistically-

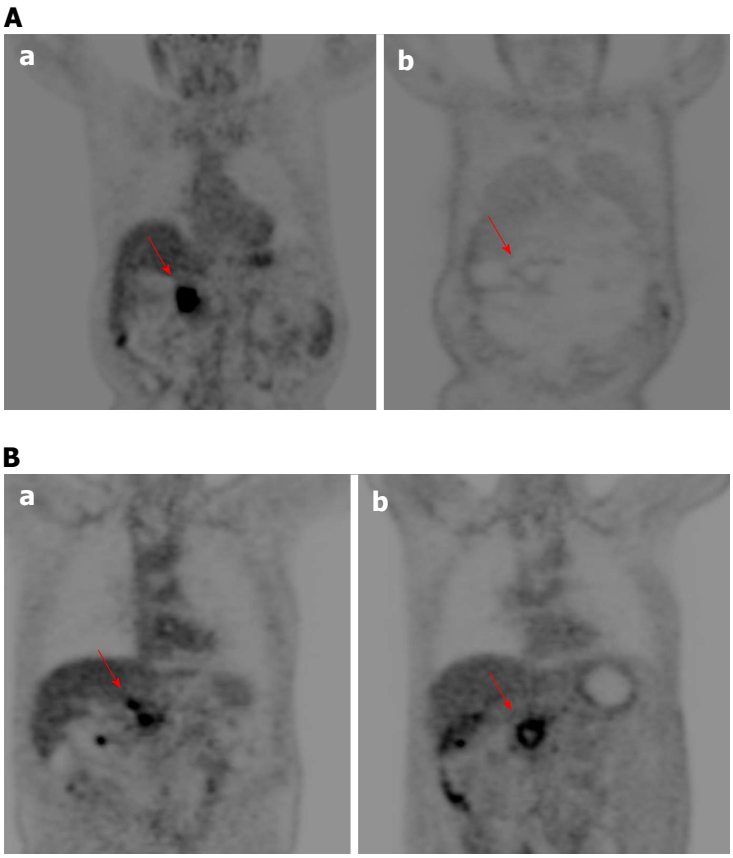

Figure 3 Positron emission tomography imaging assessment using morphological criteria for two patients (in A man 79 years old and in B man 74 years old). PET study before treatment (a) and after the treatment (b). In (b) the lesion (arrow) exhibited a reduction of glucose uptake. PET: Positron emission tomography.

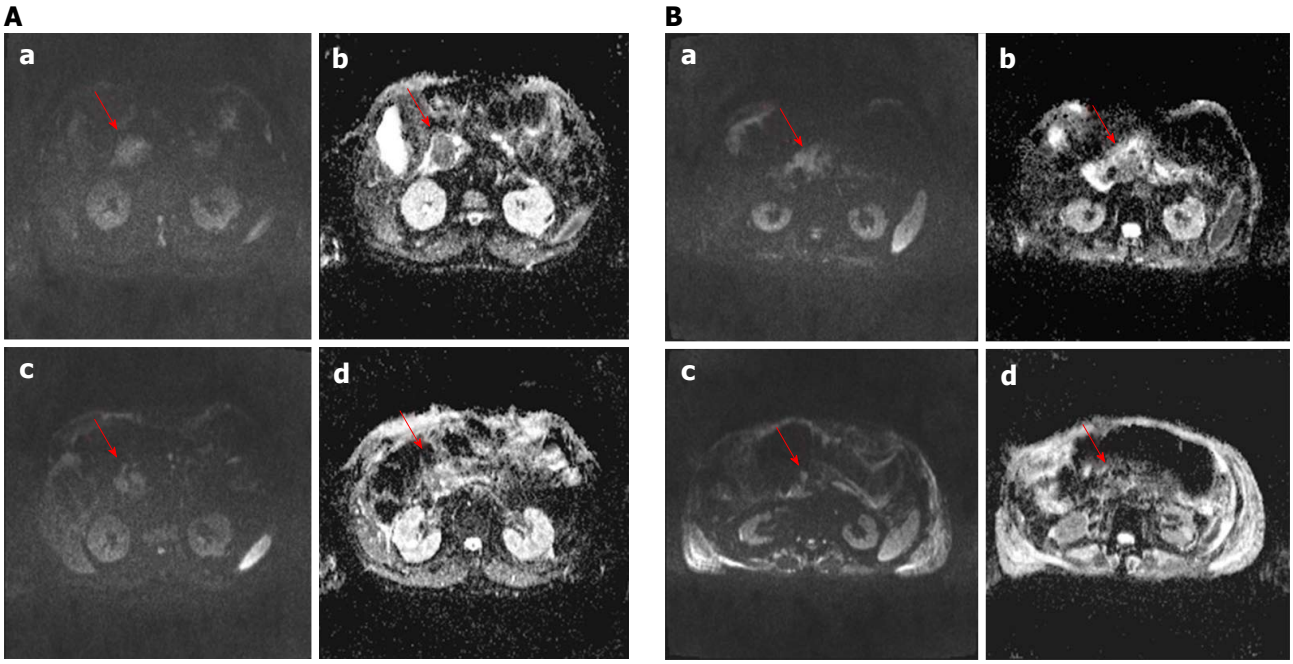

Figure 4 Diffusion weighted imaging assessment using morphological criteria for two patients (in A man 79 years old and in B man 74 years old). In a (image at *b* value 800), in b (ADC map) is showed the lesion before the treatment and in c (image at *b* value 800) and d (ADC map) is showed the lesion after the treatment; there was a difference in diffusion maps before and after treatment.

significant difference of signal intensity obtained from T1-weighted images, T2-weighted images and equilibrium-phase of contrast MR study. WIS, WOS, Dt and Dp values showed a significant reduction after

Table 5 Spearman correlation coefficient for each couple of imaging parameters

		ΔCT maximum diameter (%)	ΔHU (%)	ΔMR maximum diameter (%)	ΔWash-in (%)	ΔWash-out (%)	ΔDt (%)	Δfp (%)	ΔDp (%)	ΔSUVmax
ΔCT maximum diameter (%)	Correlation coefficient	1.000	-0.183	0.227	0.418	0.655[a]	-0.273	0.364	-0.036	-0.127
	P value		0.468	0.502	0.201	0.029	0.417	0.272	0.915	0.726
	n	18	18	11	11	11	11	11	11	10
ΔHU (%)	Correlation coefficient	-0.183	1.000	0.027	-0.373	-0.373	0.318	-0.291	0.064	0.758[a]
	P value	0.468		0.937	0.259	0.259	0.340	0.385	0.853	0.011
	n	18	18	11	11	11	11	11	11	10
ΔMR maximum diameter (%)	Correlation coefficient	0.227	0.027	1.000	-0.345	-0.082	-0.036	-0.409	-0.391	0.183
	P value	0.502	0.937		0.298	0.811	0.915	0.212	0.235	0.637
	n	11	11	11	11	11	11	11	11	9
ΔWash-in (%)	Correlation coefficient	0.418	-0.373	-0.345	1.000	0.527	-0.436	0.318	0.209	-0.150
	P value	0.201	0.259	0.298		0.096	0.180	0.340	0.537	0.700
	n	11	11	11	11	11	11	11	11	9
ΔWash-out (%)	Correlation coefficient	0.655[a]	-0.373	-0.082	0.527	1.000	-0.264	0.709[a]	0.291	-0.383
	P value	0.029	0.259	0.811	0.096		0.433	0.015	0.385	0.308
	n	11	11	11	11	11	11	11	11	9
ΔDt (%)	Correlation coefficient	-0.273	0.318	-0.036	-0.436	-0.264	1.000	0.118	-0.164	0.417
	P value	0.417	0.340	0.915	0.180	0.433		0.729	0.631	0.265
	n	11	11	11	11	11	11	11	11	9
Δfp (%)	Correlation coefficient	0.364	-0.291	-0.409	0.318	0.709[a]	0.118	1.000	0.545	-0.400
	P value	0.272	0.385	0.212	0.340	0.015	0.729		0.083	0.286
	n	11	11	11	11	11	11	11	11	9
ΔDp (%)	Correlation coefficient	-0.036	0.064	-0.391	0.209	0.291	-0.164	0.545	1.000	-0.167
	P value	0.915	0.853	0.235	0.537	0.385	0.631	0.083		0.668
	n	11	11	11	11	11	11	11	11	9
ΔSUVmax	Correlation coefficient	-0.127	0.758[a]	0.183	-0.150	-0.383	0.417	-0.400	-0.167	1.000
	P value	0.726	0.011	0.637	0.700	0.308	0.265	0.286	0.668	
	n	10	10	9	9	9	9	9	9	10

[a]$P < 0.05$ was considered statistically significant. CT: Computed tomography; HU: Hounsfield unit; MR: Magnetic resonance; WIS: Wash-in slope; WOS: Wash-out slope; Dt: Diffusivity; fp: Perfusion fraction; SUVmax: Maximum standardized uptake value.

ECT. Our results confirmed the data reported by Hjouj *et al*[41] that established as MRI might be used for brain electroporation treatment monitoring. Quantitative functional MR derived parameters such as WIS, WOS, Dt, Dp, fp, have allowed the identification of necrotic areas and of fibrotic tissue compared to any residual tumor[17] so as to overcome the limitations of RECIST 1.1. Considering the response evaluation accordance between at least 2 radiological modalities, 10 patients of our population, after one month from ECT, showed a significant reduction of viable tissue associated to a partial response, while two patients showed stable disease. We, also, demonstrated a good correlation between ΔHU and ΔSUV_{max} and between Δfp and ΔWOS, with a significant statistically difference ($P < 0.05$). Sakane *et al*[42] has demonstrated significant correlation between apparent diffusion coefficient and SUV in pancreatic cancer, and that leads us to think that in responder patients where the SUV is reduced significantly also Dt is reduced significantly as reported in our results.

The major limitations of this study are the small number of patients evaluated and the availability for all patients of the same diagnostic techniques, to compare all results obtained and to validate the potential, in term of efficacy, of perfusion and diffusion MR derived parameters, to differentiate responders by not responders after ECT with PET, CT and MR examination. The future goal is to increase the radiological data and to have a more homogeneous group in order to compare the results.

In conclusion, ECT is a promising technique for locally advanced pancreatic cancer, but there is still the issue of how to monitor the treatment response. Conventional morphologic data (RECIST criteria) obtained by CT or MR imaging were not able to differentiate partial, complete or incomplete response after ECT while the changes in functional parameters, obtained with PET (SUV_{max}), MR (wash-in and wash-out and for DCE and Dp, fp and Dt for DWI) and CT (tissue density) study

could be more suitable to assess ECT response.

COMMENTS

Background

Adenocarcinoma of pancreas is among the most aggressive forms of cancer. Surgical resection is the only potentially curative treatment for pancreatic cancer. Unfortunately, the majority of patients have grossly unresectable disease; over 80% of patients with pancreatic cancer have locally advanced or metastatic disease. Current standard therapy is chemotherapy and/or radiotherapy. The most frequently used chemotherapy agent in LAPC was Gemcitabine; moreover some studies have shown the combination of Gemcitabine with other chemotherapy agents increases overall survival. Because a limited group of patients responds to chemotherapy, additional therapies were explored in order to obtain tumor debulking or interstitial ablation.

Research frontiers

Reversible electroporation combined with low doses of chemotherapeutic drugs, known as electrochemotherapy (ECT), has been used to promote chemotherapy uptake into tumor cells reducing reducing its cytotoxic effect.

Innovations and breakthroughs

Currently, standard imaging modalities and response criteria do not always provide the adequate and necessary information to assess tumor ECT response. The innovation of the study is to report the early imaging assessment of treated area with ECT in locally advanced pancreatic cancer, emphasizing the role of new functional imaging tools in magnetic resonance imaging (MRI) compared to standard morphological response evaluation criteria in solid tumour (RECIST), Choi and positron emission tomography ERCIST criteria.

Applications

New functional imaging tools in MRI that allow diffusion and perfusion tissue assessment could be used for early ECT tumor response.

Terminology

ECT consists of the concomitant administration of low doses of chemotherapeutic drugs and reversible electroporation by means the delivering of an external electrical field to a cell membrane that induces a transient and reversible orientation of its polar molecules, with an increased permeability.

Peer-review

This paper report a single center experience of Imaging assessment in locally advanced pancreatic cancer treated with electrochemotherapy. The topic is interesting, the weakness of the manuscipt is limited by the small number of patients and heterogeneous data since not all patients underwent the same radiologic examinations

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ORIGINAL ARTICLE

Retrospective Study

Effect of initial stent position on patency of transjugular intrahepatic portosystemic shunt

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Author contributions: Luo SH and Chu JG designed the research; Luo SH and Huang H performed the research; Luo SH analyzed the data; Luo SH and Huang H wrote the paper; Yao KC revised the paper.

Institutional review board statement: This study was reviewed and approved by the Ethics Committee of Air Force General Hospital of PLA, Beijing, China.

Informed consent statement: Patients were not required to give informed consent because the analysis used anonymous clinical data that were obtained after each patient agreed to treatment by written consent.

Conflict-of-interest statement: The authors declare that there is no conflict of interest related to this study.

Data sharing statement: No additional data are available.

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Abstract

AIM

To evaluate the effect of initial stent position on transjugular intrahepatic portosystemic shunt (TIPS).

METHODS

We studied 425 patients from January 2004 to January 2015 with refractory ascites or variceal bleeding who required TIPS placement. Patients were randomly divided into group A (stent in hepatic vein, n = 57), group B (stent extended to junction of hepatic vein and inferior vena cava, n = 136), group C (stent in left branch of portal vein, n = 83) and group D (stent in main portal vein, n = 149). Primary unassisted patency was compared using Kaplan-Meier analysis, and incidence of recurrence of bleeding, ascites and hepatic encephalopathy (HE) were analyzed.

RESULTS

The mean primary unassisted patency rate in group B tended to be higher than in group A at 3, 6 and 12 mo (P = 0.001, 0.000 and 0.005), and in group D it tended to be lower than in group C at 3, 6 and 12 mo (P = 0.012, 0.000 and 0.028). The median shunt primary patency time for group A was shorter than for group B (5.2 mo *vs* 9.1 mo, 95%CI: 4.3-5.6, P = 0.013, log-rank test), while for group C it was longer than for group D (8.3 mo *vs* 6.9 mo, 95%CI: 6.3-7.6, P = 0.025, log-rank test). Recurrence of bleeding and ascites in group A was higher than in group B at 3 mo (P = 0.014

and 0.020), 6 mo (P = 0.014 and 0.019) and 12 mo (P = 0.024 and 0.034. Recurrence in group D was higher than in group C at 3 mo (P = 0.035 and 0.035), 6 mo (P = 0.038 and 0.022) and 12 mo (P = 0.017 and 0.009). The incidence of HE was not significantly different among any of the groups (P = 0.965).

CONCLUSION

The initial stent position can markedly affect stent patency, which potentially influences the risk of recurrent symptoms associated with shunt stenosis or occlusion.

Key words: Transjugular intrahepatic portosystemic shunt; Liver cirrhosis; Stent position; Portal hypertension

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Core tip: We studied a large cohort of patients with cirrhosis who underwent transjugular intrahepatic portosystemic shunt for recurrent variceal bleeding and ascites. Initial stent position at both the distal and proximal endpoints can markedly affect stent patency, which potentially influences the risk of recurrent symptoms associated with shunt stenosis or occlusion.

Luo SH, Chu JG, Huang H, Yao KC. Effect of initial stent position on patency of transjugular intrahepatic portosystemic shunt. *World J Gastroenterol* 2017; 23(26): 4779-4787 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i26/4779.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i26.4779

INTRODUCTION

Transjugular intrahepatic portosystemic shunt (TIPS) is currently used for the treatment of complications of portal hypertension[1]. The establishment of TIPS has been widely accepted as an alternative to surgery in the management of complications from portal hypertension, such as variceal bleeding, refractory ascites, Budd-Chiari syndrome, hepatorenal syndrome, hepatic hydrothorax and even hepatopulmonary syndrome[2]. After TIPS was introduced as an alternative treatment for complications related to portal hypertension, it has become progressively recognized as an effective therapeutic option in a growing number of clinical situations[3,4].

Despite its efficacy in preventing such syndromes, however, TIPS is prone to shunt stenosis or occlusion leading to shunt failure, and approximately half of all patients with TIPS require shunt revision during follow-up[5], making close surveillance and frequent costly revisions mandatory[6]. Recently, the use of a new generation of covered stents has overcome the problem of shunt dysfunction with significant improvement in TIPS patency and clinical efficacy[7]. Many experimental and clinical studies[8] have been performed with the use of covered stent grafts to improve the long-term patency of TIPS. The best results have been achieved with the use of stent grafts covered with polytetrafluoroethylene (PTFE), as reported by Nishimine *et al*[9]; and these positive results were confirmed by Haskal[10] and Jung *et al*[11]. Even with these new stents, however, post-TIPS shunt obstruction and a high clinical symptom recurrence rate remain problematic.

The purpose of this study was to evaluate the effect of initial stent position on primary shunt patency and time to recurrence of TIPS-related symptoms of ascites or variceal bleeding.

MATERIALS AND METHODS

Patient information

We retrospectively enrolled 1950 patients referred to us on an intention-to-treat basis who underwent a TIPS procedure at our institution between January 2004 and January 2015. The Ethic Institutional Review Board Committee approved the study protocol and all patients agreed to treatment by written consent. We reviewed the medical records and medical images for 436 patients to gather information regarding the underlying etiology, clinical presentation, age, sex, and severity of cirrhosis. Four hundred and twenty-five patients successfully underwent TIPS, and the demographic data were compared between the groups. Age, sex, etiology and Child-Pugh score are shown in Table 1, and there were no significant differences among the groups.

Study design

This study was a randomized, single-center, open-label trial that compared the effect of primary stent position on primary shunt patency at different stent ends, leading to different clinical manifestations. The patients were randomly divided into four groups: A (stent in hepatic vein, n = 57), B [stent extended to junction of hepatic vein and inferior vena cava (IVC), n = 136], C (stent in left branch of portal vein, n = 83) and D (stent in main portal vein, n = 149), according to the initial stent position in the distal inflow and proximal outflow ends. The inclusion criteria were portal-hypertension-related complications of recurrent variceal bleeding (n = 309) after a session of variceal sclerotherapy or refractory ascites (n = 116) or both (n = 78) that required TIPS placement. The exclusion criteria were as follows: variceal bleeding as an emergency indication, portal vein thrombosis, present history of hepatic encephalopathy (HE), severe right-sided heart failure, severe liver failure (bilirubin > 4 mg/dL), polycystic liver disease, dilated biliary ducts, age > 75 years, bilirubin level > 5 mg/dL, creatinine level > 3 mg/dL, Child-Pugh score > 11, hepatic carcinoma, sepsis, spontaneous bacterial peritonitis, and patients who underwent liver transplantation.

Table 1 Demographic characteristics of the patients

Parameter	Group				*P* value
	A	B	C	D	
n	57	136	83	149	
Male/female	24/33	64/72	42/41	70/79	0.806
Age in yr	39.35 ± 19.56	36.97 ± 15.79	34.33 ± 12.35	37.69 ± 16.69	0.959
Etiology, viral/not viral	52/5	125/11	78/5	136/13	0.806
Child-Pugh class, A/B/C/D	6/44/7	18/99/19	8/62/13	16/109/24	0.968

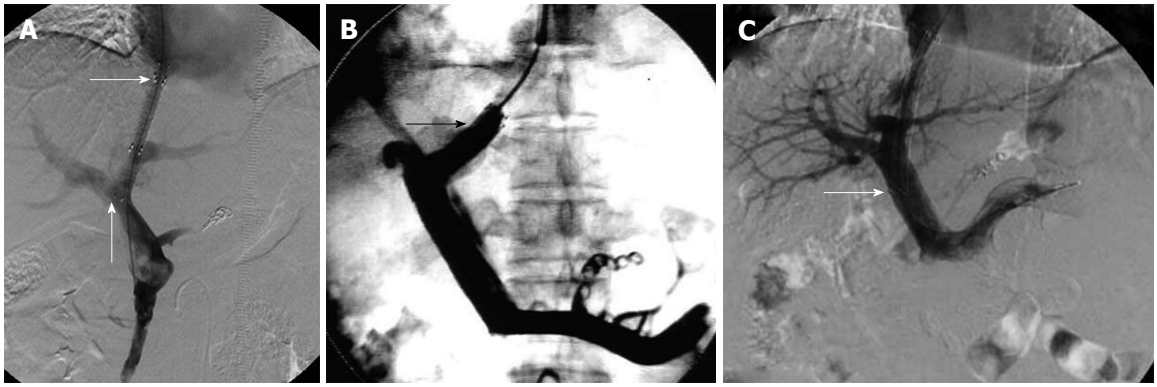

Figure 1 Stent was positioned as described in the study design for each group. A: Ideal position of the stent endpoint in the TIPS procedure. The transverse arrow indicates the proximal end of the stent at the junction of the hepatic vein and IVC. No angle was formed between the stent and left branch of the portal vein. The proximal end of the stent was located at the junction of the hepatic vein and IVC and did not cause stenosis or occlusion, and did not affect liver transplantation. The arrow pointing up indicates the distal end of the stent located at the left branch of the portal vein. The stent was in parallel to the direction of blood flow, therefore, it was not easy to cause stent stenosis or occlusion; B: The proximal end of the stent in the hepatic vein in the TIPS procedure. The arrow indicates the proximal end of the stent in the hepatic vein. The stent endpoint caused stenosis or occlusion due to blood flow shear force and vascular intimal hyperplasia; C: Distal end of the stent in the main hepatic vein in the TIPS procedure. The arrow indicates the distal end of the stent in the main hepatic vein in the transjugular intrahepatic portosystemic shunt procedure. The distal end of the stent was prone to stimulate the blood vessel wall and interfered with the portal vein blood flow. The stent endpoint caused stenosis or occlusion due to blood flow shear force and vascular intimal hyperplasia. TIPS: Transjugular intrahepatic portosystemic shunt; IVC: Inferior vena cava.

TIPS procedure

TIPS was carried out under standard local anesthesia. All of the patients were evaluated and followed up by the same medical team using a prospective protocol diagnostic work-up and surveillance strategy. Before the operation, the patients' medical histories were taken, and after TIPS, the four groups were followed up according to the same protocol.

TIPS was performed through a transjugular approach, as described previously[12]. After mesenteric artery angiography was performed, the right hepatic vein was reached using a TIPS set (RUPS-100; Cook Inc., Bloomington, IL, United States), and the left or right branch of the portal vein was punctured under the guidance of digital subtraction angiography in both the posterior anterior and lateral positions. When the branch of the portal vein was punctured and confirmed by portoangiography, a 7-8-mm balloon (Cook Inc.) dilated the hepatic tract. A 7-8-mm covered stent (Fluency; Bard, Voisins le Bretonneux, France) was used for TIPS creation and two bare 10-mm stents were used inside the Bard stent. Portosystemic gradient (PSG) and right atrial pressure were measured before and after TIPS.

The entire length of the intrahepatic tract should be covered by the stent graft. The stent was positioned as described in the study design for each group (Figure 1).

The shunts were dilated to full nominal diameter to reach a target PSG of < 12 mmHg and gastroesophageal collateral vessels observed during the TIPS procedure were embolized with coils (Cook Inc.). A covered stent (Viatorr; W.L. Gore and Associates, Flagstaff, AZ, United States) was not used because they were not approved by the State Food and Drug Administration in the Chinese Mainland for the patients included in this study. Subsequent direct portography was performed to evaluate whether the portal venous system was completely patent. After the TIPS procedure, intravenous heparin (4000 U/d; Chase Sun Pharma Co. Ltd, Tianjing, China) was given for 3 d and then oral warfarin (2.5 mg/d; Orion Pharma Co. Ltd., Orionintie, Finland) was prescribed to achieve 2 of international normalized ratio.

Follow-up

Baseline duplex sonography was performed on the day after TIPS creation. Subsequent shunt velocities were compared to this baseline result during follow-up. After TIPS, patients were placed into a routine follow-up protocol identical for each group. They were seen as outpatients 1 mo after the procedure and then every 3 mo, or whenever needed. Each consultation included a

Table 2 Mean pressure of the right atrium and portosystemic gradient value before and after transjugular intrahepatic portosystemic shunt placement

Group	Pressure of the right atrium in mmHg		*P* value	PSG in mmHg		*P* value
	Before TIPS	After TIPS		Before TIPS	After TIPS	
A	2.81 ± 1.58	2.98 ± 1.11	0.335	31.08 ± 8.11	13.81 ± 4.50	0.014
B	2.87 ± 1.58	3.01 ± 1.11	0.235	33.73 ± 7.77	15.00 ± 4.32	0.009
C	2.79 ± 1.45	3.03 ± 1.03	0.149	32.69 ± 7.55	14.57 ± 4.12	0.015
D	2.80 ± 1.44	2.95 ± 1.04	0.101	32.65 ± 7.26	14.34 ± 3.84	0.012

TIPS: Transjugular intrahepatic portosystemic shunt; PSG: Portosystemic gradient.

clinical examination, blood chemistry, and assessment of HE. Ultrasonography was performed at 1 and 4 wk after TIPS and then at 3 and 6 mo, and at 6-mo intervals thereafter, or in case of recurrent bleeding or ascites.

The primary endpoint of the study was primary unassisted patency, which was determined from the review of interventional radiology clinic records. Primary unassisted patency rate, the first stenosis or occlusion time was compared.

Shunt dysfunction that needed shunt revision during TIPS venography, or significant recurrent symptoms were used as endpoints for the loss of primary unassisted patency. TIPS angiography was performed in patients with recurrent symptoms of suspected shunt dysfunction. TIPS revision was performed when a hemodynamically significant shunt stenosis (> 50%) was present with recurrent variceal bleeding, recurrent or gradually worsening ascites, and PSG ≥ 15 mmHg unless grade Ⅲ/Ⅳ encephalopathy (West Haven Criteria) was present. Patients lost to follow-up were censored at the time of the last known imaging of the shunt (duplex ultrasonography or shunt venography).

Statistical analysis

Results are expressed as mean ± SD. Primary patency and the first stenosis or occlusion time were calculated using the Kaplan-Meier method, and the resultant curves were compared by means of the log-rank test. Logistic regression analysis was then performed for the variables. The differences between the groups were compared using one-way analysis of variance followed by least significant difference *t* tests. Differences were considered significant at $P < 0.05$. The statistical analyses were performed with SPSS version 20.0 (SPSS, Chicago, IL, United States).

RESULTS

We created a shunt between the hepatic vein, or the IVC and the portal vein, with successful deployment of the stent graft, and no patients had stents extending into the right atrium at the time of TIPS procedure. Among 436 patients, 425 (97.5%) had technically successful TIPS, and no patient died within 30 d after TIPS, with an early survival rate of 100%.

Before TIPS placement, the mean right atrial pressure in the four groups was 2.81 ± 1.58 mmHg in group A, 2.87 ± 1.58 mmHg in group B, 2.79 ± 1.45 mmHg in group C and 2.80 ± 1.44 mmHg in group D. After TIPS placement, the mean right atrial pressure was 2.98 ± 1.11 mmHg in group A ($P = 0.335$), 3.01 ± 1.11 mmHg in group B ($P = 0.235$), 3.03 ± 1.03 mmHg in group C ($P = 0.149$) and 2.95 ± 1.04 mmHg in group D ($P = 0.101$). There were no significant differences before and after TIPS placement ($P > 0.05$). After TIPS placement, the mean PSG value decreased from 31.08 ± 8.11 to 13.81 ± 4.50 mmHg in group A ($P = 0.014$), 33.73 ± 7.77 to 15.0 ± 4.32 mmHg in group B ($P = 0.009$), 32.69 ± 7.55 to 14.57 ± 4.12 mmHg in group C ($P = 0.015$) and 32.65 ± 7.26 to 14.34 ± 3.84 mmHg in group D ($P = 0.012$). There were significant differences before and after TIPS placement ($P < 0.05$) (Table 2).

In group A (Table 3), 74 patients showed stent stenosis or occlusion of the outflow endpoint *via* venography. Forty-four patients showed recurrent variceal bleeding, 43 showed ascites, and 10 showed both recurrent variceal bleeding and ascites. Of all the patients who showed stent dysfunction, 51 patients underwent balloon dilation, and in 23 the stent was replaced and extended to the IVC. Nine patients manifested HE: seven were grade Ⅰ and two were grade Ⅱ. After drug treatment, the symptoms disappeared in patients with grade Ⅰ or Ⅱ HE.

In group B, 53 patients showed stent stenosis or occlusion of the outflow endpoint *via* venography. A total of 29 patients showed recurrent variceal bleeding, 24 showed ascites, and 19 showed both variceal bleeding and ascites. Of all the patients who showed stent dysfunction, 41 underwent balloon dilation, and in 12 the stent was replaced and extended to the IVC. Twenty-two patients had HE: 13 were grade I, 6 were grade Ⅱ, and 3 were grade Ⅲ. After drug treatment, the symptoms disappeared in patients with grade Ⅰ or Ⅱ HE, and in patients with grade Ⅲ HE, the symptoms disappeared after implantation of shunt-reducing stents.

In group C, 50 patients showed stent stenosis or occlusion *via* venography, 24 showed recurrent variceal bleeding, 27 showed ascites, and 9 showed both variceal bleeding and ascites. Among the patients who showed stent dysfunction, 38 underwent balloon dilation and 12 underwent stent replacement. Thirteen

Table 3 Clinical characteristics of the patients

Characteristic		Group A	Group B	*P* value	Group C	Group D	*P* value
Unassisted	3 mo	75.4%	92.6%	0.001	88.0%	73.8%	0.012
	6 mo	57.9%	89.2%	0.000	86.0%	66.4%	0.000
Patency rate	12 mo	54.4%	75.0%	0.005	74.7%	60.4%	0.028
Median primary patency in mo		5.2	9.1	0.013	8.3	6.9	0.025
Mean primary patency		4.98	15.01	0.006	13.28	8.20	0.009
Recurrence	3 mo	15.8%	5.1%	0.014	6.0%	15.4	0.035
of bleeding in mo	6 mo	28.1%	13.2%	0.014	9.6%	20.1%	0.038
	12 mo	33.3%	18.4%	0.024	13.3%	26.8%	0.017
Median recurrent bleeding in mo		5.2	7.4	0.016	13.61	7.47	0.011
Mean recurrent bleeding in mo		4.21	6.93	0.023	8.7	6.3	0.018
Recurrence	3 mo	17.5%	6.6%	0.020	5.9%	16.4%	0.035
of ascites in mo	6 mo	22.8%	10.3%	0.019	10.5%	25.5%	0.022
	12 mo	35.1%	20.6%	0.034	19.3%	35.6%	0.009
Mean recurrent ascites in mo		6.11	11.45	0.011	14.26	7.19	0.005
Median recurrent ascites in mo		5.9	10.4	0.007	9.1	6.8	0.009
Stent dysfunction times		74	53	0.037	50	117	0.021
Hepatic encephalopathy cases		9	22		13	22	0.965

patients had HE: 8 were grade Ⅰ and 5 were grade Ⅱ. After drug treatment, the symptoms disappeared in patients with grade Ⅰ or Ⅱ HE.

In group D, 117 patients showed stent stenosis or occlusion *via* venography, 93 showed recurrent variceal bleeding, 114 showed ascites, and 30 showed both variceal bleeding and ascites. Among the patients who showed stent dysfunction, 89 underwent balloon dilation and 28 underwent stent replacement. Twenty-two patients had HE: 14 were grade Ⅰ, 6 were grade Ⅱ, and 2 were grade Ⅲ. After drug treatment, the symptoms disappeared in patients with grade Ⅰ or Ⅱ HE, and in patients of grade Ⅲ HE, the symptoms disappeared after implantation of shunt-reducing stents. There was a significant difference in stent dysfunction times between groups C and D ($P = 0.021$).

