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EDITORIAL

Serum levels of angiotensin converting enzyme as a biomarker of liver fibrosis

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Abstract

The renin angiotensin system (RAS) is classically conceived as a circulating hormonal system involved in blood pressure control and hydroelectrolyte balance. The discovery that RAS components are locally expressed in a wide range of organs and tissues, including the liver, pointed to a role for this system in the pathogenesis of several conditions including hepatic fibrosis and cirrhosis. It has been widely reported that the classical RAS axis composed by the angiotensin converting enzyme (ACE)-angiotensin (Ang) Ⅱ-Ang type 1 (AT1) receptor mediates pro-inflammatory, pro-thrombotic, and pro-fibrotic processes. On the other hand, the alternative axis comprising ACE2-Ang-(1-7)-Mas receptor seems to play a protective role by frequently opposing Ang Ⅱ action. Chronic hepatitis B (CHB) is one of the leading causes of liver fibrosis, accounting for the death of nearly one million people worldwide. Liver fibrosis is a key factor to determine therapeutic interventions for patients with CHB. However, the establishment of non-invasive and accurate methods to detect reversible stages of liver fibrosis is still a challenge. In an elegant study published in the 36th issue of the *World Journal of Gastroenterology*, Noguchi *et al* showed the predictive value of serum ACE levels in detecting not only advanced stages of liver fibrosis but also initial and

intermediate fibrotic stages. The serum levels of ACE might represent an accurate, non-invasive, widely available, and easy method to evaluate fibrosis related to CHB. Moreover, therapies involving the inhibition of the classical RAS axis components might be promising in the control of CHB-related liver fibrosis.

Key words: Renin angiotensin system; Angiotensin converting enzyme; Angiotensin Ⅱ; Angiotensin-(1-7); Chronic hepatitis B; Hepatic cirrhosis; Liver fibrosis

Core tip: The therapeutic intervention for patients with chronic hepatitis B frequently relies on the pathological classification of liver fibrosis severity in biopsy. The establishment of non-invasive and accurate methods to detect reversible stages of liver fibrosis is still a challenge. High serum levels of angiotensin converting enzyme seem to better predict intermediate liver fibrosis than other classical fibrotic markers. Non-invasive methods to detect intermediate stages of liver fibrosis with very good accuracy may permit the introduction and/or evaluation of treatments during reversible stages of the disease. Further studies are urgently necessary to fully clarify the role of RAS components in liver disease.

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INTRODUCTION

The discovery of renin-angiotensin system (RAS) components locally expressed in several organs and tissues, including the kidney, brain, and liver, challenged the classical view of the RAS as a solely circulating hormonal system involved in blood pressure control and hydroelectrolyte balance[1]. Currently, the RAS is considered a system formed by two opposing axes: the classical axis, which includes the angiotensin-converting enzyme (ACE)-angiotensin (Ang) II-Ang type 1 (AT_1) receptor, and the alternative axis, which comprises the ACE2-Ang-(1-7)-Mas receptor. In general, the classical arm seems to mediate pro-inflammatory, pro-thrombotic, and pro-fibrotic processes, mainly through the activation of AT_1 receptors[2]. On the other hand, the heptapeptide Ang-(1-7) seems to play a protective role as a biologically active RAS mediator by frequently opposing the action of Ang II *via* stimulation of Mas receptors[3,4]. In this scenario, over the past decades, an imbalance in the components of the RAS classical and alternative axes has been implicated in the pathogenesis of a wide range of conditions such as atherosclerosis, obesity, insulin resistance, asthma, renal and liver diseases[3-5]. Accordingly, many therapeutic strategies have been designed to inhibit ACE-Ang II-AT_1 receptor activity and to stimulate ACE2-Ang-(1-7)-Mas receptor activity[4,5].

Chronic hepatitis B (CHB) is one of the major causes of liver fibrosis, which, along with hepatitis C, alcohol use, and obesity-related steatohepatitis, has resulted in a significant elevation in the occurrence of cirrhosis and in the mortality of at least 800000 individuals worldwide per year[6]. Purnak *et al*[7] detected high serum concentrations of ACE in patients with CHB and they considered this RAS enzyme as a marker of fibrosis. This finding is in line with a more recent study that supported the role of serum ACE level as a noninvasive marker for the prediction of necroinflammatory activity in CHB patients[8]. Taken together, these studies point to a role of the RAS in liver injury in response to CHB, and pave the way for measuring components of this system as potential predictors of disease evolution.

It is worth mentioning that although the pathophysiology of hepatic fibrosis is still not totally clarified, current opinions have proposed that cirrhosis might be in theory reversible, above all in the compensated stage. Therefore, the evaluation of predictive biomarkers and of novel therapeutic targets is of utmost importance[9].

STUDY ANALYSIS

In the 36th issue of the *World Journal of Gastroenterology*, Noguchi *et al*[10] conducted an observational study to investigate the predictive value of serum ACE levels in CHB-associated fibrosis. A total of 100 patients diagnosed with CHB were enrolled in the study and underwent routine liver biopsy. Thirty patients with a history of hypertension, fatty liver, and alcohol abuse were excluded. The degree of hepatic fibrosis in the liver biopsy specimen was evaluated and classified based on the METAVIR score for chronic hepatitis, ranging from F0, no fibrosis, to F4, cirrhosis. The F2 degree (portal fibrosis with few septa) was considered significant liver fibrosis. Additionally, serum levels of ACE and well-known fibrotic markers including the number of platelets (PLT), the aspartate aminotransferase (AST)-to-platelet ratio index (APRI), the Mac-2 binding protein glycosylation isomer (M2BPGi) concentration, and the fibrosis index according to four factors (FIB-4) were also evaluated. For differentiating mild fibrosis (F0-F1) from substantial fibrosis ($\geqslant$ F2), the 12.8 U/L cut-off value of ACE had a high sensitivity (91.7%) with a good specificity (75%). The receiver-operating characteristic (ROC) curve analysis showed that the area under the curve (AUC) value of ACE serum level measurements was 0.871. The AUC of serum ACE

was bigger than that of other tests for liver fibrosis, including APRI, FIB-4, M2BPGi, and PLT. Importantly, CHB patients in early stages of fibrosis (F0-1) had significantly lower serum levels of ACE than those with significant, advanced fibrosis and cirrhosis (F2-4). The authors concluded that serum levels of ACE might represent an accurate, non-invasive, widely available, and easy method to evaluate fibrosis related to CHB. This conclusion is particularly true for CHB patients without other associated conditions such as fatty liver and/or habitual alcoholic consumption.

The general severity of liver fibrosis influences therapeutic clinical decisions in CHB patients. Serum levels of ACE have been previously evaluated in CHB patients as a potential marker of hepatic fibrosis[7,8]. For instance, Purnak *et al*[7] reported higher serum levels of ACE in 22 patients with advanced liver fibrosis compared with 28 patients with mild fibrosis, indicating that the utilization of measurements of serum ACE levels for CHB patients may provide further prognostic data. A more recent study in 54 patients with severe fibrosis showed that serum ACE levels, together with hepatitis B virus deoxyribonucleic acid and serum transaminase levels, might be used as noninvasive markers for predicting necroinflammation in CHB patients[8]. Even though these previous studies pave the way for the hypothesis that increased serum levels of ACE might be a marker of CHB-associated fibrosis, both included only patients at advanced stages of liver fibrosis for which treatment may not be as efficient as for earlier stages. The study of Noguchi *et al*[10] was thus designed to overcome this limitation. The authors provide a more accurate classification of the degree of hepatic fibrosis, allowing the predictive value of serum ACE levels to be investigated not only for severe fibrosis but also for initial (F1) and intermediate (F2-F3) stages. Furthermore, Noguchi *et al*[10] showed that measurement of ACE serum levels was better than other tests for detecting liver fibrosis. It should also be mentioned that the possibility of adopting a non-invasive yet accurate method to detect intermediate stages of liver fibrosis might allow the introduction and/or evaluation of treatments during reversible stages of the disease. Moreover, these findings also suggest that the therapeutic role of inhibitors of the classical RAS axis, such as ACE inhibitors and AT_1 receptor antagonists, should be more deeply investigated for controlling liver fibrosis in CHB patients.

PERSPECTIVES

The establishment of non-invasive and accurate methods to detect reversible stages of liver fibrosis remains a challenge. Many biomarkers have been investigated in this context. However, most of these biomarkers are accurate only for advanced stages of liver disease, when therapies are no longer efficient. Components of the RAS have a clear role in the pathophysiology of liver disease. Therefore, the measurement of these molecules seems to be very reasonable not only for predicting the progression of liver disease but also for establishing its prognosis, and for being tested as therapeutic targets. In this regard, Noguchi *et al*[10] clearly showed the ability of serum ACE levels to differentiate initial (F0 and F1) from intermediate (F2-F3) stages of liver fibrosis in CHB patients without other associated conditions. These findings open many different clinical and research approaches. First, measurement of serum ACE levels should be tested in larger samples of patients with CHB and other liver diseases. Second, serum levels of other RAS components, including serum levels of ACE2, Ang Ⅱ, and Ang-(1-7), should be measured in patients with liver diseases to evaluate these molecules as biomarkers of liver fibrosis and/or disease prognosis. Third, randomized clinical trials with ACE inhibitors or AT_1 receptor antagonists should be conducted in patients in reversible stages of liver diseases. Fourth, the role of components that stimulate the alternative RAS axis (ACE2 activators, Ang-(1-7) analogs, and Mas receptor agonists) should be tested in phase Ⅰ/phase Ⅱ studies of liver diseases. In conclusion, the role of RAS components in liver diseases deserves further investigation.

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MINIREVIEWS

Mechanisms of autophagy activation in endothelial cell and their targeting during normothermic machine liver perfusion

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Abstract

Ischaemia-reperfusion injury (IRI) is the leading cause of injury seen in the liver following transplantation. IRI also causes injury following liver surgery and haemodynamic shock. The first cells within the liver to be injured by IRI are the liver sinusoidal endothelial cells (LSEC). Recent evidence suggests that LSEC co-ordinate and regulates the livers response to a variety of injuries. It is becoming increasingly apparent that the cyto-protective cellular process of autophagy is a key regulator of IRI. In particular LSEC autophagy may be an essential gatekeeper to the development of IRI. The recent availability of liver perfusion devices has allowed for the therapeutic targeting of autophagy to reduce IRI. In particular normothermic machine liver perfusion (NMP-L) allow the delivery of pharmacological agents to donor livers whilst maintaining physiological temperature and hepatic flow rates. In this review we summarise the current understanding of endothelial autophagy and how this may be manipulated during NMP-L to reduce liver IRI.

Key words: Autophagy; Liver transplant; Ischaemia-reperfusion injury; Normothermic machine liver perfusion

Core tip: Liver sinusoidal endothelial cells autophagy regulates liver ischaemia reperfusion injury and this process can be targeted for therapeutic benefit using normothermic machine liver perfusion.

Boteon YL, Laing R, Mergental H, Reynolds GM, Mirza DF, Afford SC, Bhogal RH. Mechanisms of autophagy activation in endothelial cell and their targeting during normothermic machine liver perfusion**.** *World J Gastroenterol* 2017; 23(48): 8443-8451 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i48/8443.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i48.8443

INTRODUCTION

The term autophagy is derived from the Greek meaning "eating of self" and the precise cellular role of autophagy has been controversial[1]. However research over the past decade has demonstrated that the evolutionarily conserved process of autophagy is primarily a cell survival mechanism allowing cells and tissues to maintain homeostasis during periods of stress such as starvation and ischaemia[2]. Specifically autophagy eliminates damaged organelles, long-lived proteins or intracellular pathogens through the co-ordinated engulfment of the targeted cargo in a double membrane cytoplasmic structure known as an autophagosome[3,4]. Autophagosomes then fuse with lysosomes to form autophagolysosomes that degrade the engulfed cargo allowing their reuse and thus potentially negating periods of cellular stress[2,4-6]. Hence unsurprisingly autophagy is involved in number of cellular processes such as metabolism, protein synthesis and cellular transportation[4,7]. Dysregulated or uncoordinated autophagy is linked to cell injury and a number of disease processes such as neurodegenerative diseases and cancer[2,4,6,7].

Three distinct types of autophagy have been characterised. The mostly widely studied is macro-autophagy (referred to as autophagy hereafter) and is the primary focus of this review[4,5,8]. Micro-autophagy is characterized by the invagination of the target by the lysosomal membrane itself[5,9]. Chaperone-mediated autophagy targets proteins with the KFERQ motif to the lysosome via interaction with lysosomal-associated membrane protein (LAMP)[9,10]. More recently distinct macroautophagy signalling pathways have been characterised that are activated to specifically eliminate portions of the cell and/or cytoplasm leading to the characterisation of mitophagy (mitochondria), ERphagy (endoplasmic reticulum), xenophagy (microorganisms), lipophagy (lipids) and ribophagy (ribosomes). It is becoming increasingly apparent that specific forms of autophagy are important in the development and pathophysiology of different disease processes. The precise regulation of each type of autophagy is beyond the scope of this review but the reader is referred to recent excellent reviews on the subject[2,4,6,7].

The therapeutic targeting of autophagy has gathered momentum since the publication of the first clinical trials using autophagy inhibitors in treatment of cancers[11-13]. For instance autophagy inhibitors (*e.g.,* hydroxychloroquine) were used in combination with chemotherapy in patients with advanced pancreatic cancer and in those patients with increased levels of the autophagy marker LC3 in peripheral blood mononuclear cells there was an improvement in disease free and patient survival[11]. However in other trials using hydroxychloroquine in patients with glioblastoma the optimal therapeutic dose was not found due to the marked side effects with the investigators concluding that drugs with less toxicity should be awaited[12]. Although these studies have provided the impetus to manipulate autophagy it still remains to be established whether autophagy should be activated or inhibited to derive therapeutic benefit in many disease processes. Moreover many groups working on the therapeutic manipulation of autophagy are now suggesting that the targeting of autophagy during pathophysiological processes needs to tissue and possibly even cell specific[14].

The use of autophagy as a target in treating liver diseases have been the focus of intense recent research[15]. Indeed the manipulation of autophagy may be useful in treating many liver diseases. For instance induction of autophagy/lipophagy may reduce steatosis in fatty liver disease[16] and inhibiting autophagy in hepatobiliary cancers may promote cancer cell death[11,17]. The manipulation of the autophagy signalling pathway also holds significant promise in attempting to reduce the liver ischaemia-reperfusion injury (IRI). IRI is an antigen independent pro-inflammatory process that mediates liver injury following transplantation, liver surgery and haemorrhagic shock[18]. The injury occurs in two distinct phases. In the ischaemia phase blood flow to the liver is interrupted leading to tissue hypoxia and the generation of reactive oxygen species (ROS). During the reperfusion phase although blood flow to the liver is restored there is a concomitant increase in pro-inflammatory mediators, ROS and inflammatory cells that amplifies the liver injury[18]. It is well established that IRI targets and injuries the liver parenchymal cells such as hepatocytes and liver sinusoidal endothelial cells (LSEC)[19]. However early IRI is characterised by LSEC injury and dysfunction[19]. Indeed recent studies demonstrate the key role of LSEC in co-ordinating the livers response to injury whilst also mediating the recovery from liver injury[20]. Thus the targeting of autophagy in the LSEC during IRI is an attractive method with which to reduce liver IRI.

The emerging technology of normothermic machine perfusion of the liver (NMP-L) provides an exciting modality with which to target autophagy in LSEC whilst simultaneously allowing an assessment of liver function[21]. There are different NMP-L devices available allowing either selective hepatic artery perfusion or dual hepatic perfusion of donor livers. Using primarily blood based perfusion fluids, oxygen is delivered to donor livers whilst maintaining a normal

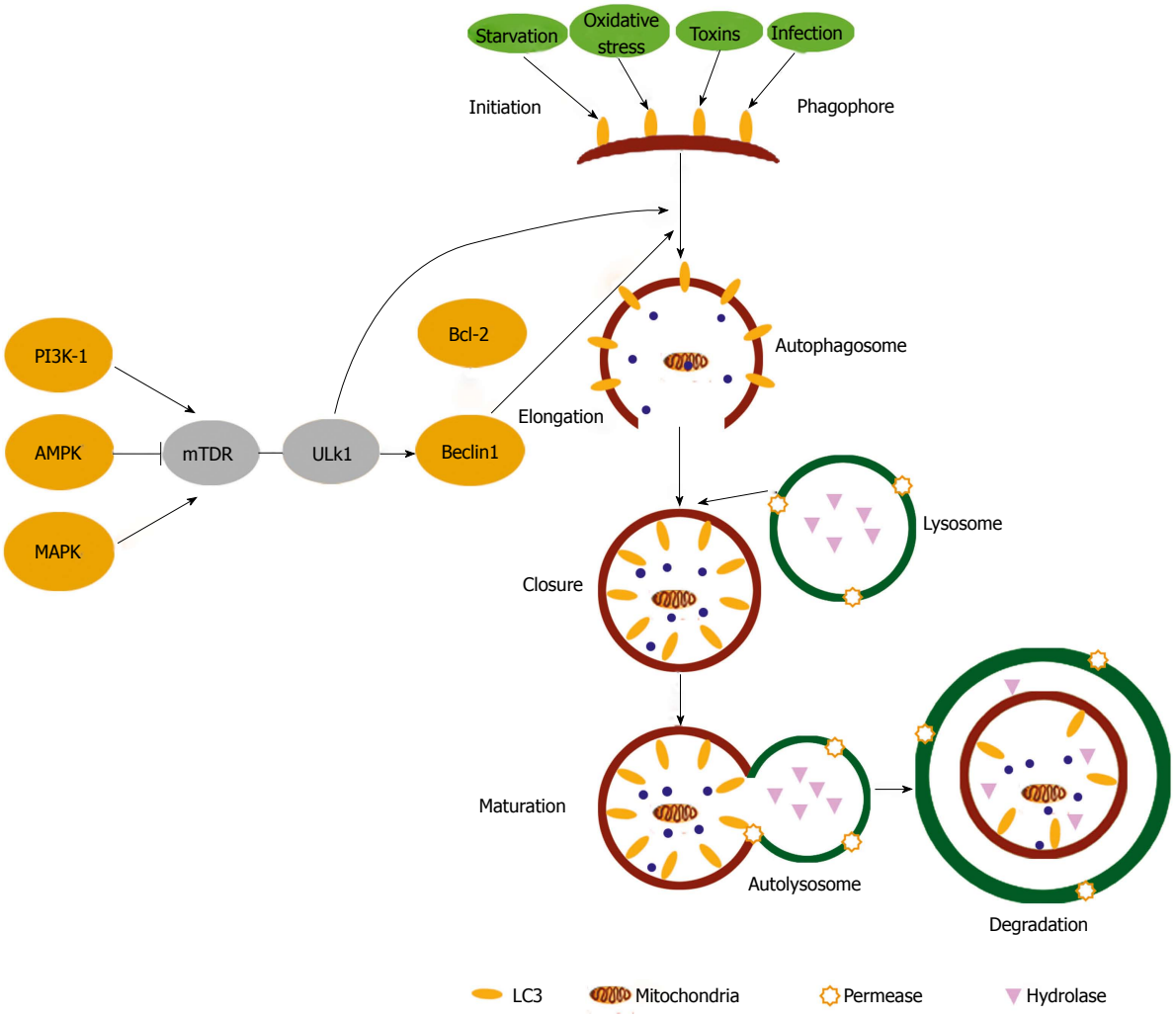

Figure 1 Autophagy signalling pathways. Upstream autophagy activation is regulated by the integration of signalling from a number of pathways including AMPK, PI-3K and the Mitogen-Activated Protein Kinases. Phagophore initiation is directly regulated by the serine/threonine protein kinases ULK1 that forms a complex with Beclin 1. Upon initiation, cytoplasmic constituents are enclosed in a double membraned isolation structure known as an autophagosome that is elongated mainly through the action of two ubiquitin-like conjugation systems. Autophagosomes fuse with lysosomes to form autophagolysosomes, where breakdown of the vesicle contents/cargo takes place along with the autophagosome inner membrane. Autophagy can be activated by many stimuli including starvation, toxins, oxidative stress and infections. (Taken from Shan NN *et al* Targeting autophagy as a potential therapeutic approach for immune thrombocytopenia therapy. *Crit Rev Oncol Hematol* 2016; **100**: 11-15. DOI: 10.1016/j.critrevonc.2016.01.011).

temperature (37 ℃). NMP-L is typically performed for 6 h and the sequential sampling of perfusates and liver tissue allows a dynamic assessment of liver function. One of the many potential benefits of NMP-L is the reduction in the liver IRI. Therefore, the manipulation of autophagy in LSEC during NMP-L is an attractive therapeutic target with which to improve donor liver organ function prior to transplantation.

AUTOPHAGY SIGNALLING PATHWAY

The autophagy signalling pathway is regulated by specific and dedicated cellular machinery. These proteins were first isolated in yeast two-hybrid screens and are now known as the Autophagy-related proteins (ATGs). To date 30 ATGs have been characterised that are essential for autophagy induction. Recent reviews by Stork *et al*[21] provide an in-depth review of the pathway whilst a brief overview is given here. In general the autophagy signalling pathway can be divided into distinct phases including the initiation, elongation, autophagosome formation, fusion and autophagolysosomal formation (Figure 1).

The formation of autophagosomes commences upon omegasomes and is known as the initiation phase[22]. This process is regulated by the ATG proteins unc-51 like autophagy activation kinase (ULK1) and WIPI1-4/ATG18[22,23]. Starvation is a classical activator of autophagy and in nutrient rich conditions the activation of autophagy is inhibited by Mammalian Target of Rapamycin (mTOR)[24]. However when autophagy is activated, the inhibitory effect of mTOR is lost allowing for the activation of ULK1 kinase complex during the initiation phase[9]. In addition autophagy can also be initiated by AMP-activated protein kinase (AMPK)[25] (Figure 1). Subsequently there is

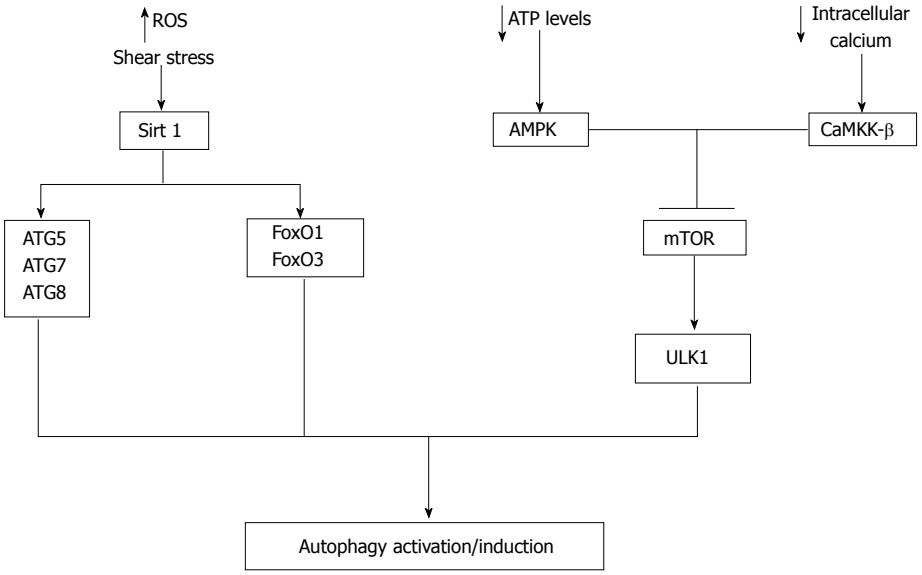

Figure 2 Autophagy activation in endothelial cells. A number of mechanisms potentially regulate autophagy activation in endothelial cells. A decrease in cellular ATP or reduction in growth factors availability leads to the activation of AMP-activated protein kinase (AMPK). Once activated AMPK can inhibit mTOR leading to the activation of ULK1 and hence autophagy activation. In addition decreases in intracellular calcium can activate CaMKK-β leading to mTOR inhibition and autophagy activation. Moreover, Sirt1 can activate autophagy *via* deacetylation of ATG5, ATG7, ATG8 and increased transcription of FoxO1 and FoxO3 that then regulate the expression ATGs *via* deacetylation of Akt. Reactive oxygen species (ROS) and shear stress are important regulators of Sirt1 activity.

recruitment of class Ⅲ phosphatidylinositol 3-kinase (PI3-K), Vps34 and Beclin-1/ATG6 to the developing autophagosome[5,9]. ULK1 is then able to activate the PI3-K complex leading to the activation of Beclin-1 and the later phases of autophagy. This pathway details the canonical autophagy signalling pathway but recent evidence suggests that autophagy can be activated by Beclin-1 independent mechanisms/non-canonical pathways too[9,26].

The elongation phase of autophagy involves two separate ATG conjugation systems. Firstly ATG12 conjugates ATG5 *via* ATG7/ATG10. The ATG12-ATG5 complex is then able to interact with ATG16 resulting in the formation of the ATG12-ATG5-ATG16L complex[5,27]. The second conjugation system involves LC3-I/ATG8 that is cleaved by ATG4 in a process requiring ATG7 and ATG3. This culminates in the formation of LC3-Ⅱ[5,28]. Both these conjugation systems are integral to autophagosome membrane expansion[4,7,9]. At the point at which appropriate membrane expansion has been reached there is closure of double layered membrane that is regulated by amongst other proteins the docking protein p62 which is located on the outer membrane of the autophagosome and is responsible for docking with the lysosome *via* a dynein-dependent mechanism[5]. Thereafter, lysosomal acid hydrolases degrade the engulfed content/cargo and lysosomal efflux permeases release the final products to the cytosol for anabolic processes (Figure 1).

AUTOPHAGY AND ENDOTHELIAL CELLS

Regulation of autophagy activation in endothelial cells

Much of our understanding of the role of autophagy in endothelial cells, such as LSEC, has come from the study of endothelial cell biology in cardiovascular disease. Recent studies have demonstrated how autophagy dysfunction in endothelial cells contributes to the pathogenesis of arthrosclerosis by regulating angiogenesis, haemostasis and nitric oxide production[9].

In endothelial cells upstream activation of the autophagy is regulated by the integration of signalling from the AMPK and mTOR-ULK1 pathways[9] (Figure 1). AMPK can detect decreases in cellular ATP levels and activate autophagy primarily to provide substrates for energy used for essential cellular processes[9,29] (Figure 2). Specifically AMPK phosphorylates the Tuberous Sclerosis 2 (TSC2) protein that inhibits mTOR and activates autophagy in an analogous mechanism to that seen during starvation[9,29-33]. In HeLa cells starvation increases intracellular calcium levels and can activate AMPK through Calmodulin-dependent protein kinase kinase-β (CaMKK-β) that then subsequently activates autophagy[9,34,35]. In endothelial cells CaMKK-β mediated AMPK activation inhibits mTOR leading to ULK1 upregulation and autophagy activation[34]. Thus in endothelial cells the activation of autophagy is a dynamic interplay between AMPK, intracellular calcium, mTOR and ULK-1.

The deacylation protein Sirt1 has attracted recent attention as an important regulator of autophagy activation. Specifically Sirt1 can deacetylate ATG5, ATG7, and ATG8[9,36] whilst activating the transcription factors Forkhead box O (FoxO) FoxO1 and FoxO3. Both FoxO1 and FoxO3 can regulate the expression of ATGs *via* deacetylation of Akt[9,35,37-39]. Indeed during liver IRI ROS can regulate the activation of Sirt1, which may have important implications for endothelial cell autophagy during NMP-L[9,40-43]. Specifically perfusion fluid used during NMP-L induces tangential force across the endothelium causing shear stress that is associated with autophagy activation *via* the Sirt1-FoxO pathway[43]. Low shear stress is associated with reduced levels of autophagy suggesting an important relationship between shear stress and autophagy[44]. Therefore autophagy activation in endothelial cell can be regulated by multiple signalling pathways and at present is remains to be established which of these pathways is important during NMP-L.

Effects of autophagy on endothelial cell function

Autophagy activation has recently been demonstrated to have a number of important effects upon endothelial cell function. Following endothelial injury exposure of the sub-intimal layer promotes glycoproteins, expressed on platelets, to bind to subendothelial von Willebrand Factors (vWF) leading to the formation of a platelet thrombus and production of thromboxane A2, serotonin and adenosine diphosphate[34]. Recent studies demonstrate that vWF is found in close proximity to autophagosomes in endothelial cells[35]. The inhibition of autophagy impairs the secretion of vWF from endothelial cells suggesting that endothelial cell autophagy may have an anti-thrombotic function[45].

In endothelial cells, endothelial nitric oxide synthase (eNOS) regulates nitric oxide (NO) production that in turn regulates vascular tone, platelet aggregation and leukocyte adhesion[46]. Indeed this may be a central function of endothelial cells during liver IRI and a potential protective mechanism induced within the liver by NMP-L. Furthermore eNOS can regulate autophagy induction[47,48]. The exact mechanisms involved in eNOS/NO-autophagy axis are not yet understood although impaired autophagy does result in reduced eNOS function reduced NO production[49].

As eluded to earlier blood flow within the liver generates shear stress across the endothelium that is associated with induction of eNOS and NO production[50]. This is in turn associated with a reduction in oxidative stress and inflammation in endothelial cells. Disturbed shear stress generated by non-laminar flow is associated with endothelial dysfunction that can be implicated in disease processes such as arthrosclerosis[50]. Hence low shear induces autophagy dysfunction[48] which is associated with insufficient autophagy activation in endothelial cells and thus endothelial dysfunction[43]. NMP-L may aid endothelial cell autophagy by ensuring laminar flow within the hepatic vasculature thereby prompting autophagy leading to increased eNOS transcription and NO production. In summary, physiologic shear stress is an essential mechanism for the maintenance of endothelial cells function and is in part mediated by autophagy activation[47].

Autophagy activation is also associated with angiogenesis in endothelial cells particularly within ischaemic microenvironments[51]. The relationship between redox signalling and autophagy is complex and out of the scope of this review although it has been recently reviewed[42]. Autophagy regulation of angiogenesis appears to be dependent upon the activation of the Akt signalling pathway in endothelial cells although the precise mechanism remains to be determined[9,51,52].

Therapeutic targeting of autophagy in endothelial cells

The evolving understanding of the autophagy signalling pathway has lead to the targeting of the pathway for potential therapeutic benefit in many clinical scenarios. However what is becoming evident from these studies is that for the successful therapeutic targeting of autophagy the timing of the intervention, the part of the autophagy pathway targeted, the cell type targeted and whether autophagy should be inhibited or activated are all critical factors. The targeting of autophagy within endothelial cells remains a nascent field with few studies investigating this area.

Epigallocatechin gallate, found in green tea, can induce the specific form of autophagy known as lipophagy in endothelial cells. Lipophagy causes the specific elimination of lipid droplets. In artheroscelosis the degradation of lipid droplets in vascular endothelial cells can potentially modulate the disease process by allowing endothelial cells to resist the effects of lipotoxicity[35,53]. In human umbilical vein endothelial cells (HUVEC) exposed to oxidative stress, vitamin D dependent autophagy activation prevents cell death by activating Beclin1 that prevents mitochondrial depolarisation and caspase activation[54]. Pterostilbene can activate AMPK to stimulate autophagy in HUVEC promoting the elimination of excess lipids and thus reducing apoptosis[53] whilst other groups have suggest that Advanced glycation end-products (AGEs) may regulate the activation of autophagy in this scenario[55]. Furthermore the autophagy activator rapamycin can increase the viability of HUVEC during starvation[56]. These limited studies do suggest that selective activation of autophagy in cells of endothelial lineage promotes cell survival.

Very few studies have addressed the role of autophagy in endothelial cells *in vivo*. Torisu *et al*[45] reported, using a Cre-lox conditional ATG7 endothelial knockdown mice, that inhibition of autophagy leads a reduction in the secretion of vWF suggesting that autophagy may have a role in preventing thrombosis.

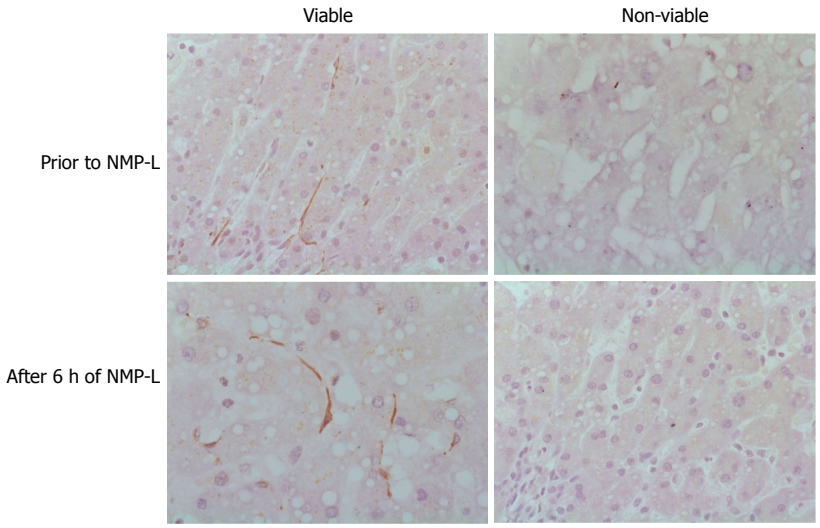

Figure 3 Autophagy and liver viability following normothermic machine liver perfusion. Immunohistochemical analysis was performed for the autophagy protein LC3B in liver tissue prior to normothermic machine liver perfusion (NMP-L) and after 6 h of NMP-L. Livers deemed viable after NMP-L demonstrated LC3B immunostaining in the hepatic sinusoids prior to commencing NMP-L and at the end of perfusion. Donor livers not fulfilling viability criteria demonstrated no LC3B Immunostaining.

However the role of endothelial cell autophagy *in vivo* need much more investigation.

THERAPEUTIC MANIPULATION OF ENDOTHELIAL CELL AUTOPHAGY DURING NMP-L

NMP-L is a novel technique that can be employed to assess and recondition donor livers prior to transplantation. One of the potential benefits of NMP-L is the potential reduction in liver IRI especially when compared to traditional static cold storage[57,58]. Recent studies have established haemodynamic and biochemical parameters that allow donor livers to be classified as viable and non-viable where viable denotes a liver that can be transplanted following NMP-L. Indeed donor livers represent conditions were autophagy is expected to be activated; relative tissue ischaemia and reduced availability of nutrients. Recent data from our laboratory has shown that donor livers that demonstrate these viability criteria shows increased levels of autophagy within hepatic sinusoids as assessed by immunohistochemical analysis of the specific autophagy marker LC3B (Figure 3). This suggests a relationship between autophagy induction and liver viability although further work is needed to fully establish this relationship.

As discussed above targeting of autophagy needs to be considered in terms of the timing of intervention, the cell type targeted and the part of the autophagy pathway targeted. During NMP-L there are two forms of intervention that could modulate autophagy in endothelial cells; mechanical or pharmacological.

Livers exposed to NMP-L are associated with increased cellular ATP levels in comparison to cold static stored livers. For instance in a porcine model of donation after cardiac death (DCD) livers exposed to 1 h warm ischaemia, followed by 2 h of cold ischaemia and then 4 h of NMP-L increased cellular ATP levels by 80% in comparison with livers maintained in cold static storage for 2 h[59]. Higher ATP levels within liver grafts prior to liver transplantation are associated with better patient outcomes in some series[60,61]. Although it must be remembered that these studies have assessed global liver ATP levels and not specifically LSEC ATP levels. Additionally, NMP-L is associated with preservation of liver architecture and integrity of the mitochondria[59]. The regulation of mitochondrial function and number is regulated in large part by the specific autophagy process termed mitophagy[62]. The regulation of mitophagy during NMP-L and liver IRI is now being actively investigated as a potential method to reduce liver injury following transplantation[62]. The manipulation of mitophagy in LSEC is emerging as a method to reduce liver injury. However despite this it remains to be established whether LSEC autophagy contributes to increased ATP levels during NMP-L and whether autophagy, through promoting survival of LSEC, improves the survival of neighbouring liver parenchymal cells.

NMP-L may also regulate autophagy activation through calcium signalling. During NMP-L the perfusion fluid is supplemented with calcium to maintain physiological extracellular levels of the ion ensuring that the electrochemical gradient of calcium is maintained across cell membranes[58]. Low intracellular levels of calcium are reported to induce CaMMk-β activation followed by mTOR inhibition and therefore ULK1/autophagy activation[34]. Physiological calcium levels during NMP-L potentially promote normal autophagy activity within LSEC and other liver cells ensuring homeostasis is maintained.

The mechanical manipulation of autophagy during

NMP-L is dependent upon shear stress. NMP-L has the advantage of providing an adjustable vascular/laminar flow rate to livers. In turn these flow rate provides a near physiological shear stress known to promote autophagy induction[43]. Activated autophagy in endothelial cells is fundamental for eNOS transcription, NO production and maintenance of vascular tone maintenance[47]. Increased NO may also reduce platelet aggregation and leukocyte adhesion in endothelial cells thus reducing IRI and hepatic microcirculatory disturbance[63].

Pharmacological modulation of autophagy during NMP-L may also allow the targeting of endothelial cells dysfunction in donor liver grafts prior to transplantation. Extended criteria liver donors (*e.g.,* DCD grafts and steatotic grafts) tend to be associated with lower cellular ATP content and increased ROS production increasing their susceptibility to IRI[57,59]. Pharmacological agents promoting activation of autophagy during NMP-L may promote elimination of damaged organelles and toxins prior to graft implantation. In particular aiding mitophagy function may be crucial to maintaining LSEC function during NMP-L and thus aiding the survival of other liver cells. As described above, many drugs are already known to regulate autophagy activation and the use of these drugs in experimental NMP-L perfusion offers an important method to evaluate these interventions. This manipulation of autophagy in LSEC using NMP-L is an exciting area of interest for research groups working on liver IRI particularly as this technology will become more widespread in addition to the development of new autophagy modulating drugs.

CONCLUSION

In summary, autophagy is a complex metabolic process that is essential for the cell survival; it promotes clearance of harmful substances and provides energy to the cell during cellular stress. The role of autophagy in endothelial cells and its manipulation using NMP-L offers significant promise in reducing liver IRI thereby improving the quality of donor liver organs used for transplantation.

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ORIGINAL ARTICLE

Basic Study

Human small intestine is capable of restoring barrier function after short ischemic periods

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Abstract

AIM

To assess intestinal barrier function during human intestinal ischemia and reperfusion (IR).

METHODS

In a human experimental model, 6 cm of jejunum was selectively exposed to 30 min of ischemia (I) followed by 30 and 120 min of reperfusion (R). A sham procedure was also performed. Blood and tissue was sampled at all-time points. Functional barrier function was assessed using dual-sugar absorption tests with lactulose (L) and rhamnose (R). Plasma concentrations of citrulline, an amino acid described as marker for enterocyte function were measured as marker of metabolic enterocytes restoration. Damage to the epithelial lining was assessed by immunohistochemistry for tight junctions (TJs), by plasma marker for enterocytes damage (I-FABP) and analyzed by electron microscopy (EM) using lanthanum nitrate as an electrondense marker.

RESULTS

Plasma L/R ratio's were significantly increased after 30 min of ischemia (30I) followed by 30 min of reperfusion (30R) compared to control (0.75 ± 0.10 *vs* 0.20 ± 0.09, $P < 0.05$). At 120 min of reperfusion (120R), ratio's normalized (0.17 ± 0.06) and were not significantly different from control. Plasma levels of I-FABP correlated with plasma L/R ratios measured at the same time points (correlation: 0.467, $P < 0.01$). TJs staining shows distortion of staining at 30I. An intact lining of TJs was again observed at 30I120R. Electron microscopy analysis revealed disrupted TJs after 30I with paracellular leakage of lanthanum nitrate, which restored after 30I120R. Furthermore, citrulline concentrations closely paralleled the histological perturbations during intestinal IR.

CONCLUSION

This study directly correlates histological data with intestinal permeability tests, revealing that the human gut has the ability of to withstand short episodes of ischemia, with morphological and functional recovery of the intestinal barrier within 120 min of reperfusion.

Key words: Intestinal ischemia-reperfusion; Intestinal barrier function; Intestinal permeability; Human; Dual-sugar absorption test; Tight Junctions; Citrulline

Core tip: Using an unique experiment human intestinal ischemia and reperfusion (IR) model, this is the first study to directly correlate histological data with intestinal permeability tests. The results reveal the ability of the intestine to withstand short episodes of ischemia, with morphological and functional recovery of the intestinal barrier within 120 min of reperfusion. These results explain why there are often no signs of inflammation or bacterial translocation after short periods of intestinal ischemia. Exploration of the mechanisms responsible for this rapid recovery might impact understanding and treatment of intestinal diseases. Data from the dual-sugar absorption tests and citrulline reflect the histological perturbations during intestinal IR, highlighting the potential diagnostic value of these tests in patients with intestinal diseases associated with intestinal barrier loss.

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INTRODUCTION

The intestinal epithelial lining is one of the largest sites of the human body exposed to the external environment and holds a dual function: the absorption of nutrients and water from the lumen and at the same time providing an effective barrier against potentially harmful compounds such as bacteria, toxins and antigens[1,2].

This is achieved by various lines of defense, including a physical barrier, formed by a mucus layer and a single rigid layer of enterocytes which are interconnected by tight junctions (TJs) and the subjacent adherens junctions (AJs)[3]. The TJs act as a fence, regulating the passive diffusion of solutes across the paracellular pathway[4]. They are composed of transmembrane proteins such as occludin and members of the claudin family, which are anchored to the cell cytoskeleton by zonula occludens proteins (ZO-1, ZO-2 and ZO-3)[5-8]. AJs are important for connecting neighbouring cells and consist, amongst others, of the transmembrane protein E-cadherin. Formation of AJs is required prior to the assembly of the TJs[9].

Loss of intestinal barrier function is considered to play a key role in the development and/or progression of intestinal inflammation and the onset of systemic inflammatory response syndrome, and is associated with intestinal diseases including celiac disease, inflammatory bowel disease (IBD) and intestinal ischemia-reperfusion (IR)[1,10]. Intestinal IR is a frequently occurring phenomenon associated with high morbidity and mortality. It is caused by various pathological conditions that involve a critical reduction of blood flow to the intestine, such as acute obstruction of mesenteric arterial blood flow and hypoperfusion associated with major vascular or abdominal surgical procedures, hemorrhagic shock, major trauma and

sepsis[11-15].

Previous research of our group revealed the striking ability of the human small intestine to reduce and restore IR-induced epithelial lining damage. Using an experimental human intestinal IR model[16], it was observed that after periods of ischemia of up to 30 min, villous sloughing with epithelial lining disruption was rapidly followed by complete histological recovery during reperfusion[17,18]. The important remaining question however is whether functional barrier restoration with closure of paracellular permeability is achieved within this time span. This is in particular important as few human experimental studies exist that directly correlate barrier function to histological appearance. Here, we set out to study the relation between histological and functional barrier recovery using a human experimental small intestinal IR model. Damage to the epithelial lining was assessed by immunohistochemistry, plasma measurement of Intestinal Fattty Acid Binding Protein (I-FABP) and electron microscopy (EM). Functional intestinal barrier loss was evaluated using lanthanum nitrate as an electrondense marker together with the currently used gold standard for the measurement of intestinal permeability in clinical practice, the dual-sugar absorption tests (DST)[19]. These tests rely on the differential intestinal paracellular and cellular permeability of larger (lactulose) and smaller (L-rhamnose) molecules. Simultaneous measurement of lactulose and L-rhamnose are used as controls for gastric emptying, intestinal fluid volume, gastrointestinal transit time, and renal excretion which are thought to affect each molecule equally. The ratio of plasma concentrations reflects small intestinal permeability. Furthermore, plasma concentrations of citrulline, an amino acid produced by enterocytes that has been described as a marker for enterocyte mass/function[20], were measured as a marker of functional, metabolic enterocyte restoration following 30 minutes of ischemia.

MATERIALS AND METHODS

Ethics

The study was approved by the Medical Ethics Committee of the Maastricht University Medical Center (METC 06-3-044) and was conducted according to the revised version of the Declaration of Helsinki (October 2008, Seoul). Written informed consent of all patients was obtained.

Patients and surgical procedures

Patients: 23 patients undergoing pancreatico-duodenectomy for benign or malignant disease were included in this study. DST data were obtained from 13 patients (10F:3M) with a median age of 69 (range 48 to 85) years included in the experimental human intestinal IR model of these 3 patients underwent a sham procedure; their intestinal barrier function was assessed using DST as described below without being exposed to ischemia. DST data were compared to histology from 10 other patients (3F:7M), with a median age of 63 (range 47 to 78) years undergoing the same human intestinal IR model without applying the DST.

Human intestinal IR protocol: The experimental protocol was performed as previously described[16]. In short, during pancreatico-duodenectomy, a variable length of jejunum is routinely resected as part of the surgical procedure. The terminal 6 cm of this jejunal segment was isolated and subjected to 30 min of ischemia by placing two atraumatic vascular clamps across the mesentery (n = 20 of which 10 patients were included in the DST-protocol (see below)). Meanwhile surgery proceeded as planned. After ischemia, one third (2 cm) of the isolated ischemic jejunum was resected using a linear cutting stapler (30I) (GIA^{tm}, Covidien, Mansfield, MA). Next, clamps were removed to allow reperfusion, as confirmed by regaining of normal pink color and restoration of gut motility. Another segment of the isolated jejunum (2 cm) was resected similarly after 30 min of reperfusion (30I30R) and 120 min of reperfusion (30I120R). Simultaneously, 2 cm of jejunum which remained untreated during surgery was resected and served as internal control tissue. Tissue samples were immediately snap frozen or formalin fixed for future analysis.

DST protocol: To study the consequences of IR on intestinal barrier function loss and recovery, a bolus of 10 mL 0.9% NaCl containing the saccharides lactulose (1 mg/mL Lactulose, Centrafarm B.V. Etten-Leur, The Netherlands) and L-Rhamnose (0.5 mg/mL, Danisco Sweeteners, Copenhagen, Denmark) was directly injected into the lumen of the isolated part of intestine of 10 patients before the induction of ischemia. The piece of jejunum with the injection site was stapled off, to prevent any possible leakage of intraluminal content towards the abdominal cavity. After the first blood sample was obtained (5 min after injecting the saccharides), the human IR protocol was initiated (see detailed protocol above). The only difference with the regular IR protocol was that no tissue was resected during IR to eliminate potential confounding effects of absorptive surface reduction and decrease in luminal presence of the saccharide solution on the outcome parameters. Three patients underwent a sham procedure during which the saccharide solution was injected in the isolated jejunum segment, without this being exposed to IR. Blood from all patients was drawn, centrifuged, aliquoted and stored according to the regular intestinal IR protocol (as mentioned below) until further analysis for plasma saccharide concentrations. In addition, luminal debris from the isolated segment was sampled at the end of the IR protocol to measure the remaining saccharide concentration and compare this to the concentration at

the beginning of the experiment.

Blood sampling: Arterial blood was sampled before ischemia, immediately on reperfusion (30I), and at 30 (30I30R) and 120 min (30I120R) after start of reperfusion. Simultaneous with each respective arterial blood sample, blood was drawn from the venule draining the isolated jejunal segment by direct puncture to assess concentration gradients across the isolated jejunal segment. All blood samples were centrifuged at 3500 rpm, 4 ℃ for 15 min to obtain plasma. Plasma was immediately stored in aliquots at -80 ℃ until analysis.

Measurement of intestinal barrier function loss

Arterial and venous plasma concentrations of lactulose and L-rhamnose were measured using High Performance Liquid Chromatography combined with Mass Spectrometry (HPLC-MS). In brief, sugars were separated using ion-exchange chromatography as described previously[21]. After separation, the column effluent was mixed with an ammonia containing solvent, which enabled the electrospray ionisation to ammonia adducts. Detection, based on the mass to charge value (m/z value) of the ammonia adducts was then performed using mass spectrometry in positive mode. Arterial-venous concentrations differences and lactulose/L-rhamnose ratios (L/R-ratio) were calculated to investigate intestinal barrier function during IR.

Morphological assessment

Histology: Tissue specimens obtained during the experimental protocol were immediately immersed in 4% formaldehyde fixative (Unifix, Klinipath, Duiven, the Netherlands) and incubated overnight at room temperature. Next, tissue samples were embedded in paraffin and 4 μm sections were cut. Sections were deparaffinized in xylene and rehydrated in graded ethanol to distilled water and stained with haematoxylin and eosin (H&E).

Immunofluorescence: Cryostat sections (4 μm) were cut and stained for ZO-1, occludin and E-cadherin. Briefly, slides were dried, fixed in 10% trichloroacetic acid for 15 min and permeabilized using 30 mmol/L glycine and 1% triton X-100 in phosphate buffered saline (PBS). Non-specific antibody binding was blocked using 30 mmol/L glycine and 3% Fetal Calf Serum in PBS for one hour. Next, slides were incubated with mouse anti-human ZO-1, rabbit anti human occludin (both Invitrogen, Eugene, OR, United States) or mouse anti human E-cadherin (Abcam, Cambridge, United Kingdom). After washing, slides were incubated with goat anti-mouse Alexa 488, goat anti-rabbit CY3, or goat anti-mouse CY3 (all Invitrogen) secondary antibodies, followed by incubation with 4',6-diamino-2-phenylindole dihydrochloride (DAPI). Next, slides were washed and mounted in VECTASHIELD® Mounting Media (Vector Laboratories, Burlingame, CA, United States) and visualized with an immunofluorescence microscope.

Electron microscopy: For electron microscopy (EM), jejunal tissue was directly immersed in 2.5% glutaraldehyde and 2% paraformaldehyde fixative buffered in 0.1M cacodylate buffer at a pH of 7.4 for at least 3 d. Samples were then washed overnight in 0.1 mol/L cacodylate buffer with 7.5% sucrose and postfixed for 1 h at 4 ℃ in 1% osmium tetroxide, containing 1% lanthanum nitrate buffered to pH 7.4 with 0.1 mol/L cacodylate. After washing in cacodylate buffer containing 7% sucrose at pH 7.4, dehydration was carried out rapidly in graded ethanol series followed by routine embedding in Epon. Ultrathin sections were cut, stained with uranyl acetate and lead citrate. A Philips CM 100 electron microscope was used to visualize the ultrastructure of the intestinal epithelial lining and the distribution of lanthanum nitrate particles. The presence of lanthanum nitrate inside the paracellular space of two adjacent enterocytes is associated with TJ and AJ function loss.

Measurement of intestinal mucosal cell damage: Damage to the epithelial lining was quantified using plasma levels of Intestinal fatty acid–binding protein (I-FABP). I-FABPs are small (14 kDa) cytosolic proteins specifically present in mature enterocytes at the tip of the villus[22]. They are released upon enterocyte membrane integrity loss into the circulation, which makes them useful as markes for enterocyte damage[23]. I-FABP-levels were measured in both arterial and venous plasma samples, to allow for calculation of arteriovenous (V-A) concentration differences, by means of an in-house enzyme-linked immunosorbent assay (ELISA). This assay was developed to measure I-FABP in human plasma samples with rabbit polyclonal antibodies, using purified human I-FABP as standard (detection window: 12.5 to 800 pg/mL). Samples were run in duplicate, and a variability of 5% between sample duplicates was accepted.