The unassisted patency rates for groups A and B were 75.4% *vs* 92.6% (3 mo, $P = 0.001$), 57.9% *vs* 89.2% (6 mo, $P = 0.000$), and 54.4% *vs* 75.0% (12 mo, $P = 0.005$), respectively, and these differences were significant ($P < 0.05$). The primary unassisted patency rates of groups C and D were 88.0% *vs* 73.8% (3 mo, $P = 0.012$), 86.0% *vs* 66.4% (6 mo, $P = 0.000$), and 74.7% *vs* 60.4% (12 mo, $P = 0.028$), respectively, and these differences were significant ($P < 0.05$).

As for the stent stenosis or occlusion time, in group A, the first symptoms were seen at 3.6 and 6.7 mo later, but the first symptoms were 5.4 and 7.4 mo later in group B. The mean shunt primary patency time was 4.98 mo in group A and 15.01 mo in group B ($P = 0.006$). The median shunt primary patency time was 5.2 mo in group A and 9.1 mo in group B (95%CI: 4.3-5.6) ($P = 0.013$, log-rank test). There was a significant difference in stent dysfunction times between groups A and B ($P = 0.037$). As for the stent stenosis or occlusion time, in group C, the first symptoms were seen at 3.6 and 6.7 mo later, but the first symptoms were seen at 5.4 and 7.4 mo later in group D. The mean shunt primary patency time was 13.28 mo in group C and 8.20 mo in group D ($P = 0.009$). The median shunt primary patency time was 8.3 mo in group C and 6.9 mo in group D (95%CI: 6.3-7.6) ($P = 0.025$, log-rank test) (Figure 2A).

Recurrent bleeding and ascites in group A were higher than in group B at 3 mo (15.8% *vs* 5.1%, $P = 0.014$; 17.5% *vs* 6.6%, $P = 0.020$), 6 mo (28.1% *vs* 13.2%, $P = 0.014$; 22.8% *vs* 10.3%, $P = 0.019$), and 12 mo (33.3% *vs* 18.4%, $P = 0.024$; 35.1% *vs* 20.6%, $P = 0.034$). The mean time to recurrent bleeding time was 4.21 mo in group A and 6.93 mo in group B ($P = 0.023$). The median time to recurrent bleeding was 5.2 mo in group A and 7.4 mo in group B (95%CI: 3.2-8.5) ($P = 0.016$, log-rank test). The mean time to recurrence of ascites was 6.11 mo in group A and 11.45 mo in group B ($P = 0.011$). The median time to recurrence of ascites was 5.9 mo in group A and 10.4 mo in group B (95%CI: 6.5-9.2) ($P = 0.007$, log-rank test) (Figure 2B and 2C).

The recurrence of bleeding and ascites in group D were higher than in group C at 3 mo (15.4% *vs* 6.0%, $P = 0.035$; 16.4% *vs* 5.9%, $P = 0.035$), 6 mo (20.1% *vs* 9.6%, $P = 0.038$; 25.5% *vs* 10.5%, $P = 0.022$), and 12 mo (26.8% *vs* 13.3%, $P = 0.017$; 35.6% *vs* 19.3%, $P = 0.009$). The mean time to recurrent bleeding was 13.61 mo in group C and 7.47 mo in group D ($P = 0.018$). The median time to recurrent bleeding was 8.7 mo in group C and 6.3 mo in group D (95%CI: 3.2-8.5) ($P = 0.011$, log-rank test). The mean time to recurrence of ascites was 14.26 mo in group C and 7.19 mo in group D ($P = 0.005$). The median time to recurrence of ascites was 9.1 mo in group C and 6.8 mo in group D (95%CI: 6.5-9.2) ($P = 0.009$, log-rank test).

In all the patients among the four groups, the incidence of HE did not differ significantly ($P = 0.965$).

DISCUSSION

It was believed previously that the shear force

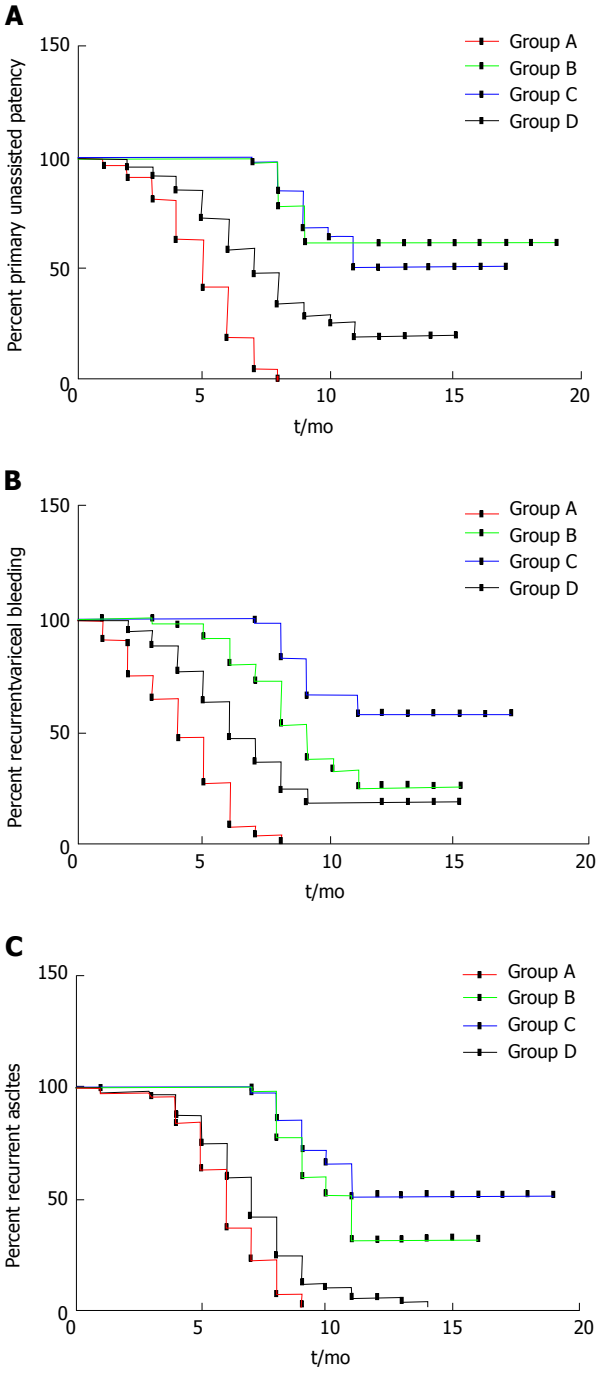

Figure 2 Primary unassisted patency (A), recurrent variceal bleeding (B) and recurrent ascites (C) in four groups of patients with transjugular intrahepatic portosystemic shunt. A: The median shunt primary patency time was 5.2 mo in group A and 9.1 mo in group B (95%CI: 4.3-5.6, *P* = 0.013, log-rank test). The median shunt primary patency time was 8.3 mo in group C and 6.9 mo in group D (95%CI: 6.3-7.6, *P* = 0.025, log-rank test); B: The median time to recurrence of bleeding was 5.2 mo in group A and 7.4 mo in group B (95%CI: 3.2-8.5, *P* = 0.016, log-rank test). The median time to recurrence of bleeding was 8.7 mo in group C and 6.3 mo in group D (95%CI: 3.2-8.5, *P* = 0.011, log-rank test); C: The median time to recurrence of ascites was 5.9 mo in group A and 10.4 mo in group B (95%CI: 6.5-9.2, *P* = 0.007, log-rank test). The median time to recurrence of ascites was 9.1 mo in group C and 6.8 mo in group D (95%CI: 6.5-9.2, *P* = 0.009, log-rank test).

of blood flow at the end of the stent, and fibrotic healing response to the injury of shunt creation leads to parenchymal stenosis, resulted in stenosis and occlusion due to the pseudointimal hyperplasia of the shunt end[13]. A previous study has suggested that the end of the stent positioned in the hepatic vein within 2 cm of the junction of hepatic vein and IVC improves the primary patency of TIPS[14] when deployed with bare metallic stent. The other factors of tract angle influence the primary patency of the TIPS[12], such as portal vein to the parenchymal tract and hepatic vein to the parenchymal tract.

Andring *et al*[15] have suggested that whether the end of the stent position in relation to the hepatic vein IVC confluence or other factors of tract angle, such as portal vein to parenchymal tract and hepatic vein to parenchymal tract, have little effect on the primary patency rate, which leads to recurrence of symptoms and related mortality following TIPS.

PTFE-covered stent grafts increase the patency of the stent for the TIPS procedure[16,17]. However, it is reported that TIPS stent dysfunction and related complications remain problems that disturb the preferred clinical outcomes[18,19]. It is recommended that the outflow of the PTFE-covered stent is connected to the junction of the hepatic vein and IVC, and the inflow to the main portal vein[10,20].

The standard of position of the stent graft in the hepatic vein in TIPS creation is based on the study by Clark *et al*[21], in which the bare-metal stents used led to the suggestion. They suggested that the hepatic venous end of the bare metal stent was positioned within 2 cm of the junction of the hepatic vein and IVC was superior primary patency during TIPS creation.

The dilemma of initial stent position during TIPS placement can have several clinical implications[22]. Andring *et al*[15] have shown that the hepatic venous end of TIPS stent graft position in relation to the hepatic vein and IVC junction has little effect on the primary patency rate following TIPS. Similarly, other factors such as whether access to the portal vein of the stent involved the inflow end also has no significant effect on primary patency, which leads to recurrence of symptoms and TIPS-related mortality. Others believe that the initial stent position within the outflow end of the TIPS stent graft is an important determinant of primary shunt patency, and have suggested that adequate stent coverage of the hepatic venous outflow affects stent patency[23,24]. For patients in whom the caudal end of the stent was not parallel to the vascular wall of the portal vein, chronic injury to the portal vein intima caused by the end of the stent graft can be responsible for the stenosis or occlusion of the portal vein[14].

Our center has been engaged in TIPS treatment since 1993, from the outset of using bare stents to stent grafts after 2004. Placement of the stent in the left branch of the portal vein decreases the risk of HE, and highly angulated and/or tortuous parenchymal tracts, affects shunt patency by creating areas of altered shear stress with potentially accelerated pseudointimal hyperplasia[25,26]. During the TIPS

procedure, we punctured as far as possible to the left branch of the portal vein and the stent was straight, avoiding the sheer force of the blood flow caused by the stent. In our study, all 425 patients had the left branch of the portal vein punctured, in an attempt to minimize the occurrence of HE.

In this study, we investigated the problem of initial stent position at the time of TIPS creation and predicted stent patency. As reported previously[27], we did not compare long-term outcomes among the four groups because patients who were later found to have TIPS shunt terminating in the hepatic vein or main portal vein underwent TIPS revision with placement of an additional stent to extend the outflow to the IVC, and/or dilated balloon.

As seen in our study, the primary unassisted patency rate in group B tended to be higher than in group A, and the median unassisted patency time was shorter in group A than in group B. We confirmed that the initial end of the stent position within the outflow of the TIPS shunt is an important determinant of shunt patency. The stenosis or occlusion sites in the cases with shunt dysfunction correlated well with their initial stent position, and we suggest that an adequate stent should be extended to the junction of the hepatic vein and IVC.

It is believed that, in patients who are potential liver transplantation candidates, the outflow position of the initial stent must be chosen with care as to avoid where it will interfere with placement of the suprahepatic clamp[28,29]. However, for orthotopic liver transplantation, the lack of liver tissue attachment to the stent, which allows an easier stent-graft removal, and the need to cover the IVC does not seem to be a contraindication in patients awaiting liver transplantation[30]. For piggy-back liver transplantation, stent placement at the junction of the hepatic vein and IVC does not influence the suprahepatic clamp[31]. So, we suggest that an adequate stent should be extended to the junction of the hepatic vein and IVC, and it should not influence liver transplantation.

Acceptance of the PTFE-covered stent (Viatorr) in the Chinese marketplace means that it will be widely deployed in TIPS placement. The inflow endpoint of the stent is the main portal vein[32]. The bare part of the stent is stiff and may cause endothelial injuries, with the subsequent development of thrombosis. A modification of the uncovered portion of the stent graft would probably be necessary to avoid portal vein stenosis.

In our study, the primary unassisted patency rate in group C tended to be higher than in group D, and the median unassisted patency time was longer in group C than group D. Our experience was consistent with the hypothesis that the end of the stent leads to chronic injury to the portal vein intima that is responsible for portal vein stenosis or occlusion. The portal blood flow is remodeled by the inflow position after stent placement, which produces a vortex and turbulence, and the shear force and uneven flow cause endometrial damage, thrombosis, intimal hyperplasia and stenosis[12,33,34]. Thus, we suggest that if improvement is needed at the front end of the stent, one should not enter the main portal vein in order to reduce the possibility of stenosis or occlusion.

The incidence of HE reported in the literature varies widely[3]. However, in our study, HE occurred at the same rate at the first year after TIPS creation. We speculate that the prevalence of HE was equal in the inpatients treated with an 8-mm stent, and shunt dysfunction needed immediate revision during TIPS venography for stent patency. We recommend the use of 8-mm stent grafts in most patients.

Our study had some limitations. First, it was a retrospective, single-center study, although there was a wide range of patient populations. We now anticipate a multicenter study. Second, we have yet to apply the PTFE-covered stent (Viatorr) stent, which has not been used in this capacity, but it is expected that some suggestions will be provided based on previous experience. Third, the specification of balloon and stent was deficient, which may have resulted in errors.

In conclusion, the initial stent position within the outflow and inflow of the TIPS creation is an important determinant of shunt primary patency. We suggest that the initial stent position of the outflow should be extended to the junction of the hepatic vein and IVC, and the inflow to the left branch of the portal vein.

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COMMENTS

Background

Transjugular intrahepatic portosystemic shunt (TIPS) is currently used for the treatment of complications of portal hypertension. Despite its efficacy in preventing syndromes, TIPS is prone to shunt stenosis or occlusion leading to shunt failure, and about 50% of patients with TIPS require shunt revision, which makes close surveillance and frequent costly revisions mandatory. Even with new stents or stent grafts covered with polytetrafluoroethylene (PTFE), post-TIPS shunt obstruction and a high rate of symptom recurrence remain problems. The purpose of this study was to evaluate the effect of selected technical factors of the primary stent position on primary shunt patency, and time to recurrence of symptoms in TIPS with a stent graft to avoid the need for repeat interventions.

Research frontiers

Previous studies based on TIPS created with bare metallic stents have suggested that the positioning of the hepatic venous end of the stent within 2 cm of the hepatic vein/inferior vena cava (IVC) junction improves the primary patency of TIPS. It is recommended that the outflow of the PTFE-covered stent (Viatorr) is placed at the junction of the hepatic vein and IVC, and the inflow at the main portal vein. These results help to explain the inflow of the stent to the portal vein, outflow of the stent to the hepatic vein and IVC, by retrospective analysis of a large patient sample and long-term case review. The results of this

study also provide useful clinical suggestions.

Innovations and breakthroughs

In this study, the initial position of the stent inflow at the junction of the hepatic vein and IVC prolongs the median primary unassisted patency rate and reduces the incidence of recurrent bleeding and ascites. These results are in agreement with previous reports. However, in this study, the initial position of the stent outflow at the left branch of the portal vein also prolonged the median primary unassisted patency rate and reduced the incidence of recurrent bleeding and ascites. This emphasizes that the initial stent position within the outflow and inflow of the TIPS is an important determinant of shunt patency, and suggests that the initial stent position of the outflow should be extended to the junction of the hepatic vein and IVC, and the inflow to the left branch of the portal vein.

Applications

The initial stent position within the outflow and inflow of the TIPS is an important determinant of shunt patency. This study suggests that the initial stent position of the outflow should be extended to the junction of hepatic vein and IVC, and the inflow to the left branch of the portal vein.

Terminology

TIPS is currently used for the treatment of complications of portal hypertension by establishing a shunt between the intrahepatic portal vein and vena cava to relieve portal hypertension.

Peer-review

The author reported 425 patients with refractory ascites or variceal bleeding treated with TIPS placement. To date this size of cohort study have never been reported and is essential to be published. Their results demonstrated that the initial stent position influences stent patency, and the risk of recurrent symptoms associated with shunt stenosis or occlusion.

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ORIGINAL ARTICLE

Observational Study

Endoscopy is of low yield in the identification of gastrointestinal neoplasia in patients with dermatomyositis: A cross-sectional study

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Abstract

AIM

To determine the prevalence of gastrointestinal neoplasia among dermatomyositis patients who underwent an esophagogastroduodenoscopy and/or colonoscopy.

METHODS

A cross-sectional study examining the results of upper endoscopy and colonoscopy in adults with dermatomyositis at an urban, university hospital over

a ten year period was performed. Chart review was performed to confirm the diagnosis of dermatomyositis. Findings on endoscopy were collected and statistical analyses stratified by age and presence of symptoms were performed.

RESULTS

Among 373 adult patients identified through a code based search strategy, only 163 patients had dermatomyositis confirmed by chart review. Of the 47 patients who underwent upper endoscopy, two cases of Barrett's esophagus without dysplasia were identified and there were no cases of malignancy. Of the 67 patients who underwent colonoscopy, no cases of malignancy were identified and an adenoma was identified in 15% of cases. No significant differences were identified in the yield of endoscopy when stratified by age or presence of symptoms.

CONCLUSION

The yield of endoscopy is low in patients with dermatomyositis and is likely similar to the general population; we identified no cases of malignancy. A code based search strategy is inaccurate for the diagnosis of dermatomyositis, calling into question the results of prior population-based studies. Larger studies with rigorously validated search strategies are necessary to understand the risk of gastrointestinal malignancy in patients with dermatomyositis.

Key words: Endoscopy; Dermatomyositis; Colon cancer; Screening

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Core tip: Dermatomyositis is associated with an increased risk of gastrointestinal (GI) malignancies based on large-population based studies. These prior studies utilized code-based search strategies and did not perform individual chart review. The yield of endoscopy in this patient population is not known. In this study, endoscopy identified no cases of malignancy and was of low yield, likely similar to the general population, in the identification of pre-malignant findings. Code-based searched strategies were inaccurate in the identification of dermatomyositis, calling into question the results of prior population-based studies. The association between increased GI malignancy and dermatomyositis may be lower than previously reported.

Kidambi TD, Schmajuk G, Gross AJ, Ostroff JW, Terdiman JP, Lee JK. Endoscopy is of low yield in the identification of gastrointestinal neoplasia in patients with dermatomyositis: A cross-sectional study. *World J Gastroenterol* 2017; 23(26): 4788-4795 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i26/4788.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i26.4788

INTRODUCTION

Patients with dermatomyositis, a common idiopathic inflammatory myopathy characterized by muscle weakness and cutaneous findings affecting approximately 1 per 100000 people are generally considered to have an increased prevalence of gastrointestinal (GI) malignancy[1,2]. However, the reported prevalence of GI malignancy varies depending on the level of chart review or diagnostic codes used to verify the diagnoses[3-9]. Large population-based studies[3-5] utilizing diagnostic codes for identifying dermatomyositis patients have reported a prevalence of GI malignancy as high as five percent. Additionally, a study using extensive testing for malignancy in dermatomyositis patients reported a 15% prevalence of GI malignancy[6]. In contrast, small retrospective studies[7-9] utilizing individual level chart review have reported the prevalence of gastric or colorectal cancer (CRC) to be closer to one percent among dermatomyositis patients.

Currently, there are no guidelines that recommend an initial endoscopic workup for evaluation of an underlying GI malignancy in dermatomyositis patients. However, given the reported increased prevalence of GI neoplasia, many providers recommend an upper esophagogastroduodenoscopy (EGD) and colonoscopy as part of routine clinical care among dermatomyositis patients despite the lack of signs, symptoms, or laboratory abnormalities to suggest a GI source. To date, there are no data regarding the utility of this practice among dermatomyositis patients. Therefore, the aim of this study was to determine the prevalence of GI neoplasia among dermatomyositis patients who underwent an EGD and/or colonoscopy.

MATERIALS AND METHODS

Study population

This retrospective, cross-sectional study was conducted at the University of California, San Francisco (UCSF), an academic, tertiary care medical center serving over 1.2 million people in the San Francisco Bay Area. Patients with dermatomyositis were referred to either UCSF's rheumatology, dermatology, or gastroenterology clinics.

Study oversight

The study was approved by the UCSF institutional review board, which waived the requirement for informed consent. The listed authors had sole responsibility for the study design, data collection, decision to submit the manuscript for publication, and drafting of the manuscript.

Study eligibility criteria

We included all dermatomyositis patients 18 years of age and above seen at UCSF between January

2005-February 2016, who had an endoscopy (EGD and/or colonoscopy) at or after their dermatomyositis diagnosis. We selected this time period because of the availability of electronic health records. To identify patients with dermatomyositis seen at UCSF, we performed a comprehensive search of our electronic medical record using International Classification of Diseases (ICD)-9 and ICD-10 diagnoses codes (Supplementary Table 1). We also reviewed a separate query of the electronic medical record initiated by UCSF's rheumatology division, which utilized a combination of ICD-9 and ICD-10 codes as well as clinic modifiers used for research purposes by the rheumatology clinic, to identify any additional dermatomyositis patients that may have been missed by our diagnostic code search. After the initial search, two reviewers (TDK, JKL) manually reviewed each chart to confirm the diagnosis of dermatomyositis. We defined a dermatomyositis diagnosis as any patient seen in either rheumatology and/or dermatology clinic with a diagnosis of dermatomyositis made by histological and/or clinical criteria; patients with an overlap rheumatologic condition that included dermatomyositis were included. For any charts where the diagnosis of dermatomyositis was unclear or with discordant results by the two reviewers, a rheumatologist was used for adjudication.

Data sources and variables

We obtained patient demographic and clinical information for each dermatomyositis patient included in this study from our electronic medical records, which included clinic notes, consultation notes, endoscopy reports, pathology reports, radiology reports, and cancer diagnoses. Age and disease duration were defined as the age at the end of the study period or the difference in years between the patients' date of birth and the end of the study period (February 2016), respectively. For patients who underwent an endoscopy, data on age and disease duration at the time of endoscopy was collected. Findings on EGD were categorized as normal, pre-malignant, malignant, or non-malignant. Gastric and duodenal erosions with non-specific inflammation on histology were included as normal. Pre-malignant findings on EGD included Barrett's esophagus (with or without dysplasia), gastric intestinal metaplasia (with or without dysplasia) and duodenal adenoma. Malignant findings on EGD included carcinoma of the esophagus, stomach or duodenum. Non-malignant findings included erosive esophagitis, infectious esophagitis (such as candidiasis or cytomegalovirus infection) and helicobacter pylori infection, all of which were confirmed on histology, as well as esophago-gastric varices. If subsequent EGDs were performed after the initial endoscopy, the findings were collected and the highest risk finding was recorded.

Findings on colonoscopy were categorized as normal, pre-malignant, malignant, or non-malignant. Diverticulosis, distal hyperplastic polyps and non-specific findings on endoscopy with normal histology (such as "thickened folds" or "nodules") were included as normal. Pre-malignant findings included any colonic adenoma, proximal serrated lesions, and were further categorized as advanced adenomas or low-risk adenomas. We defined an advanced adenoma as any adenoma of any size with high grade dysplasia or a villous component or an adenoma greater than ten millimeters in size, low risk tubular adenomas, defined as less than three adenomas not meeting criteria for an advanced adenoma, or higher risk tubular adenomas, defined as three of more tubular adenomas found on a single colonoscopy. Malignancy was defined as carcinoma of the colon, including adenocarcinoma and neuroendocrine neoplasms. Non-malignant findings included inflammatory bowel disease and microscopic colitis. If subsequent colonoscopies were performed after the initial colonoscopy, the findings were collected and the highest risk finding was recorded.

Statistical analyses

The primary outcome of the study was the yield of endoscopy to identify GI neoplasia among dermatomyositis patients. We further stratified the findings on endoscopy by age and whether signs or symptoms were present at the time of endoscopy. Statistical analyses comparing differences amongst the patients who underwent endoscopy stratified by age or presence of symptoms was performed using χ^2 test and Fisher's exact test was used for categorical variables with less than five outcomes. Results were displayed as either mean ± SD) or the number of outcomes (percentage who underwent endoscopy). All analyses were performed using SPSS (IBM version 23) and a two-tailed P value of < 0.05 was considered statistically significant.

RESULTS

Identification of study cohort

A total of 458 patients were identified by querying the electronic medical records for ICD-9 and ICD-10 codes of dermatomyositis between January 2005 and February 2016. We did not identify any additional dermatomyositis patients after reviewing the rheumatology initiated query of the electronic medical record. After chart review of the 458 patients, 295 of these patients (64%) were excluded because they either had dermatomyositis incorrectly coded (n = 210, 46%) or they were younger than 18 years of age (n = 85, 18%). Thus, a total of 163 patients with dermatomyositis were included and, of these, 79 patients had an endoscopy documented within the electronic medical record (EGD and/or colonoscopy) as shown in Figure 1.

Patient characteristics

Baseline demographic data for the patients who

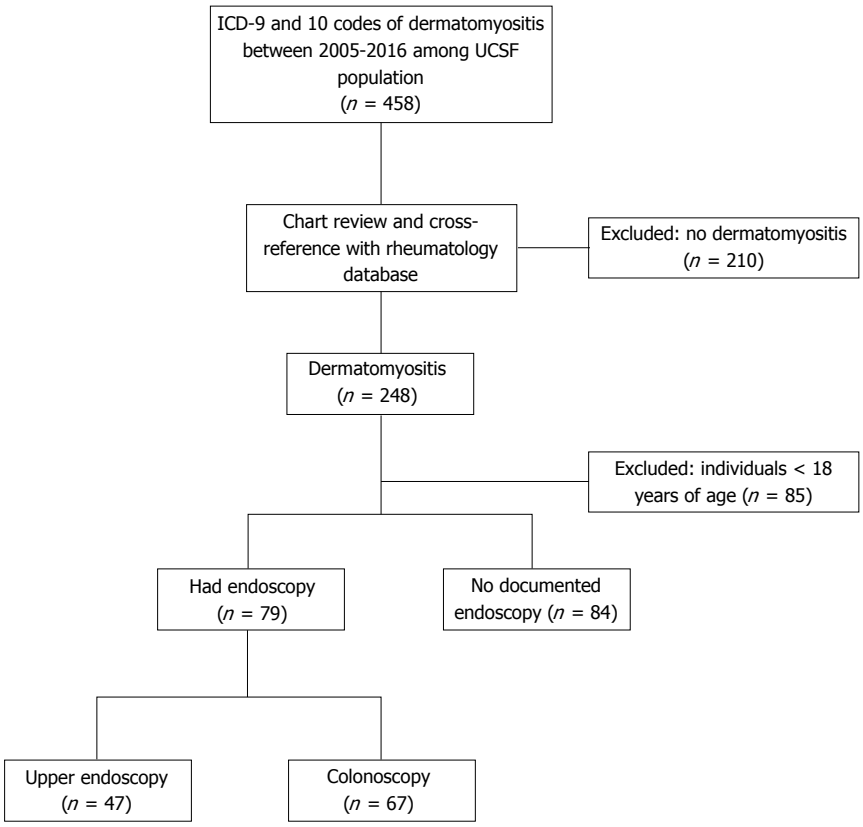

Figure 1 Attached as a powerpoint file.

underwent endoscopy is shown in Table 1. The mean age of patients at the time of endoscopy was 56.7 years with a range from 21 to 88 years and a SD of 14.4 years; 73% were women. On average, patients had dermatomyositis for 6.8 years at the time of endoscopy with a SD of 6.6 years; 39 patients (49%) had endoscopy within 5 years of their diagnosis. Of the two patients with an adenocarcinoma of unknown origin, one of these patients was diagnosed based on biopsy of a large lymph node found on cross sectional imaging while the other patient's diagnosis was made based on biopsy of a liver lesion, with genome sequencing of the tumor suggestive of a lung primary. The most common indication for EGD and colonoscopy was screening in an otherwise asymptomatic patient (40.4% and 73.1%, respectively); dysphagia was the most common symptom present at time of EGD (27.7%) and diarrhea and abdominal pain were the most common symptoms prompting colonoscopy (16.5%).

Chart review was performed on the patients with a diagnosis of dermatomyositis who did not undergo endoscopy and the results comparing demographic data for these patients compared to those who did undergo endoscopy is shown in Supplementary Table 2. Patients who underwent endoscopic evaluation were significantly older at the time of their dermatomyositis diagnosis than those who did not (50.1 ± 15.9 *vs* 40.6 ± 15.9, respectively, $P < 0.01$). No significant differences were identified in any of the other variables, including personal history of cancer. The single patient with CRC who did not undergo endoscopy was diagnosed with CRC 10 years prior to her dermatomyositis diagnosis and no records of the initial or subsequent surveillance colonoscopies were available in the electronic medical record.

Yield of endoscopy

Of the 47 patients who underwent EGD, no cases of malignancy were identified on either the initial exam, or if performed, on subsequent exams as shown in Table 2. EGD identified two cases of Barrett's esophagus without dysplasia and subsequent EGD at one year identified no progression of the Barrett's in either patients. In another patient, gastric intestinal metaplasia without dysplasia on random gastric biopsies was identified on EGD and the patient underwent a total of seven subsequent surveillance EGDs over ten years without progression of the gastric intestinal metaplasia.

There were no cases of malignancy detected in the 67 patients who underwent colonoscopy as shown in Table 2. Of the 12 patients who underwent surveillance exams, the majority underwent a single surveillance exam in the study period while one patient underwent three exams over a ten-year period for surveillance purposes because of a previous finding of an advanced adenoma (> 10 mm sessile serrated adenoma) and a family history of CRC. Seventy-nine percent of the colonoscopies were normal and at least one adenoma was identified on 14.9% of the initial colonoscopies,

Table 1 Baseline demographic and indications for endoscopy *n* (%)

Baseline characteristics	Had endoscopy (*n* = 79)
Age (mean ± SD)	56.7 (14.4)
Male gender	21 (27)
Age at dermatomyositis diagnosis (mean ± SD)	50.1 (15.9)
Disease duration (mean ± SD) at time of endoscopy	6.8 (6.6)
Personal history of cancer	8 (10.1)
Melanoma	1
Prostate	1
Endometrial	1
NCC	1
HCC	1
RCC	1
AdenoCA of unknown origin	2
Indication for EGD	Had EGD (*n* = 47)
Screening	19 (40.4)
Dysphagia	13 (27.7)
Dyspepsia/pain	9 (19.1)
IDA	2 (4.3)
Weight loss	3 (6.4)
Abnormal CT scan	1 (2.1)
Indication for colonoscopy	Had colonoscopy (*n* = 67)
Screening	49 (73.1)
Surveillance	2 (3.0)
Diarrhea, abdominal pain	11 (16.4)
IDA	2 (3.0)
Weight loss	1 (1.5)
Blood in stool	1 (1.5)
Abnormal CT	1 (1.5)

DM: Dermatomyositis; NCC: Nasal cell cancer; HCC: Hepatocellular carcinoma; RCC: Renal cell cancer; AdenoCa: Adenocarcinoma; EGD: Esophagogastroduodenoscopy; CT: Computed tomography.

with the majority being low risk tubular adenomas (9.0%).

Yield of endoscopy stratified by age and symptoms

The yield of endoscopy, stratified by age less than or greater than 50 years is shown in Table 3. We selected age 50 as the cutoff because many United States guidelines commonly recommend average-risk CRC screening at this age[10]. In addition, multiple gastroenterology societies recommend esophageal cancer or Barrett's esophagus screening for high-risk individuals (*i.e.*, long-standing gastro-esophageal reflux disease, Caucasian race, obesity, *etc.*) at age 50[11]. There were no statistical differences in the yield of EGD for pre-malignant conditions stratified by age.

The yield of endoscopy, stratified by the presence of symptoms is shown in Table 4. There were no identified differences in the yield of EGD when symptoms were present. Asymptomatic patients undergoing colonoscopy were more likely to have a normal exam than those with symptoms (86.3% *vs* 56.3%, respectively, $P = 0.01$), which was attributable to the finding of inflammatory bowel disease or microscopic colitis in symptomatic patients (25% *vs* 0% in the asymptomatic patients, $P < 0.01$).

Table 2 Yield of endoscopy *n* (%)

Findings on EGD, *n* = 47	
Malignancy	0 (0)
Pre-malignancy	3 (6.4)
Barrett's esophagus without dysplasia	2
Gastric intestinal metaplasia	1
Non-malignant findings	8 (17)
Esophageal varices	2
H. pylori gastritis	4
CMV esophagitis	1
Candida esophagitis	1
Normal	36 (76.6)
Findings on colonoscopy, *n* = 67	
Colorectal cancer	0 (0)
Any adenoma	10 (14.9)
Inflammatory bowel disease	4 (6.0)
Normal	53 (79.1)

EGD: Esophagogastroduodenoscopy.

DISCUSSION

Although screening for GI neoplasia is commonly recommended in patients with dermatomyositis, the yield of endoscopy remains unclear. To that end, we report results of the largest study of dermatomyositis patients utilizing individual chart-review and the first to examine the yield of endoscopy on identification of pre-malignant and malignant lesions of the GI tract in the United States. In our study of 79 physician-confirmed dermatomyositis patients, we found the yield of endoscopy to be low for the identification of pre-malignant and malignant GI lesions, even when stratified by age, in both asymptomatic and symptomatic patients, calling into question an aggressive diagnostic approach to identify occult malignancy. No cases of GI malignancy were identified; EGD was of very low yield in the identification of significant pathology and colonoscopy was only useful in identifying pre-malignant lesions (colonic adenomas) in the screening age population (patients 50 years and older).

To date, few studies have evaluated the prevalence of GI malignancy in dermatomyositis patients. Previous large, population-based studies have found an association between dermatomyositis and GI malignancies[3-5]. In addition, a single descriptive study[6] of 40 patients with dermatomyositis and polymositis at a French hospital over a 20 year period suggested that an extensive search for malignancy with CT scans of the chest, abdomen and pelvis increased the identification of malignancy, when compared to routine malignancy screening. In contrast, smaller studies[7-9] have found a low prevalence of GI neoplasia. These studies found gastric cancer in less than 3% of patients[8,9] and CRC in less than 2% of patients[7,8] with dermatomyositis. In our study, we found no GI malignancies after an extensive endoscopic evaluation. One potential explanation for these inconsistent findings could be the identification of dermatomyositis patients. Studies

Table 3 Yield of endoscopy stratified by age *n* (%)

	Age < 50 (*n* = 21)	Age ⩾ 50 (*n* = 26)	Age ⩾ 50 (*n* = 46)	*P* value
Findings on EGD				
Normal	16 (76.2)	20 (76.9)	-	0.95
Pre-malignancy	1 (4.8)	2 (7.7)	-	1.00
Malignancy	0 (0)	0 (0)	-	1.00
Non-malignant	4 (19.0)	4 (15.4)	-	1.00
Findings on Colonoscopy				
Normal	17 (81.0)	-	36 (78.3)	0.80
Any adenoma	1 (4.8)	-	9 (20.0)	0.15
Colorectal cancer	0 (0)	-	0 (0)	1.00
Inflammatory bowel disease	2 (9.5)	-	2 (4.3)	0.58

EGD: Esophagogastroduodenoscopy.

Table 4 Yield of endoscopy by presence of symptoms *n* (%)

	Asymptomatic		Symptomatic		*P* value
	n = 19	*n* = 51	*n* = 28	*n* = 16	
Findings on EGD					
Normal	15 (78.9)	-	21 (75.0)	-	0.75
Pre-malignancy	0 (0)	-	3 (10.7)	-	0.26
Barrett's without dysplasia	-	-	2 (7.1)	-	-
Gastric intestinal metaplasia without dysplasia	-	-	1 (3.6)	-	-
Malignancy	0 (0)	-	0 (0)	-	-
Non-malignant	4 (21.1)	-	4 (14.3)	-	0.70
Varices	2 (10.5)	-	-	-	-
Helicobacter pylori gastritis	2 (10.5)	-	2 (7.1)	-	-
Cytomegalovirus esophagitis	-	-	1 (3.6)	-	-
Candida esophagitis	-	-	1 (3.6)	-	-
Findings on Colonoscopy					
Normal	-	44 (86.3)	-	9 (56.3)	0.01
Pre-malignancy	-	7 (13.7)	-	3 (18.8)	0.69
Advanced adenoma	-	1 (2.0)	-	1 (6.3)	-
Low risk tubular adenoma	-	5 (9.8)	-	1 (6.3)	-
⩾ 3 tubular adenomas	-	1 (2.0)	-	1 (6.3)	-
Malignancy	-	0 (0)	-	0 (0)	-
Non-malignant findings	-	0 (0)	-	4 (25)	< 0.01
IBD	-	-	-	3 (18.8)	-
Microscopic colitis	-	-	-	1 (6.3)	-

EGD: Esophagogastroduodenoscopy; IBD: Inflammatory bowel disease.

utilizing ICD code based searches[3,5] and key-word search criteria[4] for the diagnosis of dermatomyositis and GI malignancy showed higher prevalence for GI malignancy compared to smaller studies[7-9], including ours, that utilized individual chart review to confirm the dermatomyositis diagnosis. In our study, we found ICD coding based diagnosis of dermatomyositis was inaccurate in 46% of the patients; this calls into question the results of these prior studies and suggests that rigorous validation is necessary for any future population-based dermatomyositis studies.

Common factors for deciding to pursue endoscopic or cross-sectional imaging workup in the general population include advancing age and symptoms. In our study, there were no significant differences in the yield of endoscopy for pre-malignant conditions stratified by age and symptoms. In the French cohort of 40 dermatomyositis and polymositis patients, six patients (15%) were diagnosed with a GI malignancy by endoscopy following an abnormal CT scan. The results of our study are inconsistent with this study, though only two patients in our study had an abnormal CT prior to endoscopy. The presence of concerning clinical symptoms (*i.e.*, constitutional symptoms, anemia, *etc.*) prompting further evaluation with CT scans in all 40 patients, likely accounts for the difference in results when compared to our study, which largely included asymptomatic patients undergoing screening endoscopy. Thus, we conclude red-flag symptoms in dermatomyositis patients, as in any patient, should prompt investigation for an underlying cause, but endoscopy is probably of similar yield in dermatomyositis patients as in age-matched patients without dermatomyositis. Unfortunately, given the retrospective nature of the study we were unable to control for all potential confounders including the presence of Epstein-Barr virus which in a prior study was shown to be associated with gastric cancer risk[12].

Our study had many strengths. Because the data were generated from routine clinical care, the findings are likely to be accurate and generalizable to other clinical centers caring for patients with dermatomyositis. A particular strength of our data is that individual chart review was conducted to verify the diagnosis of dermatomyositis as well as the findings on endoscopy and pathologic diagnoses. To our knowledge this is the largest sample size of dermatomyositis patients in a study utilizing chart review and therefore is a valuable addition to the literature. As this is the only study examining the yield of endoscopy in both symptomatic and asymptomatic dermatomyositis patients, the results are likely to help inform decisions in the routine clinical care of patients.

There were several limitations to our study. Because the study was conducted in a single tertiary care center, there was loss to follow-up and it is possible that, despite reviewing the electronic medical record including uploaded records from care received outside of UCSF, endoscopies performed elsewhere were not captured and as a result cases of cancer were missed. Furthermore, the age of patients in our study was relatively young which may have reduced the likelihood of identifying malignancy, as older age has been shown to be a risk factor for neoplasia in this patient population[13]. However, this supports our finding that the risk of neoplasia is likely similar to the general, age-matched population and screening outside of the guideline recommendations may not be needed where an adenoma detection rate of 20%-30% is expected in the United States. Additionally, a large number of patients with dermatomyositis did not undergo endoscopy so cases of cancer could have been missed. However, this was mitigated by the fact that the entire medical record was reviewed, since a cancer diagnosis likely would have been mentioned in clinic notes or within the problem lists. Furthermore, because there is wide variation in practice it is possible that patients who underwent endoscopy may have been perceived by their physicians to be at higher risk for malignancy but this would have biased our study towards an overestimate of the prevalence of malignancy, which is less of a concern given the negative findings of our study. While this was the largest study of its kind, the power to identify differences in endoscopy yield stratified by age was low. For example, to detect a 5% difference in the prevalence of colonic adenomas assuming a prevalence 25% would require over 1000 patients 50 years and older and another 1000 patients younger than 50. Given dermatomyositis is a rare disease, an adequately powered study was not feasible. Nevertheless, the absolute values and findings in our study are still meaningful, interpretable and may be more applicable to the clinician caring for dermatomyositis than the previous larger population based studies that did not utilize individual level chart review.

In conclusion, this study suggests that the yield of endoscopy in dermatomyositis for the identification of pre-malignant and malignant GI lesions is low in the United States. In fact, our study identified no cases of upper GI or colon cancers. Moreover, even when stratified by age or the presence of symptoms, endoscopy was of low yield, raising the question of whether routine use of aggressive screening for GI malignancy is necessary and justified. Further studies utilizing rigorously validated search strategies are necessary to determine the absolute risk of GI malignancy in patients with dermatomyositis to inform whether aggressive surveillance is needed beyond what is recommended in the average-risk screening population.

COMMENTS

Background

Dermatomyositis is associated with an increased risk of gastrointestinal (GI) malignancies based on large-population based studies. These prior studies utilized code-based search strategies and did not perform individual chart review.

Research frontiers

Endoscopy is now used to screen for GI neoplasias, but the yield of endoscopy in this patient population with dermatomyositis is not known. Understanding the yield of endoscopy could help inform clinical care of these patients.

Innovations and breakthroughs

By utilizing individual chart review, we provided an accurate estimate of prevalence of GI neoplasia in this population. They found that endoscopy identified no cases of malignancy and was of low yield, likely similar to the general population, in the identification of pre-malignant findings. Code-based searched strategies were inaccurate in the identification of dermatomyositis, calling into question the results of prior population-based studies.

Applications

The association between increased GI malignancy and dermatomyositis may be lower than previously reported.

Peer-review

This is an observational study attempting to quantify the actual risk of GI malignancy in patients with dermatomyositis and the yield of routine screening endoscopy in this population. The study is performed by retrospective chart review after identifying patients with dermatomyositis and reviewing endoscopy results. The paper is well written with good numbers and a thorough search method and provides evidence for management of a rare condition.

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ORIGINAL ARTICLE

Observational Study

Levels and activities of von Willebrand factor and metalloproteinase with thrombospondin type-1 motif, number 13 in inflammatory bowel diseases

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Author contributions: Butenas S and Undas A created study concept and designed the research; Cibor D, Owczarek D and Mach T collected the data; Cibor D, Owczarek D, Salapa K, Mach T and Undas A analyzed and interpretated the data; Cibor D, Owczarek D, Salapa K and Undas A drafted the manuscript; Cibor D, Owczarek D, Butenas S, Salapa K, Mach T and Undas A critically revised the manuscript.

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Abstract

AIM

To evaluate the levels of von Willebrand factor (VWF) and metalloproteinase with thrombospondin type-1 motif, number 13 (ADAMTS13) in inflammatory bowel disease (IBD) and correlate them with the disease activity.

METHODS

Consecutive patients with IBD aged 18 years or older were enrolled in the study. Forty-seven patients with ulcerative colitis (UC), 38 with Crohn's disease (CD), and 50 healthy controls were included. The white blood cell count, haematocrit, platelet count, fibrinogen, partial activated thromboplastin time, C-reactive protein, albumin, VWF antigen level (VWF:Ag), VWF ristocetin cofactor activity (VWF:RCo), VWF collagen-

binding activity (VWF:CB), and ADAMTS13 antigen level (ADAMTS13:Ag) and activity (ADAMTS13act) were measured. The following ratios were assessed: VWF:RCo/VWF:Ag, VWF:CB/VWF:Ag, VWF:Ag/ADAMTS13act, and ADAMTS13act/ADAMTS13:Ag.

RESULTS

Compared to controls, the odds ratio (OR) of an elevated VWF: Ag > 150% was 8.7 (95%CI: 2.7-28.1) in the UC group and 16.2 (95%CI: 4.8-54.0) in the CD group. VWF:CB was lower in UC patients, and active CD was associated with a higher VWF: RCo (+38%). The ORs of VWF:CB/VWF:Ag < 0.7 (a marker of acquired von Willebrand syndrome) in the UC and CD groups were 11.9 (95%CI: 4.4-32.4) and 13.3 (95%CI: 4.6-38.1), respectively. Active UC was associated with lower ADAMTS13:Ag (-23%) and ADAMTS13act (-20%) compared to UC in remission. Patients with active CD had a 15% lower ADAMTS13act than controls. The activity of UC, but not that of CD, was inversely correlated with ADAMTS13:Ag (r = -0.76) and ADAMTS13act (r = -0.81).

CONCLUSION

Complex VWF-ADAMTS13-mediated mechanisms disturb haemostasis in IBD. A reduced WVF:CB is a risk factor for bleeding, while a lower ADAMTS13 level combined with an elevated VWF:Ag could predispose one to thrombosis.

Key words: ADAMTS13; Inflammatory bowel disease; Thrombosis; Acquired von Willebrand syndrome; von Willebrand factor

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Core tip: A tightly regulated balance between von Willebrand factor and metalloproteinase with thrombospondin type-1 motif, number 13 (ADAMTS13) is important for haemostasis, and its dysregulation might predispose one to either thrombotic events or bleeding. We demonstrated a decrease in ADAMTS13act levels in Crohn's disease (CD) patients, and in ADAMTS13:Ag and ADAMTS13act in ulcerative colitis (UC) patients, in whom these parameters were negatively correlated with disease activity and inflammatory markers. We report for the first time the presence of abnormalities typical of type A2 acquired von Willebrand syndrome in inflammatory bowel disease patients. These findings provide insight into the elevated risk for thromboembolic events and bleeding observed in UC and less frequently in CD.

Cibor D, Owczarek D, Butenas S, Salapa K, Mach T, Undas A. Levels and activities of von Willebrand factor and metalloproteinase with thrombospondin type-1 motif, number 13 in inflammatory bowel diseases. *World J Gastroenterol* 2017; 23(26): 4796-4805 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i26/4796.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i26.4796

INTRODUCTION

In inflammatory bowel disease (IBD), which includes ulcerative colitis (UC) and Crohn's disease (CD), a prothrombotic state leads to increased risk for thrombosis[1-3]. The estimated risk of venous thromboembolism is threefold higher in IBD patients than in the general population[4]. A role for microvascular thrombi in the pathogenesis of IBD is supported by reports of a lower incidence of IBD in patients with inherited bleeding disorders, such as haemophilia A and B, and von Willebrand's disease (VWD)[5].

Inflammation and blood coagulation are closely related. An inflammatory state disturbs the balance between procoagulant and anticoagulant mechanisms, leading to enhanced blood coagulation, thrombin generation, and deposition of poorly lysable fibrin[6-9]. Von Willebrand factor (VWF) is an acute-phase protein and a marker of endothelial damage[10-12]. Activation of endothelial cells by inflammation impairs their anticoagulant function[13-16]. VWF is crucial for platelet adhesion and aggregation. High-molecular-weight multimers (HMWMs) are involved in platelet aggregation under high shear stress but present solely in the subendothelium and released into the bloodstream following activation of endothelial cells under normal conditions[17].

To assess VWF activity, two assays are commonly used: VWF ristocetin co-factor activity (VWF:RCo), which evaluates the ability of VWF to interact with platelet glycoprotein Ib, and VWF binding to collagen (VWF:CB), which evaluates platelet adhesion to collagen exposed following damage to the endothelium[18].

Type 2A acquired von Willebrand syndrome (AVWS) is characterised by an acquired qualitative VWF deficit associated with HMWM depletion, which increases the risk of mucocutaneous bleeding. VWF:CB is used to detect the qualitative abnormalities associated with HMWM depletion in type 2A VWD[18]. In the absence of shear stress, HMWMs are highly resistant to proteolysis by metalloproteinase with thrombospondin type-1 motif, number 13 (ADAMTS13)[19]. Regulation of the size of VWF multimers by ADAMTS13 is critical for normal haemostasis[20].

ADAMTS13 is a glycoprotein synthesised in endothelial cells and in hepatic stellate cells; it cleaves large VWF multimers to smaller, less-active multimers[17]. Impaired cleavage of large VWF multimers due to inadequate ADAMTS13 levels and/or impaired ADAMTS13 activity (ADAMTS13act) leads to thrombotic microangiopathies (TMA), which occur in several diseases such as thrombotic thrombocytopenic purpura (TTP), sepsis, malaria, malignancy, liver diseases, myocardial infarction, and ischaemic stroke[11,12,21]. In severe inflammatory states, in which the VWF level

is markedly elevated, a normal or slightly decreased ADAMTS13 level may be insufficient to control ultra-large VWF (UL-VWF) multimers[22].

A tightly regulated balance between VWF and ADAMTS13 is important for haemostasis, and its dysregulation might predispose one to either thrombotic events or bleeding. For example, patients undergoing elective coronary artery bypass grafting with lower preoperative VWF:RCo and high ADAMTS13 antigen (ADAMTS13:Ag) levels have a higher risk of high postoperative bleeding[23].

To the best of our knowledge, the association between ADAMTS13:Ag or ADAMTS13act and VWF antigen level (VWF:Ag) and activity in IBD has not been investigated in connection with the disease clinical activity. Therefore, we evaluated the plasma levels of these proteins and assessed their associations with the clinical characteristics and levels of inflammatory markers in patients with IBD.

MATERIALS AND METHODS

Study population

Consecutive patients with IBD aged 18 years or older were enrolled; the study population included 38 subjects with CD and 47 with UC. Disease was diagnosed based on classic histological, endoscopic, and radiological criteria[24]. The patients were followed-up at the Department of Gastroenterology, Hepatology and Infectious Diseases, Jagiellonian University Medical College, Krakow, Poland. The controls were 50 healthy volunteers recruited from the hospital staff and their acquaintances. The exclusion criteria were pregnancy, concomitant inflammatory disorders, and severe diseases including myocardial infarction, stroke, thromboembolism, known haemorrhagic diathesis, cancer, renal insufficiency, liver injury and diabetes. None of the patients or volunteers had been treated with aspirin, heparin, oral anticoagulants, or oral contraceptives.

The subjects provided informed written consent to participate in the study, which was approved by the Jagiellonian University Bioethics Committee (no KBET/287/B/2014). Participants gave also informed consent for data sharing. The study was performed in accordance with the ethical principles of the Helsinki Declaration of 2008.

Clinical assessment

The clinical assessment included the presence of comorbidities, cigarette-smoking habits, and medications. Body mass index was calculated. In patients with CD and UC, the following parameters were evaluated: disease duration, disease location, disease activity, complications, and past surgical procedures. Complications were defined as abscesses, fistulae, and stenoses resulting in post-obstructive symptoms. To determine the location of lesions in UC and CD patients, the Montreal classification was employed[25]. To assess CD and UC activity, the CD activity index[26] and disease activity index were used[27].

Laboratory tests

Fasting blood samples were collected from the antecubital vein in the morning. On the same day, using routine laboratory techniques, the following laboratory parameters were determined: WBC, haematocrit, platelet count, fibrinogen, partial activated thromboplastin time, C-reactive protein (CRP), and albumin.

VWF:Ag was measured by latex immunoassay using an STAR coagulation instrument (Diagnostica Stago, Asnieres, France), with a detection limit of 2 IU/dL. The intra- and inter-assay coefficients of variation were 5.2% and 5.5%, respectively. VWF:RCo was assessed turbidimetrically (Siemens, Erlangen, Germany); the intra- and inter-assay coefficients of variation were 6.9% and 7.5%. The detection threshold for the assay was 5 IU/dL. VWF:CB was determined by ELISA using type Ⅲ collagen (Sigma) diluted in acetic acid, with a limit of detection of 4 IU/dL; the intra- and inter-assay coefficients of variation were 6.1% and 6.7%. ADAMTS13:Ag and ADAMTS13act were quantified by means of fluorogenic assays (Technoclone, Wien, Austria). The intra- and inter-assay coefficients of variation were 5.8% and 6.5% for the level and 7.3% and 7.8% for the activity. The lower limit of detection of both tests was 5 IU/dL. The normal reference ranges for VWF and ADAMTS13 parameters were 50-150 IU/dL.

The following ratios were assessed: VWF:RCo/VWF:Ag, VWF:CB/VWF:Ag, VWF:Ag/ADAMTS13act, and ADAMTS13act/ADAMTS13:Ag.

Statistical analysis

The study was powered to have an 80% likelihood of detecting a 30% effect size among patients with UC, CD, and healthy controls in ADAMTS13act using a significance level $\alpha = 0.05$ and one-way analysis of variance (ANOVA; fixed effects). To demonstrate an effect size or greater in this variable, 37 patients were required in each group.

Continuous data with a skewed distribution are presented as means with standard deviations or medians with interquartile ranges. Discrete data are presented as numbers and percentages. To compare mean values, a one-way ANOVA was performed if all assumptions were met; otherwise a Kruskal-Wallis test or Welch test was used. Normality was verified using the Shapiro-Wilk test, and the Levene test was applied to investigate the heterogeneity of variance. The χ^2 test or Fisher's exact test was performed to examine associations between categorical data. Odds ratios were calculated for selected features. Correlations between continuous variables were assessed by calculating the Pearson or Spearman coefficient. A multivariate linear regression was employed to verify the impact of factors on ADAMTS13:Ag and ADAMTS13act.

Table 1 Characteristics of the studied subjects *n* (%)

	Ulcerative colitis *n* = 47	Crohn's disease *n* = 38	Control group *n* = 50
Men	22 (46.81)	20 (52.63)	35 (70)
Age, yr	37 (26-47)[b]	29.5 (22-34)[a]	36 (29-46)
BMI, kg/m^2	22.1 (20.5-25)	20.43 (18.38-22.9)	23.9 (22.2-25.3)
Smoking	9 (19.15)	15 (39.47)	17 (34)
Disease duration, yr	5 (2.5-9)	3.5 (2-6)	NA
Surgery	0 (0)[b]	11 (28.9)	NA
Complications	3 (6.4)[b]	11 (28.9)	NA
Location			
Rectum	7 (14.9)	NA	NA
Left-sided	25 (53.2)	NA	NA
Extensive colitis	15 (31.9)	NA	NA
Ileal	NA	12 (31.6)	NA
Colonic	NA	3 (7.9)	NA
Ileocolonic	NA	23 (60.5)	NA
Pregnancies, *n*	17	12	13
Miscarriages, *n*	0	1	1

[a]$P < 0.05$ *vs* the control group, [b]$P < 0.05$ *vs* the Crohn's disease group. BMI: Body mass index.

Table 2 Comparison of active and inactive ulcerative colitis groups and controls

	Ulcerative colitis inactive *n* = 21	Ulcerative colitis active *n* = 26	Control group *n* = 50
Disease activity index (points)	2.33 ± 1.68[b]	7.96 ± 1.91	NA
White blood cells, × 10^3/μL	6.89 (4.91-7.74)	8.22 (5.54-9.65)	6.75 (6.1-7.9)
Platelet coun, × 10^3/μL	286.02 (216-303)[b]	326 (252-379)[a]	229.5 (199-268)
Haematocrit (%)	43.4 (39.95-44.55)[b]	39.85 (36.8-42.2)	41.1 (39.5-43)
Haemoglobin, g/dL	13.5 ± 1.67	12.7 ± 1.5[a]	14.3 ± 1.1
Fibrinogen, g/L	2.52 (2.19-3.92)[b]	4.97 (3.83-6.99)[a]	3.05 (2.48-4.16)
CRP, mg/L	1.24 (0.79-1.89)[b]	10.7 (7.22-20.8)[a]	1.68 (.98-2.24)

[a]$P < 0.05$ *vs* the control group, [b]$P < 0.05$ *vs* active ulcerative colitis group. CRP: C-reactive protein.

Calculations were performed using the R statistical package version 3.2.2 (The R Foundation for Statistical Computing, www.r-project.org). The G*Power 3 software version 3.1.9.2 was used for the power calculation[28]. The statistical review of the study was performed by a biomedical statistician, Kinga Salapa from the Department of Bioinformatics and Telemedicine, Jagiellonian University Medical College.

RESULTS

Patient characteristics

The characteristics of the IBD patients and controls are presented in Table 1. Patients with UC were treated with mesalamine 2-4 g/d and 10 of them were on maintenance therapy with thiopurines. All patients with CD were on maintenance therapy with thiopurines and the subgroup with inflammatory lesions in the large intestine and the ileocecal region was treated with mesalamine (2 g/d). Data for the active and inactive UC and CD groups are presented in Tables 2 and 3, respectively.

VWF

VWF:Ag was higher in patients with active and inactive UC (Table 4) and CD (Table 5) as compared to controls. VWF activity, as assessed by the VWF:CB coefficient, was lower in patients with active and inactive UC compared to controls (Table 4). No such difference was evident in CD patients (Table 5). Patients with active CD had 38% higher VWF: RCo activity than the controls (Figure 1). Approximately 50% of the IBD patients (*n* = 42) had a VWF:Ag of greater than 150%, compared to 8% (*n* = 4) of the controls. In the UC group, the OR of a VWF; Ag greater than 150% was 8.7 (95%CI: 2.7-28.1), while in the CD group the OR was 16.2 (95%CI: 4.8-54.0) *vs* controls (Figure 2). None of the subjects had a deficiency in VWF; no cases of VWD were observed.

ADAMTS13

Patients with active UC had a 23% lower ADAMTS13: Ag and ADAMTS13act compared to those in remission (Table 4). No such differences were noted in the CD group, in which patients with active CD had a 15% lower ADAMTS13act than the controls (Table 5).

Both the UC and CD patients had lower VWF:CB/VWF:Ag coefficients than the controls. The mean VWF: CB/VWF:Ag values tended to be lower in patients with exacerbated disease compared to subjects in remission

Table 3 Comparison of active and inactive Crohn's disease groups and controls

	Crohn's disease inactive *n* = 16	Crohn's disease active *n* = 22	Control group *n* = 50
CDAI (points)	83.38 ± 33.30[b]	236.05 ± 63.46	NA
White blood cells, × 10^3/μL	6.74 (5.47-7.08)	6.71 (4.91-9.02)	6.75 (6.1-7.9)
Platelet count, × 10^3/μL	291.5 (209-336)[b]	324.5 (289-386)[a]	229.5 (199-268)
Haematocrit, %	42.7 (39.75-45.1)[b]	37.9 (35.8-39.8)[a]	41.1 (39.5-43)
Haemoglobin, g/dL	13.99 ± 1.36[b]	12.15 ± 1.4[a]	14.3 ± 1.1
Fibrinogen, g/L	3.81 (2.63-4.7)[b]	6.52 (4.76-8.26)[a]	3.05 (2.48-4.16)
CRP, mg/L	2.95 (1.19-5.72)[b]	33.8 (16.4-72.3)[a]	1.68 (0.98-2.24)

[a]$P < 0.05$ *vs* the control group, [b]$P < 0.05$ *vs* active Crohn's disease group. CDAI: Crohn's disease activity index; CRP: C-reactive protein.

Table 4 Characteristics of markers in active and inactive ulcerative colitis groups and controls

	Ulcerative colitis inactive *n* = 21	Ulcerative colitis active *n* = 26	Control group *n* = 50
VWF:RCo, IU/dL	120.61 ± 36.33	134.2 ± 46.7	114.03 ± 24.67
VWF:Ag, IU/dL	139.6 (110.3-166.5)[a]	144.95 (120.8-180.5)[a]	106.65 (90.7-122.5)
VWF:CB, IU/dL	84.93 ± 13.88[a]	78.87 ± 14.99[a]	97.99 ± 10.3
ADAMTS13: Ag (IU/dL)	86.15 ± 10.17[a,b]	66.39 ± 11.45[a]	103.86 ± 10.84
ADAMTS13act (IU/dL)	81.2 (79.4-92.9)[b]	65.05 (55.7-75.1)[a]	111.0 (97.8-122.8)
VWF:RCo/VWF:Ag	0.96 (0.92-0.97)	0.97 (0.87-1.01)	0.97 (0.91-1.15)
VWF:CB/VWF:Ag	0.68 (0.50-0.79)[a]	0.5 (0.45-0.70)[a]	0.91 (0.80-1.05)
VWF:Ag/ADAMTS13act	1.54 (1.34-2.07)[a]	2.22 (1.65-2.84)[a]	0.98 (0.79-1.20)
ADAMTS13act/ADAMTS13:Ag	1.01 (0.97-1.02)	1.0 (0.97-1.03)	1.02 (0.94-1.18)

[a]$P < 0.05$ *vs* the control group, [b]$P < 0.05$ *vs* active ulcerative colitis group.

Table 5 Characteristics of markers in active and inactive Crohn's disease groups and controls

	Crohn's disease inactive *n* = 16	Crohn's disease active *n* = 22	Control group *n* = 50
VWF:RCo, IU/dL	143.47 ± 46.12	157.04 ± 61.03[a]	114.03 ± 24.67
VWF:Ag, IU/dL	135.4 (116.85-175.65)[a]	182.98 (130.4-219.8)[a]	106.65 (90.7-122.5)
VWF:CB, IU/dL	98.85 (86.15-102.25)	89.3 (80.6-99.7)	98.05 (91.9-101.8)
ADAMTS13:Ag, IU/dL	104.44 ± 9.96	99.29 ± 15.33	103.86 ± 10.84
ADAMTS13act, IU/dL	104.82 ± 8.61	94.48 ± 15.74[a]	111.05 ± 17.69
VWFRCo/VWF:Ag	0.96 (0.95-1.00)	0.96 (0.79-1.01)	0.97 (0.91-1.15)
VWF:CB/VWF:Ag	0.65 (0.45-0.87)[a]	0.54 (0.45-0.65)[a]	0.91 (0.80-1.05)
VWF:Ag/ADAMTS13act	1.26 (1.12-1.62)[a]	1.88 (1.48-2.56)[a]	0.98 (0.79-1.20)
ADAMTS13act/ADAMTS13:Ag	0.99 (0.94-1.05)	0.96 (0.92-1.03)	1.02 (0.94-1.18)

[a]$P < 0.05$ *vs* the control group.

($P > 0.05$). Importantly, the OR of a VWF:CB/VWF:Ag < 0.7 in the UC group was 11.9 (95%CI: 4.4-32.4) *vs* controls, while that in the CD patients was 13.3 (95%CI: 4.6-38.1). The OR of a VWF:RCo/VWF:Ag of less than 0.7 in the UC group was 18.7 (95%CI: 1.0-337.4) and that in the CD group was 20.2 (95%CI: 1.1-370.8) *vs* control subjects.

The VWF:Ag/ADAMTS13act coefficients were higher in the two disease groups than in the control group. In the UC group, there were inverse correlations between ADAMTS13: Ag and VWF:RCo ($r = -0.35$, $P = 0.0168$) and between ADAMTS13:Ag and VWF:Ag ($r = -0.33$, $P = 0.0245$). In the CD group there were inverse associations between ADAMTS13:Ag and VWF:Ag ($r = -0.39$, $P = 0.0158$) and between ADAMTS13:Ag and VWF:CB ($r = -0.33$, $P = 0.0388$). In both groups, ADAMTS13:Ag was closely correlated with ADAMTS13act (UC: $r = 0.98$; CD: $r = 0.85$; $P < 0.0001$ for both).

VWF:CB was negatively correlated with disease activity ($r = -0.35$, $P = 0.0157$) and positively correlated with disease duration ($r = 0.38$, $P = 0.0116$) in the UC group. UC patients also showed inverse correlations between disease activity and ADAMTS13: Ag ($r = -0.76$, $P < 0.0001$) and ADAMTS13act ($r = -0.81$, $P < 0.0001$) (Figure 3).

The multivariate linear regression model showed that in the UC patients 63.5% of the ADAMTS13Ag variation was associated with three predictors: disease activity, CRP, and platelets, with the first having the greatest effect (Table 6). Moreover, disease activity explained approximately 61.4% of the variation in

Table 6 Results of multiple linear regression in ulcerative colitis patients

	ADAMTS13:Ag	ADAMTS13act
Platelets	β = -0.03 (95%CI: -0.07; -0.01) *P* = 0.044	-
C-reactive protein	β = -0.19 (95%CI: -0.33; -0.05) *P* = 0.103	β = -0.25 (95%CI: -0.38; -0.12) *P* < 0.001
Disease activity	β = -2.27 (95%CI: -3.18; -1.35) *P* < 0.001	β = -0.2.27 (95%CI: -3.48; -1.86) *P* < 0.001
adj.R^2	65.3%	69.5%

Results show the estimated value of parameter, *P*-value for the *t*-test and adjusted determinant coefficient. Additional, 95%CI of parameter's estimate when *P* < 0.05. adj.R^2: Adjusted determinant coefficient.