Assessment of circulating amino acids

To study the metabolic activity of enterocytes following 30 min of ischemia and subsequent reperfusion, arterial and venous plasma concentrations of citrulline and glutamine were measured using HPLC (Model PU-1980 pump, Jasco Easton, MD, United States) combined with MS (Model LTQ XL, Thermo Fisher Scientific, Waltham, MA, United States) as previously described[24]. A 100 μL plasma aliquot was pipetted into a 1.5 mL Eppendorf tube that already contained 5.5 mg solid sulfosalicylic acid (SSA), vortex-mixed immediately to deproteinize the plasma samples, snap frozen in liquid nitrogen and stored at -80 ℃ until analysis.

Before analysis, deproteinized plasma samples

were thawed, vortex-mixed and centrifuged for 10 min at 50000 × *g* at 4 ℃ in a Biofuge Stratos centrifuge (Heraeus, Haarlem, the Netherlands). Next, 5 μL of the clear supernatant was diluted 100-fold in ice-cold water into a 1 mL WISP-style vial (Waters, Etten-Leur, the Netherlands). Amino acid analysis was performed by HPLC after automated pre-column derivatization using ophthaldialdehyde (OPA). At the start of each cycle, 5 μL of the diluted sample, stored in the pre-chilled sample compartment of a WISP autosampler, was automatically mixed with 5 μL of OPA reagent. The resulting OPA-amino acid derivatives were separated on a 150 mm × 4.6 mm (inner diameter) Allsphere ODS 2 3 μmol/L High-Performance Liquid Chromatography (HPLC) column (Grace, Breda, the Netherlands), using an acetonitrile gradient against an aqueous citric acid buffer (25 mmol/L, pH = 6.8) and detected by fluorescence (330 nm excitation, 440 nm emission). Arterial-venous concentration differences of citrulline divided by the arterial concentration of glutamine (Cit V-A/Gln A) reflect enterocyte functional and metabolic capacity.

Statistical analysis

Statistical analysis was performed using GraphPad Prism 5.0 for Windows (GraphPad Software Inc. San Diego, CA, United States) and SPSS 23.0 (SPSS, Inc., Chicago, IL, United States). Normality was verified by using the Kolmogorov-Smirnov test. None of the parameters showed a normal distribution. A Dunn multiple comparison test was used (after significant one-way analysis of variance) to compare DST values and amino acid ratios over time. For between-group comparisons, the Mann Whitney *U* test was used. All data are expressed as mean ± SE.

Linear mixed model regression was used to analyze correlations over time between plasma I-FABP and plasma L/R ratio. Within-person correlations were computed by normalizing both data sets. This enables the assessment of the pure association of both variables by calculating the correlation coefficient. Linear regression was used to visualize the correlation. A *P*-value below 0.05 was considered statistically significant.

RESULTS

Thirty minutes of ischemia only moderately affects intestinal barrier function

As described previously, the epithelial lining was microscopically intact directly after 30 min ischemia (30I) (Figure 1A, right panel)[18]. Immunofluorescence staining of ZO-1, occludin and E-cadherin revealed structured ZO-1 and occludin staining at the apical pole of the epithelial cells and evenly distributed E-cadherin between the epithelial cells over the tips of the villi in both control tissue and jejunum after an ischemic period of 30 min (Figure 1A). To assess whether the ischemia-induced enterocyte damage resulted in increased intestinal permeability, the plasma Lactulose/L-rhamnose (L/R) ratio was determined. No statistically significant changes were observed in the plasma L/R ratio directly after 30I compared to control (0.16 ± 0.05 *vs* 0.20 ± 0.09, $P = 0.80$; Figure 1C) or sham-operated patients (0.10 ± 0.07 *vs* 0.20 ± 0.09, $P = 0.72$; supplementary Figure 1). These data suggest that a short period of ischemia alone does not result in increased intestinal permeabi lity. EM images of two adjacent enterocytes with microvilli showed that in control tissue the interconnecting TJs were intact (Figure 1B left panel, arrowhead) and the contrast dye lanthanum remains intraluminally. However, in contrast to the histological analysis, as shown by EM after 30I, lanthanum was able to penetrate the paracellular space, indicating intestinal barrier integrity loss (Figure 1B, right panel, arrowheads).

Enterocyte damage was quantified by measuring arteriovenous I-FABP concentration differences across the studied jejunum. I-FABP arteriovenous concentration differences significantly increased from 1.75 ng/mL ± 0.78 ng/mL before ischemia to 126.6 ng/mL ± 65.53 ng/mL on reperfusion ($P < 0.01$; Figure 1D).

The arterial-venous concentration differences of citrulline divided by the arterial concentration of glutamine (Cit V-A/Gln-A) ratio in 10 patients at control was 0.04 ± 0.009. During the ischemic period, a significant decline in the Cit V-A/Gln-A ratio was observed (0.02 ± 0.004, $P < 0.02$ compared to control, Figure 2).

This decreased plasma Cit-V-A/Gln-A ratio was the result of a decline in venous citrulline concentrations after ischemia (see Supplementary Table 1).

Initial reperfusion results in significant tight junction damage and functional intestinal barrier loss

After 30 min of ischemia followed by 30 min of reperfusion (30I30R), shedding of IR-damaged enterocytes from the villus tips towards the intestinal lumen was observed (Figure 3A, right panel, arrowhead). Short reperfusion led to compromised TJ integrity as immunofluorescence for ZO-1 and occludin showed an irregular staining pattern and loss of protein expression in the epithelial sheets that had lost contact with the basal membrane at the tips of the villi (Figure 3A, right panels, arrowheads). This was accompanied by disruption of E-cadherin with diffuse staining in the cell cytoplasm across the villi (Figure 3A, right panel, arrowheads). The plasma L/R ratio significantly increased after 30I30R compared to control (0.75 ± 0.10 *vs* 0.20 ± 0.09, $P < 0.01$; Figure 3C). Analysis also showed an increase of the plasma L/R ratio after 30I30R compared to sham (0.75 ± 0.10 *vs* 0.04 ± 0.01, $P < 0.02$; supplementary Figure 1). EM analysis revealed that lanthanum was still able to penetrate between two adjacent enterocytes demonstrating disruption of TJs and AJs after 30I30R (Figure 3B). These results show that the intestinal barrier function gets compromised

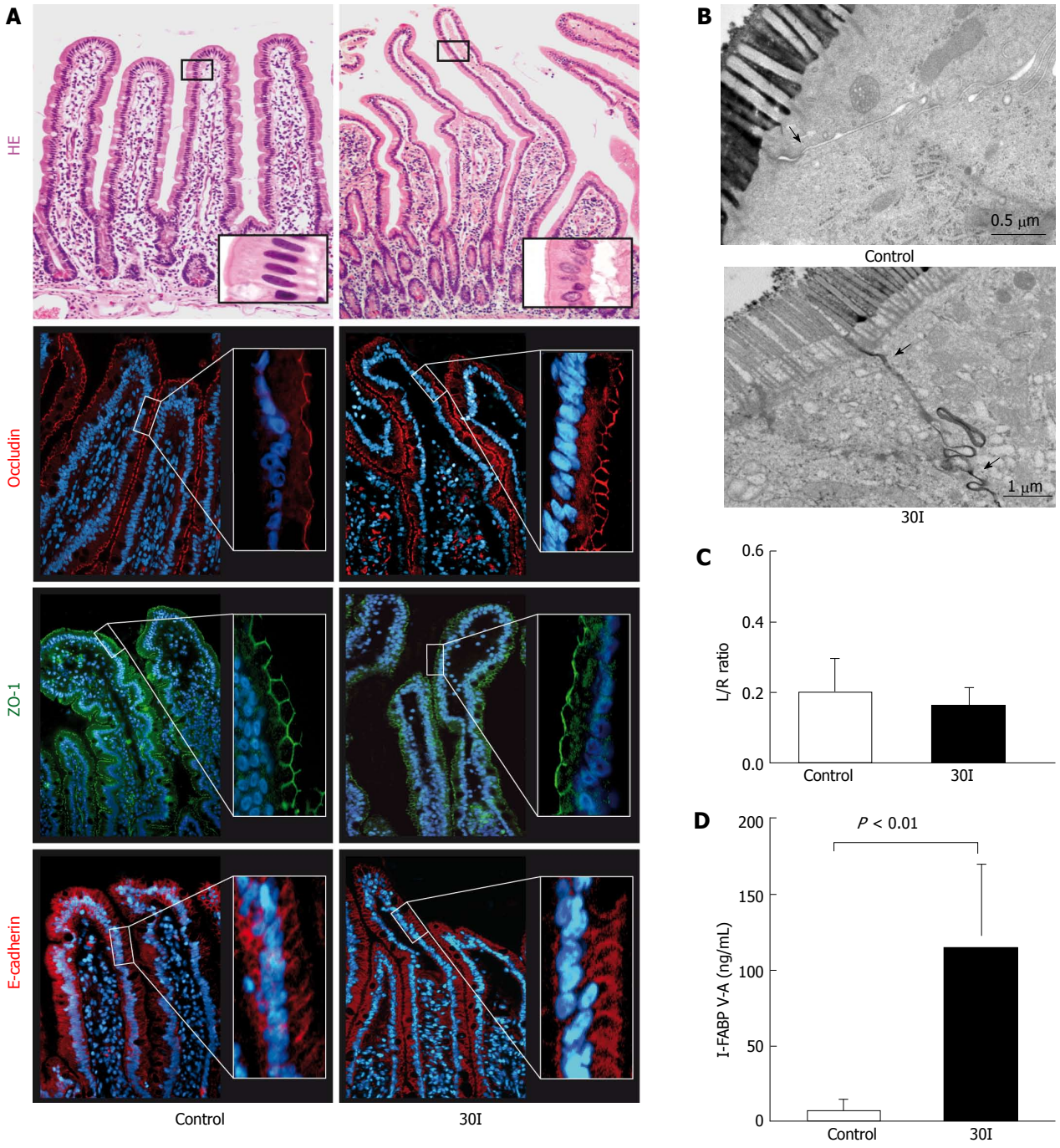

Figure 1 Changes in intestinal barrier function after 30 min of ischemia. A: HE staining showed an intact epithelial lining in control tissue. After 30 min of ischemia (30I) subepithelial spaces were visualized at the tips of the villi. Strong and evenly distributed immunofluorescent stainings for ZO-1, occludin and E-Cadherin were observed, over the tips of the villi, in both control tissue and jejunum subjected to 30I; B: Electron microscopic images of two enterocytes with interconnecting tight junctions demonstrate in control tissue that tight junctions are intact (arrow) and the contrast dye lanthanum remains intraluminally. On the contrary, after 30I, lanthanum is able to penetrate the paracellular space, indicating tight junction integrity loss (arrow); C: Plasma L/R ratio's however, remained unchanged after ischemia when compared with control; D: Mean plasma I-FABP V-A concentration differences show a significant release after ischemia when compared with control (126.6 ng/mL ± 65.53 ng/mL *vs* 1.75 ng/mL ± 0.78 ng/mL, $P < 0.01$), reflecting enterocyte damage. Electron microscopy scale bars = 0.5 μm and 1 μm respectively.

during the early stages of reperfusion after a short ischemic hit.

I-FABP V-A concentrations declined after 30I30R but were still significantly increased compared to control (27.06 ± 2.73 ng/mL *vs* 1.75 ± 0.78 ng/mL, $P = 0.01$; Figure 3D), still indicating loss of enterocyte membrane integrity during the early reperfusion phase.

The Cit V-A/Gln A ratio however, was increased after a short period of 30 minutes of reperfusion when compared with 30 min of ischemia (0.05 ± 0.09 *vs* 0.02 ± 0.004, $P < 0.001$, (Figure 2) and was no longer significantly different compared with control. This may indicate that the remaining enterocytes are already

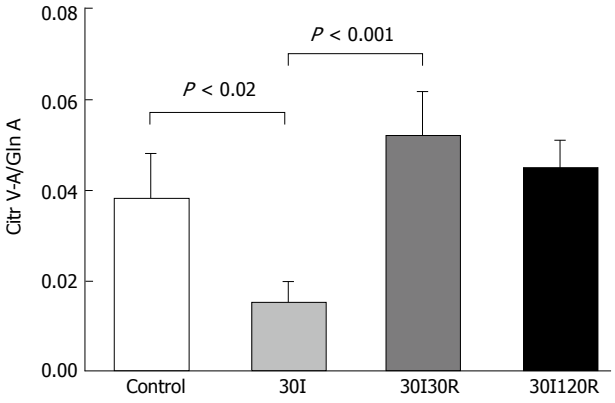

Figure 2 Mean Cit V-A/Gln A ratios in patients submitted to 30 min of ischemia followed by 30 and 120 min of reperfusion. Ratios were significantly decreased after 30I compared to control. During reperfusion ratio's return back to baseline values, which were no longer different compared to control. Results are presented as mean ± SE. A *P*-value of < 0.05 was considered significant.

viable despite the compromised intestinal barrier.

Intestinal barrier restores within 120 min of reperfusion

After 30I120R, shedding of IR-damaged cells led to shortening of the villi, with an intact epithelial lining (Figure 4A, right panel). Immunofluorescence showed that both ZO-1 and occludin again formed a continuous staining pattern around the epithelial lining near the apical region of the enterocytes. In addition, E-cadherin staining appeared normal and was visible along the entire epithelium of each villus, rebuilding epithelial cell-cell integrity (Figure 4A, right panel). In line, the L/R ratio normalized at 30I120R to 0.17 ± 0.06 and was not significantly different from control or sham (Figure 4C). In EM images, lanthanum was no longer present in paracellular spaces after 30I120R (Figure 4B, right panel) indicating restored tight junction integrity.

I-FABP levels rapidly decreased over the course of a period of 120 min reperfusion to 14.77 ng/mL ± 5.46 ng/mL and were no longer significantly elevated compared to control, demonstrating rapid and full recovery of epithelial membrane integrity (Figure 4D).

Remarkably, at 120 min of reperfusion the Cit V-A/Gln A ratio no longer differed from the value measured at control (Figure 2). The results demonstrate that, despite IR-induced reduction of enterocyte mass, the remaining epithelial cells are functional again after a short period of ischemia followed by 120 min of reperfusion

To rule out the possibility that the low concentrations of saccharides measured at later time points were due to significantly decreased intraluminal saccharides as a consequence of uptake/leakage during early ischemia and reperfusion, we measured intraluminal concentrations of saccharides at 30I120R. Although the concentrations measured at 120R were approximately 3 times lower than the administered stock (29.16 ± 0.31 *vs* 11.47 ± 1.01 $P < 0.002$) and L-rhamnose (30.62 ± 1.80 *vs* 8.22 ± 1.30 $P < 0.002$, Supplementary Figure 2), the luminal concentrations of both sugars were still 100x higher than the observed plasma lactulose and L-rhamnose concentrations at 30I120R.

Intestinal barrier function in relation to intestinal damage

The IR-induced damage to the epithelial lining was measured using plasma I-FABP. This sensitive plasma parameter of enterocyte damage is rapidly released upon intestinal ischemia. This allows us to relate the structural damage to the mucosa with the permeability changes during intestinal IR. To this end within-person correlations were studied between arteriovenous concentration differences of plasma I-FABP and L/R ratios. Plasma levels of I-FABP correlated with plasma L/R ratio's measured at the same time points [correlation: 0.467, ($P < 0.01$)], indicating a relationship between structural damage to the intestinal mucosa and the observed changes in permeability (Figure 5).

DISCUSSION

The current study demonstrated that the human intestinal epithelial barrier has the remarkable capacity to rapidly and functionally restore IR-induced tissue damage within 120 min of reperfusion. Second, we show in this study that the currently used golden standard to measure intestinal epithelial permeability *i.e.* the dual sugar test, reflects intestinal barrier function as objectified by electron microscopy in our model of acute intestinal damage and repair. These results are important for our understanding of the pathophysiology of human intestinal ischemia-reperfusion in general, and have implications for clinical practice. There is increasing evidence that the loss of intestinal barrier function after a period of ischemia or hypoperfusion is associated with the development of sepsis and multiple organ dysfunction in the acute setting, including trauma, hemorrhagic shock, following major (vascular) surgery or acute pancreatitis[2,25]. Furthermore, exposure of the intestinal epithelium to periods of ischemia followed by reperfusion results in sloughing of enterocytes at the tips of villi causing a breach in the gut barrier[12,26,27]. Disruption of epithelial integrity is associated with intestinal inflammation, bacterial translocation, the development of sepsis and multiple organ dysfunction[4,28,29]. In addition, loss of intestinal barrier function plays a role in the pathophysiology of various intestinal diseases including IBD and celiac disease[3,30]. Epithelial hypoxia can occur secondary to the inflammation, and might play a role in the pathophysiology[31] and etiology[32] of IBD.

The intestinal barrier function is dependent on an intact epithelial lining which is formed by epithelial cells, interconnected by cell to cell adhesion proteins

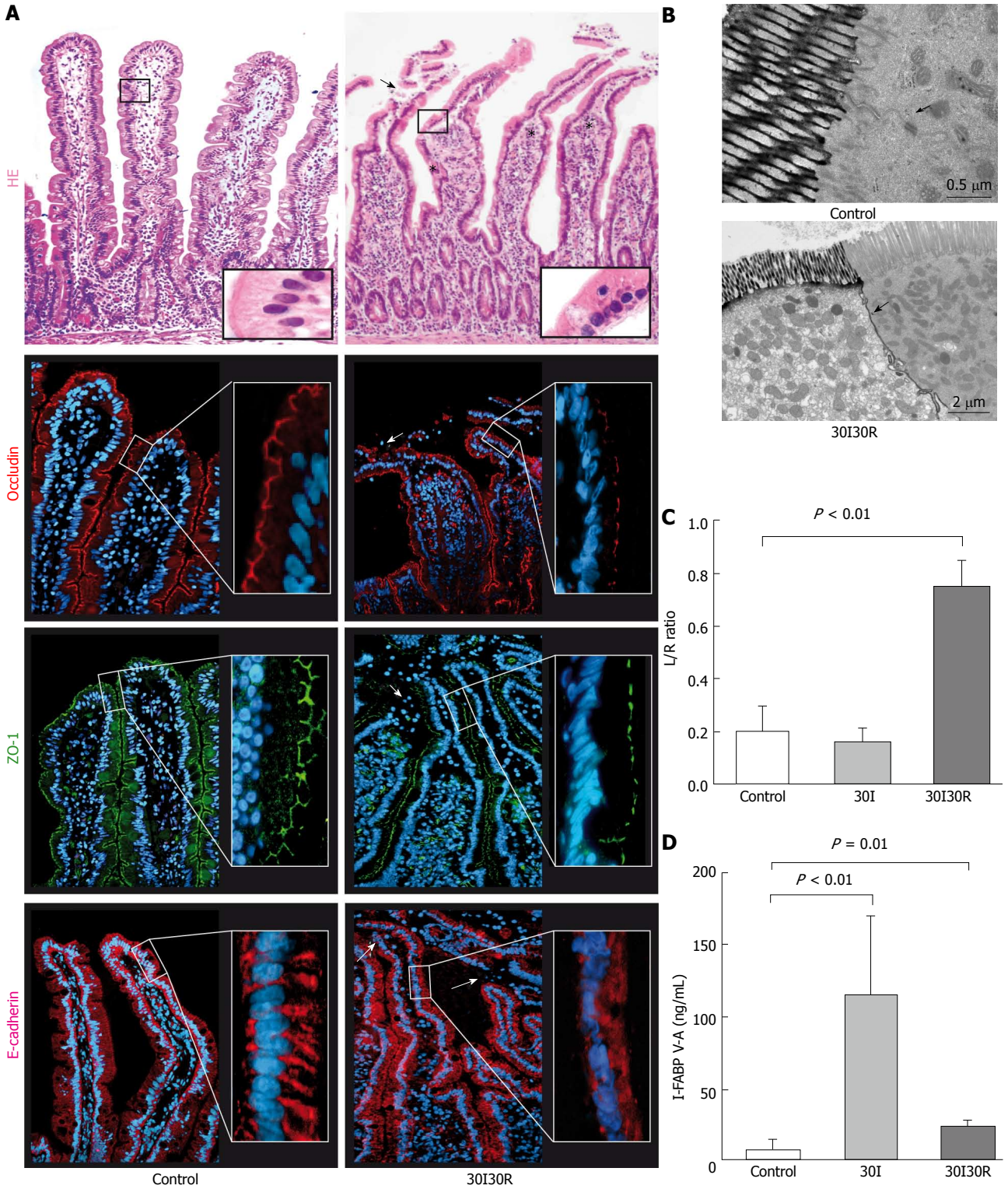

Figure 3 Thirty min of reperfusion results in tight junction damage and functional intestinal barrier loss. A: After 30 min of reperfusion (30I30R) damaged epithelial cells were pinched off and shed into the lumen (arrow). Immunofluorescence for ZO-1 and occludin revealed irregular distribution patterns (arrowheads) and loss of expression in the epithelial sheets at the tips of the villi, together with the disruption of E-Cadherin showing only diffuse staining in the cell cytoplasm; B: Lanthanum was still able to penetrate between two adjacent enterocytes (arrowhead) demonstrating disruption of TJs and AJs at 30I30R; C: Plasma L/R ratio was significantly elevated at 30I30R compared to control (0.75 ± 0.10 *vs* 0.20 ± 0.09, $P < 0.01$), indicating increased intestinal permeability after short reperfusion; D: Plasma I-FABP gradually reduce following 30I30R but was still significantly elevated compared to control (27.06 ± 2.73 ng/mL *vs* 1.75 ± 0.78 ng/mL, $P = 0.01$), indicating entrocyte membrane integrity loss. Electron microscopy scale bars = 0.5 μm and 2 μm respectively.

at different levels of the intercellular junction[3]. TJ complexes consist of transmembrane proteins, including occludin and claudins linked to the cell cytoskeleton by intracellular proteins, such as ZO-1. The TJ barrier controls the paracellular pathway *via* at least two routes allowing selective transport across

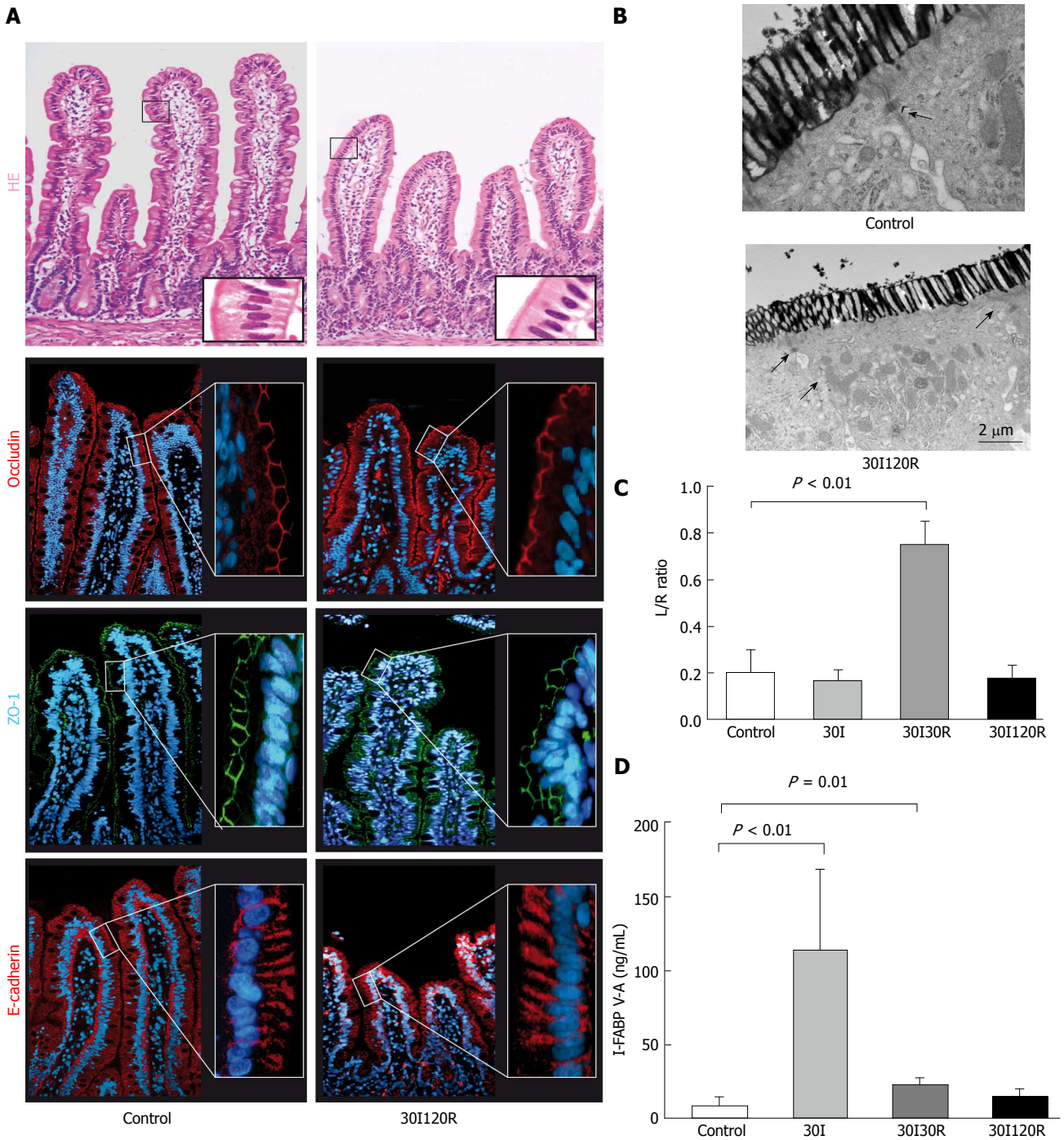

Figure 4 The intestinal barrier is restored after 30 min of ischema followed by 120 min of reperfusion. A: At 120 min of reperfusion (30I120R) the epithelial lining appeared histologically intact compared to control tissue with ZO-1, occludin and E-Cadherin normally distributed across the villi.); B: In addition, electron microscopy revealed that lanthanum was no longer present in the paracellular spaces, indicating restored tight junction integrity; C: Moreover, the observed restoration of the intestinal barrier seems to correlate with permeability with the plasma L/R ratio normalized to 0.17 ± 0.06 which was no longer significantly different from control; D: Plasma I-FABP levels, reflecting intestinal epithelial damage, also returned to 14.77 ng/mL ± 5.46 ng/mL and were no longer significantly elevated compared to control. Electron microscopy scale bars = 0.5 μm and 2 μm respectively.

the intestinal barrier. The pore pathway is a size- and charge-selective route for ions which is regulated by claudins. The leak pathway allows paracellular transport of large molecules, including proteins and bacterial lipopolysaccharides. Studies have shown that occludin and ZO-1 are mainly involved in the regulation of the leak pathway. Mainly defects in the leak pathway lead to increased epithelial permeability allowing leakage of bacterial products and antigens from the lumen into the systemic circulation[4,8].

At 30 min of ischemia, ZO-1 and occludin appeared as continuous bands along the villous tips, similar as

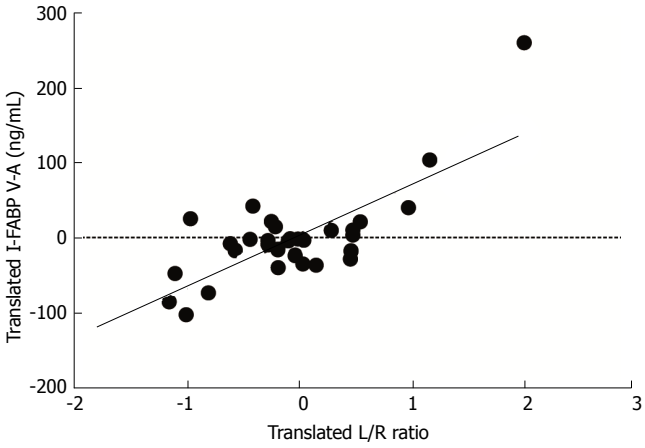

Figure 5 Translated values of plasma levels of I-FABP and translated values of L/R ratio's in patients submitted to 30 min of ischemia followed by 30 and 120 min of reperfusion. I-FABP correlated significantly with L/R ratio's [correlation: 0.467, (P < 0.01)]. Translations of both variables are specific for an individual in such a way that all within-person means correspond to the zeros in the plot. In this way the variation of individual levels are cancelled and the pure association of both variables remains.

the immunofluorescent staining of control tissue. In line, the DST showed that the intestinal barrier was functionally still intact. Similar to the human setting animal studies have shown that the appearances of sub epithelial spaces during ischemia are not accompanied by increased permeability for lactulose[33].

In contrast, shortly after reperfusion desquamation of the intestinal epithelium at the tips of the villi occurred. Although the gut possesses elegant pathways that maintain the intestinal barrier during cell shedding[34,35], excessive rates of apoptosis make these pathways to fall short resulting in intestinal barrier loss[26,36]. Indeed, TJ derangements were observed and accompanied by increased L/R ratios.

These human data are in line with previous data from animal models demonstrating that the magnitude of ischemia and reperfusion-induced intestinal mucosal damage is a result of the duration of occlusion time. This was evaluated in standardized animal models of complete segmental arterial ischemia demonstrating that 30 min of ischemia followed by 30 min of reperfusion results in massive epithelial lifting with a few denuded villi[37]. Moreover, the mechanism of intestinal barrier repair during intestinal IR is also previously described[26]. This is a highly regulated event involving villus contraction, epithelial restitution and closure of the paracellular space. The latter is considered to account for the majority of barrier recovery after intestinal injury. The net result of the above mentioned repair mechanisms is a remarkably rapid closure of mucosal wounds in the mammalian intestinal epithelial lining to prevent the onset of sepsis. The observed time scale of intestinal barrier restoration within hours after ischemia, is also in agreement with previous studies[26].

To study if the intestinal barrier is indeed functionally repaired, it is necessary to be able to actively and reliably measure intestinal barrier function. However, this has been a major challenge in clinical practice. Classically, DST are used based on the differential absorption of intraluminal sugars of different molecular weight across the intestinal barrier. Using these tests it has been shown that intestinal barrier function is involved in intestinal diseases including patients with IBD and celiac disease[38,39]. However, a pitfall of the DST is the lack of knowledge on how these tests correlate to epithelial cell damage and tight junction loss in vivo. In intestinal IR, loss of intestinal barrier function is seen as key explanation of intestinal dysfunction in patients leading to increased incidence of bacteria and toxin translocation from the intestinal lumen to the systemic circulation, causing infectious complications including sepsis and the multiple organ failure syndrome[28]. Using our *in vivo* human model, we were able to demonstrate the direct relationship between morphological barrier loss (epithelial cells and TJ proteins) and functional intestinal barrier loss (dual-sugar absorption test and leakage of lanthanum nitrate through defective tight junction complexes as visualized by EM).

I-FABP concentrations and release-patterns were comparable with previous published data from our group[23]. This shows that 30 min of ischemia is associated with rapid reversal of IR-induced structural tissue damage of the epithelial lining. Interestingly, the extent of intestinal damage, reflected by an increase in plasma I-FABP, significantly correlated with the permeability changes in the small intestine. This is in line with previous studies performed in humans undergoing splanchnic hypoperfusion because of moderate-to-high intensity exercise demonstrating that intestinal cell damage result in permeability changes including restoration of the epithelial lining[40].

Our results showed that the DST data closely reflect the morphological findings during intestinal IR. This is of importance as it supports the usefulness of DST[10,41,42].

In addition, we provide support for the use of citrulline as a marker for enterocyte function. Measurement of gastrointestinal dysfunction, following small bowel diseases, is hard to establish[43]. Citrulline has been used for this purpose as it is an amino acid mainly produced by small intestinal enterocytes by the conversion of glutamine through the glutamate to ornithine pathway, without incorporation into proteins[44]. Loss of the intestinal epithelial lining has been shown to result in declined circulating levels of citrulline[45]. Several studies showed that there is a link between low plasma citrulline concentration and intestinal barrier function[46-48]. Supply of glutamine to the enterocyte occurs from both arterial blood as well as from the intestinal lumen. As all patients were fasted, we hypothesized that the arterial-venous concentrations of citrulline divided by the arterial concentration of glutamine (Cit V-A/Gln A) ratios reflect enterocyte functional capacity. It is striking that the DST is at baseline level at 30I and only increased at 30I30R,

whereas the Citr/Gln ratio is only decreased at the 30I time point and not during reperfusion. This discrepancy is in agreement with previous studies in patients with chemotherapy-induced mucositis and patients suffering from IBD, where permeability was not correlated with changes in plasma citrulline concentrations[49,50]. This may indicate that enterocytes do not require an intact intestinal barrier to be metabolically viable. Citrulline performed better as a marker for functional epithelial cell mass in these studies where it detected impairment of intestinal epithelium and small intestinal barrier integrity earlier compared with the DST and indicated recovery more accurate.

Potential limitations of this study may include the fact that the intraluminal concentration of lactulose and L-rhamnose show a decrease during the IR protocol. This drop in concentration however, is only partly relevant since the remaining concentrations in the lumen were still 100x higher than the observed plasma lactulose and L-rhamnose concentrations at 30I120R. Furthermore the hyperosmolarity of the saccharide solution may affect the intestinal permeability for L-rhamnose directing the flux towards the intestinal lumen. To correct for this diffusion, the experimental IR protocol started 5 min after injection of the saccharides. In addition, dual-sugar absorption tests can be influenced by the presence of food-derived sugars in plasma and most of the studies of intestinal permeability do not report baseline excretion of the saccharides, which is perhaps most relevant with the use of DST. As baseline measurements were taken in the current study, this should have been accounted for.

Next, the larger part of the total paracellular conductance of intestinal epithelial lining is *via* a high linear density of TJs residing in the crypts[4]. After villus contraction, caused by the IR injury, crypt epithelium accounts for the majority of surface area remaining. As we did not focus on the role of the crypts in this study, the latter could be of influence on the results. Future studies, measuring mucosal barrier function within the crypts are warranted to investigated villus/crypt differences during IR injury.

Also, one should be careful generalizing the current findings to the whole length of the human gastrointestinal tract. First, it is important to understand that epithelial permeability of the gastrointestinal tract needs to be evaluated in a site specific manner. Several saccharide probes are destroyed by digestion processes that take place in the lumen of different parts of the gut, which limits their capabilities to detect permeability changes throughout the hole intestinal tract. For example lactulose and L-rhamnose are destroyed in the caecum and therefore provide only information regarding the small intestinal epithelium[3]. Also the expression of epithelial tight junction proteins is region-specific along the gastrointestinal tract, which determine the properties of permeability in different regions. The proximal segments of the jejunum have a higher permeability than the distal ileum segments. Next, Takeyoshi *et al*[51]. evaluated the mucosal regeneration of different parts of the small intestine during transplantation in dogs. They showed that the regenerative capacity was twice as fast in the jejunum than in the ileum. This more pronounced recovery in jejunal tissue could have a beneficial effect on our data.

Last, important to note is that the dual-sugar tests have not yet gained a place in everyday practice for diagnosis and follow up of the several patients groups, mainly because the detection methods are complex and not widely available. Taken together, these findings reflect the ability of the intestine to withstand short episodes of ischemia, with morphological and functional recovery of the intestinal barrier within 120 minutes of reperfusion. These results explain why there are often no signs of inflammation or bacterial translocation after short periods of intestinal ischemia. Further exploration of the mechanisms responsible for this rapid morphological and functional recovery might impact treatment of intestinal diseases associated with barrier recovery loss. Next, data from the DST and citrulline glutamine ratios closely reflect the histological perturbations during intestinal IR highlighting the potential diagnostic value of these tests in the follow-up of patients with intestinal disease associated with intestinal barrier loss.

COMMENTS

Background

Human small intestine is frequently exposed to ischemia without severe complications. Intestinal hypoxia and loss of intestinal barrier function are associated with the onset of sepsis and multiorgan failure or intestinal diseases including celiac disease and inflammatory bowel disease. Rapid morphological recovery occurs in human ischemia/reperfusion-exposed small intestine by a zipper like constriction of the epithelium.

Research frontiers

Patients with intestinal ischemia may suffer from sepsis, however we showed in previous human studies that short periods of ischemia led to fast structural recovery of damaged mucosa. Animal studies suggest that this is accompanied by barrier function recovery. In humans, we still have to elucidate whether structural ischemic damage and recovery correlates with barrier function. This is of importance, since complete knowledge of pathophysiological sequelae of human intestinal ischemia-reperfusion will help us to guide new therapeutic options and develop better diagnostic possibilities for the patients suffering from intestinal ischemia.

Innovations and breakthroughs

The author's results show that short periods of human intestinal ischemia are followed by mucosal damage, which is rapidly followed by herstel. Most important, we add to this knowledge that the gut has the remarkable capacity of functional recovery after short periods of ischemia. Furthermore, our results indicate that the dual-sugar absorption tests and plasma citrulline may have a potential diagnostic value in detecting and monitoring patients with intestinal diseases associated with intestinal barrier loss.

Applications

There is great need for better insight in the pathophysiology of small intestinal IR, because of the high morbidity and mortality rates, while preventive

and/or therapeutic approaches are lacking. Results of this study will add important knowledge to our understanding of the pathophysiology of intestinal ischemia. This will also help to early detect intestinal ischemia and its serious consequences. Next it will help open new opportunities to develop therapeutic interventions.

Terminology

Intestinal ischemia-reperfusion: Pathological condition characterized by an initial undersupply of blood to the intestine followed by a restoration of perfusion and reoxygenation Reperfusion and reoxygenation is commonly associated with an exacerbation of tissue injury and a profound inflammatory response. This often leads to systemic inflammatory response syndrome (SIRS), sepsis and multiple organ failure (MOF), causing the high morbidity and mortality rates associated with intestinal IR. Intestinal barrier: The protective component of the intestine shielding us against the invasion of bacteria toxins and antigens, but on the other hand enabling us to absorb nutrients

Peer-review

The study is well designed and sound, the manuscript is interesting. Maintenance of the intestinal barrier is an important defense against invasion of luminal pathogens, and assessment of barrier function is relevant in a number of diseases of the gut where the barrier is compromised. The paper presents some interesting in vivo data showing the ability of the jejunal epithelium to recover rapidly following ischemic injury. Overall, the study is well designed and sound.

This is a straight-forward paper associating results from lactulose/rhamnose ratios with histology and microscopic observations of the intestine following ischemia reperfusion. Much of what is reported has been demonstrated in animal models, but not before in human tissue.

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ORIGINAL ARTICLE

Basic Study

Stable gastric pentadecapeptide BPC 157 in the treatment of colitis and ischemia and reperfusion in rats: New insights

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Abstract

AIM

To provide new insights in treatment of colitis and ischemia and reperfusion in rats using stable gastric pentadecapeptide BPC 157.

METHODS

Medication [BPC 157, L-NAME, L-arginine (alone/combined), saline] was bath at the blood deprived colon segment. During reperfusion, medication was BPC 157 or saline. We recorded (USB microscope camera) vessel presentation through next 15 min of ischemic colitis (IC-rats) or reperfusion (removed ligations) (IC + RL-rats); oxidative stress as MDA (increased (IC- and IC + RL-rats)) and NO levels (decreased (IC-rats); increased (IC + RL-rats)) in colon tissue. IC + OB-rats [IC-rats had additional colon obstruction (OB)] for 3 d (IC + OB-rats), then received BPC 157 bath.

RESULTS

Commonly, in colon segment (25 mm, 2 ligations on left colic artery and vein, 3 arcade vessels within ligated segment), in IC-, IC + RL-, IC + OB-rats, BPC 157 (10 μg/kg) bath (1 mL/rat) increased vessel presentation, inside/outside arcade interconnections quickly reappeared, mucosal folds were preserved and the pale areas were small and markedly reduced. BPC 157 counteracted worsening effects induced by L-NAME (5 mg) and L-arginine (100 mg). MDA- and NO-levels were normal in BPC 157 treated IC-rats and IC + RL-rats. In addition, on day 10, BPC 157-treated IC + OB-rats presented almost completely spared mucosa with very small pale areas and no gross mucosal defects; the treated colon segment was of normal diameter, and only small adhesions were present.

CONCLUSION

BPC 157 is a fundamental treatment that quickly restores blood supply to the ischemically injured area and rapidly activates collaterals. This effect involves the NO system.

Key words: Ischemic colitis; Blood flow rescue; Collaterals; BPC 157; L-NAME; L-arginine; Oxidative stress; NO; Rats

Core tip: We rescued rat ischemic colitis. The gastric pentadecapeptide BPC 157, which has been used in clinical trials for ulcerative colitis, exerted rapid cyto-protective endothelium rescue against the disabled left colic artery and vein after blood deprivation via two ligations and during reperfusion (ligations removed). By bypassing obstructions, quickly rescuing blood supply, rapidly activating collaterals, and restoring arcade interconnections, as a new integrative beneficial effect, BPC 157 prevented the occurrence of pale lesions without mucosal folds and normalized the levels of NO and MDA, two oxidative stress markers, in tissues. BPC 157 showed effectiveness over the NO-system background, immobilized (L-NAME + L-arginine), (over)stimulated (L-arginine) or blocked (L-NAME). Likewise, later application of BPC 157 in a bath treatment to rats with pertinently obstructed vessels that underwent additional colon obstruction for three days produced a similar beneficial effect.

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INTRODUCTION

The colon is particularly susceptible to insufficient vascular perfusion[1]. In this work, we focused on the prototype cytoprotective anti-ulcer peptide stable gastric pentadecapeptide BPC 157, which has been used in trials for ulcerative colitis and now for multiple sclerosis, in the treatment of colitis and ischemia in rats[2-8], seeking new insights into ischemic colitis (IC), ischemia and reperfusion and therapy. The harmful events were on the left colic artery and vein, such as two ligations (IC rats) or injuries from removed ligations (RL) (IC + RL rats) (there was always gross vessel presentation failure), and the combination of two obstructions, ligation of the vessels and additional colon obstruction (OB) (IC + OB rats). IC was assessed at short (minutes) (IC rats, IC + RL rats) or more prolonged (3- and 10-d) intervals (IC + OB rats).

The main focus of the intervention was that BPC 157 rapidly activates collaterals due to its particular direct and rapid effect on vessel presentation, the bypassing of one or more of the vascular obstructions and thereby achieving a therapeutic effect.

The next focus was on NO-system, the effect of NO system agents in IC rats, NOS blocker L-NAME and/or NOS substrate, L-arginine[2-8]; in colon tissue, assessing NO levels and oxidative stress (MDA levels) (as result of the lysis of endothelial cells[9,10]) and ischemia/reperfusion injury, both as a spontaneous course [when blood supply was deprived *via* ligation (IC rats)] and a more exaggerated course (after ligation removal (IC + RL rats)) in immediate post-ligation time. Notably, although the NO system is largely implicated in stomach cytoprotection and colitis lesions[2-4], the application of L-NAME (a vasoconstrictor)[11] and/or L-arginine (a vasodilator)[11] has not been investigated with respect to the immediate presentation of the

blood vessels after a segment of left colic artery and vein was occluded by two ligations. By contrast, BPC 157 largely interacts with the NO system in various models and species, as shown in cytoprotection studies, in particular, studies using both L-NAME and L-arginine as individual agents or in combination[2-4].

The shared essential effect of cytoprotective agents, which was originally noted in the stomach, is regarded as a rapid endothelial protective mechanism that can be used to prevent and resolve adjacent ischemic mucosal lesions[12-16]. It may be possible to extend the rapidly occurring beneficial cytoprotective effect of these agents and to use them to prevent ischemic colitis lesions, in which specific activation of the collateral circulation can circumvent obstructions and reestablish the continuity of blood flow. This effect must be a long-lasting effect that is exerted within the immediate post-injury time period (a factor that has not investigated in vascular studies of ischemic colitis[17-19]); it must also be applicable in the later period, even after a considerable period of additional colon obstruction has occurred.

Unlike standard cytoprotective agents that exhibit only prophylactic effectiveness (shared limitation of activity)[9,10,12,13,15,16,20], BPC 157 represents a prototype of a more effective class of cytoprotective agents with both prophylactic and therapeutic ability[7,16]. BPC 157, as a novel mediator of Robert's cytoprotection[2-8,20], is native and stable in human gastric juice and maintains gastrointestinal mucosal integrity[2-8]. BPC 157 additionally maintains the gross presentation of stomach blood vessels under harmful conditions[21,22] when these vessels would otherwise disappear. In addition to its beneficial effect, BPC 157 counteracts the vessels' disappearance[21,22]. Furthermore, this additional rapid cytoprotective vascular recovery and presentation leads to a consequent strong angiogenic effect in subsequent days[2,22-28]. Its angiogenic response[2,22-28] in combination with its healing effects and its interaction with several molecular pathways[11,25-29] is more profound than the angiogenesis of standard anti-ulcer agents[23].

Previously, BPC 157 also counteracts colitis (in various models of colitis[30-35]) and its complications, such as fistulas[36-38], failed healing of anastomoses[30,34,38-40] and other gastrointestinal lesions that were otherwise poorly healed[2-8], given parenterally or per-orally. For further clarification and to show a direct beneficial effect, medication was given once as a bath to the segment of left colic artery and vein obstructed by two ligations. Consequently, beneficial effects can be directly related to the reversal of obstructive injury outcome consequences, in both early (IC rats; IC + RL rats) and later (IC + OB rats) periods, and may be triggered shortly after injury initiation [IC rats (vessels ligation)]; IC + RL rats (ligations removed, exaggerated reperfusion)) or later, with an already-advanced injury course (IC+OB rats). Then, to focus on the initial ligation-course (blood vessel presentation), pentadecapeptide BPC 157, L-NAME, L-arginine were given to in IC rats alone and/or in combination. Alternatively, BPC 157 was given post-ligation. At these points, colon tissue levels of MDA and NO were assessed, and results showed NO colon tissue levels and oxidative stress (MDA) and ischemia/reperfusion injury during the ligation course; these effects were even worse after ligation removal and post-ligation. Likewise, as a therapeutic effect that is also applicable in the later period, BPC 157 was given to the rats with ligated left colic artery and vein, and they underwent additional colon obstruction for three days (IC + OB rats).

MATERIALS AND METHODS

Animals

Male Albino Wistar rats, 200 g b.w., were used in the experiments. The animals were randomly assigned to groups of at least 6 rats per group. The experiments were approved by the local ethics committee. The surgical procedure was performed in rats that had food and water ad libitum before the procedure and until the end of the experiment, and assessments were performed by an observer unaware of the treatments used.

Drugs

The pentadecapeptide Gly-Glu-Pro-Pro-Pro-Gly-Lys-Pro-Ala-Asp-Asp-Ala-Gly-Leu-Val, M.W. 1419, named BPC 157, which is a part of the sequence of human gastric juice protein, coded BPC, freely soluble in water at pH 7.0 and in saline, was prepared (Diagen, Ljubljana, Slovenia) as described before[2-7,34]. L-NAME and L-arginine were commercially purchased (Sigma, United States).

Experimental protocol and assessment

The purpose of the experiments was to counteract both immediate and late consequences of vessel ligation injury in blood-deprived colon segments and to produce and assess rapid cytoprotective recovery.

Initial assessment in rats with two ligations at the left colic artery and vein for 15 min (IC rats) and in rats that had two ligations at the left colic artery and vein for 15 min, and then were reperfused for the next 15 min (IC + RL rats)

The surgery was conducted in deeply anesthetized rats. A segment of the left colic artery and vein was occluded by 2 ligations (Premilene 7/0, Braun), 3 arcade vessels within the ligated segment, the 25-mm blood-flow-deprived descending colon segment. Medication (/kg, 1 mL bath/rat) applied to the 25-mm blood-flow-deprived colon segment, included BPC 157 (10 μg), the NOS blocker L-NAME (5 mg), the NOS substrate L-arginine (100 mg) alone or in combination or a saline bath of equal volume (control) at 1 min of ligation time. In rats that received two ligations of the left colic artery

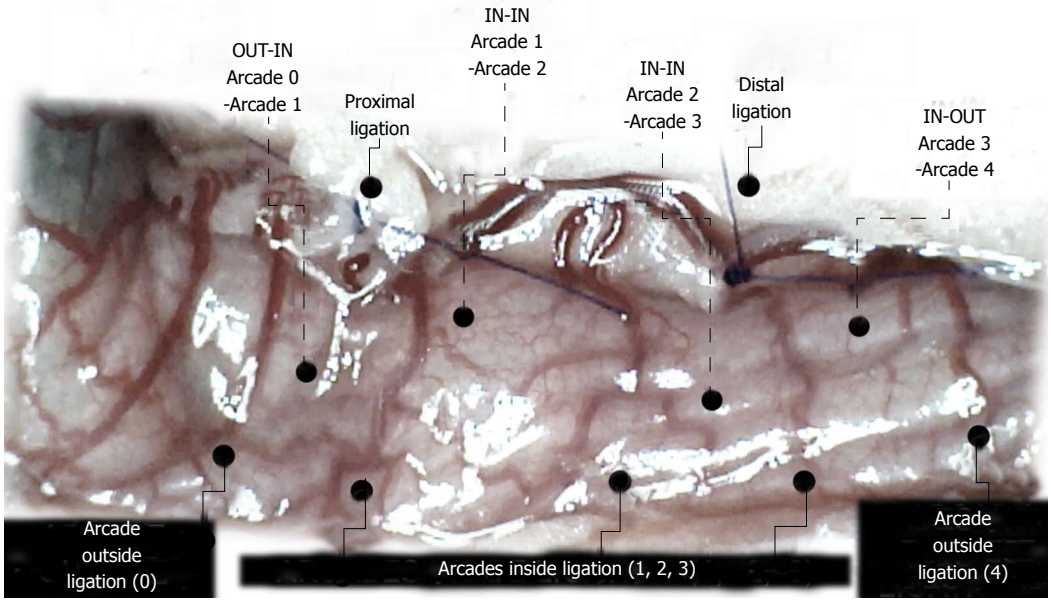

Figure 1 Assessment of arcade vessels (arcade 0; arcade 1, 2, 3; arcade 4) (dorsal colon side before the initiation of therapy, USB microscope camera). The ligations include three major arcades within the ligations (arcade 1, 2, 3), and thereby the possibility of bypassing both obstructions caused by the proximal and distal ligations (full lines) by an additional network of vessels located between the arcades (outside (arcade 0, arcade 4) and inside ligation (arcade 1, 2, 3); outside (arcade 0)-inside (arcade 1); inside -inside(arcade 1-arcade 2; arcade 2-arcade 3); inside (arcade 3)-outside (arcade 4) (dashed lines) at the both ventral and dorsal sides of the colon. Note that there is no contact between inside arcade 3 and outside arcade 4. The appearance of the arcade vessels at specific time points [A (1 minute before therapy), B (next 5 min), C (the next 5 minutes, until the 10th min), D (until the end of the 15th min)] was assessed between (1) the last arcade proximal to first ligation (arcade 0) at the first arcade distal to first ligation, inside the ligated segment (arcade 1) (OUT-IN) (0-1, Figure 2, ♦--, Figures 5, 6, 13, 14); (2) between the next arcade (arcade 2), middle arcade inside ligations (IN-IN) (1-2, Figure 2; ▲ , Figures 5, 6, 13, 14) and the following arcade (2-3, Figure 2; --X--, Figures 5, 6, 13, 14), and finally presentation to the first outside arcade distal to the second ligation (arcade 4) (IN-OUT) (3-4, Figure 2; ■, Figures 5, 6, 13, 14).

and vein for 15 min, the ligations were removed, and the area was then reperfused for next 15 min (IC + RL rats), medication at that colon segment during reperfusion included BPC 157 (10 μg) and saline bath of equal volume (control) at 1 min of reperfusion time.