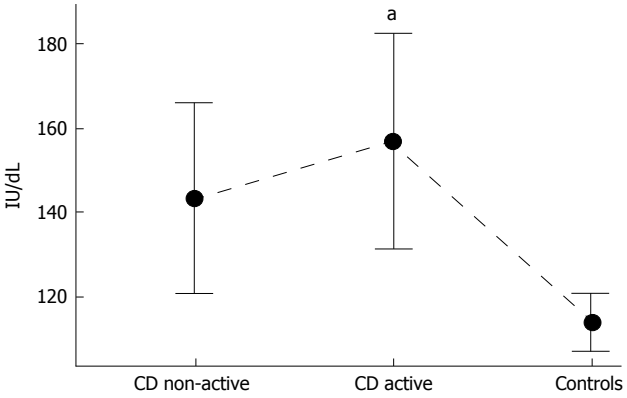

Figure 1 Activity of vWF: RCo in the Crohn's disease group and the controls. CD: Crohn's disease.

ADAMTS13act in UC patients.

In the UC group, ADAMTS13: Ag was inversely correlated with platelet counts (r = - 0.57, P < 0.0001), fibrinogen (r = -0.62, P < 0.0001), and CRP levels (r =-0.67, P < 0.0001). Similar associations were observed for ADAMTS13act in this group (r = -0.55, r = -0.7, and r = -0.72, all P < 0.0001, respectively).

In the CD group, there were no associations between ADAMTS13:Ag and inflammatory markers. However, ADAMTS13act was inversely correlated with fibrinogen (r = -0.36, P = 0.0286) and CRP (r = -0.45, P = 0.0048).

DISCUSSION

This is the first report of the effects of novel parameters associated with the structure and function of VWF on haemostasis in IBD. On the one hand, ADAMTS13:Ag was lower in IBD patients than in controls, particularly in subjects with UC; this resulted in an increased number of circulating multimers of VWF and thus an elevated thrombotic risk. The prothrombotic effects are enhanced by markedly elevated VWF:Ag. On the other hand, the incidence of AVWS, which leads to increased risk for bleeding, was higher in IBD patients. These findings provide insight into the elevated risk for thromboembolic events and bleeding observed in UC and less frequently in CD. The dysregulation of the balance between VWF function and ADAMTS13 reported herein might contribute to the complex haemostatic abnormalities observed in IBD.

Data on VWF and ADAMTS13 in IBD are scarce. Feys *et al*[29] reported no differences in VWF:Ag, ADAMTS13:Ag, and ADAMTS13act between UC and CD patients compared to healthy controls; the ADAMTS13:Ag was lower only in those with a CRP > 10 mg/L. However, that study included only 27 patients with CD and 17 with UC. Moreover, the ADAMTS13act/ADAMTS13:Ag ratio exhibited no intergroup differences. Similarly, we did not find any differences in the ADAMTS13act/ADAMTS13:Ag coefficient between our IBD and control groups. Lack of these differences may point to the quantitative nature of the defect[30].

A congenital or acquired deficit in ADAMTS13 has been reported in numerous diseases accompanied by TMA (*e.g.,* TTP, ischaemic stroke). For example, Klonizakis *et al*[31] reported that ADAMTS13 is a prognostic marker of microthrombotic manifestations in systemic lupus erythematosus. Other authors have demonstrated that the levels of ADAMTS13: Ag and ADAMTS13act in disseminated intravascular coagulation are associated with disease severity and mortality[32]. Enooku *et al*[33] showed in a Japanese population that the ADAMTS13 concentration was negatively correlated with the levels of inflammatory markers, arterial pressure, and pulse pressure. ADAMTS13 plays a role in inflammatory processes, oxidative stress, and atherosclerosis[33,34]. A reduction in ADAMTS13act and increases in VWF:Ag may increase risk for ischaemic stroke, cerebral infarction, and myocardial infarction[35,36]. In patients with infectious and noninfectious systemic inflammatory response syndrome, ADAMTS13 levels are negatively correlated with platelet count[12]. Such an association in UC patients is our novel finding.

It is possible that during inflammation, there is an increase in the number of large VWF multimers and a decrease in the level and activity of ADAMTS13:Ag, while the degree of disturbance in the balance of these markers is related to the severity of inflammation and the degree of organ failure[16,21]. An acquired decrease in ADAMTS13 level can be caused by several mechanisms[33]. These include excessive consumption

Figure 2 Occurrence of vWF:Ag > 150%, vWF:RCo/vWF: Ag < 0.7 and vWF: CB/vWF:Ag < 0.7 in patients with ulcerative colitis, Crohn's disease and in the control group.

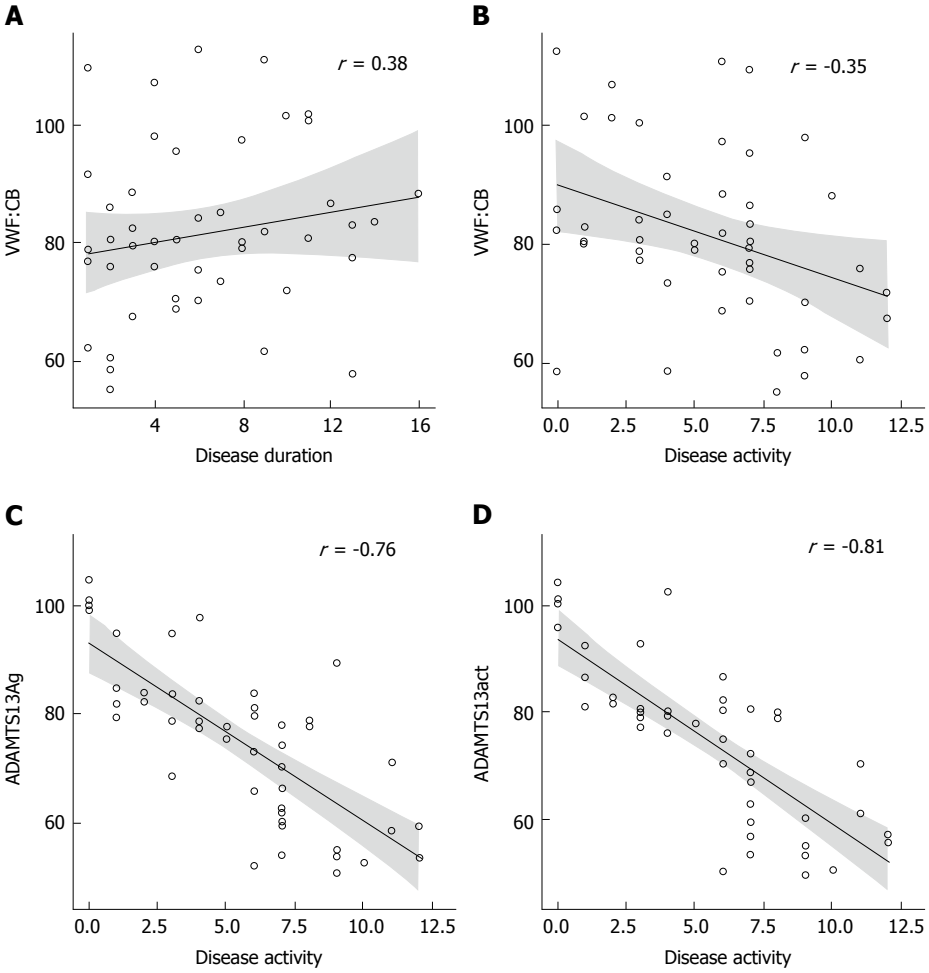

Figure 3 Correlation between selected haemostatic parameters and disease activity, and its duration. A: Correlation between disease duration and vWF: CB in patients with UC; B: Correlation between disease activity and vWF: CB in patients with ulcerative colitis (UC); C: Correlation between disease activity and ADAMTS13: Ag in patients with UC. D: Correlation between disease activity and ADAMTS13act in patients with UC.

of ADAMTS13 due to increased HMWM release from activated endothelium. ADAMTS13 becomes inactive after cleaving its substrate[37]. In addition, inhibition of ADAMTS13 synthesis by proinflammatory cytokines (interleukin 6 or TNF-alpha), or inactivation of ADAMTS13 by proteases released from neutrophils during inflammation occur in inflammatory diseases[38,39]. Interleukin 6 also has the ability to degrade ADAMTS13[21]. ADAMTS13 activity may be regulated by coagulation proteinases; thrombin, plasmin, and factor Xa activity may lead to inactivation of ADAMTS13[38,39].

Based on a mouse model, Chauchan *et al*[40] sug-

gested that by cleaving UL-VWF multimers, ADAMTS13 downregulates inflammation, while its deficit leads to increased leukocyte adhesion in vessels with inflammatory lesions, as well as increased extravasation of leukocytes. The diversity of mechanisms affecting the level and activity of ADAMTS13 confirms the interdependence of inflammation and coagulation.

A decrease in ADAMTS13act in association with an increase in the VWF:Ag/ADAMTS13act coefficient may be a risk factor for thrombosis and coagulopathy[41]. Claus *et al*[21] reported that significant disturbances in the balance between VWF:Ag and ADAMTS13act can result in the formation of UL-VWF multimers. The authors proposed that the VWF:Ag/ADAMTS13act ratio should be defined as the TMA index. High TMA index values in patients with inflammation and sepsis suggest that the index may be employed to diagnose highly prothrombotic states, and that its diagnostic value may be higher than that of VWF multimers alone[21]. *In vitro* studies have demonstrated a correlation between the presence of UL-VWF multimers and VWF:RCo[42].

Administration of recombinant ADAMTS13 in a mouse model of acquired TTP prevents development of symptoms; in contrast, in a mouse model of cerebral ischaemia it decreases the extent of stroke and improves the functional state[43,44]. Straat *et al*[45] showed that administration of frozen fresh plasma improved endothelial damage and inflammatory parameters, which was related to an increase in ADAMTS13 levels. Therefore, ADAMTS13 may be a new, alternate therapeutic agent for diseases involving disturbances of the balance between VWF and ADAMTS13.

We showed that the VWF:Ag/ADAMTS13act ratio was higher in patients with UC and CD compared to controls. These results confirm that hypercoagulability occurs in IBD, to which VWF is an important contributor. Notably, in approximately 50% of IBD patients, the VWF:Ag value was greater than 150%, compared to 8% in the controls. The inflammatory nature of IBD is associated with increased levels of VWF:Ag, which our findings support.

In contrast, the VWF:CB level in the present study was lower in the UC group than the control and CD groups. This suggests reduced collagen binding by VWF, leading to impaired platelet adhesion to damaged endothelium, as collagen itself has limited platelet-binding ability[13]. Clinically, this may manifest as gastrointestinal bleeding, which is more common in UC patients.

The VWF:CB/VWF:Ag ratio was lower in the UC and CD groups than the control group, and the OR of a VWF:CB/VWF:Ag ratio lower than 0.7 was 12- and 13-fold higher in the UC and CD groups, respectively. The VWF:RCo/VWF:Ag ratio did not differ among the CD, UC, and control groups; however, the IBD group included patients with a VWF:RCo/VWF:Ag coefficient below 0.7, while the control group did not.

VWF:RCo/VWF:Ag and VWF:CB/VWF:Ag coefficients of less than 0.7 are seen in patients with type 2A VWD, indicating substantial depletion of HMWM[45]. Riddell *et al*[18] reported that VWF:CB assessment is a more sensitive method for diagnostic management of type 2A VWD. Thus, the findings of our study suggest that the risk for AVWS in IBD patients is almost 20-fold higher than that in healthy individuals. This is of practical importance, for example, in patients undergoing antithrombotic prophylaxis during periods of IBD exacerbation, as it enables the identification of patients at increased risk for gastrointestinal bleeding.

This study had several limitations. First, the groups contained relatively few patients. Second, the presence of large VWF multimers in plasma was not analysed. Associations do not necessarily indicate a causal relationship; therefore, *in vitro* and animal model studies are necessary to elucidate the molecular mechanisms underlying our findings. Finally, this was a case-control study and patients were not followed in terms of thromboembolic events or the duration and severity of bleeding.

To our knowledge, the present study is the first to demonstrate a decrease in ADAMTS13act levels in CD patients, and in ADAMTS13:Ag and ADAMTS13act in UC patients, in whom these parameters were negatively correlated with disease activity and the levels of inflammatory markers. ADAMTS13:Ag represents a link between blood coagulation and inflammation in IBD. ADAMTS13:Ag deficit in IBD is quantitative by nature, possibly due to increased proteolysis of large VWF multimers. The elevated TMA index in IBD patients indicates increased risk for thromboembolic complications. In this group, the use of anticoagulation prophylaxis might be considered during disease flares not only in patients being hospitalised.

We also report for the first time the presence of abnormalities typical of type 2A AVWS in IBD patients. Determination of the VWF:Ag concentration and VWF activity may facilitate the identification of IBD patients at risk for bleeding complications and the management of patients with exacerbated disease, particularly when anticoagulation prophylaxis is recommended. In this group, a specific for AVWS treatment might be implemented during invasive procedures, especially surgery. Further studies involving larger patient populations and long-term follow-up are needed to validate our finding of a role for VWF in IBD.

COMMENTS

Background

Inflammation and blood coagulation are closely related. A tightly regulated balance between Von Willebrand factor (VWF) and ADAMTS13 is important for haemostasis, and its dysregulation might predispose to either thrombotic events or bleeding. Impaired cleavage of large VWF multimers due to inadequate ADAMTS13 levels and/or impaired ADAMTS13 activity leads to thrombotic microangiopathies. In severe inflammatory states, in which the VWF level is markedly elevated, a normal or slightly decreased ADAMTS13 level may be insufficient to control ultra-large VWF multimers.

Research frontiers

Inflammatory bowel disease (IBD) patients are at threefold higher risk for thromboembolic complications than the general population. This risk is especially high during hospitalizations, surgery or active disease and results from various hereditary and acquired factors.

Innovations and breakthroughs

This study demonstrated a decrease in ADAMTS13 activity levels in Crohn's disease patients and in ADAMTS13 antigen and ADAMTS13 activity in ulcerative colitis patients, in whom these parameters were negatively correlated with disease activity and inflammatory markers. On the other hand, the study showed for the first time the presence of abnormalities typical of type A2 acquired von Willebrand syndrome in IBD patients.

Applications

If further studies confirm the clinical relevance of these results, they may facilitate the individualization of antithrombotic therapy in patients with IBD. The elevated VWF antigen/ADAMTS13 activity ratio indicates an increased risk for thromboembolic complications. In this group, the use of anticoagulation prophylaxis might be considered not only in patients undergoing surgery or hospitalizations due to disease flare-ups but also in outpatients with disease exacerbation. On the other hand, determination of the VWF antigen concentration and VWF activity may facilitate the identification of IBD patients at higher risk for bleeding complications and the management of patients with exacerbated disease, particularly when anticoagulation prophylaxis is recommended. In this group, a specific for acquired von Willebrand syndrome treatment might be implemented during invasive procedures, especially surgery.

Terminology

VWF is an acute-phase protein and a marker of endothelial damage. Its function is crucial for platelet adhesion and aggregation. ADAMTS13 is a glycoprotein that cleaves large VWF multimers to smaller, less-active multimers. Type 2A acquired von Willebrand syndrome is characterised by an acquired qualitative VWF deficit associated with high-molecular-weight multimers depletion, which increases the risk of mucocutaneous bleeding.

Peer-review

The manuscript Levels and activities of von Willebrand factor and metallo-proteinase with thrombospondin type-1 motif, number 13 in IBD is an interesting original research with clear objectives.

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ORIGINAL ARTICLE

Observational Study

Predictors of esophageal varices and first variceal bleeding in liver cirrhosis patients

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Abstract

AIM

To assess "predictors" of esophageal varices (EV) and variceal bleeding using non-invasive markers in Albanian patients diagnosed with liver cirrhosis.

METHODS

One hundred thirty-nine newly diagnosed cirrhotic patients without variceal bleeding were included in this analysis. Model for end-stage liver disease (MELD), aspartate aminotransferase (AST) to alanine aminotransferase (ALT) ratio (AST/ALT), AST to platelet ratio index (APRI), platelet count to spleen diameter (PC/SD), fibrosis-4-index (FIB-4), fibrosis index (FI) and King's Score were measured for all participants. All patients underwent endoscopic assessment within two days of hospitalization. The major end point was the first esophageal variceal bleeding (EVB) event. The diagnostic performance of "predictors" for the presence of EV and EVB were assessed by sensitivity and specificity values obtained from the receiver operating characteristics procedure.

RESULTS

FIB-4 was the only strong and significant "predictor" of esophageal varices (multivariable-adjusted OR = 1.57 for one unit increment; 95%CI: 1.15-2.14). Furthermore, a cut-off value of 3.23 for FIB-4 was a significant predictor of esophageal varices, with a sensitivity of 72%, a specificity of 58% and a proportion of area under the curve (AUC) of 66% (P = 0.01). During the

follow-up (median: 31.5 mo; interquartile range: 11-59 mo), 34 patients (24%) experienced a first EVB. FIB-4 was a poor predictor of EVB (the AUC was only 51%) for a cut-off value of 5.02. Furthermore, the AUC of AST/ALT, APRI, PC/SD, FI, MELD and King's Score ranged from 45% to 55%. None of the non-invasive markers turned out to be a useful predictor of EVB.

CONCLUSION

Despite the low diagnostic accuracy, FIB-4 appears the most efficient non-invasive liver fibrosis marker which can be used as an initial screening tool for cirrhotic patients.

Key words: Albania; Esophageal varices; Liver cirrhosis; Non-invasive biomarkers; Variceal bleeding

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Core tip: Non-invasive markers of liver fibrosis constitute a simple and practical alternative to predict the presence of esophageal varices in cirrhotic patients. However, no single or a combination of non-invasive markers has been widely evaluated for predicting the variceal bleeding, to date. Our findings from a study conducted in Albania indicate that, despite the low diagnostic accuracy, fibrosis-4-index appears the most efficient non-invasive marker which can be used as an initial screening tool for cirrhotic patients in the areas with lacking endoscopy facilities. Yet, none of the non-invasive markers was a useful predictor of esophageal variceal bleeding in Albanian cirrhotic patients.

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INTRODUCTION

Liver cirrhosis is the 13th leading cause of death globally, with increasing mortality rate worldwide[1]. Portal hypertension is a frequent consequence in the progression of liver cirrhosis and plays a crucial role in the clinical manifestations of disease[2,3]. One of the most serious complications of portal hypertension is the development of esophageal varices (EV) caused by increased hepatic vascular resistance related to hepatic fibrosis and regenerative nodules[3]. In addition, the variceal bleeding due to the rupture of varices is still the most common lethal complication of cirrhosis[4]. Therefore, assessing the presence of esophageal varices in cirrhotic patients is clinically important in prevention of their bleeding. To date, the upper gastrointestinal endoscopy remains the golden diagnostic methods for EV and the recent Baveno VI Meeting Consensus recommends the endoscopy screening for all cirrhotic patients at the time of their diagnosis and periodical endoscopy examination in patients with EV[4]. However, routine endoscopy screening may not be cost-effective, as less than 50% of all patients with cirrhosis have EV[5]. Furthermore, there is a low prevalence of varices which requires primary prophylaxis[5]. Also, the upper endoscopy is an invasive and uncomfortable procedure which may not be acceptable for the patients. Hence, predicting the presence of EV through non-endoscopic and non-invasive markers is important in order to identify the patients who benefit from routine endoscopy screening and may reduce considerably the number of avoidable endoscopies[6].

Recently, various non-invasive markers, such as model for end-stage liver disease (MELD), aspartate aminotransferase (AST) to alanine aminotransferase (ALT) ratio (AST/ALT), AST to platelet ratio index (APRI), platelet count to spleen diameter (PC/SD), fibrosis-4-index (FIB-4), fibrosis index (FI) and King's score, have been demonstrated as a simple, non-invasive and easier practical alternative to predict the presence of EV in cirrhotic patients[7-14]. However, the findings of these previous studies are controversial and their utility in clinical practice is uncertain. The conclusions of these studies vary in different populations and in different etiologies of liver cirrhosis[13-16]. In this context, our prospective study aimed to assess the non-invasive markers, composed on routine laboratory parameters that could predict the presence of EV in liver cirrhosis patients in Albania, an endemic area of hepatitis B virus infection in Southeastern Europe and a Mediterranean country with a high consumption of domestic alcoholic beverages[17,18].

Despite the large number of studies, to date, no single or combination of non-invasive markers has been widely evaluated for predicting the first variceal bleeding in patients with cirrhosis[19,20]. Therefore, the second objective of our study was to identify the cirrhotic patients with a high risk for variceal bleeding using non-invasive markers.

MATERIALS AND METHODS

Study design

This was a prospective study conducted at the University Hospital Center "Mother Teresa" in Tirana, which constitutes the only academic and tertiary hospital center in Albania. Our study included all consecutive patients newly diagnosed (first time hospitalized) with liver cirrhosis hospitalized at the University Clinic of Gastro-Hepatology during 2005-2007 who were followed-up for five years (or, until an adverse event occurred during the follow-up period). The study was approved by the Department of

Internal Medicine of the University of Medicine, Tirana, Albania; all participants gave written informed consent after being explained in detail the aim and procedures of the study.

Study population and patients' follow-up

One hundred and thirty-nine newly diagnosed cirrhotic patients (Child-Turcotte-Pugh of at least 5-13) aged 18-70 years without variceal bleeding were enrolled in the study. Liver cirrhosis was diagnosed based on clinical data, laboratory test, liver imaging and/or histological criteria.

The exclusion criteria were as follows: (1) previous upper gastrointestinal hemorrhage; (2) using beta blockers or nitrates therapy; (3) current or past history of treatment for chronic B or C hepatitis (because these conditions may alter the hematological and biochemical parameters); (4) previous portosystemic shunt; (5) presence of gastric varices at endoscopy; (6) history of gastrointestinal surgery and/or gastrointestinal malignancies including hepatocellular carcinoma (HCC); (7) thrombosis of portal or splenic vein; (8) current or previous history of lympho-proliferative diseases; and (9) severe diseases of other organs or infections that could affect liver or spleen size.

After enrollment, all patients were treated according to the recent recommendations[21]. Patients with high risk EV (large, medium or small varices with red signs or in Child-Pugh class C) were administered non-selective beta blockers (propranolol), if not contraindicated[4]. Treatment compliance of the patients was monitored by measuring the resting pulse rate and interviewing them periodically if any side effects occurred. During the follow-up period, none of the patients were prescribed antiplatelet or anticoagulant agents. All patients were followed up for five years (or, until an adverse event occurred during the follow-up period). Adverse events were considered one of the following complications: the occurrence of severe ascites or hepatic encephalopathy requiring hospitalization (n = 5), a diagnosis of HCC (n = 9), upper gastrointestinal hemorrhage from variceal bleeding confirmed by endoscopy (n = 34), as well as deaths (n = 2). Ten patients were lost during the follow-up period. The outcome of interest was the occurrence of upper gastrointestinal hemorrhage from variceal bleeding confirmed by endoscopy and requiring hospitalization. We aimed to assess whether the non-invasive markers could predict the risk of first variceal bleeding.

Laboratory and endoscopic evaluation

All patients underwent detailed clinical and laboratory evaluation, ultrasonography and endoscopic assessment within two days of hospitalization. For each patient, data on age, sex, medical history, and use of medication, etiology of cirrhosis, and presence or absence of ascites, pedal edema, jaundice and hepatic encephalopathy were collected. Laboratory data included hemoglobin, platelet count, AST and ALT level, serum albumin, total serum bilirubin, gamma glutamyltranspeptidase (GGT), gamma globulin, prothrombin time with international normalized ratio (INR) and serum creatinine. In addition, all patients were classified according the Child-Turcotte-Pugh (CTP) class.

Also, the following non-invasive markers were calculated for each patient: AST/ALT, APRI, PC/SD, FIB-4, FI and King's score[9,12]. MELD score was determined by using the UNOS Internet site MELD calculator (http://www.unos.org/). At endoscopy, the presence and size of varices were classified as large, medium or small according to the proposed guidelines by a single experienced endoscopist who was unaware (blinded) of the values of non-invasive markers[5]. Presence of red signs was also recorded in all patients.

Statistical analyses

Fisher's exact test was used to compare the distribution of sex, etiology of cirrhosis, type of disease and CTP between patients with and without esophageal varices and, subsequently, between patients with and without esophageal variceal bleeding.

Conversely, Mann-Whitney *U*-test was employed to compare mean values of age, biochemical parameters (hemoglobin, creatinine, AST, ALT, GGT, gamma-globulin, INR and albumin) and "predictors" of esophageal varices and esophageal variceal bleeding (FIB-4, fibrosis index, APRI, PL/SD, AST/ALT, King's score and MELD).

Binary logistic regression was used to assess the association between non-invasive markers and esophageal varices. Initially, crude (unadjusted) models were run. Crude ORs, their respective 95%CI and *P*-values were calculated. Subsequently, multivariable-adjusted logistic regression were run, with demographic characteristics (age and sex), etiology of cirrhosis, type of disease, CTP, biochemical parameters (hemoglobin, creatinine, AST, ALT, GGT, gamma-globulin, INR and albumin) and "predictors" of esophageal varices (FIB-4, fibrosis index, APRI, PL/SD, AST/ALT, King's score and MELD) introduced all in a backward stepwise elimination procedure with a *P*-value to exit set at > 0.10. Multivariable-adjusted ORs and their respective 95%CIs were calculated.

The diagnostic performance of "predictors" (FIB-4, fibrosis index, APRI, PL/SD, AST/ALT, King's score and MELD) for the absence or presence of esophageal varices and/or esophageal variceal bleeding was assessed by sensitivity and specificity values and area under the curve (AUC) obtained from the receiver operating characteristics procedure (ROC curve).

In all cases, a P-value $\leqslant 0.05$ was considered as statistically significant. All the statistical analyses were conducted in SPSS (Statistical Package for Social Sciences), version 19.0. The statistical review of the study was performed by a biomedical statistician (GB,

Table 1 Distribution of baseline characteristics by esophageal variceal status

Variable	Total (n = 139)	Without EV (n = 26)	With EV (n = 113)	P value
Upper panel: Categorical variables[2]				
Sex				
Men	109 (78.4)[1]	18 (69.2)	91 (80.5)	0.289
Women	30 (21.6)	8 (30.8)	22 (19.5)	
Etiology of cirrhosis				0.005
Alcoholic	67 (48.2)	10 (38.5)	57 (50.4)	
HBV	32 (23.0)	5 (19.2)	27 (23.9)	
HCV	8 (5.8)	-	8 (7.1)	
Alcoholic + viral	11 (7.9)	1 (3.8)	10 (8.8)	
Other	21 (15.1)	10 (38.5)	11 (9.7)	
EV				-
None	26 (18.7)	26 (100.0)	-	
SEV	44 (31.7)	-	44 (38.9)	
MEV	39 (28.1)	-	39 (34.5)	
LEV	30 (21.6)	-	30 (26.5)	
Red signs				0.034
Without varices	26 (18.7)	22 (21.0)	4 (11.8)	
Yes	19 (13.7)	10 (9.5)	9 (26.5)	
No	94 (67.6)	73 (69.5)	21 (61.8)	
CTP				0.047
A (5-6)	43 (30.9)	13 (50.0)	30 (26.5)	
B (7-9)	63 (45.3)	10 (38.5)	53 (46.9)	
C (10-15)	33 (23.7)	3 (11.5)	30 (26.5)	
Lower panel: Numerical variables[3]				
Age (yr)	51.5 ± 13.1	47.6 ± 15.4	52.3 ± 12.4	0.106
Hemoglobin (mg/dL)	11.8 ± 2.3	11.6 ± 2.4	11.8 ± 2.3	0.916
Creatinine (mg/dL)	0.89 ± 0.34	0.89 ± 0.44	0.89 ± 0.31	0.141
AST (UI/L)	107.6 ± 156.1	153.5 ± 331.6	97.0 ± 69.9	0.412
ALT (UI/L)	73.1 ± 161.9	125.5 ± 336.7	61.0 ± 78.9	0.742
GGT (UI/L)	264.9 ± 302.7	282.0 ± 389.7	261.0 ± 280.9	0.709
Gama globulin (g/L)	27.1 ± 10.3	26.9 ± 15.3	27.2 ± 8.8	0.310
INR	1.65 ± 0.47	1.38 ± 0.34	1.71 ± 0.47	< 0.001
Albumin	3.19 ± 0.65	3.27 ± 0.71	3.17 ± 0.64	0.058
FIB-4	5.83 ± 5.33	3.98 ± 3.06	6.26 ± 5.64	0.011
Fibrosis index	3.15 ± 1.30	2.83 ± 1.08	3.22 ± 1.33	0.012
King score	82.9 ± 135.2	66.5 ± 137.4	86.7 ± 135.1	0.002
APRI	2.63 ± 3.79	2.52 ± 4.74	2.66 ± 3.57	0.014
PL/SD	1146 ± 780	1395 ± 784	1089 ± 771	0.028
AST/ALT	2.18 ± 1.62	1.93 ± 1.47	2.23 ± 1.66	0.339
MELD	15.7 ± 5.2	13.6 ± 4.8	16.1 ± 5.2	0.027

[1]Absolute numbers and *column* percentages (in parentheses); [2]P values from Fisher's exact test; [3]P values from Mann-Whitney's U-test. AST/ALT: Aspartate aminotransferase (AST) to alanine aminotransferase (ALT) ratio; APRI: AST to platelet ratio index; CTP: Child-Turcotte-Pugh score; EV: Esophageal varices; FIB-4: Fibrosis-4-index; GGT: Gamma glutamyltranspeptidase; HBV: Hepatitis B virus; HCV: Hepatitis C virus; INR: International normalized ratio; LEV: Large esophageal varices; MEV: Medium esophageal varices; MELD: Model for end-stage liver disease; PC/SD: Platelet count to spleen diameter; SEV: Small esophageal varices.

the senior author of this article).

RESULTS

Table 1 presents the distribution of baseline characteristics in patients with (n = 113) and without (n = 26) esophageal varices. Among patients with esophageal varices, 19 (17%) presented red signs compared with 94 (83%) patients without red signs. There was no sex-difference in the prevalence of esophageal varices between the two groups (upper panel). An alcoholic and/or viral etiology of cirrhosis was significantly more prevalent among patients with esophageal varices compared with their counterparts without esophageal varices. Conversely, grade A of CTP was significantly more prevalent among patients without varices than those with esophageal varices (50% *vs* 27%, respectively).

Mean age was not significantly different between patients with and without esophageal varices (Table 1 - lower panel). Likewise, there were no group differences regarding mean levels of hemoglobin, creatinine, AST and ALT, GGT and gamma globulin. Conversely, mean value of INR was significantly higher among patients with varices than in those without varices (1.7 *vs* 1.4, respectively; P < 0.001). Similarly, mean levels of most of the non-invasive markers were significantly higher in patients with varices than in those without variances (FIB-4: 6.3 *vs* 4.0, respectively; fibrosis index: 3.2 *vs* 2.8, respectively; King's score: 87 *vs*

Table 2 Association of non-invasive markers with presence of esophageal varices in liver cirrhosis

Non-invasive markers	Left panel: Unadjusted models		Right panel: Multivariable-adjusted models[2]	
	OR (95%CI)[1]	*P*[1] vaule	OR (95%CI)	*P* vaule
FIB-4	1.18 (1.01-1.38)	0.032	1.57 (1.15-2.14)	0.005
Fibrosis index	1.25 (0.90-1.74)	0.177		
King score	1.00 (0.99-1.01)	0.501		
APRI	1.01 (0.90-1.14)	0.866		
PL/SD	1.00 (0.99-1.00)	0.078		
AST/ALT	1.15 (0.84-1.57)	0.395		
MELD	1.11 (1.01-1.22)	0.026		

[1]Odds ratios (OR: Esophageal varices *vs* no esophageal varices), 95%CI and *P* values from binary logistic regression. [2]Models adjusted for age, sex, etiology of liver cirrhosis, CTP, VPT, hemoglobin, creatinine, AST, ALT, GGT, gama globulin, INR, albumin, FIB 4, fibrosis index, king score, APRI, PL/SD, AST/ALT and MELD. All variables were entered in a backward stepwise elimination procedure with a p-value to exit set at > 0.10. Empty cells refer to the variables excluded from the multivariable-adjusted logistic regression models. AST/ALT: Aspartate aminotransferase (AST) to alanine aminotransferase (ALT) ratio; APRI: AST to platelet ratio index; FIB-4: Fibrosis-4-index; MELD: Model for end-stage liver disease; PC/SD: Platelet count to spleen diameter.