Using a camera attached to a USB microscope (Veho discovery VMS-004 deluxe), we recorded the vessel presentation (fulfilled/appearance or cleared out/disappearance) between the arcade vessels on the ventral and dorsal sides throughout the following 15 min [with regard to the point immediately before therapy (as 100%)] at selected time points before and after therapy [see the assessment shown in Figure 1 (IC rats)]. The extent of pale areas without mucosal folds was recorded upon colon opening and after sacrifice as % of total area of the deprived colon segment (IC rats) or as % of total area of the reperfused colon segment (IC + RL rats). Oxidative stress was assessed by quantifying thiobarbituric acid (TBA) reactivity as malondialdehyde (MDA) equivalents and by determining the NO levels in the colon tissue. Representative tissue sections were processed for further histological analysis as described previously[2-7,34].

Oxidative stress

At the end of the experiment and at 15 min of ligation time or at 15 min reperfusion time, oxidative stress in the collected tissue samples was assessed by quantifying thiobarbituric acid-reactive species (TBARS) as malondialdehyde (MDA) equivalents. The tissue samples were homogenized in PBS (pH 7.4) containing 0.1 mmol/L butylated hydroxytoluene (BHT) (TissueRuptor, Qiagen, United States) and sonicated for 30 s in an ice bath (Ultrasonic bath, Branson, United States). Trichloroacetic acid (TCA, 10%) was added to the homogenate, the mixture was centrifuged at 3000 rpm for 5 min, and the supernatant was collected. Then, 1% TBA was added, and the samples were boiled (95 ℃, 60 min). The tubes were then kept on ice for 10 min. Following centrifugation (14000 rpm, 10 min), the absorbance of the mixture at the wavelength of 532 nm was determined. The concentration of MDA was read from a standard calibration curve plotted using 1,1,3,3'-tetraethoxy propane (TEP). The extent of lipid peroxidation was expressed as MDA using a molar extinction coefficient for MDA of 1.56×10^5 mol/L/cm. The protein concentration was determined using a commercial kit. The results are expressed in nmol per mg of protein.

NO determination in colon tissue

At the end of the experiment and at 15 min ligation

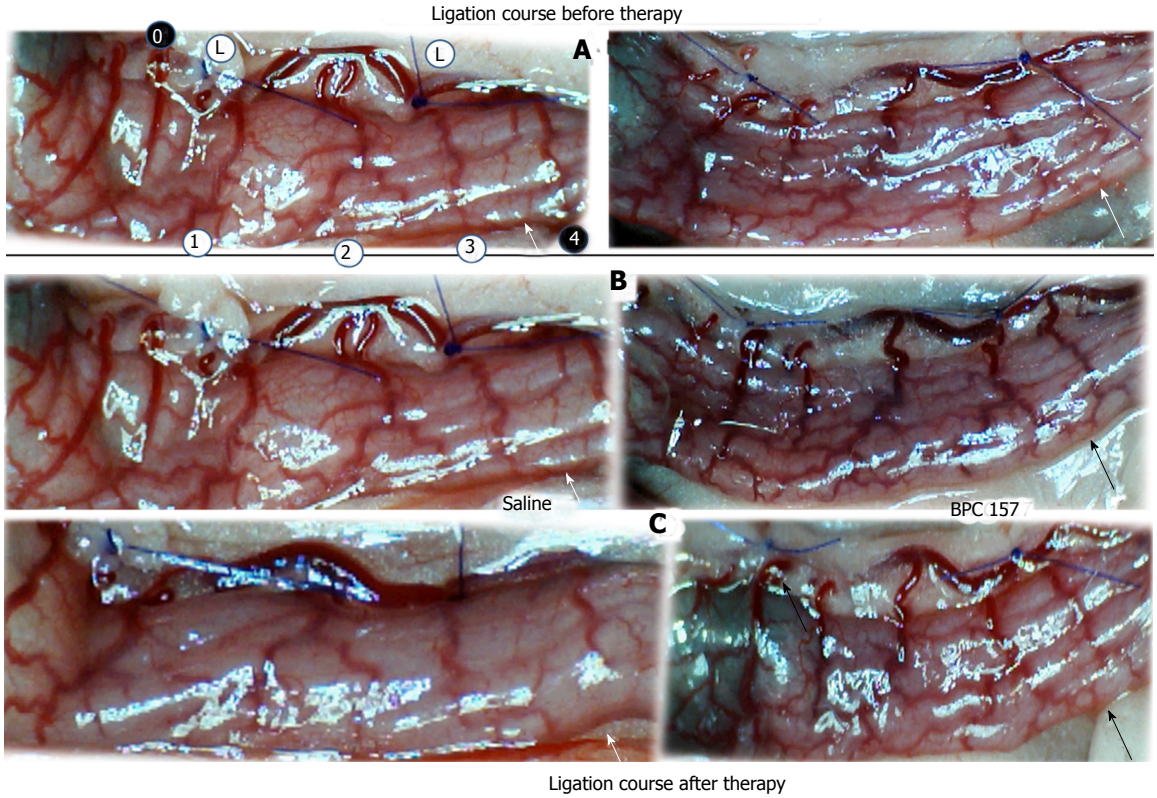

Figure 2 Progressive disappearance (A, saline bath; B, C, left) or recovery (BPC 157 bath; B, C, right) of blood vessels after ligation of the dorsal colon of IC rats (USB microscope camera). Ligation course before therapy (A); ligation course after therapy (B, C). A. Left colic artery and vein major arcade vessels obstructed by two ligations, proximal (left) and distal (right) (L), arcade vessels outside 0, 4 (black circles) or inside 1, 2, 3 (white circles), inside-outside connection absent (arcade 3-arcade 4) (white arrows). B. Five minutes later: inside-outside connection still absent (saline bath, left) (white arrow) or reestablished (black arrow); vessel presentation markedly increased (BPC 157 bath, right). C. From the 10th minute through the end of the 15th minute. Progressive vessel disappearance (saline bath, left) (white arrow); recovered blood vessels (BPC 157 bath, right) (black arrow).

time or at 15 min reperfusion time, we determined the NO levels in colon tissue samples using the Griess reaction (Griess Reagent System, Promega, United States). Sulfanilamide was added to the homogenized tissue, the mixture was incubated, and N-1-naphthylethylenediamine dihydrochloride was added. The Griess reaction is based on the diazotization reaction in which acidified nitrite reacts with diazonium ions and, in a further step, is coupled to N-1-naphthylethylenediamine dihydrochloride, forming a chromophoric azo derivative. Absorbance was measured at 540 nm, using sodium nitrite solution as a standard. NO levels are reported in μmol/mg protein. Protein concentrations were determined using a commercial kit (BioRad Protein DR Assay Reagent Kit, United States).

Late assessment: rats with ligated left colic artery and vein that underwent additional colon obstruction for three days (IC + OB rats)

In deeply anesthetized rats, in addition to the left colic artery and vein obstruction by two ligations performed as described above, which resulted in a 25-mm blood-deprived colon segment, an additional colon obstruction was made close to the distal ligation using a plastic ring 4 mm in diameter. The same BPC 157 protocol (/kg, 1 mL bath/rat) was applied at the 25-mm blood-flow-deprived colon segment after the adhesions were gently removed. BPC 157 (10 μg) or a saline bath of equal volume for the controls was applied on day 3 immediately after the ring was removed. Using a USB camera (Veho discovery VMS-004 deluxe) attached to a microscope, we recorded the vessel presentation for the 15 min immediately following treatment, as described above. In a separate group of rats, the colon was opened and the extent of pale areas without mucosal folds was recorded as described previously. At day 7 after ring removal and bath-therapy application, the deprived colon segment diameter, the colon was opened, and pale areas without mucosal folds were assessed as % of total area of the deprived colon segment and number of gross mucosal defects. Representative tissue sections were processed for further histological analysis as described previously[2-7,34]. The severity of adhesions was described as previously[34,37,39,40] and scored on a scale of 0 to 2 (0-no adhesion; 1 - fatty tissue adhering to colon; 2 - small intestine adhering to colon).

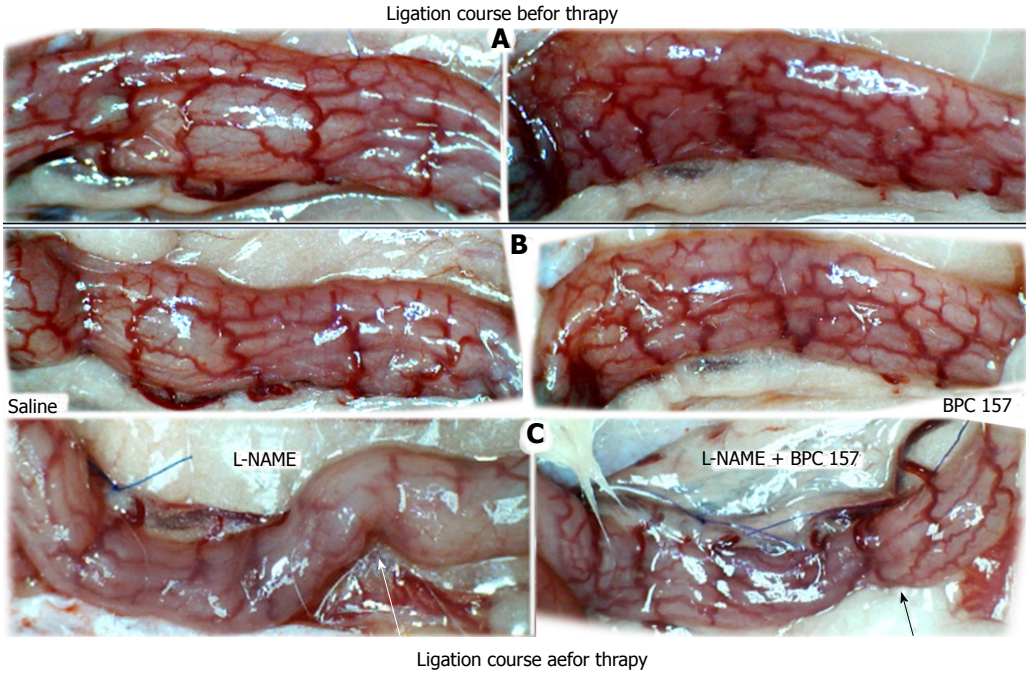

Figure 3 Blood vessels after ligation in IC rats (ventral colon side of the colon, before application of medication). A: Progressive disappearance of blood vessels (saline bath, B, ventral colon side; L-NAME, C, dorsal colon side, left) or recovery (BPC 157 bath, B, ventral colon side; L-NAME + BPC 157, C, dorsal colon side, right). Ligation course before therapy (A); ligation course after therapy (B, C). A. Ventral side of the colon showing the presence of an abundant collateral network with inside-outside contact, unlike the relatively scarce arcade collateral network and absent inside-outside contact on the dorsal side (see Figure 2). B. Five minutes later, the number of vessels on the ventral side of the colon is markedly decreased; the inside-outside connection is still present (saline bath, left); or vessel presentation is markedly increased (BPC 157 bath, right). C. From the 10th minute through the end of the 15th minute. Dorsal side of the colon showing progressive vessel disappearance, inside-outside contact largely absent (L-NAME bath, left) (white arrow), unlike recovered blood vessels and inside-outside contact (L-NAME+BPC 157 bath, right (black arrow).

Statistical analysis

Statistical analysis was performed by parametric one-way ANOVA with *post hoc* Newman-Keuls test and non-parametric Kruskal-Wallis and subsequent Mann-Whitney *U*-test to compare groups. Values are presented as the mean ± SD and as the minimum/median/maximum. $P < 0.05$ was considered statistically significant.

RESULTS

We investigated the maintenance of vessel function upon the first innate reperfusion (*i.e.*, during the initial recovery after blood supply was interrupted by the placement of two ligations on the left colic artery and vein (Figure 1) in IC rats. This method was also used much later in animals that had been subjected to the additional bowel obstruction (IC + OB rats) and, subsequently, upon massive reperfusion occurring after the removal of the vascular obstruction(s) (IC + RL rats).

In these experiments, ischemic colitis therapy was administered once at early ligation time (IC rats) or alternatively at the early post-ligation time (IC + RL rats). In other experiments, the therapy was administered once at a later ligation time after additional colon obstruction (IC + OB rats).

In general, we obtained results that showed the presence of increasing/decreasing collateralization between the arcade vessels inside and outside ligations or previous ligations. Subsequently, we demonstrated a markedly attenuated course of ischemic colitis in BPC 157-treated rats, IC rats, IC + RL rats, and IC + OB rats; conversely, an aggravated course of ischemic colitis was observed in L-NAME-treated rats, L-arginine-treated rats, and in IC rats (Tables 1-3 and Figures 1-25).

We focused on the 25-mm blood-deprived segment of the descending colon. Within this ligated area, there were three major arcades. Thus, it was possible for both the proximal and distal obstructions to be bypassed by an additional vessel network running between the arcades (outside-inside ligation, outside-inside, inside-inside and inside-outside) at both the ventral and dorsal sides of the colon. The particular arcade vessel presentation at specific subsequent time points [A (1 min before therapy), B (the first 5 min following therapy), C (the next 5 min, until the 10th min), D (until the end of the 15th min)] demonstrates how the obstructions in the tissues were bypassed. However, the alternative arcade pathway operated poorly at best in the blood-deprived colon segment. Nevertheless, the alternative arcade pathway was found to respond

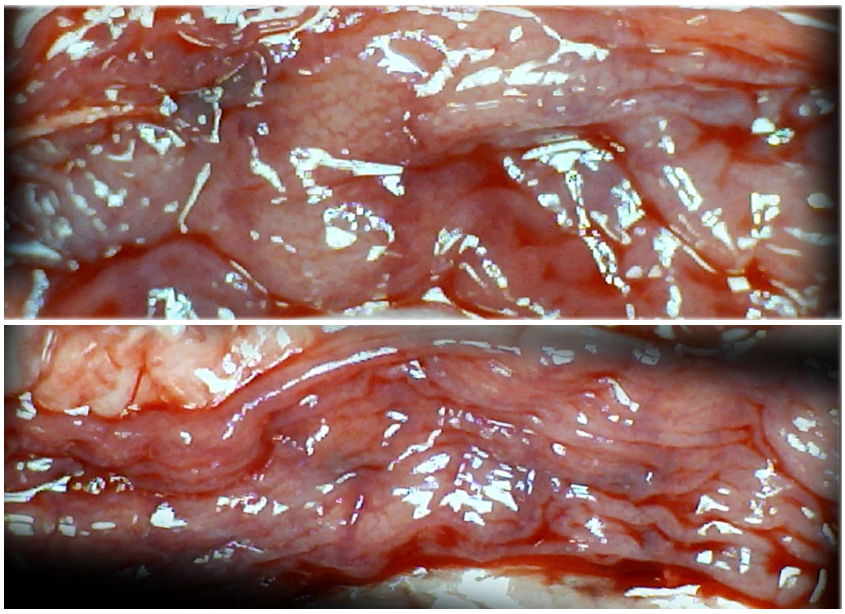

Figure 4 Characteristic appearance of the colon in IC rats 15 min after ligation, upon colon opening before sacrifice; USB microscope camera. Extremely large pale areas without mucosal folds were observed after saline bath treatment (upper), in contrast to the preserved mucosal folds seen after BPC 157 bath treatment (lower) in rats that underwent obstruction of the left colic artery and vein for 15 min. The animals initially received bath medication consisting of saline (upper) or BPC 157 (lower).

Table 1 Assessment of colon lesions in rats subjected to obstruction of the left colic artery and vein by two ligations for 15 min (pale flat areas without mucosal folds as a percentage of the area of the blood-deprived colon segment; mean ± SD)

Medication (/kg, 1-ml bath/rat) at the 25-mm blood-flow-deprived colon segment	Pale areas without mucosal folds as a percentage of the area of the blood-deprived colon segment, mean ± SD
0.9% NaCl (control)	46 ± 8
BPC 157 (10 μg)	7 ± 2[a]
L-NAME (5 mg)	80 ± 10[a]
L-arginine (100 mg)	65 ± 6[a]
L-NAME (5 mg) + L-arginine (100 mg)	50 ± 8
L-NAME (5 mg) + BPC 157 (10 μg)	10 ± 4[a]
L-arginine (100 mg) + BPC 157 (10 μg)	6 ± 2[a]
L-NAME (5 mg) + L-arginine (100 mg) + BPC 157 (10 μg)	8 ± 2[a]

The following medications or treatments (/kg, 1-mL bath/rat) were applied to the 25-mm blood-flow-deprived colon segment at 1 min post-injury: BPC 157 (10 μg); the NOS blocker L-NAME (5 mg) and the NOS substrate L-arginine (100 mg), alone or combined; equal volume of saline bath (controls). [a]$P < 0.05$ at least *vs* control.

quickly to therapy with BPC 157 *vs* L-NAME and L-arginine.

The appearance of the vessels was assessed (see also Figure 1) between (1) the last arcade proximal to first ligation (arcade 0) and the first arcade distal to the ligation (or to the previous ligation) (arcade 1) (0-1, Figure 2, Figures 5-8, and Figures 21 and 22); (2) from arcade 1 to the next arcade (arcade 2), middle arcade inside ligations (or previous ligation) (1-2, Figures 2, 5-8, 21 and 22); (3) from arcade 2 to the next arcade (arcade 3) to the last arcade inside the ligations (or the previous ligation) (2-3, Figures 2, 5-8, 21 and 22); and finally, (4) from the first arcade distal to the second ligation (or the previous ligation) (arcade 4) (3-4, Figures 2, 5-8, 21 and 22).

Initial assessment of rats that underwent two ligations of the left colic artery and vein for 15 min (IC rats)

Regular course (saline bath, control): Frequently, once major vessels were obstructed, the alternative arcade only partially appeared in the blood-deprived colon segment (Figures 2, 3, 5 and 6). After a saline bath was applied to the colon segment that had been deprived of its blood supply, arcade vessels gradually disappeared from the proximal to the distal arcades. Loose connections between inside-outside blood vessels were quickly seen at the dorsal side. As a result, there was a rapid appearance of pale flat areas and a rapid disappearance of mucosal folds (Table 1 and Figures 2-4). This dorsal/ventral side distinction may be related to the relatively scarce dorsal side arcade

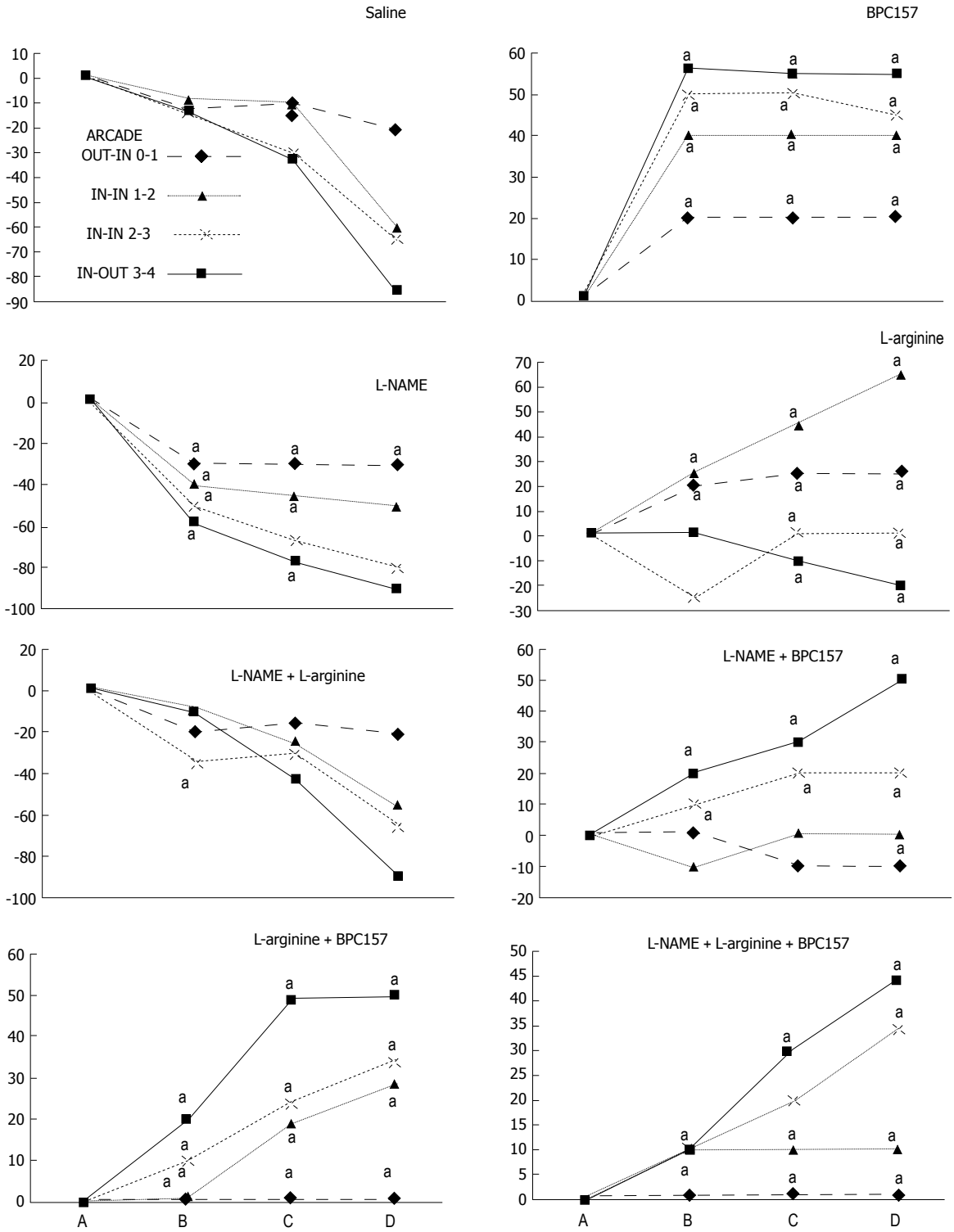

Figure 5 IC rats. Per cent of vessels present between arcade vessels next to the proximal (arcade 0) and distal (arcade 4) ligatures and within the ligated area (*arcade 1, 2, 3*) on the dorsal side of the colon at 15 min following therapy (as 100%); mean ± SD. The gross appearance of the tissue was recorded using a USB microscope camera. The following time points were assessed: A - after ligation and before therapy (1 min); B - 5 min after the application of medication; C - between 5 and 10 min after the application of medication; D - from 10 min after the application of medication until the end of the observation at 15 min. At 1 min post-injury, medication (/kg, 1 ml bath/rat) consisting of BPC 157 (10 μg), the NOS blocker L-NAME (5 mg) and the NOS substrate L-arginine (100 mg) alone or combined, or an equal volume of a saline bath (controls) was applied to the 25-mm blood-flow-deprived colon segment; the rats were sacrificed at 15 minutes. For clarity, the SD is not shown on the graph; the SD was never higher than 10% of the mean. $^{a}P < 0.05$ at least *vs* control.

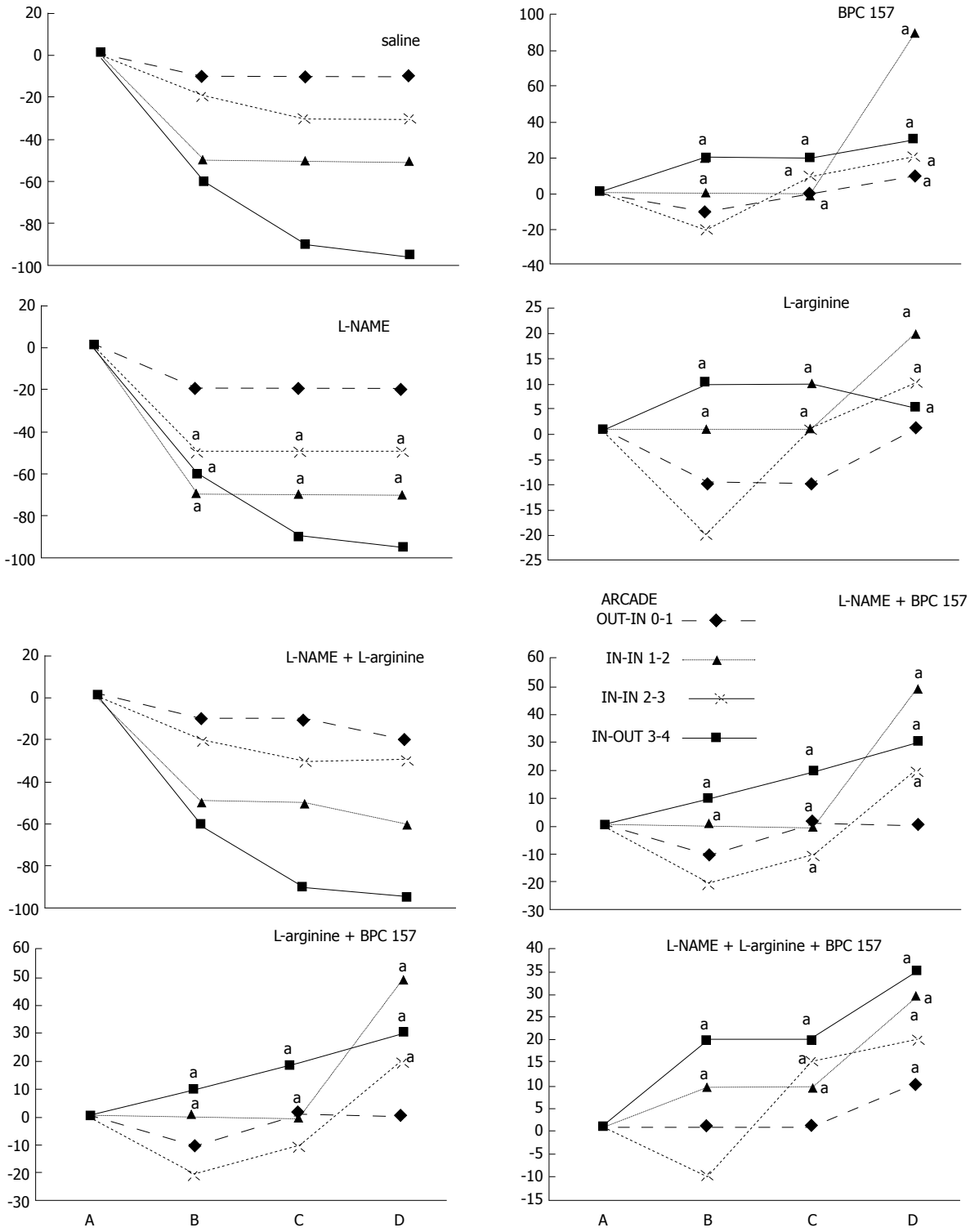

Figure 6 IC rats. Per cent of vessels present between arcade vessels, next to the proximal ligature (arcade 0), next to the distal ligature (arcade 4), and within the ligated area (arcade 1, 2, 3), on the ventral side of the colon at 15 min following therapy (as 100%); mean ± SD. The gross appearance of the tissue was recorded using a USB microscope camera. The following time points were assessed: A - after ligation and before therapy (1 min); B - 5 min after the application of medication; C - between 5 and 10 min after the application of medication; D - from 10 min after the application of medication until the end of the observation at 15 min. At 1 min post-injury, medication (/kg, 1 mL bath/rat) consisting of BPC 157 (10 μg), the NOS blocker L-NAME (5 mg) and the NOS substrate L-arginine (100 mg), alone or combined, or an equal volume of a saline bath (controls) was applied to the 25-mm blood-flow-deprived colon segment; the rats were sacrificed at 15 min. For clarity, the SD is not shown on the graph; the SD was never higher than 10% of the mean. [a]*P* < 0.05 at least *vs* control.

a rapid disappearance of mucosal folds (Table 1 and Figures 2-4). This dorsal/ventral side distinction may be related to the relatively scarce dorsal side arcade collateral network; thus, once the final connection disappeared, it could not be easily reestablished (Figures 2 and 5). By contrast, at the ventral side,

Table 2 Assessment of colon lesions in rats subjected to two ligations of the left colic artery and vein for 15 min followed by reperfusion for next 15 min (IC + RL rats) (pale flat areas without mucosal folds were measured and are shown as a percentage of the area of the blood-deprived and the reperfused colon segment; mean ± SD)

Medication (/kg, 1 ml bath/rat) applied to the 25-mm blood-flow-deprived and then reperfused colon segment	Pale areas without mucosal folds as a percentage of the area of the blood-flow-deprived and then reperfused colon segment (mean ± SD)
0.9% NaCl (control)	86 ± 8
BPC 157 (10 μg)	10 ± 2[a]

At 1 min reperfusion time, BPC 157 (10 μg/kg, 1 mL bath/rat) or an equal volume of saline bath (control) was applied to the 25-mm blood-flow-deprived colon segment. [a]$P < 0.05$ at least *vs* control.

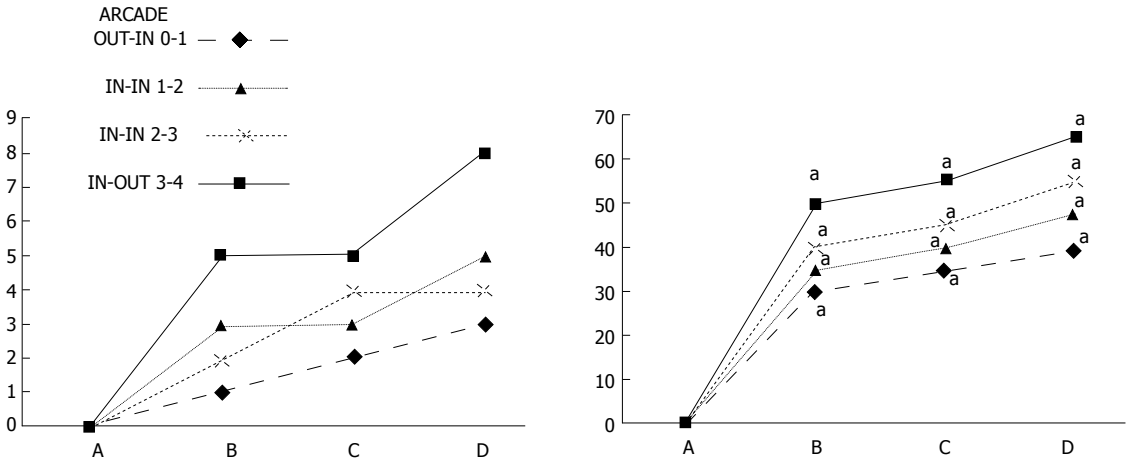

Figure 7 IC + RL rats. Per cent of vessel presentation between arcade vessels, next to the previous proximal ligature (arcade 0), next to the previous distal ligature (arcade 4), and within the previous ligated area (arcade 1, 2, 3) on the dorsal side of the colon at 15 min after therapy (as 100%); mean ± SD. The gross appearance of the tissue was recorded using a USB microscope camera. The following time points were assessed: A: After ligation and before therapy (1 min); B: 5 min after the application of medication; C: Between 5 and 10 min after the application of medication; D: From 10 min after the application of medication until the end of the observation at 15 min. At 1 min post-injury, BPC 157 (10 μg /kg, 1 mL bath/rat) or an equal volume of a saline bath (controls) was applied to the reperfused 25-mm colon segment; the rats were sacrificed at 15 min. For clarity, the SD is not shown on the graph; the SD was never higher than 10% of the mean. [a]$P < 0.05$ at least *vs* control.

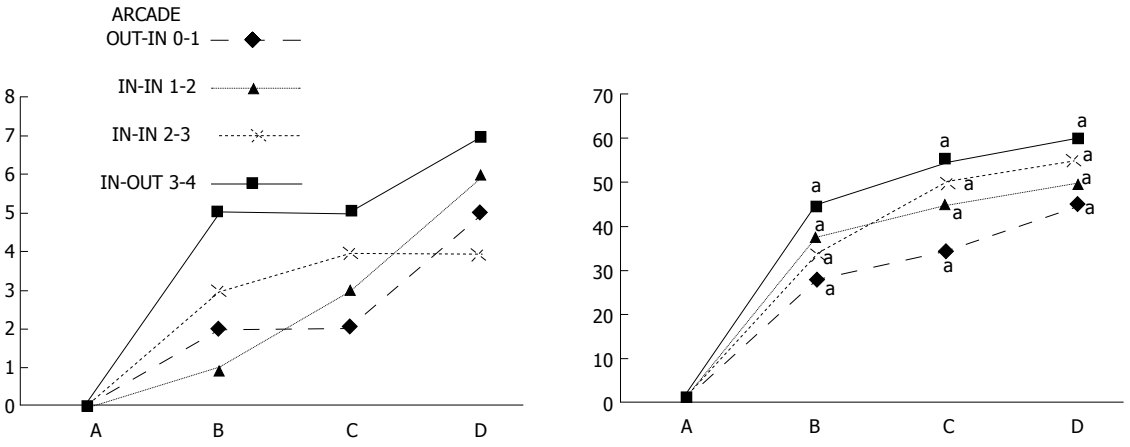

Figure 8 IC + RL rats. Per cent of vessels present between arcade vessels, next to the previous proximal ligature (arcade 0), next to the previous distal ligature (*arcade 4*), and within the previous ligation (arcade 1, 2, 3) on the ventral side of the colon 15 min following therapy (as 100%); mean ± SD. The gross appearance of the tissue was recorded using a USB microscope camera. The following time points were assessed: A: After ligation and before therapy (1 min); B: 5 min after the application of medication; C: Between 5 and 10 min after the application of medication; D: From 10 min after the application of medication until the end of the observation at 15 min. At 1 min post-injury, medication (BPC 157, 10 μg/kg, 1 mL bath/rat) or an equal volume of a saline bath was applied to the reperfused 25-mm colon segment; the rats were sacrificed 15 min later. For clarity, the SD is not shown on the graph; the SD was never higher than 10% of the mean. [a]$P < 0.05$ at least *vs* control.

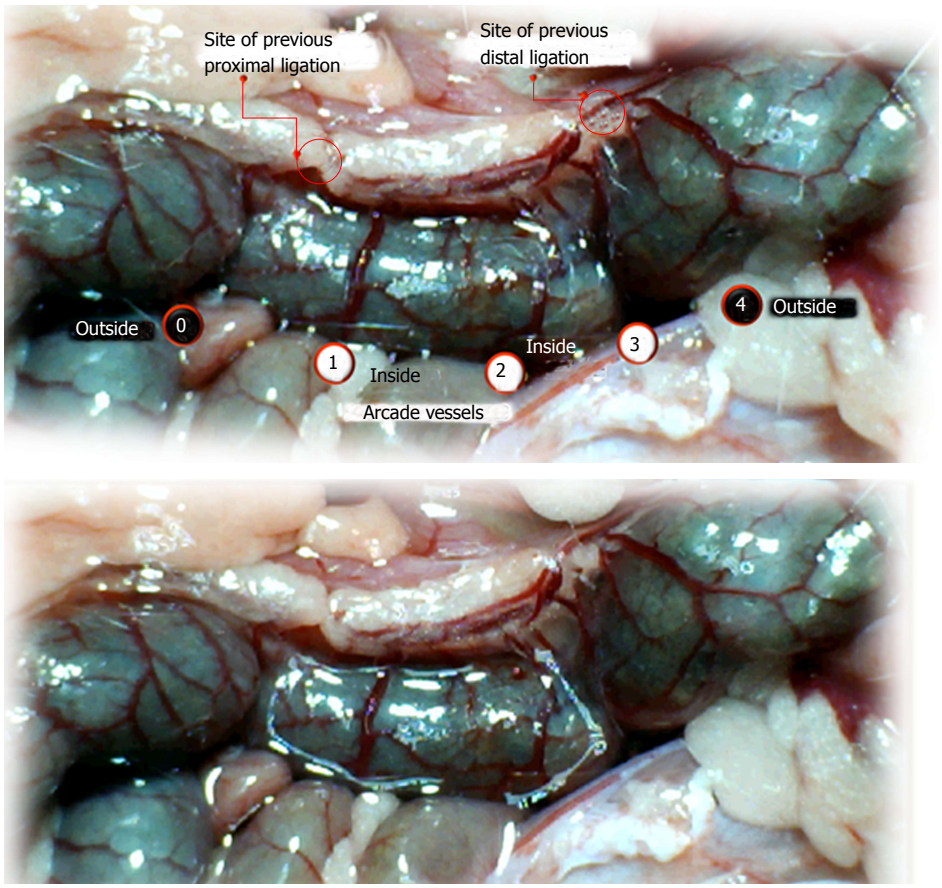

Figure 9 IC + RL rats (controls). Vessel presentation between arcade vessels next to the previous proximal ligature (arcade 0), next to the previous distal ligature (arcade 4), and within the previous ligation (arcade 1, 2, 3) on the dorsal side of the colon at 1 min reperfusion time and prior to saline bath application (upper). Similar vessel presentation is apparent immediately after application of a saline bath (1 mL bath/rat) (controls) to the reperfused 25-mm colon segment (lower).

collateral network; thus, once the final connection disappeared, it could not be easily reestablished (Figures 2 and 5). By contrast, at the ventral side, which displayed a much more abundant collateral network, despite a considerable decrease in inside-inside contact, inside-outside contact persisted (Figures 3 and 6). Along with the appearance of a considerable number of endothelial lesions following ligation, an initial blood flow deprivation and a presumably inadequate response, decreased NO levels (Figure 7) and increased MDA levels (Figure 8) were observed in the colon tissue.

BPC 157 bath: By contrast, blood vessels propagated toward the injury obstruction, bypassing it, interconnecting collaterals between arcades (Figures 2, 3, 5 and 6), recovering blood flow and thereby attenuated/counteracted ischemia/reperfusion injury[9,10]. Mucosal folds were present, and there were markedly fewer pale areas (Table 1, Figure 4). In particular, these effects all appeared in conjunction with BPC 157 application.

Notably, in animals that received the BPC 157 bath, vessel presentation was markedly increased at the re-established inside-outside connective point in particular (arcade 3-arcade 4) (Figures 2, 3, 5 and 6); in this way, blood flow was quickly restored. In support of this finding, increased MDA levels and decreased NO levels in colon tissue were found to be normal in rats that received BPC 157 bath application (Figures 13 and 14).

NO agents, L-NAME bath, L-arginine bath: After the application of NO agents, particular responses were observed; however, neither of the responses to the agents was effective with respect to the extent of the pale areas (Table 1, Figures 3, 5 and 6).

The application of L-NAME appeared to worsen the already debilitated vascular response. A larger proportion of the blood vessel network disappeared, in particular at the dorsal side (Figures 3, 5 and 6). Consequently, the pale areas and areas of mucosal fold disappearance were markedly larger (Table 1).

L-arginine increased vessel presentation but did not re-establish the broken inside-outside connection (Figures 5 and 6). Pale areas and mucosal fold disappearance were again increased (Table 1).

Combination studies, L-NAME + L-arginine bath; BPC 157 + L-NAME bath; BPC 157 + L-arginine bath; BPC 157 + L-NAME + L-arginine bath: In combination, we have mutual counteraction (L-NAME +

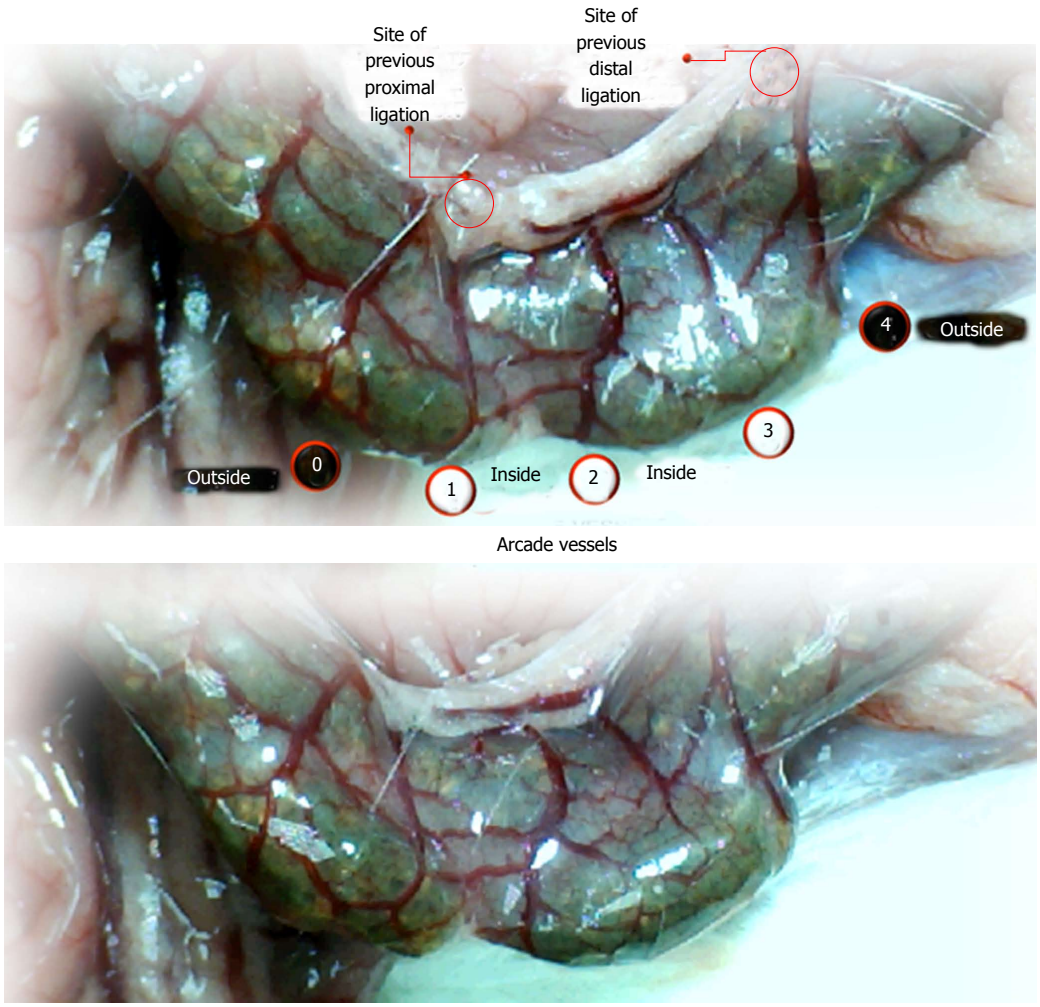

Figure 10 IC + RL rats (BPC 157-treated rats). Vessel presentation between arcade vessels, next to the previous proximal ligature (arcade 0), next to the previous distal ligature (arcade 4), and within the previous ligation (arcade 1, 2, 3) on the dorsal side of the colon at 1 minute reperfusion time and before saline bath application (upper). Increasing vessel presentation forming "honeycomb" structures between arcades can be seen immediately after BPC 157 treatment (BPC 157 (10 μg/kg), 2 μg/mL bath/rat) of the reperfused 25-mm colon segment (lower).

L-arginine) or presentation similar to BPC 157-treated rats (BPC 157 + L-NAME; BPC 157 + L-arginine; BPC 157 + L-NAME + L-arginine) (Table 1, Figures 3, 5 and 6).

Initial assessment of rats subjected to two ligations at the left colic artery and vein for 15 min, followed by reperfusion for next 15 min (IC + RL rats)

Regular course (saline bath, control): After a saline bath was applied to the reperfused colon segment, the arcade vessels were poorly established from proximal to distal arcades. The connections between inside-inside and inside-outside blood vessels that had been lost were poorly reestablished (Figures 7-9 and 11). As a result, there was an additional rapid appearance of pale flat areas and a disappearance of mucosal folds (Table 2, Figure 11).

BPC 157 bath: The application of BPC 157 after the initiation of full reperfusion with both ligations removed resulted in increased vessel presentation and arcade interconnections; the mucosal folds were recovered, and the pale areas were small and markedly reduced in area (Figures 7, 8, 10 and 12). Consistently, the otherwise increased MDA and NO levels in colon tissue were found to be normal in rats that received BPC 157 bath treatment (Figures 15 and 16).

Late assessment: rats with ligated left colic artery and vein that underwent additional colon obstruction for three days (IC + OB rats)

In these experiments, treatments were administered to blood-deprived colon segments that had additionally been challenged with ring obstruction. Notably, ligation of the left colic artery and vein caused extensive damage, and additional obstruction of the colon for three days led to even more severely disabled rats in which an extremely large pale flat area without mucosal folds occupied the entire ligated segment; thus, an advanced level of disability was present before therapy could be applied (Figure 19). If not reversed, this

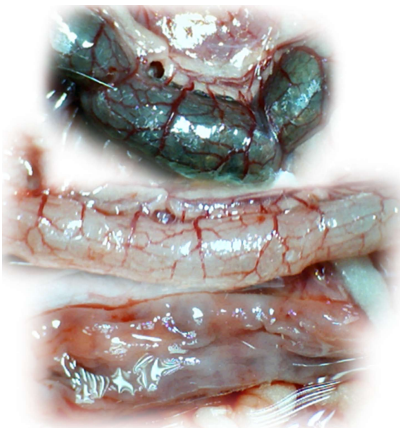

Figure 11 IC + RL rats (controls) at 15 min reperfusion time. At 1 min reperfusion time, saline (1 mL bath/rat) was applied to the reperfused 25-mm colon segment (lower). Relatively poor vessel presentation between arcade vessels is apparent in the full colon segment (upper); colon segment cleaned with water (middle); progressively appearing, extremely large pale areas without mucosal folds upon colon opening before sacrifice (lower).

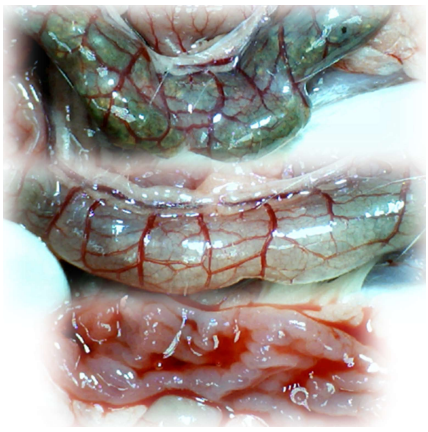

Figure 12 IC + RL rats (BPC 157-treated rats) at 15 min reperfusion time. At 1 min reperfusion time, BPC 157 (10 μg/kg, 2 μg/mL bath/rat) was applied to the reperfused 25-mm colon segment (lower). Increasing vessel presentation between arcade vessels is apparent in the full colon segment (upper); colon segment cleaned with water (middle); fully recovered mucosa and mucosal folds upon colon opening before sacrifice (lower).

procedure consistently results in a perilous outcome (Table 3, Figures 17 and 19-22). We used the same BPC 157 protocol that was shown above to be effective when applied to the 25-mm blood-flow-deprived colon segment shortly after blood vessel obstruction by two ligations. We demonstrated that the BPC 157 treatment protocol was also efficacious when it was applied on day 3, immediately after the constricting ring was removed (Table 3, Figures 18 and 20-22), as shown by its subsequent rapid effect on the appearance of the arcade vessels. An extended beneficial effect was also apparent one week later, indicating rescue from ischemic colitis.

Immediate effect (day 3): Whereas the controls exhibited an increasing disappearance of arcade vessels, particularly at the dorsal side of the colon (Figures 9, 13 and 14), after BPC 157 bath treatment the arcade vessels vigorously reappeared together with interconnected collaterals between arcades, in particular, quickly rescuing blood flow (a finding especially seen at the dorsal side of the colon) (Figures 18, 21 and 22).

Final effect (day 10): On day 10, the blood-deprived colon segment contained extremely large pale areas without mucosal folds that occupied the entire area between the ligations (Table 3, Figure 20). These pale areas were present even outside the area that was ligated, and many gross mucosal defects were present in the enlarged colon segment (Table 2, Figure 20). Adhesions to the small intestine were commonly noted (Table 3). By contrast, the areas that had been treated with BPC 157 presented almost completely spared mucosa with very small pale areas and no gross mucosal defects, a colon segment of normal diameter, and few adhesions (Table 3, Figure 20).

Microscopy

Microscopically, at 15 min after the beginning of ligation in IC rats, there was failed vascular presentation, with gross pale flat areas and the disappearance of mucosal folds (Figure 2). Severe edema of the lamina propria and continuous diffuse edema of the submucosa was also evident (Figure 23A). In addition, there was pronounced dilatation and stasis in the submucosal blood vessels (Figure 23A). By contrast, in BPC 157-treated rats, there was recovered vascular presentation, with mucosal folds present and markedly fewer pale areas (Figure 2), mild edema of the lamina propria and focally present mild-to-intermediate edema of the submucosa (Figure 23B). Moreover, stasis of the submucosal blood vessels was present, but the dilatation of veins appeared to be less pronounced (Figure 23B).

In IC + RL rats after 15 min of ligation and 15 min of full reperfusion, there was failed vascular presentation leading to an increasing number of gross pale flat areas and the disappearance of mucosal folds (Figure 12). Microscopically, even more, mucosal and submucosal edema, as well as more pronounced cyanosis, were evident. These effects were consistently counteracted by the application of BPC 157 after reperfusion initiation (Figure 24).

In IC + OB rats on day 3, immediately after removing the additional colon obstruction and the obstructing ring, grossly large pale flat areas without mucosal folds occupying the entire ligated segment and failed vascular presentation appeared (Table 3, Figures 17 and 20-22), with even more pronounced severe edema of the lamina propria and continuous diffuse edema of the submucosa as well as pronounced dilatation and stasis of the submucosal blood vessels. This effect was associated with mild edema of the

Table 3 Assessment of lesions in rats with obstruction of the left colic artery and vein for 10 d *via* two ligations; the animals underwent additional colon obstruction for the first 3 d

Medication (/kg, 1 ml bath/rat) at the 25-mm blood-flow-deprived colon segment	Pale areas without mucosal folds as a percentage of the entire area of the blood-deprived colon segment means ± SD	Number of gross mucosal defects, mean ± SD	Colon diameter, mean ± SD	Adhesion severity scored 0-2, Min/Med/Max
0.9% NaCl (control)	125 ± 10	4 ± 1	36 ± 8	2/2/2002
BPC 157 (10 μg)	10 ± 2[a]	0 ± 0[a]	9 ± 1[a]	0/1/1[a]

They received BPC 157 or saline bath on day 3, immediately after the additional colon obstruction was removed. The animals were assessed one week thereafter, on day 10 after ligation. Pale flat areas without mucosal folds were measured and are expressed as the percentage of the entire area of the blood-deprived colon segment (mean ± SD). Number of gross mucosal defects; mean ± SD; colon diameter, mean ± SD; adhesion severity, scored 0-2 (Min/Med/Max, respectively). [a]$P < 0.05$ at least *vs* control.