Table 3 Performance of fibrosis-4-index for prediction of esophageal varices (*n* = 139; esophageal varices: *n* = 113)

Cut-off	Sensitivity	Specificity	PPV	NPV	AUC (95%CI)[1]	*P* value
3.23	72%	58%	88%	32%	0.66 (0.54-0.78)	0.011

[1]AUC (area under the curve) obtained from the receiver operating characteristic. FIB-4: Fibrosis-4-index; NPV: Negative predictive value; PPV: Positive predictive value.

67, respectively; APRI: 2.7 *vs* 2.5, respectively; and MELD index: 16.1 *vs* 13.6, respectively). On the other hand, mean level of PL/SD was lower in patient with varices than in those without varices (1089 *vs* 1395, respectively; *P* = 0.03) (Table 1 - lower panel).

In crude (unadjusted) binary logistic regression models (Table 2 - left panel), there was a significant association between esophageal varices and FIB 4 (OR = 1.18 for one unit increment; 95%CI: 1.01-1.38), and MELD score (OR = 1.11 for one unit increment; 95%CI: 1.01-1.22). On the other hand, there was no evidence of any significant association of esophageal varices with the other non-invasive markers (fibrosis index, King's score, APRI, PL/SD and AST/ALT). Upon adjustment for demographic characteristics, clinical parameters and all the biomarkers in a backward step-wise elimination procedure (right panel), FIB 4 was the only strong and significant "predictor" of esophageal varices (OR = 1.57 for one unit increment; 95%CI: 1.15-2.14). Furthermore, a cut-off value of 3.23 for FIB 4 was a significant predictor of esophageal varices (Table 3), with a sensitivity of 72%, a specificity of 58% and a proportion of area under the curve of 66% (*P* = 0.01).

During follow-up (median: 31.5 mo; interquartile range: 11-59 mo), 34 patients (24%) experienced first esophageal variceal bleeding. Of these, 4 (11.8%) patients were without EV upon enrollment, but experienced EV in the course of the follow-up. There was no evidence of any significant differences in the distribution of baseline characteristics in patients with (*n* = 34) and those without (*n* = 105) hemorrhagic events (Table 4).

The performance of the non-invasive markers for prediction of esophageal variceal bleeding is presented in Table 5, as an additional objective of our analysis was to identify the cirrhotic patients with high risk for variceal bleeding employing these non-invasive markers. Yet, none of the non-invasive markers turned out to be a useful predictor of esophageal variceal bleeding in this sample of Albanian patients. The area under the curve was pretty close to 50% for all of the non-invasive markers. For PL/SD there was evidence of a poorer prediction than even by chance (the area under the curve was 45%). FIB-4, which was shown a powerful predictor of esophageal varices, was nevertheless a very poor predictor of esophageal variceal bleeding (the area under the curve was only 51%) for a cut-off value of 5.02. The analysis was also restricted to individuals with variceal prophylactic therapy (VPT) (*n* = 83); similarly though, none of the non-invasive markers appeared to be a useful predictor of esophageal variceal bleeding (Table 6).

DISCUSSION

Nowadays, clinicians are interested to identify some "ideal" non-invasive biochemical markers which may be cheaper and easy to obtain, but with a high sensitivity and specificity for reducing the number of upper endoscopy needed for screening and management of EV in patients with liver cirrhosis. Such non-invasive tools are especially needed in developing countries with shortage of endoscopy, limited resources and a strong appeal for rationalization of funding. Therefore, we tried to explore whether any of the non-invasive markers (including AST/ALT, APRI, PC/SD, FIB-4, FI and King's Score) could predict the presence

Table 4 Baseline characteristics of patients with esophageal variceal bleeding during follow-up *n* (%)

Categorical variables	Total (*n* = 139)	No EVB (*n* = 105)	EVB (*n* = 34)	*P* value
Upper panel: Categorical variables[2]				
Sex				
Men	109 (78.4)[1]	80 (76.2)	29 (85.3)	0.341
Women	30 (21.6)	25 (23.8)	5 (14.7)	
Etiology of cirrhosis:				0.762
Alcoholic	67 (48.2)	49 (46.7)	18 (52.9)	
HBV	32 (23.0)	23 (21.9)	9 (26.5)	
HCV	8 (5.8)	6 (5.7)	2 (5.9)	
Alcoholic + viral	11 (7.9)	9 (8.6)	2 (5.9)	
Other	21 (15.1)	18 (17.1)	3 (8.8)	
EV				0.176
None	26 (18.7)	22 (21.0)	4 (11.8)	
SEV	44 (31.7)	36 (34.3)	8 (23.5)	
MEV	39 (28.1)	25 (23.8)	14 (41.2)	
LEV	30 (21.6)	22 (21.0)	8 (23.5)	
Red signs				0.034
Without varices	26 (18.7)	22 (21.0)	4 (11.8)	
Yes	19 (13.7)	10 (9.5)	9 (26.5)	
No	94 (67.6)	73 (69.5)	21 (61.8)	
CTP				0.968
A (5-6)	43 (30.9)	33 (31.4)	10 (29.4)	
B (7-9)	63 (45.3)	47 (44.8)	16 (47.1)	
C (10-15)	33 (23.7)	25 (23.8)	8 (23.5)	
Lower panel: Numerical variables[3]				
Age (yr)	51.5 ± 13.1	52.1 ± 13.5	49.7 ± 11.6	0.402
Hemoglobin (mg/dL)	11.8 ± 2.3	12.0 ± 2.2	10.9 ± 2.5	0.017
PL (n/mm^3)	165539 ± 101181	168742 ± 107419	155647 ± 79459	0.801
Creatinine (mg/dL)	0.89 ± 0.34	0.88 ± 0.34	0.92 ± 0.32	0.336
AST (UI/L)	107.6 ± 156.1	112.2 ± 175.7	93.4 ± 65.8	0.852
ALT (UI/L)	73.1 ± 161.9	80.3 ± 189.9	50.6 ± 34.5	0.930
GGT (UI/L)	264.9 ± 302.7	267.1 ± 311.4	258.2 ± 278.1	0.737
Gama globulin (g/L)	27.1 ± 10.3	26.6 ± 9.0	28.6 ± 13.6	0.555
INR	1.65 ± 0.47	1.65 ± 0.48	1.65 ± 0.43	0.862
Albumin	3.19 ± 0.65	3.17 ± 0.72	3.25 ± 0.38	0.608
FIB-4	5.83 ± 5.33	5.93 ± 5.80	5.50 ± 3.56	0.881
Fibrosis index	3.15 ± 1.30	3.13 ± 1.41	3.22 ± 0.84	0.901
King score	82.9 ± 135.2	89.6 ± 152.2	62.1 ± 53.7	0.978
APRI	2.63 ± 3.79	2.80 ± 4.25	2.13 ± 1.70	0.550
PL/SD	1146 ± 780	1189 ± 826	1014 ± 605	0.398
AST/ALT	2.18 ± 1.62	2.17 ± 1.64	2.23 ± 1.57	0.628
MELD	15.7 ± 5.2	15.5 ± 5.3	16.2 ± 4.8	0.435

[1]Absolute numbers and *column* percentages (in parentheses); [2]*P* values from Fisher's exact test; [3]*P* values from Mann-Whitney's *U*-test. AST/ALT: Aspartate aminotransferase (AST) to alanine aminotransferase (ALT) ratio; APRI: AST to platelet ratio index; CTP: Child-Turcotte-Pugh score; EV: Esophageal varices; FIB-4: Fibrosis-4-index; GGT: Gamma glutamyltranspeptidase; HBV: Hepatitis B virus; HCV: Hepatitis C virus; INR: International normalized ratio; LEV: Large esophageal varices; MEV: Medium esophageal varices; MELD: Model for end-stage liver disease; PC/SD: Platelet count to spleen diameter; SEV: Small esophageal varices.

of EV in cirrhotic patients in Albanian, a transitional Mediterranean country. We found that among all clinical and biochemical features assessed in univariate analyses, FIB-4 and MELD score significantly correlated with the presence of EV. However, in multivariable-adjusted logistic regression models, only FIB-4 remained a significant independent predictor of EV. It is interesting that only FIB-4, a fibrosis marker based on parameters linked to liver dysfunction or advanced diseases (AST and ALT), linked to portal hypertension (platelet count) and age could predict the EV despite the non-invasive markers that we included in our analysis. One plausible explanation for the lack of prediction of the other markers may consist of the age of the patients included in our study. Considering that liver fibrosis is a dynamic process and there is a close relationship between liver fibrosis, portal hypertension and development of EV, older patients are more likely to have EV. Indeed, mean age of our patients was relatively high and most of the patients were admitted in an advanced liver failure stage. Furthermore, previous studies investigating FIB-4 as a predictor of EV in liver cirrhosis patients showed similar results. In another prospective studies, for a cut-off value of 3.98 and 2.8, the AUC for predicting the EV was 62% and 78%, respectively[22,23], whereas Sebastiani *et al*[10] showed an AUC of 64% for the prediction of EV at a cut-off value of 3.5 in a retrospective study. However, their sensitivities and specificities were relatively low, ranging from 66% to 76% and from

Table 5 Performance of non-invasive markers for prediction of esophageal variceal bleeding (n = 34)

Non-invasive markers	Cut-off value	Sensitivity	Specificity	AUC (95%CI)[1]	*P* value
FIB-4	5.02	53%	54%	0.51 (0.40-0.62)	0.881
Fibrosis index	3.12	53%	45%	0.52 (0.40-0.61)	0.901
King score	37.16	65%	44%	0.50 (0.40-0.60)	0.978
APRI	1.66	59%	54%	0.53 (0.43-0.64)	0.550
PL/SD	828	53%	39%	0.45 (0.34-0.56)	0.398
AST/ALT	1.71	59%	54%	0.53 (0.42-0.64)	0.628
MELD	15.5	56%	52%	0.55 (0.44-0.65)	0.436

[1]AUC (area under the curve) obtained from the receiver operating characteristic. AST/ALT: Aspartate aminotransferase (AST) to alanine aminotransferase (ALT) ratio; APRI: AST to platelet ratio index; FIB-4: Fibrosis-4-index; MELD: Model for end-stage liver disease; PC/SD: Platelet count to spleen diameter.

Table 6 Performance of non-invasive markers for prediction of esophageal variceal bleeding among patients with variceal prophylactic therapy (n = 83; esophageal variceal bleeding: n = 23)

Non-invasive markers	Cut-off value	Sensitivity	Specificity	AUC (95%CI)[1]	*P* value
FIB-4	5.02	57%	42%	0.42 (0.28-0.56)	0.257
Fibrosis index	3.43	57%	40%	0.41 (0.28-0.55)	0.215
King score	46.15	57%	28%	0.39 (0.26-0.52)	0.112
APRI	1.65	57%	58%	0.45 (0.32-0.57)	0.439
PL/SD	736	52%	43%	0.49 (0.34-0.63)	0.843
AST/ALT	1.79	57%	58%	0.53 (0.42-0.64)	0.628
MELD	16.5	57%	58%	0.49 (0.35-0.63)	0.879

[1]AUC (area under the curve) obtained from the receiver operating characteristic. AST/ALT: Aspartate aminotransferase (AST) to alanine aminotransferase (ALT) ratio; APRI: AST to platelet ratio index; FIB-4: Fibrosis-4-index; MELD: Model for end-stage liver disease; PC/SD: Platelet count to spleen diameter; VPT: Variceal prophylactic therapy.

54% to 80%, respectively. Similarly, a recent meta-analysis demonstrated that AUC of FIB-4 for prediction the presence of EV was 77%[24]. In our study, a cut-off value of 3.23 of FIB-4 was proposed for diagnosis of esophageal varices. At this cut-off, the sensitivity was 72%, specificity was 58%, PPV was 88%, NPV was 32% and the AUC was 66%.

Esophageal variceal bleeding is the most important complication of liver cirrhosis and the major cause of death in cirrhotic patients. Therefore, prevention of esophageal variceal bleeding by using prophylactic treatment is an important goal to be achieved in cirrhotic patients with high risk esophageal varices. In our study, the prophylactic treatment with non-selective beta blockers was introduced in almost 60% of cirrhotic patients based on endoscopic criteria and liver dysfunction. Nonetheless, during the five years of follow-up, 11 patients (20%) among 56 cases without VPT and 23 patients (28%) among 83 cases who underwent VPT experienced upper gastrointestinal hemorrhage from variceal bleeding confirmed by endoscopy. Thus, these patients may require other prophylactic treatments such as band ligations of esophageal varices. Furthermore, identifying the non-invasive markers to predict EVB during the follow-up may be an important tool for a better management of cirrhotic patients. From this point of view, we conducted the present study under the assumption that progression of EV and ultimately EVB is caused directly by portal hypertension which correlates with liver fibrosis and may be assessed by non-invasive markers. Our study demonstrated insufficient accuracy of AST/ALT, APRI, PC/SD, FIB-4, FI and King's score for the prediction of EVB which ranged from 0.45 to 0.55. These findings suggest that none of the non-invasive markers is a useful predictor of esophageal variceal bleeding in this sample of Albanian patients.

To our knowledge, this is the first study exploring the role of non-invasive biomarkers as predictors of EVB in a longitudinal study. These non-invasive biomarkers are based on regular laboratory data, require no extra cost, specialized devices or additional biochemical tests and differ from other noninvasive markers which may not be easily accessible[25]. This fact may be very important in developing and transitional countries with rather limited resources. However, only FIB-4 turned out to be a useful predictor in this sample of Albanian patients and, therefore, the usefulness and applicability of these noninvasive markers should be considered cautiously. Furthermore, our data were collected at a single center in which the standard of care did not change substantially during the period under study. Also, the study participants were representative of the population of cirrhotic patients with different etiologies of liver cirrhosis avoiding selection bias pertinent to different etiologies or subgroups. In addition, the relatively large number of participants and the reasonable follow-up period constitute other strength of this study.

Multivariate modeling is frequently used to predict

the risk or prognosis of diseases and treatment's response. In our study, the multivariate model allowed us to assess important variables most of which, nevertheless, did not predict the presence of EV and the risk of first variceal bleeding in cirrhotic patients. However, multi-center prospective studies are needed to confirm our findings and ensure that they are applicable to diverse populations with different etiologies of liver cirrhosis. In addition, we cannot exclude the possibility that other predictors could induce hematological changes such as antiviral treatment or continued consumption of alcohol during the follow-up period - factors which were not accounted for in our analysis. Also, assessment of the sensitivity and specificity upon a second-time measurement would have provided an additional insight into the predictive power of the non-invasive parameters included in our analysis.

In conclusion, our results, based on cirrhotic patients with different etiologies, suggest that the FIB-4 is the most reliable predictor of esophageal varices in liver cirrhosis patients. Despite the low diagnostic accuracy, FIB-4 is the most efficient non-invasive liver fibrosis marker which can be used as an initial screening tool for cirrhotic patients in the areas with lack of endoscopy facilities. However, none of the non-invasive markers assessed in this sample of Albanian patients was a useful predictor of esophageal variceal bleeding. Thus, these markers may not be adequate for the replacement of upper endoscopy. Future large prospective studies are warranted to further define the diagnostic accuracy of non-invasive markers in the diagnosis of EV and prediction of EVB in countries where there is a shortage of endoscopy.

COMMENTS

Background

Portal hypertension is a frequent consequence in the progression of liver cirrhosis. Esophageal varices are one of the most serious complications of portal hypertension and variceal bleeding is the most common lethal complication. For this reason, assessing the presence of esophageal varices in cirrhotic patients is clinically important in order to prevent bleeding. This study aimed to assess the non-invasive markers, based on routine laboratory parameters, which could predict the presence of esophageal varices and first variceal bleeding in liver cirrhosis patients in Albania.

Research frontiers

Endoscopy screening for esophageal varices is currently recommended for all cirrhotic patients. On the other hand, the upper endoscopy is an invasive and uncomfortable procedure which may not be acceptable for the patients. Therefore, various non-invasive markers have been demonstrated as a simple and easier practical alternative to predict the presence of esophageal varices in cirrhotic patients. However, the findings of previous studies are controversial and their utility in clinical practice is uncertain. Furthermore, no single or a combination of non-invasive markers has been widely evaluated for predicting the variceal bleeding, to date.

Innovations and breakthroughs

They findings from a study conducted in Albania indicate that, despite the low diagnostic accuracy, fibrosis-4-index (FIB-4) appears the most efficient non-invasive marker which can be used as an initial screening tool for cirrhotic patients in the areas with lacking endoscopy facilities. Yet, none of the non-invasive markers was a useful predictor of esophageal variceal bleeding in Albanian cirrhotic patients.

Applications

These non-invasive biomarkers are based on regular laboratory data, require no extra cost, specialized devices or additional biochemical tests and differ from other noninvasive markers which may not be easily accessible. This fact may be very important in developing and transitional countries with rather limited resources. Nevertheless, future large prospective studies are warranted to further define the diagnostic accuracy of non-invasive markers in the diagnosis of esophageal varices and prediction of esophageal variceal bleeding in countries where there is a shortage of endoscopy.

Peer-review

The paper by Kraja Bledar *et al* demonstrated FIB-4 was strong predictor of EV in patients with liver cirrhosis; however, there was no association between FIB-4 and esophageal variceal bleeding. They concluded that FIB-4 is useful for initial screening tool for cirrhotic patients in the areas with lack of endoscopy facilities.

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ORIGINAL ARTICLE

Observational Study

Extreme liver resections with preservation of segment 4 only

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Abstract

AIM

To evaluate safety and outcomes of a new technique for extreme hepatic resections with preservation of segment 4 only.

METHODS

The new method of extreme liver resection consists of a two-stage hepatectomy. The first stage involves a right hepatectomy with middle hepatic vein preservation and induction of left lobe congestion; the second stage involves a left lobectomy. Thus, the remnant liver is represented by the segment 4 only (with or without segment 1, ± S1). Five patients underwent the new two-stage hepatectomy (congestion group). Data from volumetric assessment made before the second stage was compared with that of 10 matched patients (comparison group) that underwent a single-stage right hepatectomy with middle hepatic vein preservation.

RESULTS

The two stages of the procedure were successfully carried out on all 5 patients. For the congestion group, the overall volume of the left hemiliver had increased 103% (mean increase from 438 mL to 890 mL) at 4 wk after the first stage of the procedure. Hypertrophy of the future liver remnant (*i.e.*, segment 4 ± S1) was higher than that of segments 2 and 3 (144% *vs* 54%, respectively, $P < 0.05$). The median remnant liver volume-to-body weight ratio was 0.3 (range, 0.28-0.40)

before the first stage and 0.8 (range, 0.45-0.97) before the second stage. For the comparison group, the rate of hypertrophy of the left liver after right hepatectomy with middle hepatic vein preservation was 116% ± 34%. Hypertrophy rates of segments 2 and 3 (123% ± 47%) and of segment 4 (108% ± 60%, $P > 0.05$) were proportional. The mean preoperative volume of segments 2 and 3 was 256 ± 64 cc and increased to 572 ± 257 cc after right hepatectomy. Mean preoperative volume of segment 4 increased from 211 ± 75 cc to 439 ± 180 cc after surgery.

CONCLUSION

The proposed method for extreme hepatectomy with preservation of segment 4 only represents a technique that could allow complete resection of multiple bilateral liver metastases.

Key words: Hepatectomy; Colorectal liver metastases; Hepatic congestion; Liver regeneration; Liver resection; Liver failure

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Core tip: Extreme hepatic resections with preservation of one segment only may be required for complete resection of multiple bilobar liver metastases. We evaluated a new technique of two-stage hepatectomy with preservation of segment 4 only. Stage one involves a right hepatectomy with middle hepatic vein preservation and associated left lobe congestion through reduction of the left hepatic vein diameter. This combination optimizes segment 4 regeneration while allowing the left lobe (to be resected) to maintain function with reduced hypertrophy. Stage two involves a left lobectomy. Hypertrophy rates of non-congested segment 4 were significantly greater than in congested left lobe.

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INTRODUCTION

Complete surgical resection of colorectal liver metastases (CLM) leads to long-term survival for selected patients with isolated hepatic disease or resectable associated extrahepatic disease[1-5]. Technical improvements, mainly in blood flow modulation (such as portal vein embolization, portal vein ligation, associating liver partition and portal vein ligation (ALPPS), and simultaneous portal and hepatic vein embolization), have extended the limits of resection for CLM[6-8]. Stimulation of hepatic regeneration has also made liver resection feasible in patients who would otherwise have a small future liver remnant (FLR). However, these methods are not adequate for cases requiring bilateral anatomical liver resection with preservation of only a small central liver segment, as they cannot provide sufficient selective FLR hypertrophy.

Extensive bilateral hepatic involvement by CLM that requires complete resection of the right hemiliver and of the left lateral section, but which shows minimal or no involvement of segment 4, is an unusual as well as challenging clinical situation. Usually, the volume of liver segment 4 is insufficient to meet the minimal requirements for safe resection of all other segments. Additionally, the commonly applied methods for stimulating liver regeneration were designed to induce hypertrophy in lateral segments of the liver. Deprivation of portal flow to the entire right liver plus to the left lateral section has not yet been reported but would be expected to lead to liver failure if the FLR was very small.

Right portal vein embolization (with or without hepatic vein deprivation) induces parenchymal hypertrophy of both FLR (segment 4) and the left lateral section[9-12]. The ALPPS method was developed to induce hypertrophy of the left lateral section, in particular[13,14]. Recently, our group published a technical alternative option that optimizes hypertrophy of segment 4, while avoiding hypertrophy of the left lateral section and maintaining its function[15]. In this study, we aimed to target hypertrophy to segment 4 by generating a congestion of segments 2 and 3 after performance of a right hepatectomy. The safety and efficacy outcomes of this method are presented herein.

MATERIALS AND METHODS

Study design and patients

From January 2013 to December 2015, patients (Table 1) with multiple bilateral CLM who underwent a two-stage hepatectomy with preservation of segment 4 [± segment 1 (S1)] only, using a technique previously described by our team[15], were selected for study inclusion and designated as the "congestion group". For comparative analysis, patients that underwent right hepatectomy were selected to fit a 2: 1 case-matched proportion and included as the "comparison group". Inter-group comparisons were made for the hypertrophy rates of each left side hepatic segment after a right hepatectomy only (in the comparison group) and after a right hepatectomy associated with left lobe congestion (*i.e.*, the first-stage procedure in the congestion group). Pre- and postoperative hepatic triple-phase contrast-enhanced computed tomography (CT) images were used for comparative volumetric assessment of all patients.

Table 1 Patient characteristics of the congestion group

	Sex	Age, yr	Weight, kg	Preoperative chemotherapy	Comorbidities	CLM
Patient 1	Female	52	55	Oxaliplatin	None	Metachronous
Patient 2	Female	61	63	Oxaliplatin	SAH	Metachronous
Patient 3	Female	39	98	Oxaliplatin + bevacizumab	None	Synchronous
Patient 4	Female	54	66	Oxaliplatin	None	Metachronous
Patient 5	Female	65	46	Oxaliplatin	DM, SAH	Metachronous

CLM: Colorectal liver metastases; DM: Diabetes mellitus; SAH: Systemic arterial hypertension.

Congestion group

The congestion group included 5 patients with multiple bilateral CLM who had been selected for resection according to fulfillment of the following criteria: (1) hepatic segment 4 (± S1) without lesions or with only superficial lesions appropriate for atypical resection; (2) right hemiliver and left lateral sector both considered ineligible for surgical preservation due to the presence of multiple deep and/or sectorial or segmental hepatic pedicle tumor involvement; and (3) absence of detectable extrahepatic metastases (Table 1).

Abdominal and thoracic contrast-enhanced CT, abdominal magnetic resonance imaging (MRI), and positron emission tomography (PET)-CT were used to detect hepatic and extrahepatic disease. Hepatic metastases were defined as metachronous if they were diagnosed at least 6 mo after colorectal tumor resection. All patients received chemotherapy with oxaliplatin-based standard protocols and showed no hepatic disease progression prior to the surgical resection.

At the time of presentation of the metastatic liver disease, none of the patients were considered candidates for surgery as their FRLs were too small. Individual case discussion was carried out by the multidisciplinary tumor board, with alternatives for ensuring achievement of complete hepatic resection of the metastatic disease being considered. In this scenario, simultaneous embolization of the right portal vein and left lateral sector portal branches was deemed to be too risky because of the very small FLR (segment 4 ± S1). Of note, the left lateral lobe was evaluated for preservation but complete cleanup was deemed impossible, even with application of local ablation methods.

Comparison group

The comparison group consisted of 10 patients that had been randomly selected from a large database so that each patient was matched according to the following criteria: age (classified according to decades), sex, American Society of Anesthesiologists (commonly known as ASA) score, CLM diagnoses, preoperative chemotherapy (chemotherapeutic agents and duration), and type of resection (right hepatectomy with middle hepatic vein preservation).

Rationale for segmental congestion and technical aspects

Extended hepatic resection with preservation of segment 4 only represents a significant clinical challenge. The usual options for resection in patients with multiple bilateral metastases include methods of portal vein privation with or without local ablation techniques. However, when segment 4 is the only one to be preserved, performance of isolated right portal vein embolization (or even in conjunction with right hepatic vein occlusion) induces hypertrophy of both segment 4 and the left lateral sector, namely of both the segments to be preserved and the segments to be respected. Thus, the increase of segment 4 is not optimal, and it might not be sufficient for successful outcome. On the other hand, coincident embolization of the left lateral sector portal branches (in addition to the right portal vein embolization) is a risky procedure since the non-embolized segment is too small.

Thus, to optimize hypertrophy of the FLR (*i.e.*, segment 4), our team conceived of a two-stage procedure (Figure 1) in which the initial surgery consists of a right hepatectomy with middle hepatic vein preservation and induction of congestion of the left lobe, and the second stage is a left lateral sectionectomy.

Since parenchymal resection provides the highest expected rate of remnant liver hypertrophy, a major hepatic resection (right hepatectomy) is performed in the first stage. Additionally, to promote future hypertrophy of segment 4 and to avoid hypertrophy of the left lateral sector, congestion of left lobe is induced. To induce the desired segmental congestion, a silicone tube is wrapped around the left hepatic vein, outside of the liver. The diameter of the looping silicone is progressively diminished around the vein. When macroscopic signs of congestion become evident in segments 2 and 3, a Doppler study is performed to ensure that hepatopetal flow is still present in these segments. Finally, the silicone tube is tied-off in order to maintain the diameter of the vessel according to the size of the loop. The loop is left *in situ* until the second stage is performed. It is important to ensure the hepatopetal flow despite congestion, as this will help maintain the function of the congested segments but will limit their hypertrophy rates.

At 4 wk after the first stage, a CT with volumetric assessment is performed to evaluate parenchymal

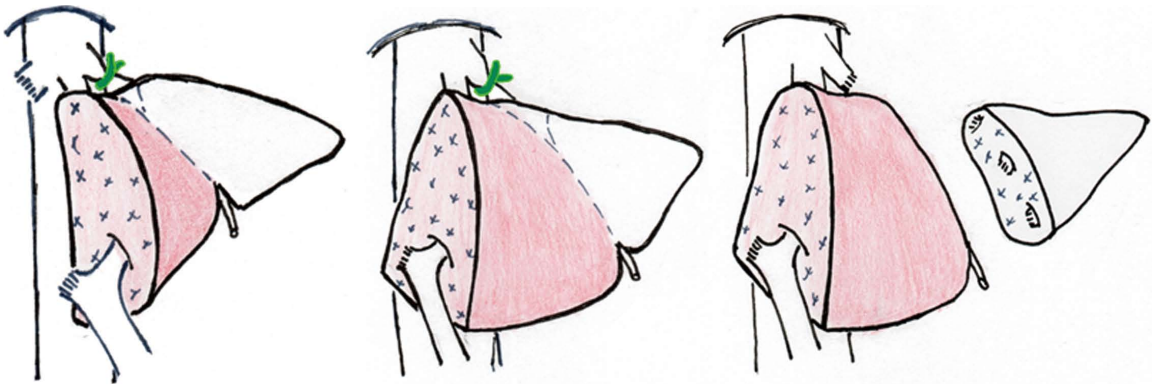

Figure 1 Schematic representation of two-stage hepatectomy with preservation of segment 4 only using outflow modulation. A: During the first stage, a right hepatectomy with middle hepatic vein is performed along with an associated induction of congestion in the left lobe; B: Redistribution of portal flow (increased inflow to segment 4 after territorial congestion of segments 2/3) optimizes hypertrophy of segment 4 and avoids hypertrophy of segment 2 and 3; C: The second stage of procedure, a left lobectomy, is performed.

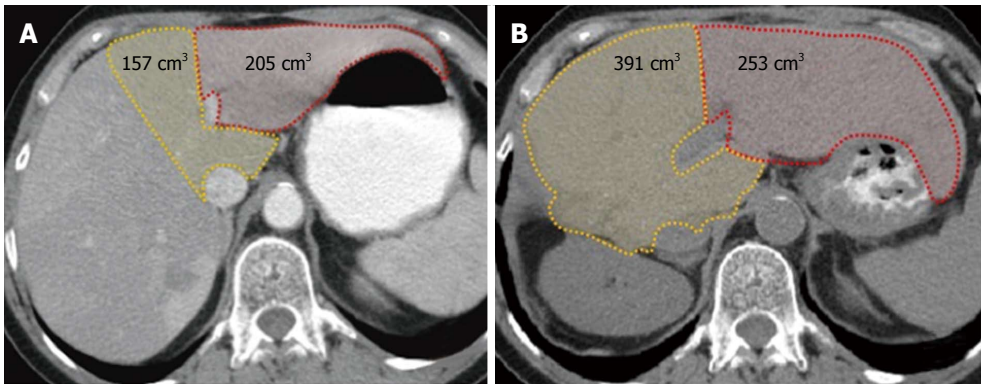

Figure 2 Computed tomography-based volumetry. A: Before the first stage of liver resection; B: Before the second stage of the procedure. Modulated congestion of the left lobe associated with right hepatectomy led to a significant hypertrophy rate of segments 4 and 1 (156%) but only a modest hypertrophy rate of segments 2 and 3 (24%). Modified from Balzan *et al*[15].

hypertrophy (Figure 2), after which a left lateral sectionectomy is performed if the FLR is considered sufficient. When lesions are also present on segments 4 and/or 1, the tumors are resected during the first stage, thereby "cleaning" the FLR for the subsequent procedure.

Both the first and second stages were performed with a J-shaped incision. Intraoperative ultrasound was used to verify the transection plane and assure preservation and permeability of the middle hepatic vein. The left hepatic vein was dissected and encircled by the standard technique. Parenchymal transection was performed using a saline-irrigated bipolar device, and all vascular and biliary structures sized ⩾ 3 mm were divided between ligatures. Intermittent Pringle maneuver (15 min of clamping with 5 min intervals) was used as needed.

Measurement of liver volumetry

Volumetry of segments 4 and 1 (or of only segment 4 if S1 should be resected) and of segments 2 and 3 (left lobe) was performed preoperatively and at 4 wk after the right hepatectomy (Figure 2). The regeneration rate (percent hypertrophy) was calculated with the following formula: [(segmental volume after surgery - segmental volume before surgery) × 100]/segmental volume before surgery. Volumetries were determined by contrast-enhanced CT acquisitions and by use of the Osirix for IOS medical imaging viewer software (Pixmeo SARL, Bernex, Switzerland, 2012).

Statistical analysis

Data are expressed as mean ± SE of the mean or median. A *t*-test was used to compare means. *P* value < 0.05 indicated statistical significance. All statistical analyses were performed by the SPSS statistical software package, version 10.1 (SPSS Inc, Chicago, IL, United States).

RESULTS

Liver regeneration in the congestion group

The overall left liver increased by 103% (mean increase from 438 mL to 890 mL) in the 4 wk after performance of the first stage of the procedure. Hypertrophy of the FLR (*i.e.*, segment 4 ± S1) was greater than that of segments 2 and 3 (144% *vs* 54%, respectively, $P < 0.05$). Table 2 shows the individual volumes

Table 2 Hypertrophy rate of congested and non-congested segments

	Segments 2/3			Segment 4 (± S1)			*P* vaule
	1st stage, in cc	2nd stage, in cc	Hypertrophy rate	1st stage, in cc	2nd stage, in cc	Hypertrophy rate	
Case 1	205	253	24%	157	391	149%	
Case 2	228	331	45%	192	505	163%	
Case 3	309	679	120%	272	823	203%	
Case 4	248	407	64%	225	641	185%	
Case 5	186	214	15%	167	206	23%	
mean ± SD	235 ± 42	377 ± 165	54 ± 37	203 ± 42	513 ± 211	144 ± 63	< 0.05

1st stage: Volumetry before 1st stage; 2nd stage: Volumetry before second stage (after right hepatectomy and segments 2 and 3 congestion).

and hypertrophy rates for each patient. The median remnant liver volume-to-body weight ratio was 0.3 (range, 0.28-0.40) before the first stage and 0.8 (range, 0.45-0.97) before the second stage.

Liver regeneration in the comparison group

The overall hypertrophy rate of the left liver at 4-6 wk after right hepatectomy with middle hepatic vein preservation in this group was 116% ± 34%. The hypertrophy rates were proportional for segments 2 and 3 and segment 4 (123% ± 47% *vs* 108% ± 60%, $P > 0.05$). The mean preoperative volume of segments 2 and 3 was 256 ± 64 cc, which increased to 572 ± 257 cc after the right hepatectomy. The mean preoperative volume of segment 4 increased from 211 ± 75 cc to 439 ± 180 cc after the surgery.

Morbidity and mortality rates

Both of the two stages of the novel procedure were successfully completed in all 5 patients. One patient (case #3) developed postoperative ascites after the first stage, requiring prolonged hospital stay. Another patient (case 5, a 65-year-old female) died at 30 d after the second stage, due to liver failure that likely resulted from small-for-size syndrome. In this patient, ascites and jaundice developed on postoperative day 7 and increased progressively with no evidence of biliary obstruction. She had been receiving systemic oxaliplatin-based chemotherapy for 8 mo prior to performance of the first stage. Her hypertrophy rate at 4 wk after the first stage was below expected, for both congested and non-congested segments (15% and 23%, respectively).

All other patients experienced uneventful postoperative recovery (*i.e.*, no Dindo-Clavien complication of grade 2 or more) after the first and second stages.

DISCUSSION

The present study demonstrates that modulation of outflow allows for targeted liver regeneration. The method reported is particularly useful for patients in whom only segment 4 (± S1) can be preserved. In such cases, as described herein, the hypertrophy rate of segment 4 (± S1) after right hepatectomy was optimized when partial outflow deprivation of segments 2/3 was employed.

The classical paradigm of liver resectability comprises the preservation of at least two contiguous functional liver segments, with appropriate portal and arterial inflow, as well as venous outflow and biliary drainage. Right and left trisectionectomies are the most extensive liver resections routinely performed[16]. Moreover, two-stage hepatectomy for bilateral CLM may provide long-term outcomes that are comparable to those in patients treated with a planned single-stage hepatectomy[16-20]. However, extreme resections with preservation of only a single liver segment have been reported rarely[21-23]. Two cases of hepatic resection with only segment 4 preservation were reported by Adam *et al*[21], and both used staged procedures. For these, the first stage involved a major hepatectomy (right hepatectomy) in one of the patients and a minor hepatectomy (left lobectomy) in the other one. Schadde *et al*[19] reported 6 cases with left lobectomy performed during the first stage, in the context of ALPPS, followed by right hepatectomy in the second stage.

Our technique comprises a right hepatectomy (with or without wedge resections on segment 4) during the first stage of the procedure and a left lobectomy as the second stage. The potential disadvantages of performing a major liver resection first include: (1) submitting the patient to a higher-risk procedure initially, although this is offset by the chance that the patient may eventually not be a candidate for the second stage due to disease progression; and (2) subjecting the surgeon of the second stage to a procedure with increased technical difficulty if adhesions involving the diaphragm have formed. However, other positive aspects should be considered: (1) a major resection performed as the initial procedure induces a greater extent of hepatic regeneration than a minor hepatectomy; (2) anatomical right hepatectomy is a standardized procedure with acceptable morbidity; and (3) lysis of adhesions from the diaphragm to the cut section of the liver is not routinely required to perform the second stage procedure (left lobectomy). Besides these, no oncological benefit has been demonstrated for one strategy over another.

Extensive parenchymal resection results in the highest rate of regeneration of the remaining liver, as

compared to other reported methods that induce FLR hypertrophy. In our study, the overall volume increase of the left liver after the first stage was 103%, and the FLR (*i.e.*, segment 4) increased 144% before the second stage. In ALPPS, the reported hypertrophy rates of FLR before the second stage have ranged from 47% to 110%[13,24-26]. Likewise, in portal vein embolization or ligation alone, or when associated with ipsilateral hepatic vein occlusion, even more modest rates of hypertrophy have been reported (from as low as 8.2% and up to 46%)[27-31].

Hypertrophy of the remaining liver after major parenchymal hepatic resection is fast and powerful, and involves a complex process of signaling pathways[32]. The role of portal flow in the process of regeneration after major hepatectomy is unclear, but induction of regeneration seems to occur due to: (1) the increase of portal flow per unit mass itself; and (2) the large incoming amounts (per hepatocyte) of signaling molecules usually present in portal blood[33]. According to these concepts, the temporary increase of portal flow to some hepatic territories would strongly stimulate the regenerative process of these hepatocytes, while the maintenance of a usual portal flow would prevent regeneration.

This phenomenon explains the disproportional rate of hypertrophy that is seen between the left lobe and segment 4 after right hepatectomy, with or without preservation of the middle hepatic vein. It is known that after right hepatectomy with middle hepatic vein resection, the rate of regeneration of segment 4 is lower than that of segments 2 and 3. On the other hand, after right hepatectomy with middle hepatic vein preservation, all remnant segments show a similar hypertrophy[33-35]. In our technique, this knowledge was applied in a reverse manner, specifically a reduction of outflow from the left lobe after the right hepatectomy with preservation of middle hepatic vein. In fact, the induced congestion of the left lobe prevents the expected increase of portal flow to this hepatic area, maintaining its function while avoiding hypertrophy. In the meantime, the portal flow is redirected to non-congested segments (segments 4 and 1), *i.e.*, the FLR, optimizing its regeneration.

Thus, induced congestion of the left lobe (which is to be resected) during the first stage of the procedure aims to prevent its hypertrophy while optimizing that of segment 4 (which is to be preserved). Our results confirm a significant disproportional hypertrophy rate of the congested left lobe (54%) compared to the non-congested segment 4 (144%), allowing for safe resection of the left lobe in the second stage of the procedure. The consistency of this strategy is evidenced by the hypertrophy rate of the matched comparison group of patients that underwent a right hepatectomy with middle hepatic vein preservation. In this comparison group, in the context of single-stage hepatectomy for CLM, a proportional hypertrophy rate was observed for left lobe and segment 4.

The interval between performance of the first and second stages ranged from 6 wk to 8 wk, and all patients underwent the second stage of the procedure. One patient died following the second stage. This patient presented an unexpected low rate of hypertrophy after the first stage (15% for the left lobe and 23% for segment 4) and underwent the second stage with a FRL of 206 cc (0.45% of the body weight). The restricted rate of hypertrophy could have been related to the long period of preoperative chemotherapy (8 mo) that the patient had undergone. The postoperative clinical findings were compatible with small-for-size syndrome and started to develop after postoperative day 7, with progressive hyperbilirubinemia and voluminous ascites. The patient died of liver failure on postoperative day 30.

Future studies should take into account both the expected rate of hypertrophy and the kinetic rate of hypertrophy. These rates should probably be considered as contraindications for the second procedure, as in other strategies used to induce liver hypertrophy. Also, the ideal interval between the two stages remains to be determined, as well as the benefit of chemotherapy during the interval period. To evaluate the viability of performing the second stage early, our group recently initiated a prospective protocol that includes volumetric assessment on day 7 after performance of the first stage.

In summary, this novel method for extreme hepatectomy with preservation of only segment 4 represents a new understanding of the clinical treatment that could refine the assignment of patients with multiple bilateral liver metastases to achieve a complete resection.

COMMENTS

Background

Complete resection is the only potential curative treatment for colorectal liver metastases (CLM). Resection of multiple bilateral lesions represents a technical challenge and a real risk of postoperative hepatic failure when segment 4 is the only hepatic segment to be preserved.

Research frontiers

The usual technical options for resection of multiple bilateral CLM are preoperative portal vein embolization, associating liver partition and portal vein ligation, and two-stage hepatectomy. Unfortunately, these are rarely useful for extreme resection with preservation of segment 4 only, due to the common very-small future liver remnant. Thus, a method allowing safe hepatic resection with preservation of only segment 4 will benefit a significant number of patients.

Innovations and breakthroughs

A two-stage procedure that can overcome the risk of postoperative hepatic failure among patients with preservation of segment 4 only is presented. The first stage involves a right hepatectomy (to accomplish the higher expected hypertrophy rate) and a reduction of the left hepatic vein diameter (venous congestion to preclude proper hypertrophy). This strategy allows for an extraordinary hypertrophy rate of segment 4 (future liver remnant), while the left lobe maintains its function with a much less extent of increase in size. Then, the second stage can be safely accomplished through a left lobectomy. Compared to conventional right hepatectomy, the use of outflow modulation optimizes the

hypertrophy rate of the target area.

Applications

This surgical technique represents a useful and safe method to perform extreme resections when the only hepatic segment to be preserved is segment 4.

Peer-review

This is a retrospective study, limited by the small number of patients. However, the use of this innovative technique allows extensive liver resection in patients with multiple bilateral liver metastases in a scenario that cannot be treated by conventional approaches. The comparison group in this paper clearly shows that venous congestion is a useful tool to optimize liver hypertrophy of the future liver remnant. The spreading of such technique should encourage other centers to perform it. Its benefit in a larger number of patients shall become apparent with further series.

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ORIGINAL ARTICLE

Observational Study

Predictive factors for body weight loss and its impact on quality of life following gastrectomy

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Abstract

AIM

To determine the predictive factors and impact of body weight loss on postgastrectomy quality of life (QOL).

METHODS

We applied the newly developed integrated questionnaire postgastrectomy syndrome assessment scale-45, which consists of 45 items including those from the Short Form-8 and Gastrointestinal Symptom Rating Scale instruments, in addition to 22 newly selected items. Between July 2009 and December 2010, completed questionnaires were received from 2520 patients with curative resection at 1 year or more after having undergone one of six types of gastrectomy for Stage I gastric cancer at one of 52 participating institutions. Of those, we analyzed 1777 eligible questionnaires from patients who underwent total gastrectomy with Roux-en-Y procedure (TGRY) or distal gastrectomy with Billroth-I (DGBI) or Roux-en-Y (DGRY) procedures.

RESULTS

A total of 393, 475 and 909 patients underwent TGRY, DGRY, and DGBI, respectively. The mean age of patients was 62.1 ± 9.2 years. The mean time interval between surgery and retrieval of the questionnaires was 37.0 ± 26.8 mo. On multiple regression analysis, higher preoperative body mass index, total gastrectomy, and female sex, in that order, were independent predictors of greater body weight loss after gastrectomy. There was a significant difference in the degree of weight loss (P < 0.001) among groups stratified according to preoperative body mass index (< 18.5, 18.5-25 and > 25 kg/m^2). Multiple linear regression analysis identified lower postoperative body mass index, rather than greater body weight loss postoperatively, as a certain factor for worse QOL (P < 0.0001) after gastrectomy, but the influence of both such factors on QOL was relatively small (R^2, 0.028-0.080).

CONCLUSION

While it is certainly important to maintain adequate body weight after gastrectomy, the impact of body weight loss on QOL is unexpectedly small.

Key words: Quality of life; Gastrectomy; Weight loss; Postgastrectomy syndrome assessment scale-45

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Core tip: Our study of almost 1800 gastrectomy patients revealed that higher preoperative body mass index, total gastrectomy, and female sex were independent predictors of greater body weight loss after gastrectomy. Moreover, we determined lower postoperative body mass index, rather than greater postoperative weight loss, as a certain factor of worse quality of life (QOL), although the effect was not substantial. We believe that this contribution is theoretically and practically relevant in the current context of gastric cancer treatment and recovery because early diagnosis and improved treatments have led to increased long-term survival postgastrectomy, highlighting the need for better QOL.

Tanabe K, Takahashi M, Urushihara T, Nakamura Y, Yamada M, Lee SW, Tanaka S, Miki A, Ikeda M, Nakada K. Predictive factors for body weight loss and its impact on quality of life following gastrectomy. *World J Gastroenterol* 2017; 23(26): 4823-4830 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i26/4823.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i26.4823

INTRODUCTION

Despite its gradually decreasing incidence, gastric cancer remains the second leading cause of cancer death in the world[1]. Surgical resection and regional lymphadenectomy are the only curative options for patients with localized gastric tumors[2-4]. As early diagnosis and improved treatment have led to longer-term survival, patients are now more aware of the morbidities associated with gastrectomy, which is called postgastrectomy syndrome. Indeed, the gastrectomized patients may experience various nutritional and functional problems that interfere with their quality of life (QOL)[5-7]. Loss of body weight is a common complaint after gastrectomy, and is thought as one of few objective indices to measure the well-being of postgastrectomy patients. Some reports suggest that the type of gastrectomy is a certain predictor of postoperative weight loss[6,8,9], however, other predictive factors for postoperative weight loss has yet not been determined. Though the low body mass index (BMI) as well as body weight loss is often identified after gastrectomy and may affects the QOL after gastrectomy[10], their detail implication on the QOL has not been clarified.

The aim of the present study was to determine the predictive factors for postoperative weight loss and to investigate the impact of body weight loss and low BMI on the QOL in patients after gastrectomy using the Postgastrectomy Syndrome Assessment Scale (PGSAS)-45, which was established specifically to assess symptoms, living status and QOL among patients after gastrectomy[11].

MATERIALS AND METHODS

The PGSAS study, a surveillance study involving 52 institutions, was conducted by the Japanese Post-gastrectomy Syndrome Working Party (JPGSWP) and approved by the institutional review boards of all participating institutions. After completion of the informed consent process, patients were enrolled in this study if they met the following eligibility criteria: 20-75 years of age, histologically proven Stage I gastric cancer based on the 13th edition of the Japanese

Table 1 Characteristics of patients after conventional gastrectomy

Sex [male: *n* (%)]	1188 (66.9)
Age (yr, mean ± SD)	62.1 ± 9.2
Type of gastrectomy (*n*: TGRY/DGBI/DGRY)	393/909/475
Period after gastrectomy (mo: mean ± SD)	37.0 ± 26.8
Change in body weight (%, mean ± SD)	-9.5 ± 8.0
Preoperative BMI (kg/m^2, mean ± SD)	22.8 ± 3.1
Postoperative BMI (kg/m^2, mean ± SD)	20.6 ± 2.8
Approach (*n*, open/laparoscopic)	1102 ± 664
Preservation of celiac branch of vagus (Y/N)	173/1567

BMI: Body mass index.

classification of gastric carcinoma[12], curative resection at least 1 year after surgery, no signs of recurrence at the point of assessment, and no other active malignancy.

The PGSAS-45 questionnaire consists of 45 questions, with 8 items from the Short Form-8 (SF-8)[13], 15 items from the Gastrointestinal Symptom Rating Scale[14], and 22 clinically important items determined by the JPGSWP. Patients were given the questionnaire together with a stamped and addressed envelope in the outpatient clinic and were asked to complete questionnaire and return it by post to the data center. Of the 2922 patients to whom questionnaires were given during July 2009 to December 2010, 2520 (86%) responded and 2368 (81%) were confirmed to be eligible for the original study. Of these, the data from 1777 patients who underwent total gastrectomy with Roux-en-Y (TGRY) and distal gastrectomy with Billroth-I (DGBI) or Roux-en-Y (DGRY) were analyzed in this study.

Statistical analysis

The degree of body weight loss was compared among the three relevant preoperative BMI groups (BMI, < 18.5, 18.5-25 and > 25 kg/m^2) by multiple comparisons. Multiple regression analysis was performed to determine the factors affecting body weight loss after surgery, and to study the impact of the change in body weight and postoperative BMI on QOL. A P value of < 0.05 was considered to indicate statistical significance. To evaluate effect sizes, Cohen's d, standardization coefficient of regression (β) and coefficient of determination (R^2) were used. Interpretation of effect sizes were ⩾ 0.2 small, ⩾ 0.5 medium, and ⩾ 0.8 large in Cohen's d; ⩾ 0.1 small, ⩾ 0.3 medium, and ⩾ 0.5 large in β; ⩾ 0.02 small, ⩾ 0.13 medium, and ⩾ 0.26 large in R^2. All statistical analyses were performed by biostatisticians who primarily used Stat View for Windows Ver. 5.0 (SAS Institute Inc., Cary, NC, United States).

RESULTS

Patient characteristics

A CONSORT flowchart of the PGSAS study is shown in Figure 1. A total of 1777 patients (1188 men; 66.9%) who underwent conventional gastrectomy were enrolled in this study. The mean age of patients was 62.1 ± 9.2 years. The numbers of patients undergoing each operative procedure were as follows: TGRY, 393; DGRY, 475; and DGBI, 909. The mean time interval between surgery and retrieval of the questionnaires was 37.0 ± 26.8 mo, and the mean body weight loss among postgastrectomy patients was 9.5% ± 8.0% at that time (Table 1).

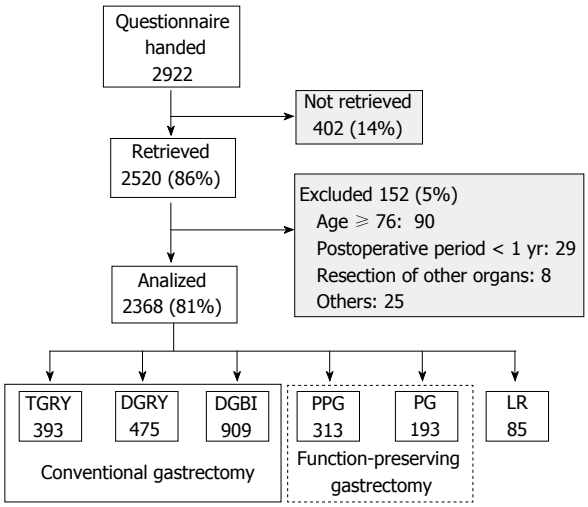

Figure 1 CONSORT flowchart of the Postgastrectomy Syndrome Assessment Study (PGSAS study). TGRY: Total gastrectomy with Roux-en-Y reconstruction; DGRY: Distal gastrectomy with Roux-en-Y reconstruction; DGBI: Distal gastrectomy with Billroth-I reconstruction; PPG: Pylorus-preserving gastrectomy; PG: Proximal gastrectomy; LR: Local resection.

QOL measures in the PGSAS-45

The PGSAS-45 is an integrated questionnaire for assessing the symptoms, the living status and the QOL in patients after gastrectomy, as described previously[11]. The structure of the PGSAS-45 is shown in Table 2. QOL scores in the PGSAS-45 were obtained for two subdomains: dissatisfaction and the SF-8 items. The dissatisfaction subdomain consists of four outcome measures based on symptoms (item 43), meals (item 44), working (item 45), and daily life subscale (mean of the item 43-45). The SF-8 consists of eight items and generates two summary measures, the physical component summary and the mental component summary. The mean values of main outcome measures are shown in Table 3.

Factors affecting postoperative weight loss

To clarify the predictive factors affecting change in body weight after surgery, multiple regression analysis was performed. In order of significance, higher preoperative BMI, type of gastrectomy (TGRY) and female sex were the independent predictors for postoperative weight loss (Table 4).

Relationship between preoperative BMI and change in body weight

Considering that preoperative BMI was the most

Table 2 Structure of postgastrectomy syndrome assessment scale-45 (domains/subdomains/items/subscales)

Domains	Subdomains		Items	Subscales
QOL	SF-8 (QOL)	1	Physical functioning[1]	Physical component summary[1] (item 1-8)
		2	Role physical[1]	Mental component summary[1] (item 1-8)
		3	Bodily pain[1]	
		4	General health[1]	
		5	Vitality[1]	
		6	Social functioning[1]	
		7	Role emotional[1]	
		8	Mental health[1]	
Symptoms	GSRS (Symptoms)	9	Abdominal pains	Esophageal reflux subscale (item 10, 11, 13, 24)
		10	Heartburn	Abdominal pain subscale (item 9, 12, 28)
		11	Acid regurgitation	Meal-related distress subscale (item 25-27)
		12	Sucking sensations in the epigastrium	Indigestion subscale (item 14-17)
		13	Nausea and vomiting	Diarrhea subscale (item 19, 20, 22)
		14	Borborygmus	Constipation subscale (item 18, 21, 23)
		15	Abdominal distension	Dumping subscale (item 30, 31, 33)
		16	Nausea and vomiting	
		17	Increased flatus	Total symptom scale (above seven subscales)
		18	Decreased passage of stools	
		19	Increased passage of stools	
		20	Loose stools	
		21	Hard stools	
		22	Urgent need for defecation	
		23	Feeling of incomplete evacuation	
	Symptoms	24	Bile regurgitation	
		25	Sense of foods sticking	
		26	Postprandial fullness	
		27	Early satiation	
		28	Lower abdominal pains	
		29	Number and type of early dumping symptoms	
		30	Early dumping general symptoms	
		31	Early dumping abdominal symptoms	
		32	Number and type of late dumping symptoms	
		33	Late dumping symptoms	
Living status	Meals (amount) 1	34	Ingested amount of food per meal[1]	
		35	Ingested amount of food per day[1]	
		36	Frequency of main meals	
		37	Frequency of additional meals	
	Meals (quality)	38	Appetite[1]	Quality of ingestion subscale[1] (item 38-40)
		39	Hunger feeling[1]	
		40	Satiety feeling[1]	
	Meals (amount) 2	41	Necessity for additional meals	
	Social activity	42	Ability for working	
QOL	Dissatisfaction (QOL)	43	Dissatisfaction with symptoms	Dissatisfaction for daily life subscale (item 43-45)
		44	Dissatisfaction at the meals	
		45	Dissatisfaction at working	

[1]Higher scores indicate better conditions. Each subscales is calculated as the mean of its composite items or subscales, except the physical and mental component summaries of SF-8. Items 29 and 32 do not have scores; these were analyzed separately. PGSAS-45: Postgastrectomy syndrome assessment scale-45; SF-8: Short form-8; QOL: Quality of life; GSRS: Gastrointestinal symptom rating scale.

Table 3 Main outcome measures of postgastrectomy syndrome assessment scale-45 quality of life domain in patients after conventional gastrectomy (n = 1777)

Subdomains	Item in PGSAS-45	Main outcomes measures	Scale	mean ± SD
Dissatisfaction	43	Dissatisfaction with symptoms	Five-point Likert scale	1.87 ± 0.95
	44	Dissatisfaction at the meals		1.13
	45	Dissatisfaction at working		1.79 ± 0.97
	43-45	Dissatisfaction for daily life subscale		0.87
SF-8	1-8	Physical component summary[1]	Five or six-point Likert scale	50.4 ± 5.6
	1-8	Mental component summary[1]		49.7 ± 5.8

[1]Higher score indicating better condition. Integrated subscales (SS) are underlined in the Table. PGSAS-45: Postgastrectomy syndrome assessment scale-45; SF-8: Short form-8.

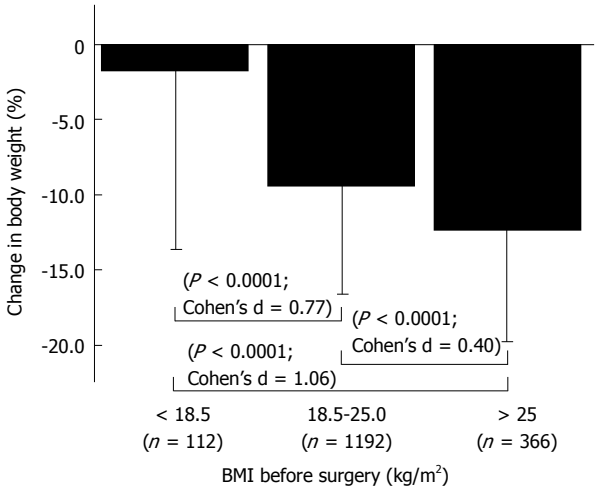

Figure 2 Preoperative body mass index strongly influences change in body weight postoperatively. Bars represent the mean change in body weight (mean ± SD); effect size for group difference are reported as Cohen's *d* ($P < 0.0001$). BMI: Body mass index.

influential factor affecting weight loss postoperatively, we compared the degree of weight loss among three relevant preoperative BMI groups: < 18.5; 18.5-25; and 25 < (kg/m^2) (Figure 2). There was a significant difference between each group ($P < 0.0001$) with a certain effect size in terms of Cohen's d. The patients with higher BMI (> 25 kg/m^2) exhibited the greatest weight loss (12.3%) among the groups, while the degree of weight loss in patients with lower BMI < (18.5) was spare (2%).

Impact of change in body weight and postoperative BMI on QOL

Finally, we performed multiple regression analysis to compare the influence on postoperative QOL between body weight loss and low postoperative BMI (Tables 5 and 6). The low postoperative BMI significantly affected on all QOL outcome measures with small but clinically meaningful effect size in terms of standardized partial regression coefficient (β), while the body weight loss only affected on some of QOL outcome measures with smaller effect size in β (approximately of half value compared to that of postoperative BMI). In addition, coefficient of determination R^2, which indicates the aggregated impact of body weight loss and low postoperative BMI on the QOL, were relatively small for each QOL outcome measures.

DISCUSSION

This study identified the causal factors affecting body weight loss after gastrectomy and investigated the impact of body weight loss on the postoperative QOL using the PGSAS-45 questionnaire, which was recently developed to assess the QOL following gastrectomy. Our results identified higher preoperative BMI as the most influential factor affecting postoperative weight loss, followed by the type of gastrectomy performed (TGRY) and female sex, in order of significance. Moreover, the patients with higher BMI (> 25 kg/m^2) preoperatively exhibited the largest postoperative weight loss among three relevant preoperative BMI groups. The patients with low postoperative BMI experienced worse QOL than those with greater body weight loss, though the aggregated impact of low BMI and excess body weight loss on the QOL postoperatively was relatively smaller than generally considered.

Loss of body weight after gastrectomy is thought to be caused by multiple factors, including decreased serum ghrelin[15], reduced food intake due to various abdominal symptoms, and disorder of digestive and absorptive function due to pancreatic exocrine insufficiency or postcibal pancreaticobilliary asynchrony. The degree of weight loss was also affected by the type of gastrectomy employed[15-19]. Additionally, body weight loss is also related to tumor progression or chemotherapy after surgery. In this study, we focused on Stage Ⅰ patients in order to exclude the influence of other factors that may influence the postoperative body weight, and to isolate the effect of the surgical procedures. The findings of present study that patients undergoing TGRY had a greater body weight loss compared to those undergoing DGBI or DGRY were compatible with the previous reports[19,20]. However, the influence of the other surgical procedures such as laparoscopic approach or preservation of celiac branch of vagus, which maintains the postprandial motility of the duodenum and jejunum[21] and attenuates a dumping syndrome[22], were insignificant as for effect size, β.

Recent analyzes of specific disease processes,

Table 4 Factors influencing body weight loss after gastrectomy (multiple regression analysis)

Variables	Change in body weight	
	β	*P* value
Type of gastrectomy (DGBI)	0.204	< 0.0001
Type of gastrectomy (DGRY)	0.116	< 0.0001
Postoperative period (mo)	(-0.02)	NS
Age (yr)	(-0.04)	0.0746
Gender (male)	0.120	< 0.0001
Preoperative BMI (kg/m^2)	-0.356[1]	< 0.0001
Approach (Laparoscopic)	(0.01)	NS
Celiac branch of vagus (Preserved)	(0.074)	0.0010
R^2 (*P* value)	0.216	< 0.0001
The interpretation of effect size	β	R^2
None-very small	< (0.100)	< (0.020)
Small	> 0.100	> 0.020
Medium	> 0.300[1]	> 0.130[1]
Large	> 0.500	> 0.260

[1]Integrated subscales. Higher score indicative of a better condition. If β is positive, the score of the outcome measure of the patients belonging to the category in (brackets) is higher in cases when the factor is a nominal scale, and the score of outcome measure of the patients with larger values is higher in cases when the factor is a numeral scale. DGBI: Distal gastrectomy with Billroth-I; DGRY: Distal gastrectomy with Roux-en-Y.

Table 5 Impact of postoperative lower body mass index and body weight loss on the quality of life (multiple regression analysis)

Variables	Ability for working		Dissatisfaction with symptoms		Dissatisfaction at the meals		Dissatisfaction at working		Dissatisfaction for daily life subscale		PCS		MCS	
	β	*P* value	β	*P* value	β	*P* value	β	*P* value	β	*P* value	β	*P* value	β	*P* value
Postoperative BMI (kg/m^2)	-0.134	< 0.0001	-0.189	< 0.0001	0.216	< 0.0001	-0.185	< 0.0001	-0.231	< 0.0001	0.148	< 0.0001	0.109	< 0.0001
Change in body weight (%)	(-0.081)	0.0018	(-0.073)	0.0040	-0.112	< 0.001	(-0.097)	<0.0001	-0.109	< 0.0001	(0.047)	0.066	(0.025)	NS
R^2 (*P* value)	0.031	< 0.0001	0.048	< 0.0001	0.073	< 0.001	0.054	< 0.0001	0.080	< 0.0001	0.028	< 0.0001	(0.014)	< 0.0001

BMI: Body mass index; QOL: Quality of life; PCS: Physical component summary; MCS: Mental component summary.

Table 6 Regression analysis of effect size

The interpretation of effect size	β	R^2
None-very small	< (0.100)	< (0.020)
Small	> 0.100	> 0.020
Medium	> 0.300	> 0.130
Large	> 0.500	> 0.260

including sarcopenia and metabolic diseases, have identified the importance of evaluating not only BMI but also body component composition, such as body fat and skeletal muscle[23-26]. Siervo *et al*[27] also reported that body composition varies with BMI, age and sex. Although a significant reduction in body fat has been reported after gastrectomy, several studies indicated that the reduction in skeletal muscle mass was smaller than reductions in the volume of body fat[28-31]. These previous findings may, in part, explain the smaller body weight loss in patients with low BMI (< 18.5), in which, the proportion of the skeletal muscle supposed to be larger than those of the other relevant preoperative BMI groups.

Body weight loss is considered to be one of the objective index which resulting in worse QOL after gastrectomy[5,8,32,33], and also loss of body weight is associated with intolerance to adjuvant chemotherapy[34]. However, in clinical setting, excess body weight loss is not always accompanied with worse QOL, therefore, precise features of the impact of body weight loss on the postoperative QOL should be investigated. For this purpose, we studied the impact of body weight loss as well as postoperative BMI on the postgastrectomy QOL using the PGSAS-45 questionnaire, which is the first questionnaire developed to specifically measure QOL in gastrectomized patients[11,35-38], by multiple regression analysis. The results of our study demonstrated that the preoperative BMI rather than the degree of body weight loss was the most influential predictor of worse QOL after gastrectomy. The low postoperative BMI significantly affected on all QOL outcome measures, though the body weight loss only affected few QOL outcome measures with smaller effect size in terms of β. The aggregated impact of low BMI and body weight loss was unexpectedly small for each QOL outcome measures in terms of R^2. There may be other factors influencing worse QOL postgastrectomy, and future work should focus on investigation of other possible factors.