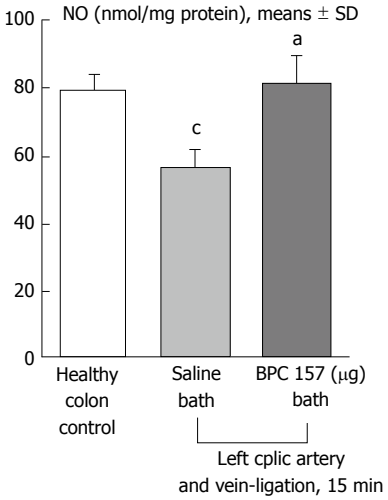

Figure 13 NO levels in the colon tissue of IC rats at 15 min ligation time determined using the Griess reaction. BPC 157 (10 μg/kg, 1 mL bath/rat) or an equal volume of a saline bath (controls) was applied to the 25-mm blood-flow-deprived colon segment. [a]$P < 0.05$ *vs* saline; [c]$P < 0.05$ *vs* healthy colon.

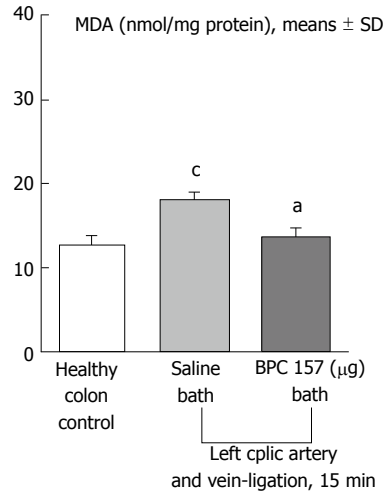

Figure 14 Malondialdehyde levels in the colon tissue of IC rats at 15 min ligation time, determined by quantifying thiobarbituric acid reactivity as malondialdehyde equivalents. BPC 157 (10 μg/kg, 1 ml bath/rat) or an equal volume of a saline bath (controls) was applied to the 25-mm blood-flow-deprived colon segment. [a]$P < 0.05$ *vs* saline; [c]$P < 0.05$ *vs* healthy colon.

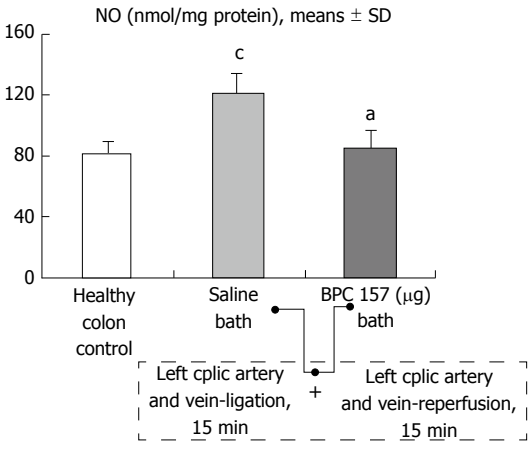

Figure 15 NO levels in the colon tissue of IC + RL rats after 15 min of reperfusion, determined using the Griess reaction. At 1 min reperfusion time, BPC 157 (10 μg/kg, 2 μg/1 mL bath/rat) or an equal volume of a saline bath (controls) was applied to the reperfused 25-mm colon segment. [a]$P < 0.05$ *vs* saline; [c]$P < 0.05$ *vs* healthy colon.

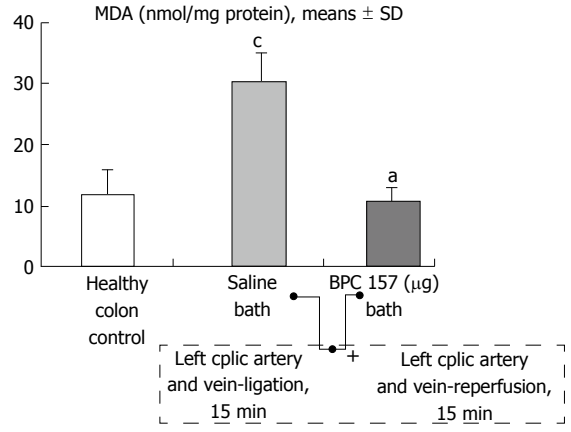

Figure 16 Malondialdehyde levels in the colon tissue of IC + RL rats after 15 min of reperfusion, determined by quantifying thiobarbituric acid reactivity as malondialdehyde equivalents. At 1 min reperfusion time, BPC 157 (10 μg/kg, 2 μg/1 mL bath/rat) or an equal volume of a saline bath (controls) was applied to the reperfused 25-mm colon segment. [a]$P < 0.05$ *vs* saline; [c]$P < 0.05$ *vs* healthy colon.

lamina propria and diffuse mild-to-intermediate edema of the submucosa, with collagen formation, one week later on day 10 after ligation (Figure 25A). Stasis of the submucosal blood vessels was also present (Figure 25A). In these samples, the rugae are broadened and flattened, so the mucosa appears macroscopically flattened (Figure 17). By contrast, BPC 157-treated

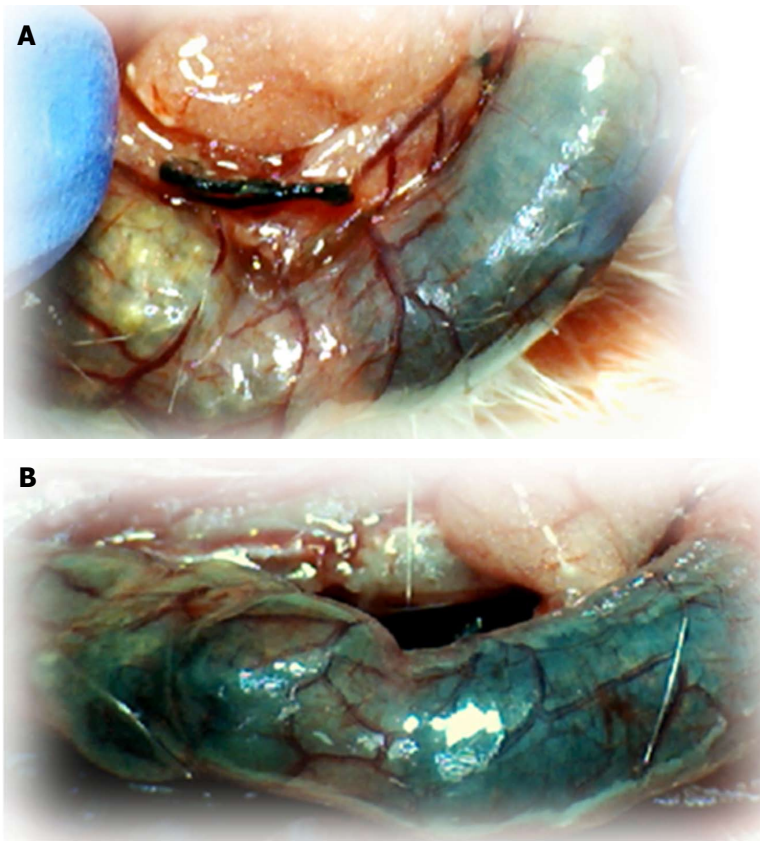

Figure 17 IC + OB rats that underwent additional colon obstruction for three days presented severely impaired gross arcade vessels immediately after the additional colon obstruction was removed (A, before therapy, upper); after saline bath application, they exhibited an increasing disappearance of arcade vessels, particularly on the dorsal side of the colon (B, 5 min after therapy, lower). The images were obtained using a USB microscope camera.

rats grossly presented almost completely spared mucosa (Figure 20), with mild edema of the lamina propria and practically no edema of the submucosa (Figure 25B). Stasis of the submucosal blood vessels was also present but to a much lesser extent than in controls. The rugae were histologically well formed, only occasionally displaying slight broadening. There was the minimal apparent formation of new collagen fibers in the submucosa (Figure 25B).

In summary, these results show that BPC 157 protects colon tissue from the endothelial damage induced by ischemia and reperfusion. The cytoprotective effect of BPC 157 against colonic ischemia/reperfusion-mediated mucosal damage is associated with the activation of the collateral circulation, which circumvents obstructed sites and results in the resolution of the obstruction, reduction of oxidative stress and normalization of NO synthesis.

DISCUSSION

We demonstrated that well-placed arcade vessels respond poorly to the increased demands that occur upon blood supply deprivation, reperfusion or additional bowel obstruction. Thus, the particular susceptibility of the colon to insufficient vascular perfusion[1] mandates that the main focus of the treatment of such conditions should be bypassing one or more of the vascular obstructions. Then, the main focus is maintaining vessel function upon the first innate reperfusion (as evidenced by an initial innate recovery while the blood supply is deprived) in IC rats [which is also applicable much later with additional bowel obstruction (IC + OB rats)], and subsequently upon massive reperfusion following the removal of vascular obstruction(s) (IC + RL rats).

Based on this reasoning, therapy for ischemic colitis was administered once at early ligation-time (IC-rats) or alternatively at early post-ligation-time (IC + RL rats). In some animals, the therapy was administered once at a later time after additional colon obstruction (IC + OB rats). BPC 157 therapy was shown to cure rat ischemic colitis in both the very early and late time points and under diverse harmful conditions (short-lasting blood deprivation (IC rats) *vs* reperfusion (IC + RL rats) *vs* long-lasting blood deprivation and additional bowel obstruction (IC + OB rats)). Obviously, such BPC 157 therapy involves analogous cytoprotection/endothelium mechanisms[2-8,12-16,20], such as the cytoprotective response that was previously highlighted in original stomach cytoprotection studies of cytoprotective

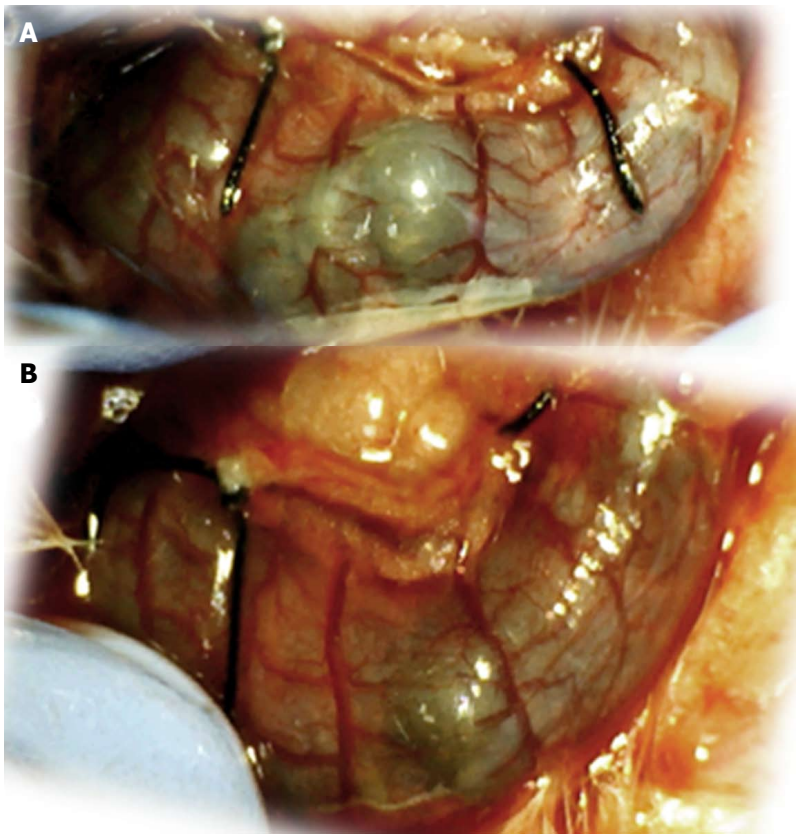

Figure 18 IC + OB rats that underwent additional colon obstruction for the first three days presented severely impaired gross arcade vessels immediately after the additional colon obstruction was removed (A, before therapy, upper); by contrast, after bath treatment with BPC 157, all of the arcade vessels vigorously reappeared, together with interconnecting collaterals between the arcades. Rapid recovery of blood flow also occurred, and this was especially evident on the dorsal side of the colon (B, 5 min after therapy, lower). The images were obtained using a USB microscope camera.

agents in general and of BPC 157 specifically[12-16,20] in the immediate post-injury time period[12-16,20]. These results may underlie the aforementioned response for both ischemic and reperfusion injuries. The effects of BPC 157 may be usefully upgraded and accordingly rendered advantageous for arcade collateralization and for bypassing obstructive defects, thereby resulting in recovery throughout the course of ischemic colitis.

Thus, influencing not only the early period (which has not been validated before in studies of ischemic colitis lesions[17-19]) but also the later period should also be crucial in determining how to treat more complex ischemic colitis lesions. Interestingly, in rats with a prolonged obstruction of blood vessels that underwent additional colon obstruction due to a ring around the colon (IC + OB rats), the evidence suggests that a bath administration of BPC 157 immediately (IC rats) or shortly thereafter (IC + RL rats) produced a beneficial effect and that the effect was even greater when BPC 157 was administered later (IC + OB rats).

Notably, a post-injury course that is not reversed always presents a devastating progression. In cases in which the blood vessels disappeared with blood deprivation and did not recover with reperfusion, extremely large pale mucosal areas, flat and without folds, were found. In IC and IC + RL rats, ischemia/reperfusion injury[9,10] increased and increased dramatically, respectively, whereas the MDA oxidative stress levels progressively increased, NO tissue values decreased with ligation and increased with ligation removal.

The course of development described above is in sharp contrast to the observed course of ischemic colitis course in rats that received BPC 157 therapy. Instead of regularly failing in either experiment, the gross presentation of the blood vessels was recovered, and collateral blood vessels made interconnections that propagated toward the obstruction injury and bypassed it, rescuing blood flow. Severe edema of the lamina propria and continuous diffuse edema of the submucosa, pronounced dilatation and stasis in the submucosal blood vessels were all markedly attenuated. The animals in which BPC 157 therapy attenuated/counteracted the ischemia/reperfusion injury[9,10] showed no MDA oxidative stress and displayed normalized NO tissue values. Mucosal folds remained present (IC rats) or were recovered (IC + RL rats, IC + OB rats), and there were markedly fewer pale areas in both the short and long periods after ligation. At the end of the experiment, these animals displayed mucosa that was almost completely spared, with no ulceration, and the otherwise regular perilous

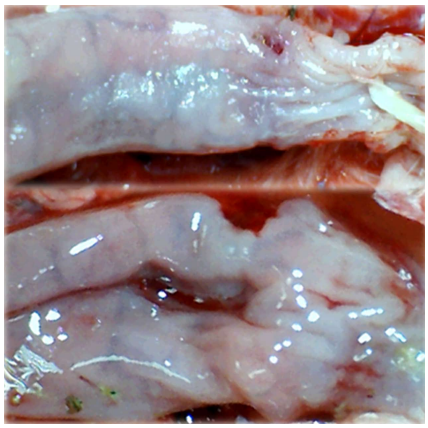

Figure 19 In IC + OB rats that underwent additional colon obstruction for three days, colon opening revealed pale flattened areas without mucosal folds immediately after additional colon obstruction was removed. The images were obtained using a USB microscope camera.

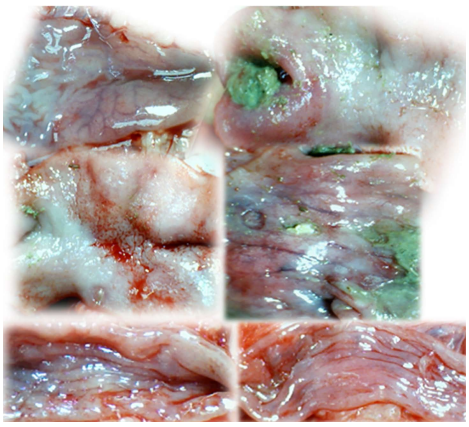

Figure 20 IC + OB rats underwent additional colon obstruction for three days; the colon was then opened before sacrifice on day 10 [*i.e.*, one week after the additional colon obstruction had been removed and bath therapy applied (saline, upper and middle; BPC 157, lower)]. The examination revealed progressive worsening in the saline-treated animals (upper and middle) and recovery in the BPC 157-treated animals (lower) (see Figure 11). The control rats exhibited extremely large pale areas without mucosal folds covering the entire area between ligations (middle); such areas were present even beyond the area of the ligation (upper), together with ulcerations in the deprived, apparently enlarged colon segment (middle). BPC 157-treated rats exhibited almost completely spared mucosa (very small pale areas) and no ulceration; the previously ligated colon segment was of normal diameter (lower). The images were obtained using a USB microscope camera.

outcome of extensive pale and flattened mucosal areas with severe ulcerations was avoided. In addition, microscopic examination showed only mild edema of the lamina propria and practically no edema of the submucosa; the rugae were well formed and only occasionally slightly broadened, and there was minimal formation of new collagen fibers in the submucosa. These aforementioned points, with or without therapy, would have a NO background.

Notably, for quick endothelium rescue, operating collaterals (there are rich anastomoses between individual vessels on both surfaces of the large intestine[41]) were needed for blood flow reorganization. This process occurred in rats with obstructed vessels and with vessels that used to be obstructed, after BPC 157 therapy. By contrast, in the experiments in which the course of ischemia was not reversed, decreased NO levels in the colon tissue appeared as an immediate heavy loss of endothelial cells from the vascular wall, which resulted in a lower eNOS production ability[42]. Similarly, the endothelium has general significance; failure[43] due to oxidative stress regularly appears as a result of the lysis of endothelial cells[9,10], and it may be further aggravated by additional reperfusion. Together, these reflect first a sudden decrease of colonic blood supply[1] and inadequate eNOS system function[2]. Subsequently, when the ligation is removed, the massive induced reperfusion likely shifts the tissue toward a state of noxious inducible isozyme or vascular wall and other tissue cell damage[44]. These developments can hardly be prevented spontaneously unless a specific agent such as BPC 157[2-8] is used as a therapeutic to produce a rapid and persistent recovery of vascular function.

BPC 157 was shown to restore endothelial integrity and to actually reverse most of the oxidative damage, even during reperfusion and exaggerated reperfusion. This effect was noticeable in IC rats and even more noticeable in IC + RL rats. The effect of BPC 157 is not like reperfusion injury, which must be regularly ameliorated by interventions initiated at the end of the ischemic period[45]. BPC 157 was previously shown to have a beneficial effect and to induce NO release in gastric mucosa from rat stomach tissue homogenates[46,47]. This effect occurred even under conditions in which L-arginine was not effective, a finding that could be generalized to other tissues[3,46,47]. Additionally, various free-radical-induced lesions in other organs were counteracted by BPC 157 administration[45,48,49]. The pentadecapeptide BPC 157 contains four carboxylic groups that may act as possible antioxidants; all of them could be active in the scavenger process, and if they are reactivated (by, *e.g.*, glutathione or enzymes), the overall antioxidant activity could be very high. Additionally, BPC 157 is present in most tissues, where it can bind reactive free radicals and inactivate them at crucial positions that other antioxidants cannot reach[50].

Finally, to envisage all possibilities for the involvement of the NO system in colonic ischemic injury (opposing healing and bleeding capabilities; L-NAME opposes healing and decreases bleeding time, whereas L-arginine promotes healing and prolongs bleeding time[3,51]), we conducted a parallel investigation of the effects of NOS blockade (L-NAME) and NOS substrate (L-arginine) administration, which is an important point that should be addressed[3].

In addition to experiments in which blood flow is instantly interrupted by means of two ligations in rats

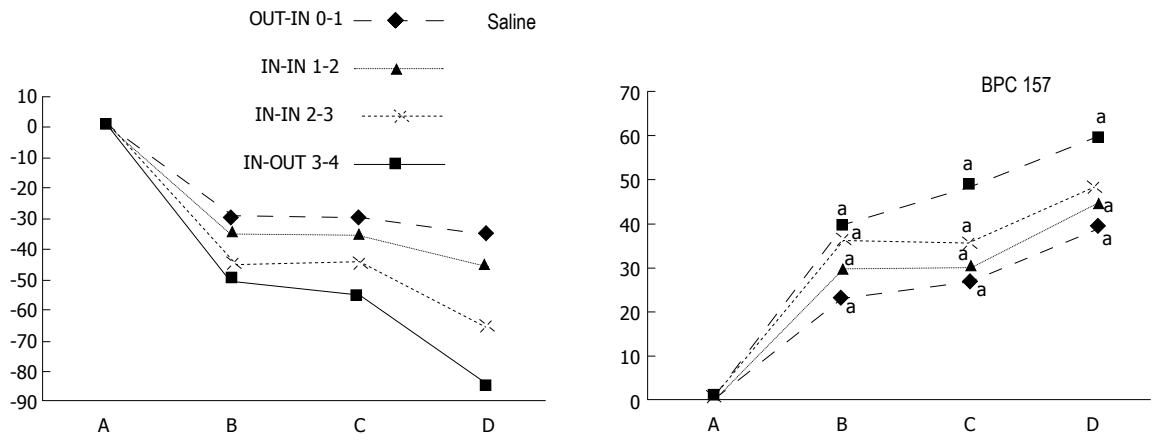

Figure 21 IC + OB rats. Per cent of vessels present between arcade vessels, next to the proximal ligature *(*arcade 0*)*, next to the distal ligature (arcade 4), and within the ligation (arcade 1, 2, 3) on the dorsal side of the colon at 15 min after therapy (as 100%); mean ± SD. The gross presentation was recorded using a USB microscope camera. After additional colon obstruction for three days, BPC 157 (10 μg/kg, 1 mL bath/rat) or an equal volume of a saline bath was applied to the 25-mm blood-flow-deprived colon segment 1 min after the colon obstruction was removed. The rats were sacrificed 15 min later, and the following time points were assessed: A: after ligation and before therapy (1 min); B: 5 min after the application of medication; C: between 5 and 10 min after the application of medication; D: from 10 min after the application of medication until the end of the observation at 15 min. For clarity, the SD is not shown on the graph; the SD was never higher than 10% of the mean. [a]$P < 0.05$ at least *vs* control.

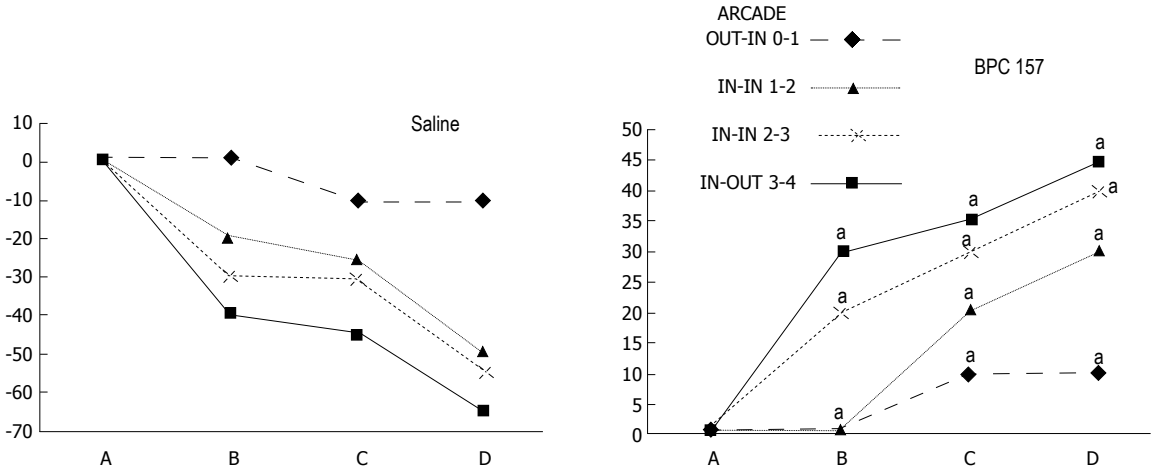

Figure 22 IC + OB rats. Per cent of vessels present between arcade vessels, next to the proximal ligature (arcade 0), next to the distal ligature (arcade 4), and within the ligated area (arcade 1, 2, 3) on the ventral side of the colon 15 min after therapy (as 100%); mean ± SD. The gross presentation was recorded using a USB microscope camera. After additional colon obstruction for three days, BPC 157 (10 μg/kg, 1 mL bath/rat) or an equal volume of a saline bath was applied to the 25-mm blood-flow-deprived colon segment 1 min after the colon obstruction was removed. The rats were sacrificed 15 min later, and the following time points were assessed: A - after ligation and before therapy (1 min); B - 5 min after the application of medication; C - between 5 and 10 min after the application of medication; D - from 10 min after the application of medication until the end of the observation at 15 min. For clarity, the SD is not shown on the graph; the SD was never higher than 10% of the mean. [a]$P < 0.05$ at least *vs* control.

that underwent interventions to produce ischemic colitis, the relationship between BPC 157 and NO has been established in various experimental models and species. The results of these studies suggest that BPC 157 might interfere with the effects of NOS blocking agent or NOS substrate application[3]. As noted in this study, the complex action of BPC 157 tends to rule out a simple effect of either the vasoconstrictor (L-NAME) or the vasodilator (L-arginine)[3] in the noted rapid presentation of blood vessels that bypass the two obstructions of major vessels and reestablishing the blood flow that was interrupted by ligation in rats which subjected to the ischemic colitis procedure. Namely, upon encountering the regular vicious course of double obstruction of vessels, as individual agents, both L-NAME and L-arginine affect the course of the lesion in a particular way, and neither appears to be completely effective. L-NAME initially caused all vessels to disappear more rapidly, thereby inducing larger pale areas, whereas L-arginine increased the number of vessels present but also induced larger pale areas (note that L-arginine, unlike BPC 157, did not produce proper collateral arcade interconnection).