Despite above mentioned results, both to maintain postoperative body weight and to avoid low BMI seem yet important for better QOL after gastrectomy, therefore, enhanced perioperative nutritional management should be required particularly in patients with low preoperative BMI.

Several limitations of our study should be acknowledged. This study was not a prospective study and the investigation was performed at a single point in time postoperatively. We focused on long-term QOL, more than 1 year after gastrectomy based on previous findings that most QOL measures are stable at > 1 year postoperatively[39]. However, such QOL measurements at a single point in time may be insufficient to reflect the true impact of body weight loss. Further prospective and chronological studies assessing QOL over short- and longer-term periods after gastrectomy are required.

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COMMENTS

Background

Body weight loss, a common complaint after gastrectomy, is likely associated with various factors such as tumor progression and chemotherapy. While several reports indicated that the type of gastrectomy may be a determinant of postoperative weight loss, other risk factors have yet to be determined. In the present study, they focused only on patients with Stage I gastric cancer, so as to evaluate the impact of the surgical procedure without the confounding effect of other factors.

Research frontiers

Previous reports indicated that the type of gastrectomy is a certain postoperative weight loss, suggesting that total gastrectomy resulted in greater weight loss. Additionally, patients with excess weight loss after gastrectomy were shown to have lower performance status and difficulty in continuing chemotherapy. However, few reports have analyzed the relationship between postgastrectomy body weight loss and quality of life (QOL).

Innovations and breakthroughs

The authors aimed to determine the predictive factors and clarify the quality-of-life impact of postgastrectomy body weight loss and low body mass index. For this purpose, the authors used the postgastrectomy syndrome assessment scale-45, which was established specifically to evaluate QOL following

gastrectomy. Interestingly, the authors found that postoperative body mass index, rather than the degree of weight loss, was a predictor of worse QOL after gastrectomy, but the effect was relatively mild.

Applications

To minimize the negative effects on QOL after gastrectomy, it is better to maintain the postoperative body weight and avoid low body mass index. Postgastrectomy syndrome is a group of disorders and complications following gastrectomy. It includes early/late dumping syndrome, reflux gastritis, diarrhea, anemia, malabsorption, reflux gastritis, and weight loss.

Peer-review

The authors have conducted a well-written observational study. The case enrollment and variable choices were appropriate. Despite this study has the limit that QOL measures are conducted only at a single point after surgery, it has some new insights.

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ORIGINAL ARTICLE

Prospective Study

Divergent expression of bacterial wall sensing Toll-like receptors 2 and 4 in colorectal cancer

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Author contributions: Mäkelä J and Karttunen TJ contributed equally to this work; Paarnio K, Mäkelä J and Karttunen TJ designed the research; Paarnio K and Väyrynen S analyzed the tissue samples; Klintrup K recruited the patients and organized the collection of the tissue samples; Ohtonen P and Paarnio K analyzed the data; Paarnio K, Mäkinen MJ, Karttunen TJ and Mäkelä J wrote the paper.

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Abstract

AIM

To characterize the expression of toll-like receptors (TLR) 2 and 4 in colorectal cancer (CRC) and in normal colorectal mucosa.

METHODS

We analysed tissue samples from a prospective series of 118 unselected surgically treated patients with CRC. Sections from formalin fixed, paraffin embedded specimens were analysed for TLR2 and TLR4 expression by immunohistochemistry. Two independent assessors evaluated separately expression at the normal mucosa, at the invasive front and the bulk of the carcinoma, and in the lymph node metastases when present. Expression levels in different locations were compared and their associations with clinicopathological features including TNM-stage and the grade of the tumour and 5-year follow-up observations were analysed.

RESULTS

Normal colorectal epithelium showed a gradient of expression of both TLR2 and TLR4 with low levels in the crypt bases and high levels in the surface. In CRC, expression of both TLRs was present in all cases and in the major proportion of tumour cells. Compared to normal epithelium, TLR4 expression was significantly weaker but TLR2 expression stronger in carcinoma cells. Weak TLR4 expression in the invasive front was associated with distant metastases and worse cancer-specific survival at 5 years. In tumours of the proximal colon the cancer-specific survival at 5 years was 36.9% better with strong TLR4 expression as compared with those with weak expression (P = 0.044). In contrast, TLR2 expression levels were not associated with prognosis. Tumour cells in the lymph node metastases showed higher TLR4 expression and lower TLR2 expression than cells in primary tumours.

CONCLUSION

Tumour cells in CRC show downregulation of TLR4 and upregulation of TLR2. Low expression of TLR4 in the invasive front predicts poor prognosis and metastatic disease.

Key words: Colorectal Cancer; Toll-like receptor 2; Toll-like receptor 4; Inflammation; Prognosis

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Core tip: Toll-like receptor 4 (TLR4) is downregulated in colorectal cancer (CRC), suggesting a role as a tumour suppressor. Low expression of TLR4 in the invasive front marks poor prognosis and is associated with metastatic disease. The most significant finding was the association between weak TLR4 expression in the tumour front and 36.9% (P = 0.044) lower cancer-specific survival in cancer of the proximal colon as compared with strong TLR4 expression. Weak TLR4 staining in tumour front was present in 50% (11/22) of the cases with metastases and only in 17% (16/94) of those without metastases (P = 0.001). TLR2 is upregulated in CRC but not associated with prognosis.

Paarnio K, Väyrynen S, Klintrup K, Ohtonen P, Mäkinen MJ, Mäkelä J, Karttunen TJ. Divergent expression of bacterial wall sensing Toll-like receptors 2 and 4 in colorectal cancer. *World J Gastroenterol* 2017; 23(26): 4831-4838 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i26/4831.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i26.4831

INTRODUCTION

Colorectal cancer (CRC) is the third most common cancer in both Europe[1] and the United States[2]. It evolves through stepwise accumulation of genetic mutations and the effects of environmental factors. Recent research has focused on the significance of tumour-associated inflammation in the development and progression of carcinoma. Inflammation and immunity are related to the pathogenesis of cancer by several mechanisms, including alterations in the intestinal microbiome, oncogene activation-induced immune reaction, and a role of local inflammation in tumour progression[3]. An elevated systemic inflammatory response is associated with poor outcome[4], but local inflammation at the invasive front of the tumour is linked to better survival[5].

Toll-like receptors (TLRs) are a family of receptors with a significant role in defence against pathogens. They recognize microorganisms, and ligand binding to TLRs initiates signalling cascades leading to the inflammatory response[6]. TLR4 detects lipopolysaccharide from Gram-negative bacteria[7], and previous studies concerning its role in CRC are controversial[8-10]. TLR2 recognizes several bacterial, fungal, and viral proteins[11], and in CRC, higher TLR2 expression has been found compared to normal mucosa but with no link to prognosis[8]. Both are expressed in normal colorectal epithelium and in immune system cells[12].

We have characterized TLR2 and TLR4 tissue expression in a series of colorectal carcinomas and their metastases, evaluating tumour properties that affect expression and the influence of expression on prognosis. Our hypothesis was that TLR2 and TLR4 expression levels would change with tumour progression and show an association with prognosis.

MATERIALS AND METHODS

Patients

A case series consisting of patients with CRC was collected prospectively between April 2006 and January 2010 at Oulu University Hospital. The prospective study (Kantola *et al*[13]) was explained to all newly diagnosed CRC patients who underwent surgery in our hospital during that time (n = 344). A total of 149 patients who were both eligible for the study and had signed informed consent to participate were included in the study. The Regional Ethical Committee of North Ostrobothnia Hospital District has accepted both the original study design and the follow-up study (58/2005, 184/2009, 60/2012). The study was conducted in collaboration between the Department of Surgery and the Department of Pathology.

The clinical details and follow-up information for the patients were obtained from clinical records and information about time and cause of death from Statistics Finland. The preoperative staging of CRC was made by whole body computed tomography scan and local staging for rectal cancer by magnetic resonance imaging scan. The patients with T3 or T4 rectal cancer received preoperative neoadjuvant radiation or chemoradiation therapy (n = 31) and were excluded from the final analysis, leaving a total of 118 patients. The histological features of the tumours were obtained from pathological records. The classifications

used were TNM6[14] for staging and the WHO 2010 classification[15] for grading. In addition, serrated adenocarcinomas were diagnosed according to WHO 2010 criteria, as described in more detail[15-17]. All these histopathological analyses were performed by experienced pathologist.

We defined disease-free survival as the time interval between the primary operation and detection of recurrence. Disease-free survival analysis was based on the data for patients whose primary operation was curative and who did not die for a cause other than CRC during the 5-year follow-up period (83 patients).

Immunohistochemistry

Immunohistochemistry was performed on formalin fixed, paraffin-embedded tissue sections. Tissue microarrays were conducted using the primary tumour specimens[18], and a human kidney tissue specimen was included in the multi-tissue block as a positive control for TLR2 staining[19]. Macrophages served as positive control for TLR4 staining. Samples representing the normal mucosa and lymph node metastases were stained and analysed separately.

For antigen retrieval, sections were treated at a high temperature in Tris-EDTA buffer for 15 min. Immunostaining was performed with Dako Autostainer (Dako Copenhagen, Denmark) using mouse monoclonal antibodies (Abnova MAB0066 Clone 1030A5.138 for TLR2 and Abnova H00007099-M02 Clone 3B6 for TLR4; Abnova, Taoyuan City, Taiwan), at dilutions of 1:50 and 1:1000, respectively. For detection, we used Dako Envision kit (Dako) and diaminobenzidine (Dako basic DAB-kit) as a chromogen. For negative controls, we omitted the primary antibody and replaced the primary antibody with a mouse primary antibody isotype control.

Assessment of TLR2 and -4 expression

The two independent researchers (Paarnio K and Väyrynen S) analysed the expression of TLR2 and TLR4 with a close guidance by an experienced gastrointestinal pathologist (Karttunen TJ). The assessors were blinded to the clinical data and the results of assessment of other patient specimens, such as normal mucosa or metastases. We assessed separately the intensity and extent of the staining. Carcinoma cells at the invasive front and the bulk of the primary tumour, lymph node metastases if present, and epithelial cells in the normal mucosa were separately assessed. The intensity of the staining was assigned using a four-point scale [negative (0), weak (1), moderate (2), and strong (3)], and the extent of staining was assigned as a percentage of positive cells (0%-100%). If there was more than one step difference in the intensity score or over 30% difference in the percentages between the two assessors, a consensus was reached after joint re-evaluation[20]. Otherwise, we used the mean values of the two assessors. Histoscore (0-300) for tissue samples was obtained by multiplying the intensity score by the percentage of positive cells.

Statistical analysis

The statistical methods of this study were reviewed by biostatistician Pasi Ohtonen, MSc, Division of Operative Care, Oulu University Hospital. Summary statistics are presented as medians with 25th-75th percentiles unless stated otherwise. The χ^2 test or Fisher's exact test was used to analyse categorical data, the Student's *t*-test for continuous data, and the paired samples *t*-test to compare mean differences between different sample sites. The Cox proportional hazards model was used to estimate the impact of TLR2 and TLR4 on 5-year survival. In the Cox model, age and sex, along with tumour size, location (distal/proximal), and stage (0-Ⅱ/Ⅲ-Ⅳ) were used as adjusting factors and left in the model only if their $P < 0.05$ or the impact on the -2*Log Likelihood function was significant. Interaction terms with TLR2 and TLR4 were calculated and found to be non-significant. HRs with 95%CIs are presented as results of the Cox models. Two-tailed *P*-values are presented. Analyses were performed using SPSS for Windows (Released 2012. IBM SPSS Statistics for Windows, Version 21.0. IBM Corp., Armonk, NY, United States).

RESULTS

TLR2 and TLR4 expression in normal colorectal mucosa and in CRC

Patient characteristics and tumour features are presented in Tables 1 and 2. In the normal mucosa, the expression of TLR2 and -4 was cytoplasmic and present in all epithelial cells and in lymphocytes of the lamina propria (Figure 1). There was a constant gradient of the expression of both, with the crypt base cells showing weaker expression than cells in the upper parts of the crypts and surface.

In carcinomas, both TLR2 and TLR4 showed cytoplasmic expression (Figure 2), and the expression was present in all cases. In the majority of the cases, the expression was present in most tumour cells.

TLR2 expression was increased in carcinoma cells compared with normal colon epithelium (Table 3). Expression did not differ between the tumour bulk and the front, but levels were lower in lymph node metastases than in the primary tumours.

The TLR4 expression pattern was distinct from that of TLR2 and showed the highest levels in the normal mucosa; in cancer, the tumour bulk had lower TLR4 expression levels than the tumour front and lymph node metastases (Table 3).

The comparison between TLR2 and TLR4 expression levels is shown in Table 4. The intensity of staining did not differ in the invasive front, but TLR2 staining was significantly stronger in the tumour bulk, as was TLR4 staining in both the lymph node metastasis and the normal mucosa.

Weak intensity (less than 2) of TLR4 staining in

Table 1 Characteristics of 118 patients with colorectal carcinoma *n* (%)

	Whole study group	Survival in 5-yr follow-up
Gender		
Male	56 (47.5)	35 (62.5)
Female	62 (52.5)	36 (58.1)
Age, median (range)	69 (36-89)	
Other morbidities		
No	27 (22.9)	16 (59.3)
Yes	91 (77.1)	55 (60.4)
Other neoplasm		
No	109 (92.4)	62 (56.9)
Sex organs or breast cancer	2 (1.7)	2 (100.0)
Other cancer	3 (2.5)	3 (100.0)
Colon adenoma	4 (3.4)	4 (100.0)
Cancer in family		
No/not known	104 (88.1)	59 (56.7)
CRC	8 (6.8)	7 (87.5)
HNPCC family	2 (1.7)	2 (100.0)
Other HNPCC-associated cancer	3 (2.5)	2 (66.7)
Other cancer	1 (0.8)	1 (100.0)
Type of operation		
Radical[1]	94 (80.3)	69 (73.4)
Palliative[2]	23 (19.7)	2 (8.7)
Location of tumour		
Proximal colon	46 (39.0)	27 (58.7)
Distal colon	39 (33.1)	25 (64.1)
Rectum	32 (27.1)	18 (56.3)
Multiple tumours	1 (0.8)	1 (100.0)

[1]Radically operated distant metastases in a second operation in one case; [2]Metastases treated non-operatively in two cases (both alive at 5-year follow-up).

Table 2 Tumour features *n* (%)

	Whole study group	Survival in 5-yr follow-up
Stage		
Ⅰ	18 (15.3)	15 (83.3)
Ⅱ	48 (40.7)	37 (77.1)
Ⅲ	30 (25.4)	16 (53.3)
Ⅳ	22 (18.6)	3 (13.6)
Grade		
Ⅰ	17 (14.4)	13 (76.5)
Ⅱ	86 (72.9)	53 (61.6)
Ⅲ	14 (11.9)	5 (35.7)
Data missing	1 (0.8)	0 (0)
Lymph node metastasis		
No	72 (61.0)	53 (73.6)
Yes	46 (39.0)	18 (39.1)
Distant metastasis		
No	96 (81.4)	68 (70.8)
Yes	22 (18.6)	3 (13.6)
Mucinous carcinoma		
No	99 (83.9)	59 (59.6)
Yes	10 (8.5)	6 (60.0)
Data missing	9 (7.6)	

the invasive front was associated with metastasis and higher TNM stage. Weak staining was present in 50% (11/22) of the cases with metastases, 17% (16/94) of those without metastases (P = 0.001), 23.5% (4/17) in stage Ⅰ, 19.1% (9/47) in stage Ⅱ, 10.0% (3/30) in stage Ⅲ, and 50.0% (11/22) in stage Ⅳ (P = 0.009). Weak staining in lymph node metastases also was associated with advanced disease: weak intensity was present in 0% (0/1) of lymph node metastases in stage Ⅱ, 7.4% (2/27) in stage Ⅲ, and 40.0% (6/15) in stage Ⅳ (P = 0.028).

TLR2 and TLR4 expression was similar in conventional and serrated carcinomas (data not shown). There also was no association with the presence of a mucinous component. However, tumours with a low WHO grade showed a tendency to strong TLR2 expression in the tumour front and lymph node metastases. In the invasive front, TLR2 expression was strong in 80% of grade Ⅰ, 87.2% of grade 2, and 64.3% of grade 3 tumours (P = 0.078); in the lymph node metastases, strong TLR2 expression was seen in 100% of grade 1, 63.6% of grade 2, and 25% of grade 3 tumours (P = 0.063).

TLR2 and TLR4 expression and survival

To assess the effect of TLR2 and TLR4 expression on overall survival, cancer-specific survival, and disease-free survival, we grouped cases according to the intensity of staining into two categories: weak staining, with an intensity score less than 2, and strong staining, with an intensity score of 2 or higher.

The most significant finding was the association between strong TLR4 expression in the tumour front and 36.9% (P = 0.044) better cancer-specific survival with cancer of the proximal colon compared with weak TLR4 expression (Table 5). Strong TLR4 expression in the invasive front also was linked to 14.8% (62.9% *vs* 48.1%, P = 0.19) better overall survival compared with weak expression; in the tumour bulk, the difference in survival was 18.4% (65.8% *vs* 47.4%, P = 0.071). The same tendency was seen even more distinctly in the proximal colon: strong expression in both the front and bulk was linked to 28.3% (64.7% *vs* 36.4%, P = 0.16) better survival; in lymph node metastases, the difference in survival was 50% (50% *vs* 0%, P = 0.23).

In a stage-adjusted Cox model, strong TLR4 expression showed a trend to association with a better prognosis; in the tumour front, the HR for overall survival was 1.8 (P = 0.074, 95%CI: 0.9-3.3), and in the tumour bulk, it was 1.7 (P = 0.059, 95%CI: 1.0-3.1). There was no significant association between TLR expression and disease-free survival.

TLR2 expression did not associate with overall, cancer-specific, or disease-free survival. In the tumour front, weak TLR2 expression showed some non-significant trend to an association with better disease-free survival than strong expression (92.3% *vs* 75%, P = 0.28); for expression in lymph node metastases, the difference was even higher (66.7% *vs* 36.8%, P = 0.23). Similarly, there was a tendency to a better outcome with weak TLR2 expression in lymph node metastasis in the cancer of the distal colon or rectum; the overall survival was 30.6% (60% *vs* 29.4%, P =

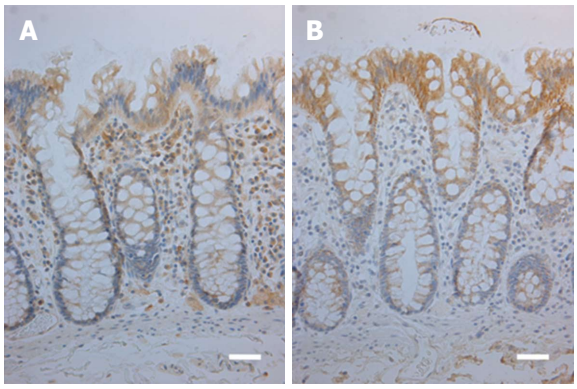

Figure 1 Immunohistochemical stainings for toll-like receptor 2 (A) and toll-like receptor 4 (B) in normal colorectal mucosa. Weak TLR2 expression is present in the deeper parts of the crypt epithelium and moderate expression in uppermost part of the crypts and the surface (A). TLR4 expression shows a similar gradient, however, with a moderate expression in the lower parts of the crypts and strong in the surface (B). Reference lines, 50 μm. TLR: Toll-like receptor.

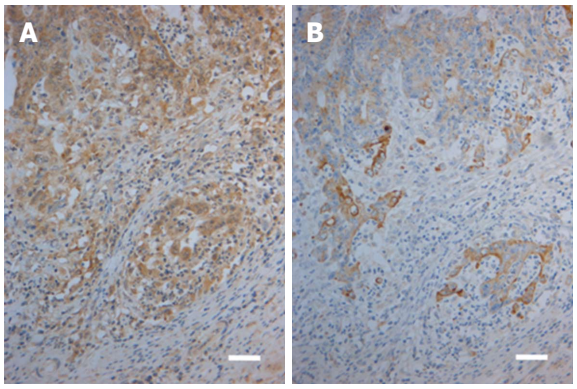

Figure 2 Immunohistochemical stainings for toll-like receptor 2 (A) and toll-like receptor 4 (B) in a case of colorectal carcinoma. Moderate to strong TLR2 expression in the carcinoma cells is present in both the invasive front (lower part of the Figure) and in the tumor bulk (upper part of the Figure; A). In contrast, corresponding view showing TLR4 staining indicates that expression is moderate to weak in the front (lower part) and weak to negative in the bulk (upper part; B). Reference lines, 50 μm. TLR: Toll-like receptor.

Table 3 Mean intensities and histoscores of immunostaining

	TLR2 intensity, mean ± SD	*P* value	TLR2 histoscore, mean ± SD	*P* value	TLR4 intensity, mean ± SD	*P* value	TLR4 histoscore, mean ± SD	*P* value
Invasive front	2.3 ± 0.6	< 0.001	224 ± 67	< 0.001	2.2 ± 0.6	< 0.001	223 ± 66	0.024
Normal mucosa	1.4 ± 0.7		129 ± 72		2.6 ± 0.6		243 ± 77	
Invasive front	2.2 ± 0.7	0.006	210 ± 77	0.054	2.2 ± 0.6	0.060	216 ± 68	0.048
Lymph node	1.8 ± 0.7		180 ± 72		2.4 ± 0.7		241 ± 70	
Tumour bulk	2.3 ± 0.6	< 0.001	224 ± 65	< 0.001	2.0 ± 0.7	< 0.001	197 ± 73	< 0.001
Normal mucosa	1.4 ± 0.7		129 ± 72		2.6 ± 0.6		244 ± 78	
Tumour bulk	2.2 ± 0.6	0.007	216 ± 65	0.011	2.0 ± 0.6	0.001	194 ± 69	0.001
Lymph node	1.8 ± 0.7		180 ± 72		2.4 ± 0.7		241 ± 70	
Lymph node	1.8 ± 0.7	0.011	179 ± 70	0.003	2.4 ± 0.7	0.017	238 ± 70	0.40
Normal mucosa	1.4 ± 0.7		127 ± 71		2.7 ± 0.5		251 ± 72	
Invasive front	2.3 ± 0.6	0.55	225 ± 66	> 0.9	2.3 ± 0.6	< 0.001	223 ± 66	< 0.001
Tumour bulk	2.3 ± 0.6		225 ± 64		2.0 ± 0.7		197 ± 73	

Mean values vary due to missing samples. TLR: Toll-like receptor.

Table 4 Comparison of mean intensities of immunostainings between toll-like receptors 2 and 4

	Intensity, mean ± SD	*P* value	Histoscore, mean ± SD	*P* value	*n*
Tumour bulk					
TLR2	2.3 ± 0.6	< 0.001	225 ± 64	< 0.001	117
TLR4	2.0 ± 0.7		198 ± 73		117
Invasive front					
TLR2	2.3 ± 0.6	0.55	225 ± 66	0.83	116
TLR4	2.3 ± 0.6		223 ± 66		116
Lymph node					
TLR2	1.8 ± 0.7	< 0.001	180 ± 71	< 0.001	43
TLR4	2.4 ± 0.7		241 ± 70		43
Normal mucosa					
TLR2	1.4 ± 0.7	< 0.001	129 ± 72	< 0.001	114
TLR4	2.6 ± 0.6		243 ± 79		114

N values vary due to missing samples. TLR: Toll-like receptor.

0.22), and cancer-specific survival was 24.7% (60% *vs* 35.3%, *P* = 0.26) higher than with strong TLR2 expression.

DISCUSSION

In this study, we found that weak TLR4 expression in the invasive front was associated with poor prognosis in CRC, linked to distant metastases and worse cancer-specific survival. Loss of TLR4 in carcinomas in comparison to normal mucosa supports the idea of a potential tumour suppressor role for the protein. In contrast, TLR2 expression was increased in carcinomas independent of stage, which is in agreement with previous reports[8]. The association with weak TLR2 expression in lymph nodes and better prognosis did not reach significance at the 5% level, even though 30.6% better overall survival seems clinically notable for subgroup analysis; however, a bigger sample size would be needed to make any conclusions. Considering that both TLR2 and TLR4 receptors activate the same cascade[21], TLR2 upregulation and TLR4 downregulation during progression from normal mucosa to carcinoma indicate the occurrence of a distinct set of alterations in the innate immune response during the development

Table 5 5-year cancer-specific survival in percent (number of cases) *vs* intensity of staining in the tumour bulk, invasive front, and lymph node metastases *n* (%)

		Intensity of staining	Whole colon	*P* value[1]	Proximal colon	*P* valu[1]	Distal colon and rectum	*P* value[1]
TLR2	Tumour bulk	Weak	14 (63.6)	0.43	4 (66.7)	0.65	9 (60.0)	0.33
		Strong	71 (74.7)		29 (74.4)		42 (75.0)	
	Invasive front	Weak	14 (73.7)	> 0.9	6 (66.7)	0.68	8 (80.0)	0.71
		Strong	70 (72.2)		27 (75.0)		42 (70.0)	
	Lymph node	Weak	9 (50.0)	0.55	3 (37.5)	> 0.9	6 (60.0)	0.26
		Strong	10 (40.0)		3 (42.9)		6 (35.3)	
TLR4	Tumour bulk	Weak	25 (65.8)	0.27	7 (63.6)	0.45	18 (66.7)	0.59
		Strong	60 (75.9)		26 (76.5)		33 (75.0)	
	Invasive front	Weak	17 (63.0)	0.33	5 (45.5)	0.044	12 (75.0)	> 0.9
		Strong	67 (75.3)		28 (82.4)		38 (70.4)	
	Lymph node	Weak	2 (25.0)	0.27	0 (0.0)	0.23	2 (40.0)	> 0.9
		Strong	17 (48.6)		6 (50.0)		10 (45.5)	

[1]χ^2 test/Fisher's exact test. N values vary due to missing samples; One case with multiple tumours excluded from location-based analysis; Weak intensity < 2 and strong intensity ⩾ 2.

of CRC.

TLR4 expression was significantly higher in normal mucosa compared to any cancer samples. Reports on inflammatory bowel disease-associated cancers have yielded contrasting results, and higher TLR4 expression in CRC compared to normal mucosa has been found[9,22]. The increased TLR4 expression compared to normal tissue also has been reported in sporadic CRC[23], and another study demonstrated a correlation between high expression of TLR4 and advanced disease[10]. Yet results also have been published showing that the loss of TLR4 correlates with increased metastatic status[24], and TLR4 expression by tumour cells has been significantly associated with a lower rate of tumour recurrence[25]. Nihon-Yanagi *et al*[8] showed equally weak TLR4 expression in both cancerous and noncancerous tissue in general, but higher expression in the cancer of the proximal colon.

In addition to variable detection methods used, the differences in the analysis schemes are likely explanations for the conflicting results. Previous studies have not examined expression separately in the tumour bulk and invasive front, which we considered to be crucial because tumour infiltration and progression occur in the invasive front. Furthermore, the peritumoural immune reaction and tumour budding at the invasive front have a profound effect on survival in CRC. Functional polymorphisms of TLR2 and -4 show association with the risk and features of CRC, such as differentiation, advanced stage, lymph node status, and metastasis, and also affect expression levels[26,27]. Accordingly, occurrence of TLR polymorphisms may contribute to contradictory results of expression analyses and warrant further studies.

In normal colorectal mucosa in the current work, gradual accumulation of TLR2 and TLR4 were associated with the location of the epithelial cells, with the highest expression in the surface. In addition to possibly being linked to maturation of epithelial cells, this gradient might reflect the abundance of ligands originating from the luminal flora in this region. Also in CRCs, there was a distinct variation in expression in specific tumour compartments. We found that TLR4 expression in the invasive front was significantly stronger than in the tumour bulk and that lymph node metastases expressed TLR4 more strongly than either the bulk or the front. These findings suggest that areas of tumour with active invasion have higher TLR4 expression, which we speculate might be related to induction by endogenous ligands released from dead or damaged cells and matrix[28].

Again, TLR2 was different, showing similarity between the tumour bulk and the invasive front, and lymph node metastases showed less intensive expression than the primary lesion. However, our findings suggest (*P* = NS) that if TLR2 expression in lymph node metastases is strong, the prognosis weakens. Because intratumoural or intranodal differences in the supply of microbiological ligands to these TLRs do not likely differ, the observed location-related differences might be related to other factors regulating TLR expression, like mediators from the cells in the microenvironment, including endogenous TLR ligands released along with invasion[29]. We speculate that the sum effect of these factors is different in terms of expression of TLR4 and TLR2. Our finding of increased TLR4 expression and decreased TLR2 expression in metastatic carcinoma cells suggests either some selection based on TLR expression levels during the metastatic cascade or that regulatory signals from the lymph node parenchyma cells have opposite effects on TLR2 and TLR4 expression.

High TLR4 expression in the invasive front was related to better prognosis. In the proximal colon, the benefit in terms of cancer-specific survival was 36.9% (*P* = 0.044), and in overall survival, it was 28.3% (*P* = 0.16). Tumour bulk showed the same pattern, and in lymph node metastasis, the overall survival benefit was as high as 50%, but likely because of our small sample size, these clinically highly notable differences

did not reach statistical significance. In line with our findings, Simiantonaki *et al*[24] showed that a loss of TLR4 correlated with the presence of metastases, and Eiró *et al*[25] found that TLR4 expression was associated with a lower rate of recurrence.

Mechanisms linking high TLR4 expression with better survival and rarity of metastases remain speculative. Our earlier studies[30] have shown that high-grade peritumoural inflammation is associated with good prognosis, and this finding has also been used in Klintrup-Mäkinen grading; thus, it would be plausible that stronger TLR4 expression would contribute to a stronger inflammatory cell response, eventually protecting against cancer progression. Simiantonaki *et al*[24] suggested that during CRC development, immune-mediated signals downregulate the level of TLR4 expression and that epithelial cells consequently do not respond to lipopolysaccharide with an inflammatory reaction. Thus, the lack of immune response in the tumour would lead to tumour progression. Additionally, they suggested that the loss of TLR4 is linked to the lack of an anti-tumoural immune response[24]. Considering the complexity of the relationship among inflammation, immunity, and cancer, it is not surprising that other studies have reported contrasting findings. In the tumour microenvironment, the inflammatory component may either destroy neoplastic cells or potentiate tumour progression depending on the molecular combinations and expression levels.

Both TRL2 and TLR4 recognize bacteria, and TLR2 also fungal and viral proteins[7,11]. Recent findings suggest, that gut microbes may play a role in CRC development[31], but a lot of questions remain, and need further investigation. When it comes to viruses, a recent meta-analysis supports the association of cytomegalovirus infection with colorectal tumour formation[32]. Supporting possible role of Cytomegalovirus, levels of Cytomegalovirus protein were reported to correlate with those of TLR2 and TLR4 in CRC, and Cytomegaloovirus infection in a colorectal carcinoma cell line induced TLR2 production[33].

In conclusion, our study shows that TLR2 is upregulated and TLR4 downregulated in CRC. Low expression of TLR4 at the invasive front associates with short survival, but for TLR2, possible value as prognostic markers could not be established. Further studies with larger study groups are needed to clarify the role of these receptors in the development and prognosis of CRC.

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COMMENTS

Background

Colorectal cancer (CRC) is a very common disease all over the world. Peritumoural inflammation is linked to CRC development and prognosis. Toll-like receptor 2 (TLR2) and TLR4 are receptors detecting bacteria and activating the signalling cascade leading to inflammation response.

Research frontiers

The previous knowledge of the role of TLR2 and TLR4 in CRC was scarce or conflicting. However, there were some results suggesting TLR2 upregulation in CRC, but the studies concerning the role of TLR4 in CRC were controversial.

Innovations and breakthroughs

This study shows TLR4 downregulation and TLR2 upregulation in CRC, and low expression of TLR4 in the invasive front of the tumour predicts poor prognosis and metastatic disease.

Applications

These findings give new information of the role of TLR2 and TLR4 in CRC development and prognosis, and can serve as a ground for further studies aiming for better understanding of the factors affecting the course of CRC and more effective and individualized treatment strategies.

Peer-review

In this study the expression of TLR2 and TLR4 in colorectal carcinoma have been investigated. It is observed that TLR4 expression was significantly weaker but TLR2 expression stronger in carcinoma cells when compared to normal mucosa. Moreover, down-regulation of TLR4 in the invasive front of CRC predicted poor prognosis and metastatic disease. Basically this is a well-written study of an interesting topic.

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ORIGINAL ARTICLE

Prospective Study

Non-invasive assessment of liver fibrosis using two-dimensional shear wave elastography in patients with autoimmune liver diseases

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Abstract

AIM

To determine the diagnostic accuracy of two-dimensional shear wave elastography (2D-SWE) for the non-invasive assessment of liver fibrosis in patients with autoimmune liver diseases (AILD) using liver biopsy as the reference standard.

METHODS

Patients with AILD who underwent liver biopsy and 2D-SWE were consecutively enrolled. Receiver operating characteristic (ROC) curves were constructed to assess the overall accuracy and to identify optimal cut-off values.

RESULTS

The characteristics of the diagnostic performance were determined for 114 patients with AILD. The areas under the ROC curves for significant fibrosis, severe fibrosis, and cirrhosis were 0.85, 0.85, and 0.86, respectively, and the optimal cut-off values associated with significant fibrosis (≥ F2), severe fibrosis (≥ F3), and cirrhosis (F4) were 9.7 kPa, 13.2 kPa and 16.3 kPa, respectively. 2D-SWE showed sensitivity values of 81.7% for significant fibrosis, 83.0% for severe fibrosis,

and 87.0% for cirrhosis, and the respective specificity values were 81.3%, 74.6%, and 80.2%. The overall concordance rate of the liver stiffness measurements obtained using 2D-SWE *vs* fibrosis stages was 53.5%.

CONCLUSION

2D-SWE showed promising diagnostic performance for assessing liver fibrosis stages and exhibited high cut-off values in patients with AILD. Low overall concordance rate was observed in the liver stiffness measurements obtained using 2D-SWE *vs* fibrosis stages.

Key words: Autoimmune liver disease; Liver fibrosis; Two-dimensional shear wave elastography; Ultrasound; Liver stiffness

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Core tip: The study determined the diagnostic accuracy of two-dimensional shear wave elastography (2D-SWE) for the non-invasive assessment of liver fibrosis in patients with autoimmune liver diseases (AILD) using liver biopsy as the reference standard. The characteristics of the diagnostic performance were determined for 114 patients with AILD. The areas under the receiver operating characteristic curves for significant fibrosis, severe fibrosis, and cirrhosis were 0.85, 0.85 and 0.86, respectively, and the optimal cut-off values were 9.7 kPa, 13.2 kPa and 16.3 kPa, respectively. 2D-SWE showed promising diagnostic performance in assessing liver fibrosis stages and exhibited high cut-off values in patients with AILD.

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INTRODUCTION

Autoimmune liver diseases (AILD) are a group of diseases characterized by an anomalous immune response that is directed at hepatocytes or bile ducts along with the presence of serum antimitochondrial antibodies and a potential tendency to progress to cirrhosis. Autoimmune hepatitis (AIH), primary biliary cholangitis (PBC), and primary sclerosing cholangitis (PSC) are the three major forms of AILD, which differ according to the focus of the autoimmune injury, the pattern of inflammation, and the clinical phenotype[1,2]. These diseases are important because they may result in chronic liver damage with fibrosis and cirrhosis. An evaluation of the degree of liver fibrosis is important for determining medical management and prognosis. Liver biopsy (LB) is still considered the reference standard for the evaluation of liver fibrosis[3]. However, LB is an invasive procedure, limiting its use for screening and frequent follow-up. The demand for a non-invasive and reliable test to evaluate liver fibrosis in patients with AILD has increased.

Two-dimensional shear wave elastography (2D-SWE), an ultrasound elastography technique based on shear waves that is available on a clinical diagnostic ultrasound scanner, has been used to non-invasively measure liver fibrosis[4]. The 2D-SWE technique creates a real-time, two-dimensional quantitative map of liver tissue stiffness under the guidance of very-high-frame-rate B mode imaging[5]. This method has good diagnostic accuracy for the staging of liver fibrosis in patients with chronic liver diseases[6-11]. However, studies on the use of 2D-SWE to assess histologically confirmed liver fibrosis in patients with AILD have not been published. AILD are considered a relatively uncommon etiology in the Asia-Pacific region, where viral hepatitis is the primary diagnosis in the majority of patients with chronic liver diseases. However, based on recent findings, the prevalence of both AIH and PBC is increasing worldwide[12]. Despite advances in the understanding and treatment of AILD, areas of unmet need remain.

Therefore, the goal of this study was to assess the diagnostic accuracy of 2D-SWE for the non-invasive staging of liver fibrosis in patients with AILD. LB samples that were scored with the histology-based METAVIR staging system were used as the diagnostic reference standard.

MATERIALS AND METHODS

Study design and participants

Informed consent was obtained from all patients, and the study including the 2D-SWE examination was approved by the clinical medical research ethics committee of our hospital. One hundred and thirty-nine patients with AILD who underwent LB and 2D-SWE examinations were enrolled consecutively between April 2011 and March 2016. The diagnoses of AIH, PBC and PSC in all patients were confirmed by histological evidence[13-18]. All patients were not under immunosuppressive treatment at time of the LB and 2D-SWE examinations. The time intervals between LB and 2D-SWE were less than three days. The exclusion criteria included: patients younger than 18 years; a lack of consent for the 2D-SWE examination or LB; chronic liver disease accompanied by hepatitis virus infection or another disease; and LB samples less than 15 mm long or with fewer than 6 portal tracts under the microscopic examination. The following data were collected from all patients: liver stiffness measurements (LSM) obtained using 2D-SWE; fibrosis stages; necroinflammatory activity grades; age; gender; weight; height; alanine aminotransferase, aspartate aminotransferase, serum alkaline phosphatase (ALP), gamma-glutamyl transpeptidase, total bilirubin, and serum albumin con-

centrations; platelet count; and prothrombin activity. Body mass index was calculated as body weight (kg)/[height (m^2)].

2D-SWE

2D-SWE was performed within three days of LB. Two radiologists (Zeng J and Zheng J) performed the 2D-SWE examinations. Both radiologists had at least 6 mo of experience in performing 2D-SWE examinations. The radiologists were blinded to the patients' clinical information and pathology results. 2D-SWE was performed using the Aixplorer US system (SuperSonic Imagine, France) with a convex broadband probe (SC6-1, 1-6 MHz). All patients had fasted for at least 6 h prior to the examination. 2D-SWE measurements were obtained from the right lobe of the liver through the intercostal spaces when the patient was lying in the supine position and the right arm was positioned in maximal abduction. The operator positioned the target area of the liver under the guidance of conventional, real-time B-mode imaging. When the target area was located, SWE was launched, and the patient was asked to hold his or her breath for approximately 5 s during a quiet breathing period. The elasticity image box, which was approximately 4 cm × 3 cm, was set 1-2 cm deeper than Glisson's capsule of the liver and in an area of the liver parenchyma that was free of large vessels.

A circular region of interest with a 2-cm diameter was drawn inside the elasticity image box, and the mean, minimum and maximum liver stiffness and SD were calculated (Figure 1). The mean value was used to represent the LSM for each 2D-SWE image. Five consecutive 2D-SWE images were obtained for each patient. Each measurement was performed during a separate breath hold. The mean value of the five 2D SWE measurements was calculated for statistical analysis. The entire 2D-SWE examination lasted three to five minutes for each patient. Five consecutive 2D-SWE images were obtained from each patient. Measurements were considered failures when no or little signal was obtained in the SWE box for any of the acquisitions[8].

Analysis of liver histology

Ultrasonography-assisted percutaneous LB was performed in the right liver lobe using a 16-gauge Magnum needle (Bard, Tempe, AZ). The LB specimens were fixed in formalin and embedded in paraffin. The biopsy specimens were analyzed by two expert liver pathologists with more than 10 and 20 years of experience, respectively, and who were blinded to the results of 2D-SWE but not to the clinical and biochemical data for each patient. Liver fibrosis and necroinflammatory activity were semiquantitatively evaluated using the METAVIR scoring system[19]. Liver fibrosis was staged on a scale from 0 to 4 according to the METAVIR scoring system: stage F0: no fibrosis; F1: portal fibrosis without septa; F2: portal fibrosis and few septa; F3: numerous septa without cirrhosis; and F4: cirrhosis[19]. Significant fibrosis was defined as stage F2 or higher, and severe fibrosis was defined as stage F3 or higher. Necroinflammatory activity was graded as follows: A0 = none; A1 = mild; A2 = moderate; and A3 = severe[19].

Statistical analysis

The demographic, clinical, and laboratory values are summarized using descriptive statistics. The data were first tested for normality using the one-sample Kolmogorov-Smirnov test. Spearman's rank coefficient was used to determine the correlation between two study variables. Receiver operating characteristic (ROC) curves were constructed to assess the overall accuracy of the LSMs and to identify optimal cut-off values. Areas under the ROC curves (AUROCs) were compared using the method described by DeLong *et al*[20] for correlated data. The optimal cut-off values were the points with the highest Youden's index[21]. The sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) were calculated from the same data. The positive likelihood ratio (LR+) and negative likelihood ratio (LR-) were calculated from the respective sensitivity and specificity values. All factors collected from patients with AILD were entered in a multivariate logistic model to analyze the disagreement between LSMs obtained using 2D-SWE and significant fibrosis or cirrhosis. ORs were estimated from the model and are presented with their 95%CIs. Continuous variables, such as age, were dichotomized around the median (46 years), unless a cut-off was considered clinically relevant.

All statistical tests were two-sided, and the alpha value was set at 0.05. Statistical analyses were performed using SPSS software for Windows, version 13.0 (SPSS, Chicago, IL, United States), and MedCalc software, version 12.7.0 (MedCalc Software, Mariakerke, Belgium).

RESULTS

Patients

One hundred and thirty-nine patients were enrolled in the study. Twenty-two patients were not included based on the exclusion criteria, including 2 patients who were younger than 18 years, 14 patients with biopsy samples less than 15 mm long or with fewer than six portal tracts under the microscope, 1 patient with a hepatitis A virus coinfection, 3 patients with hepatitis B virus coinfections, and 2 patients with hepatitis C virus coinfections. Therefore, 117 patients with AILD defined by a reliable reference standard were consecutively enrolled in the study. The 2D-SWE examination failed in three patients. Therefore, 114 patients with reliable LSMs obtained using 2D-SWE were used to assess diagnostic accuracy. The characteristics of the 114 patients are summarized in Table 1. Fibrosis conditions that were scored as significant fibrosis (≥ F2), severe fibrosis (≥ F3),

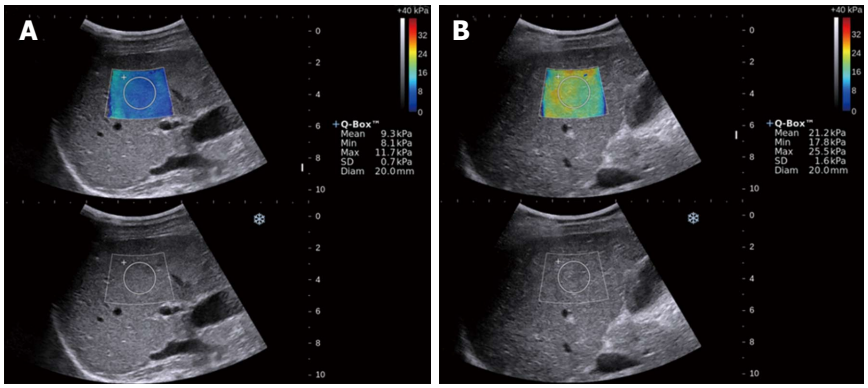

Figure 1 Two-dimensional shear wave elastography measurements in the liver. A: Fibrosis stage F1; B: Fibrosis stage F4.

Table 1 Demographic data, blood tests and histological results in patients with autoimmune liver diseases *n* (%)

Characteristic	*n* = 114
Age, yr (SD; range)	45.6 (12.5; 18-74)
Gender, male, *n* (%)	21 (18.4)
BMI, kg/m^2 (SD; range)	21.6 (3.0; 15.4-36.4)
AST, IU/L (IQR; range)	84.0 (51.3-148.0;16.0-473.0)
ALT, IU/L (IQR; range)	78.5 (49.5-142.0; 9.0-920.0)
Alkaline phosphatase, IU/L (IQR; range)	208 (110.0-353.0; 47.0-873.0)
Gamma-glutamyl transferase, IU/L (IQR; range)	252.0 (111.0-523.8; 16.0-1535.0)
Total bilirubin, umol/L (IQR; range)	20.3 (13.6-43.6; 3.6-375.8)
Serum albumin, g/L (SD; range)	37.6 (5.0; 25.9-47.0)
Platelets count, 10^9/L ((SD; range)	188.2 (69.5; 28.0-414.0)
Prothrombin activity, % (SD; range)	106.6 (22.6; 50.0-158.0)
METAVIR fibrosis stage[1]	
F0	4 (0.04)
F1	28 (24.6)
F2	35 (30.7)
F3	24 (21.1)
F4	23 (20.2)
METAVIR activity grade[2]	
A0	2 (0.02)
A1	17 (14.9)
A2	48 (42.1)
A3	47(41.2)

[1]F0, no fibrosis; F1, portal fibrosis without septa; F2, portal fibrosis and few septa; F3, numerous septa without cirrhosis; F4, cirrhosis; [2]A0, none; A1, mild; A2, moderate; A3, severe. IQR: Inter quartile range; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; BMI: Body mass index.

and cirrhosis (F4) were detected in 71.9%, 41.2%, and 20.2% of patients, respectively. The numbers of patients with different fibrosis stages of AILDs are reported in Table 2.

Liver stiffness measured by 2D-SWE at each stage of fibrosis

The median values, interquartile ranges, ranges, and *P* values of the measurements obtained for each fibrosis stage with 2D-SWE are shown in Table 3. As the fibrosis stage progressed, the median LSM of the fibrosis stage increased on 2D-SWE (Figure 2). The LSMs of patients

Table 2 Number of patients with autoimmune liver diseases at different fibrosis stages

	F0-F1 (*n*)	F2 (*n*)	F3 (*n*)	F4 (*n*)
AIH	19	18	9	16
PBC	8	10	8	4
PSC	1	1	0	1
PBC-AIH	4	6	7	2
Total	32	35	24	23

AIH: Autoimmune hepatitis; PBC: Primary biliary cholangitis; PSC: Primary sclerosing cholangitis; PBC-AIH: PBC-AIH overlap syndrome.

Table 3 Median values, inter quartile range, ranges, and *P* values of measurements obtained for each fibrosis stage using two-dimensional shear wave elastography

METAVIR stage	F0-F1	F2	F3	F4
Median value, kPa	7.7	11.7	14.7	22.0
IQR	6.5-9.2	8.3-15.4	12.3-22.3	18.8-33.3
Range	4.9-25.8	4.6-51.7	8.7-67.2	10.1-45.3
P value[1]		< 0.001	0.009	0.011

[1]*P* values refer to differences between consecutive fibrosis stages. IQR: Inter quartile range.

by 2D-SWE at a given fibrosis stage had significantly higher median LSMs obtained using 2D-SWE than patients with less fibrosis ($P < 0.05$). Spearman's correlation coefficients for LSMs and fibrosis stages were 0.68 ($P < 0.001$) (95%CI: 0.57-0.77).

Diagnostic performance of 2D-SWE for predicting significant fibrosis, severe fibrosis and cirrhosis

The AUROCs of 2D-SWE for significant fibrosis, severe fibrosis, and cirrhosis were 0.85 (95%CI: 0.77-0.91), 0.85 (95%CI: 0.77-0.92), and 0.86 (95%CI: 0.78-0.92), respectively (Figure 3). The optimal cut-off values for the different stages of fibrosis were determined by analyzing the ROCs. The optimal cut-off values associated with significant fibrosis (≥ F2), severe fibrosis (≥ F3), and cirrhosis (F4) were 9.7 kPa, 13.2 kPa and 16.3 kPa, respectively.

Table 4 Performance characteristics of two-dimensional shear wave elastography for staging liver fibrosis in patients with autoimmune liver diseases

	Cutoff, kPa	Se, % (95%CI)	Sp, % (95%CI)	PPV, % (95%CI)	NPV, % (95%CI)	LR+, ratio (95%CI)	LR-, ratio (95%CI)
≥ F2	9.7[1]	81.7 (71.6-89.4)	81.3 (63.6-92.8)	91.8 (83.0-96.9)	63.4 (46.9-77.9)	4.4 (3.6-5.3)	0.23 (0.10-0.5)
	7.2[2]	93.9 (86.3-98.0)	40.6 (23.7-59.4)	80.2 (70.8-87.6)	72.2 (46.5-90.3)	1.6 (1.0-2.4)	0.15 (0.06-0.4)
	7.1[3]	93.9 (86.3-98.0)	37.5 (21.1-56.3)	79.4 (70.0-86.9)	70.6 (44.0-89.7)	1.5 (1.0-2.4)	0.16 (0.07-0.4)
≥ F3	13.2[1]	83.0 (69.2-92.4)	74.6 (62.5-84.5)	69.6 (55.8-81.3)	86.2 (74.6-93.9)	3.3 (2.7-4.0)	0.23 (0.1-0.5)
	9.1[2]	95.7 (85.5-9.5)	52.2 (39.7-64.6)	58.4 (46.6-69.6)	94.6 (81.5-99.4)	2.0 (1.6-2.5)	0.08 (0.02-0.3)
	8.7[3]	97.9 (88.7-99.9)	49.3 (36.8-61.8)	57.5 (45.9-68.5)	97.1 (84.7-99.9)	1.9 (1.5-2.5)	0.04 (0.01-0.3)
F4	16.3[1]	87.0 (66.4-97.2)	80.2 (70.6-87.8)	52.6 (35.6-69.2)	96.1 (88.9-99.2)	4.4 (3.6-5.3)	0.16 (0.05-0.5)
	11.7[2]	95.7 (78.1-99.9)	57.1 (46.3-67.5)	36.1 (24.2-49.4)	98.1 (89.8-100.0)	2.2 (1.8-2.7)	0.08 (0.01-0.5)
	10.4[3]	95.7 (78.1-99.9)	48.4 (37.7-59.1)	31.9 (21.2-44.2)	97.8 (88.2-99.9)	1.9 (1.5-2.3)	0.09 (0.01-0.6)

[1]Cut-off values in patients with autoimmune liver diseases; [2]Cut-off values in patients with chronic hepatitis B; [3]Cut-off values in patients with chronic hepatitis C. Se: Sensitivity; Sp: Specificity; PPV: Positive predictive value; NPV: Negative predictive value; LR+: Positive likelihood ratio; LR-: Negative likelihood ratio.

Table 5 Analysis of concordance of liver stiffness measurements obtained using two-dimensional shear wave elastography *vs* METAVIR stages

	METAVIR stage					Concordance rate
	F0-F1	F2	F3	F4	Total	
F0-F1, ≤ 9.7kPa	26	13	2	0	41	63.4%
F2, > 9.7 to ≤ 13.2 kPa	3	7	4	2	16	43.8%
F3, > 13.2 to ≤ 16.3 kPa	1	9	8	1	19	42.1%
F4, > 16.3 kPa	2	6	10	20	38	52.6%
Cumulative concordance						53.5%

The sensitivity, specificity, PPV, NPV, LR+, and LR- for each METAVIR stage are shown in Table 4. Using the optimal cut-off values, 2D-SWE showed sensitivity and specificity values of 81.7% and 81.3% for significant fibrosis, 83.0% and 74.6% for severe fibrosis, and 87.0% and 80.2% for cirrhosis, respectively. The corresponding optimal cut-off values for patients with chronic hepatitis B (CHB) were 7.2 kPa, 9.1 kPa and 11.7 kPa, and the corresponding values for patients with chronic hepatitis C (CHC) were 7.1 kPa, 8.7 kPa and 10.4 kPa, respectively[8,22]. Using the cut-off values for CHB and CHC, the sensitivity values for assessing the stages of fibrosis were greater than 90%. However, trade-offs were observed: the accompanying specificity values for predicting significant fibrosis were less than 50%, and the accompanying specificity values for predicting severe fibrosis and cirrhosis fibrosis were approximately 50%.

Concordance rates of the LSMs obtained using 2D-SWE vs METAVIR stages

Table 5 shows the concordance rates of the LSMs obtained using 2D-SWE *vs* METAVIR stages. Overall, 2D-SWE correctly classified 61 of 114 (53.5%) patients. 2D-SWE returned the highest rates of correctly classified patients at the F0-1 stage at 63.4%. 2D-SWE showed lower rates of correctly classified patients at other stages, particularly the F2 and F3 stages. The concordance rates of the F2 and F3 stages were less than 50%.

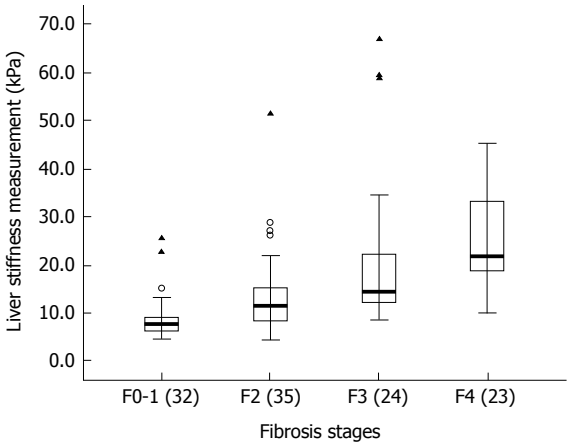

Figure 2 Liver stiffness measurements obtained using two-dimensional shear wave elastography in patients with autoimmune liver diseases. The box plots show the interquartile ranges m (boxes), medians (thick lines), ranges (thin lines), outside values (circles), and outliers (triangles).

Analysis of factors associated with the disagreement between LSMs obtained using 2D-SWE and significant fibrosis or cirrhosis

According to the multivariate logistic regression analysis, the disagreement between LSMs obtained using 2D-SWE and cirrhosis was independently associated with the following factors: age greater than 46 years (OR = 7.5, 95%CI: 1.7-33.3, P = 0.008), abnormal ALP levels (OR = 14.0, 95%CI: 1.5-129.1, P = 0.02) and abnormal serum albumin levels (OR = 11.6, 95%CI: 3.2-42.0, P < 0.001). No factors were significantly associated with the disagreement between LSMs obtained using 2D-SWE and significant fibrosis.

DISCUSSION

This study included a cohort of patients with AILD and aimed to evaluate the diagnostic accuracy of 2D-SWE for the non-invasive staging of hepatic fibrosis using LB as the reference standard. AILD are divided into two groups; the first group is predominantly characterized by hepatocellular damage and its prototype is

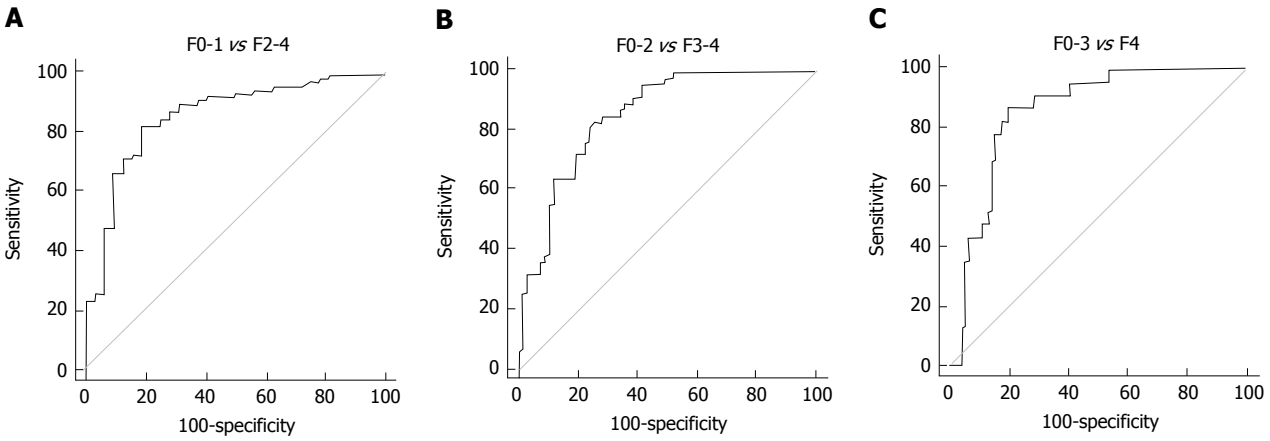

Figure 3 Area under the receiver operating characteristic curves for two-dimensional shear wave elastography in assessing liver fibrosis in patients with autoimmune liver diseases. A: Significant fibrosis (≥ F2); B: Severe fibrosis (≥ F3); C: Cirrhosis (F4).

AIH. The second group is characterized by cholestasis and includes PBC and PSC[23]. AIH, PBC and PSC represent complex disorders, as they result from interactions between genetic and environmental factors. In AIH, autoimmune injury affects hepatocytes, leading to the histological manifestation of interface hepatitis. In PBC, autoimmune injury affects the small, interlobular bile ducts, causing the typical appearance of non-suppurative, destructive cholangitis. In PSC, autoimmune or immune-mediated injury affects the medium-sized intra- and extrahepatic bile ducts, causing concentric and obliterative fibrosis and multifocal bile duct structuring[1]. However, AIH, PBC and PSC share pathophysiologic mechanisms[24,25]. Overlap syndromes encompass a small group of patients within the spectrum of AILD that may have characteristics of cholestasis (PBC or PSC) in combination with AIH[17]. In our study, 62 patients were diagnosed with AIH and 30 patients diagnosed with PBC. Overlap syndromes of AIH and PBC were diagnosed in 19 (16.7%) of 114 patients. Therefore, we used the patients with AILD as the study cohort.

Although 2D-SWE has been widely recognized as a reliable method to assess liver fibrosis in recent years, the diagnostic performance of 2D-SWE for assessing liver fibrosis stages in patients with AILD remains unclear. The 2D-SWE examinations performed in our study were successful in all but 3 of the 117 patients. We concluded that 2D-SWE provides a very high percentage of interpretable test results. Our results regarding the rate of successful 2D-SWE measurement in patients with AILD were similar to those reported in a previous study using 2D-SWE in patients with CHB[22].

Previous studies reported the diagnostic accuracy of 2D-SWE for the detection of fibrosis stage in patients with CHB or CHC when the histology-based METAVIR staging system was used as the diagnostic reference standard[8,11]. The METAVIR staging system is simple and practical and is widely used for liver fibrosis staging in patients with chronic liver diseases. In our study, we also used the METAVIR staging system as the diagnostic reference standard. AUROCs for the identification of significant fibrosis and cirrhosis were approximately 0.85 and 0.86, respectively. Stage F2 or greater indicates the beginning of progressive liver disease and therefore suggests a stronger indication to initiate treatment; thus, 2D-SWE may serve as a screening tool to differentiate patients with significant fibrosis from patients without. Cirrhosis is the end stage of chronic liver disease. 2D-SWE may also be used to differentiate patients with cirrhosis from those without. According to previous studies, AUROCs of 2D-SWE for predicting significant fibrosis, severe fibrosis, and cirrhosis were 0.88, 0.93 and 0.98 in patients with CHB and 0.92, 0.98 and 0.98 in patients with CHC, respectively[8,11]. In our study, moderate diagnostic performance for assessing liver fibrosis stages was observed in patients with AILD, with AUROCs of approximately 0.85. The AUROCs in patients with AILD were lower than those in patients with CHB or CHC. In our series, 2D-SWE correctly classified 53.5% of patients with AILD. The concordance rates were also lower for 2D-SWE *vs* METAVIR stages in patients with CHB or CHC[8,22].

The optimal cut-off values associated with significant fibrosis, severe fibrosis and cirrhosis in patients with AILD determined by the ROC analysis were 9.7 kPa, 13.2 kPa and 16.3 kPa, respectively. The corresponding optimal cut-off values in patients with CHB were 7.2 kPa, 9.1 kPa and 11.7 kPa, and the corresponding values for patients with CHC were 7.1 kPa, 8.7 kPa and 10.4 kPa, respectively[8,22]. Thus, the optimal cut-off values in patients with AILD were markedly higher than the values in patients with CHB or CHC. Using the optimal cut-off values calculated from the ROC curves in our study, 2D-SWE showed sensitivity values of greater than 80% and the corresponding specificity values were approximately 80%. Using the cut-off values for CHB or CHC, the sensitivity values for assessing the stages of fibrosis were greater than 90%, but the accompanying specificity values

were very low. Fibrosis stages would be overestimated using the cut-off values for CHB or CHC in patients with AILD. Therefore, the cut-off values for CHB and CHC were not appropriate for patients with AILD. The lower diagnostic accuracy and higher cut-off values might be characteristics of 2D-SWE for assessing liver fibrosis in patients with AILD, and differences in patient populations and etiology might explain the differences in these results.

This study has certain limitations that warrant discussion. First, our study used patients with AILD as its cohort. Patients with AIH, PBC and PSC were not separately analyzed. Second, the distribution of patients across all fibrosis stages was not uniform, as a very low percentage of enrolled patients were at stage F0. Third, the LB did not meet the American Society for the Study of Liver Diseases criteria of being at least 2-3 cm in length with at least 11 portal tracts[26]; also, agreement between the readers of the liver biopsies was not assessed in our study. Fourth, although other ultrasound techniques have been used to evaluate liver fibrosis, such as transient elastography (FibroScan; Echosens) and acoustic radiation force impulse (ARFI; Siemens Healthcare), we only evaluated 2D-SWE.

In conclusion, 2D-SWE showed promising diagnostic performance for assessing the liver fibrosis stages and exhibited high cut-off values in patients with AILD; however, the concordance rates of the LSMs obtained using 2D-SWE *vs* METAVIR stages were low. Our study opens possibilities for a non-invasive assessment of fibrosis progression in patients suffering from ALID.

COMMENTS

Background

Autoimmune liver diseases (AILD) are a group of diseases characterized by an anomalous immune response that is directed at hepatocytes or bile ducts along with the presence of serum antimitochondrial antibodies and a potential tendency to progress to cirrhosis. These diseases are important because they may result in chronic liver damage with fibrosis and cirrhosis. An evaluation of the degree of liver fibrosis is important for determining medical management and prognosis. The demand for a non-invasive and reliable test to evaluate liver fibrosis in patients with AILD has increased.

Research frontiers

Two-dimensional shear wave elastography (2D-SWE) has been used to non-invasively measure liver fibrosis. This method has good diagnostic accuracy for the staging of liver fibrosis in patients with chronic liver diseases. However, studies on the use of 2D-SWE to assess histologically confirmed liver fibrosis in patients with AILD have not been published.

Innovations and breakthroughs

2D-SWE showed promising diagnostic performance for assessing liver fibrosis stages and exhibited high cut-off values in patients with AILD; however, the concordance rates of the liver stiffness measurements obtained using 2D-SWE *vs* METAVIR stages were low.

Applications

2D-SWE is beneficial for assessing liver fibrosis stages in patients with AILD as this method is non-invasive and reproducible in the clinical setting. The optimal cut-off values are high but appropriate for patients with AILD.

Terminology

2D-SWE, an ultrasound elastography technique based on shear waves that is available on a clinical diagnostic ultrasound scanner, has been used to non-invasively measure liver fibrosis. The 2D-SWE technique creates a real-time, two-dimensional quantitative map of liver tissue stiffness under the guidance of very-high-frame-rate B mode imaging.

Peer-review

A well-written manuscript dealing with an interesting topic. Authors could ameliorate the description of results and give some important additional information to apply these data into clinical practice.

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