This effect is likely a specific aspect of the dual role of the NO system (L-NAME *vs*. L-arginine vs. combination of the two) (for a review, see[3,52,53]). Each

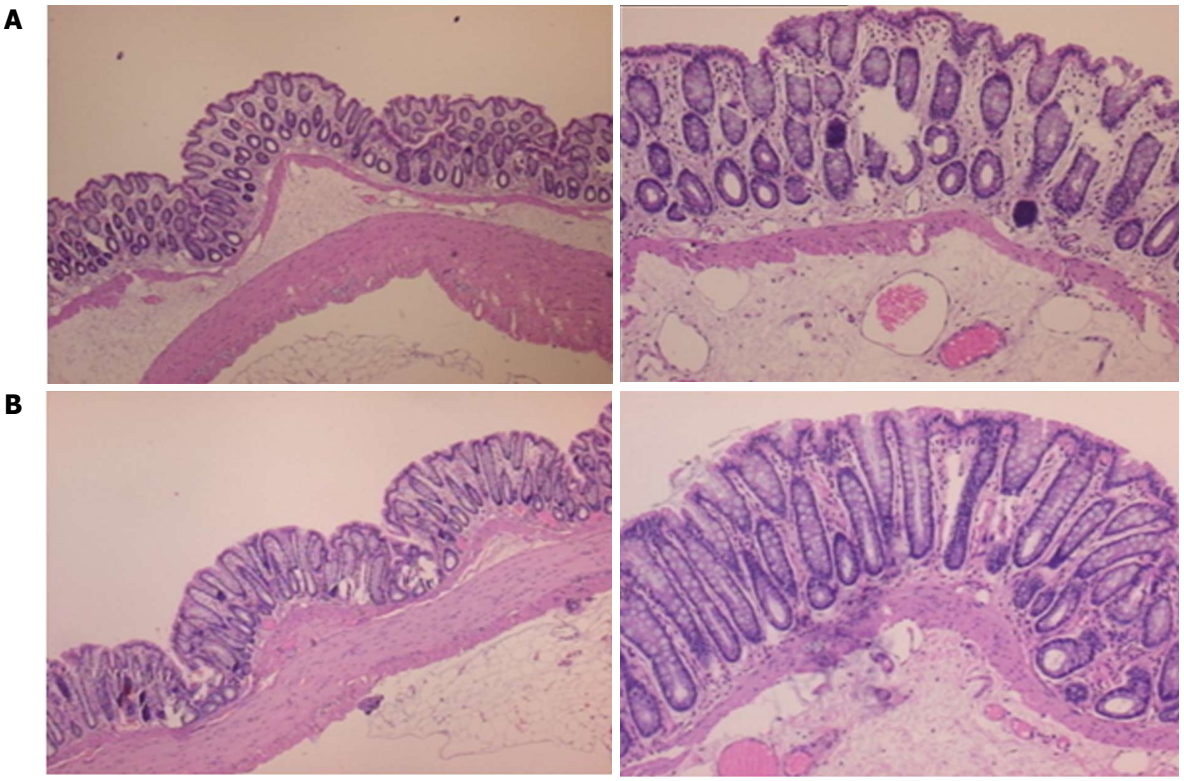

Figure 23 Characteristic microscopic appearance of the colon in IC (A, control) rats and BP 157-treated IC rats (B) 15 min after ligation. HE, 4 × objective (left) and 10 × objective (right). A: Severe edema of the lamina propria and continuous diffuse edema of the submucosa can be observed. Pronounced dilatation and stasis of the submucosal blood vessels is also present. B: Mild edema of the lamina propria and focally present mild-to-intermediate edema of the submucosa can be observed. Although stasis of the submucosal blood vessels is present, the dilatation of the veins appears to be less pronounced.

of these effects is specific (*i.e.*, NO-related) because when they were administered together, L-NAME and L-arginine regularly attenuated or antagonized each other's responses[3,52,53]. Likewise, the remaining serious pathology in the animals treated with L-NAME + L-arginine indicates that other system(s) (*i.e.*, the BPC 157 system) may function along with the NO system, which was previously supposed to be inactivated by the combined action of L-NAME and L-arginine[3,52,53]. In this view, BPC 157 would be effective regardless of whether the NO system is inactivated (L-NAME + L-arginine), overstimulated (L-arginine) or blocked (L-NAME). Thus, BPC 157 may consolidate the stimulatory and inhibitory effects of the NO system to produce more effective healing (*i.e.*, by promoting the interconnection of arcade vessels to bypass major obstructions)[3]. This rapid recruitment of existing blood vessels that would otherwise respond poorly to increased demands following ongoing harmful events may indicate that BPC 157 also affects several other molecular pathways[11,25-29], the effects which were seen at the later time point. In particular, the immediate recovery of collaterals and bypassing of the obstruction, a feature noted in the present study, and the rapid reestablishment of blood flow in both the ischemic and reperfusion conditions may be responsible for its subsequent strong angiogenic effect and its healing effects[2,22-28], which are more pronounced than those of standard anti-ulcer agents[23].

The special feature noted in the present study could be responsible, in particular, for the increased expression and internalization of VEGFR2, which is known to be essential for endothelial function, and the activation of the VEGFR2-Akt-eNOS signaling pathway[25]. Previous studies have substantiated that BPC 157 induces an acceleration of blood flow recovery and vessel number within days in rats with hind limb ischemia[23]. Other studies have demonstrated the expression of growth hormone receptor at both the mRNA and protein levels and have shown that BPC 157 regulates the phosphorylation of extracellular-signal-regulated kinases 1 and 2 (ERK1/2) as well as their downstream targets, including c-Fos, c-Jun, and Egr-1, key molecules involved in cell growth, migration, and angiogenesis[11,25-29]. BPC 157 also induced expression of the *Egr-1* gene and its co-repressor gene *NaB2*, suggesting that it may serve as part of a feedback system[29]. Together, these findings indicate how complex the BPC 157 pathways may be, pathways that remain to be further defined.

The above considerations suggest that two pharmacologically distinct mechanisms with opposite

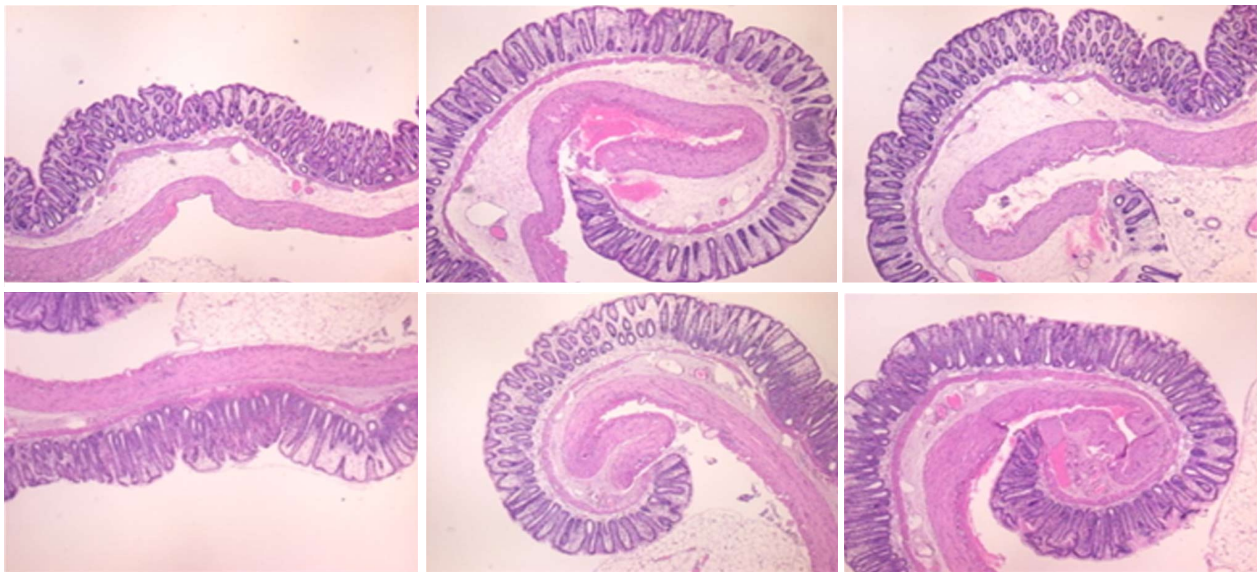

Figure 24 In IC + RL rats, 15 min of ligation followed by 15 min of full reperfusion period resulted in the appearance of even more mucosal and submucosal edema as well as more pronounced cyanosis (controls, upper, HE, 4 × objective). This was consistently attenuated by BPC 157 treatment after the initiation of reperfusion (lower, HE, 4 × objective).

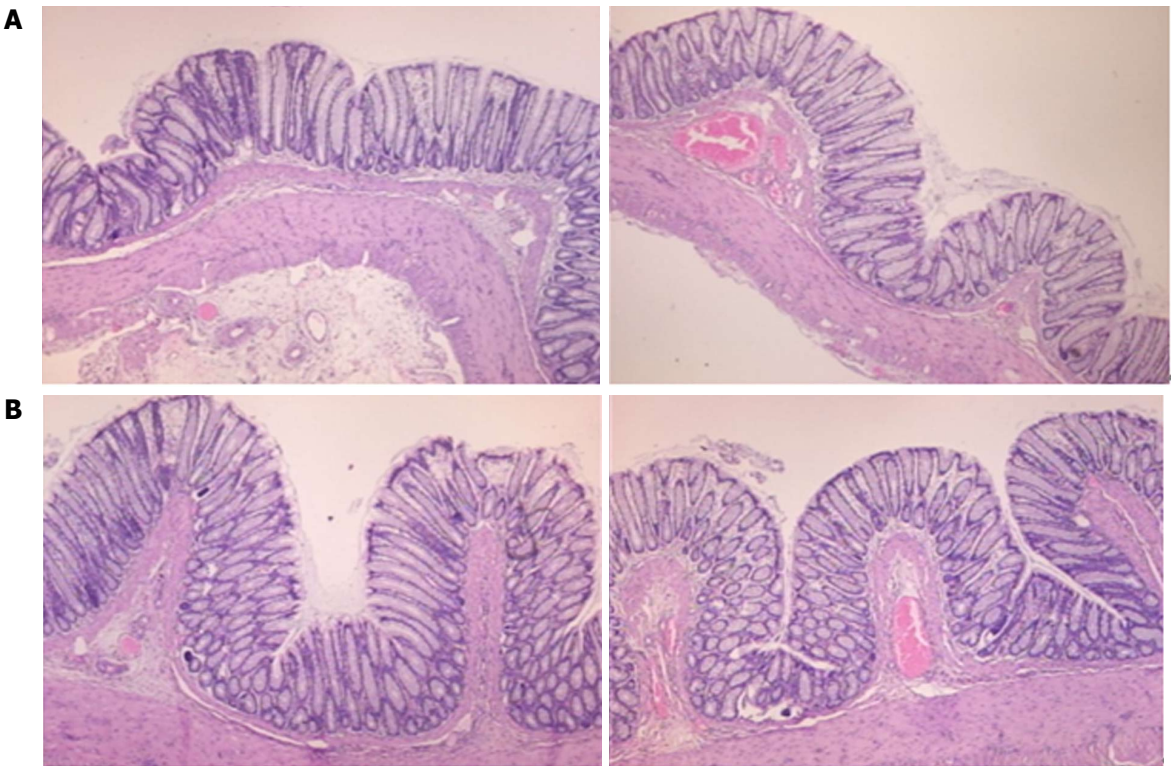

Figure 25 Microscopic appearance of the colon in IC + OB rats (A, control) and BPC 157-treated IC + OB rats (B) after additional colon obstruction for three days one week after the additional colon obstruction was removed. Control (saline bath); 10 d; BPC 157, 10 d. HE, 4 × objective (left); 10 × objective (right). A: Mild edema of the lamina propria and diffuse mild-to-intermediate edema of the submucosa, along with the formation of collagen, can be observed. Stasis of the submucosal blood vessels is also present. The rugae are broadened and flattened, so the mucosa appears flattened macroscopically. B: Mild edema of the lamina propria and practically no edema of the submucosa is observed. Stasis of the submucosal blood vessels is also present but to a much lesser extent than in the controls. The rugae are histologically well formed and only occasionally slightly broadened. There is minimal formation of new collagen fibers in the submucosa.

effects on the same signaling pathway are involved in a normally unresolvable physiological failure (complete obstruction (ligation) of vessels and ischemia due to a cause that could be not removed, inadequate rescue and unable to heal). Additionally, in practice, this means that two distinct end points should both be overwhelmed to produce the effects seen in rats that received BPC 157 application[3]. Notably,

BPC 157 instantly prevents and reverses L-NAME-induced hypertension as well as L-arginine-induced hypotension[46]. After amputation and/or anticoagulant application, BPC 157 counteracts prolonged bleeding and thrombocytopenia; it also counteracts the side effects of both L-NAME-(thrombocytopenia) and L-arginine (prolonged bleeding, thrombocytopenia)[51]. Additionally, in pupil dilation assays, a common point of action of L-NAME/L-arginine was noted (in miosis, both L-NAME and L-arginine antagonized the response to the other); this effect can be explained by an interaction with the cholinergic system[54]. BPC 157 counteracts the actions of both L-NAME (miosis) and L-arginine (miosis); interestingly, BPC 157 counteracts atropine-induced mydriasis as well[54].

The pentadecapeptide BPC 157 was also shown to counteract peritonitis and adhesion formation and to have a beneficial effect in diverse intestinal lesion models[30,36-40]. This particular activity is supported by the variety of beneficial effects of BPC 157 noted in colitis lesion studies[2-7]. A likely generalization follows the association of models of chemically induced inflammation (trinitrobenzenesulfonic acid and dextran sodium sulfate) with a marked reduction in blood flow[17-19]. A further generalization may be that BPC 157 effectiveness over the background of the NO-system[3,46,51,54], immobilized (L-NAME + L-arginine), (over)stimulated (L-arginine) or blocked (L-NAME), BPC 157 prevented MDA oxidative stress and normalized the NO tissue values. In addition to its use in ulcerative colitis clinical trials[2-7] and its effectiveness in various models of colitis[30-35], BPC 157 reduces high myeloperoxidase (MPO) activity in colonic tissue[33], heals fistulas[36-38], rescues failed anastomosis healing[30,34,39,40] and markedly improves intestinal adaptation following massive bowel resection[40]. It is likely that the particular molecular pathways[11,25-29] that have been suggested to be involved in the healing effects of BPC 157, as well as its recently demonstrated ability to cause the increased expression and internalization of VEGFR2 and the activation of the VEGFR2-Akt-eNOS signaling pathway[25], contribute to its action.

Finally, the aforementioned rapid and successful recruitment of the blood vessels during harmful events suggests that the application of BPC 157 may offer a fundamental treatment by providing the cytoprotection/endothelium protection that is essential[2-8,12-16,20] to quickly restore blood supply to the ischemically injured area and rapidly activate collaterals during various harmful conditions such as vascular obstruction, short-lasting blood deprivation, reperfusion, long-lasting blood deprivation and additional bowel obstruction.

COMMENTS

Background

Stable gastric pentadecapeptide BPC 157 would provide new insights in the treatment of colitis and ischemia and reperfusion. Likely, gastric cytoprotection and ischemic colitis (IC) lesions may have analogous therapy. Thereby, prototype cytoprotective agent gastric pentadecapeptide BPC 157, which has been used in trials for ulcerative colitis and now for multiple sclerosis, would rescue *IC* lesions in rats (IC rats). We studied the cytoprotective mechanism of BPC 157 because cytoprotection and cytoprotective agents restore the integrity of damaged stomach epithelium by rapidly rescuing damaged endothelium. This effect would involve the NO system. BPC 157 would be subsequently shown to be effective in IC rats underwent full reperfusion (removed ligations (RL) (IC + RL rats) and IC rats with additional colon obstruction (OB) (IC + OB rats).

Research frontiers

The main focus of the intervention was that BPC 157 rapidly activates collaterals due to its particular direct and rapid effect on vessel presentation, the bypassing of one or more of the vascular obstructions and thereby achieving a therapeutic effect.

The next focus was on NO-system, the effect of NO system agents in IC rats, NOS blocker L-NAME and/or NOS substrate, L-arginine; in colon tissue, assessing NO levels and oxidative stress (MDA levels) (as result of the lysis of endothelial cells) and ischemia/reperfusion injury, both as a spontaneous course [when blood supply was deprived via ligation (IC rats)] and a more exaggerated course [after ligation removal (IC + RL rats)] in immediate post-ligation time. Notably, although the NO system is largely implicated in stomach cytoprotection and colitis lesions, the application of L-NAME (a vasoconstrictor) and/or L-arginine (a vasodilator) has not been investigated with respect to the immediate presentation of the blood vessels after a segment of left colic artery and vein was occluded by two ligations. By contrast, BPC 157 largely interacts with the NO system in various models and species, as shown in cytoprotection studies, in particular, studies using both L-NAME and L-arginine as individual agents or in combination.

Innovations and breakthrough

We rescued rat ischemic colitis. The gastric pentadecapeptide BPC 157, which has been used in clinical trials for ulcerative colitis, exerted rapid cytoprotective endothelium rescue against the disabled left colic artery and vein after blood deprivation via two ligations and during reperfusion (ligations removed). By bypassing obstructions, quickly rescuing blood supply, rapidly activating collaterals, and restoring arcade interconnections, as a new integrative beneficial effect, BPC 157 prevented the occurrence of pale lesions without mucosal folds and normalized the levels of NO and MDA, two oxidative stress markers, in tissues. BPC 157 showed effectiveness over the NO-system background, immobilized (L-NAME + L-arginine), (over)stimulated (L-arginine) or blocked (L-NAME). Likewise, later application of BPC 157 in a bath treatment to rats with pertinently obstructed vessels that underwent additional colon obstruction for three days produced a similar beneficial effect.

Applications

BPC 157 is a fundamental treatment that quickly restores blood supply to the ischemically injured area and rapidly activates collaterals. This effect involves the NO system.

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ORIGINAL ARTICLE

Basic Study

Exploring pathogenesis of primary biliary cholangitis by proteomics: A pilot study

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Abstract

AIM

To explore the pathogenesis of primary biliary cholangitis (PBC) by identifying candidate autoantibodies in serum samples by proteomics and bioinformatics.

METHODS

Nine antimitochondrial antibody (AMA)-positive PBC patients and nine age- and sex-matched AMA-negative PBC patients were recruited. Antigen enrichment technology was applied to capture autoantigens of human intrahepatic biliary epithelial cells (HiBECs) that

are recognized by autoantibodies from the sera of PBC patients. Candidate autoantigens were identified by label-free mass spectrometry. Bioinformatics analysis with MaxQuant software (version 1.5.2.8), DAVID platform, and Cytoscape v.3.0 allowed illustration of pathways potentially involved in the pathogenesis of PBC.

RESULTS

In total, 1081 candidate autoantigen proteins were identified from the PBC patient pool. Among them, 371 were determined to be significantly differentially expressed between AMA-positive and -negative PBC patients ($P < 0.05$). Fisher's exact test was performed for enrichment analysis of Gene Ontology protein annotations (biological processes, cellular components, and molecular functions) and the Kyoto Encyclopedia of Genes and Genomes pathways. Significantly different protein categories were revealed between AMA-positive and -negative PBC patients. As expected, autoantigens related to mitochondria were highly enriched in AMA-positive PBC patients. However, lower levels of AMA were also detected in AMA-negative PBC patients. In addition, autoantigens of AMA-negative PBC patients were mainly involved in B-cell activation, recognition of phagocytosis, and complement activation.

CONCLUSION

AMA-negative PBC individuals may not exist, but rather, those patients exhibit pathogenesis pathways different from those of AMA-positive PBC. Comprehensive research is needed to confirm these observations.

Key words: Anti-mitochondrial antibody; Bioinformatics; Pathogenesis; Primary biliary cholangitis; Proteomics

Core tip: The pathogenesis of primary biliary cholangitis (PBC) is still unclear. Related studies have focused on genes, immune cells, and pathology. However, little research has been conducted to establish pathogenesis-related autoantibodies. In this study, we unraveled the pathogenesis of PBC by detecting novel autoantibodies, using proteomics. Our results suggest that the dysfunction of three pathways in human intrahepatic biliary epithelial cells might be causative in the pathogenesis of antimitochondrial antibody (AMA)-negative PBC. More interestingly, we identified AMA-negative pathology as a potential misnomer, as we detected low levels of AMA in sera of AMA-negative patients. Comprehensive research is needed to confirm these observations.

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INTRODUCTION

Primary biliary cholangitis (PBC) is a chronic and progressive intrahepatic cholestatic disease that can ultimately progress to severe hepatic illnesses, including hepatic cirrhosis, hepatic failure, and even hepatocellular carcinoma[1]. Currently, the pathogenesis of PBC is still unclear, but interactions between genetic predisposition and environmental triggers are considered to play an important role[2]. Several studies have found that PBC risk alleles tend to occur in loci that are related to immune function[3]. Pathological examination confirmed that the immune system is dysregulated in PBC patients. Most intriguingly, portal triads are heavily infiltrated by T cells that are responsive to mitochondrial and nuclear antigens, resulting in autoantibodies such as anti-mitochondrial antibody (AMA) found in humoral immune responses[4].

Elevated levels of serum autoantibodies are characteristic of autoimmune disease. The appearance and fluctuation of the autoantibodies not only represent the disease status but also further our understanding of the pathogenesis of the disease[5]. For example, the specific diagnostic biomarker of PBC, AMA, was confirmed to participate in injuring intrahepatic biliary epithelial cells (iBECs) and perpetuating the destructive process[6]. Interestingly, AMA cannot be detected in 10% of PBC cases, which prompted us to determine whether other pathogenesis-associated and AMA-like autoantibodies exist in AMA-negative PBC patients.

In recent years, proteomics and bioinformatics approaches have been applied to several fields, including the exploration of diagnostic markers, predictive factors, and disease pathogenesis[7-9]. Previously, we applied proteomic technologies, including high-throughput human proteome microarrays and two-dimensional difference gel electrophoresis, to explore biomarkers for PBC[7-11] in order to understand the pathogenesis of PBC. Our results were confirmed by another research team[12], which verified the scientific value of proteomics.

Dysfunction and loss of iBECs are one of the most important pathological features of PBC. A few studies have focused on the mechanism of damage to iBECs. Molecular cholangiocyte defects stymie defense against toxic bile acids, and defects in biliary bicarbonate secretion were a particular focus of investigation toward unraveling the pathogenesis of PBC[13].

In this context, we sought to perform an exploratory study by combining enrichment of antigens from iBECs, label-free mass spectrometry, and bioinformatics to explore autoantibody candidates for AMA-negative PBC.

This may aid in decrypting the pathogenesis of PBC. In this pilot study, we aimed to ascertain the feasibility of the experimental approach and to obtain preliminary results that will serve as a guide for the future study.

MATERIALS AND METHODS

Study patients

From 2014 to 2015, 18 PBC patients were recruited from the Peking Union Medical College Hospital. They included nine AMA-positive and nine AMA-negative PBC cases, with pairs matched for age and sex. AMA and the M2 subtype of AMA were detected by an indirect immunofluorescence assay and an enzyme-linked immunosorbent assay, respectively. All enrolled patients were treatment-naïve and diagnosed according to the criteria proposed by Heathcote *et al*[14]. The Ethics Committee of the Peking Union Medical College Hospital approved this study. The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki. Written informed consent was obtained from all participants.

Sample collection

Blood samples were collected under fasting conditions in the morning. Samples were drawn from the cubital vein and dispensed into 5-mL pro-coagulation tubes with gel (Becton, Dickinson and Company, United Kingdom). Blood samples were centrifuged at 1000 *g* for 5 min within 6 h of collection. Sera were frozen at -80 ℃ until use.

Cell culture

Human iBECS (HiBECs) were purchased from PriCell (Wuhan, China), where they were isolated from human livers obtained *via* organ donation from donor tissues that were not suitable for organ transplantation. HiBECs were subcultured when they reached 90%-95% confluency, and the culture medium was removed and discarded. The cell layer was rinsed briefly with 5 mL of phosphate-buffered saline solution to remove all traces of serum. Next, 1 mL of 0.25% (w/v) trypsin and 0.53 mmol/L EDTA solution was added to the flask, which was incubated in a 37 ℃ incubator for 5 min. Cells were observed under an inverted microscope until the cell layer dispersed. Cells were aspirated by gentle pipetting and washed twice with complete growth medium (PriCell, Wuhan, China). Cells were fed every other day with complete growth medium at 37 ℃ in a humidified atmosphere containing 5% CO_2 and 95% air until ready for assay. HiBECs were verified by an immunofluorescence microscopic method with an antibody against cytokeratin 19.

Enrichment of target antigens of potential autoantibodies

Target antigens of potential autoantibodies were enriched using the Pierce Crosslink Immunoprecipitation Kit (Pierce Biotechnology, Rockford, United States). Briefly, equal volumes of three serum samples from AMA-positive PBC patients were pooled, resulting in three AMA-positive PBC sample pools. AMA-negative PBC samples were handled identically. All pooled samples were diluted and incubated with protein A/G agarose to capture antibodies. Then, the antibodies were cross-linked to prevent co-elution with antigens.

HiBECs were lysed using the immunoprecipitation lysis and wash buffers provided in the immunoprecipitation kit. Control agarose resin was used to pre-clear the lysate, adsorbing non-specific binding entities. To ensure the formation of target complexes, cleared lysates with excess antigens were incubated with the antibody-agarose complexes. The target antigens were eluted from these complexes, using low-pH elution buffer provided with the kit. Then, 1 M Tris pH 9.5 was added to the eluate to neutralize the pH. The final collections were stored at -80 ℃ until proteomic analysis.

Liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis

Total target antigens (50 μg) from each sample were resuspended in 100 μL of 4% SDS and 0.1 mol/L DTT in 0.1 mol/L Tris HCl, pH 7.6 at 25 ℃ following a filter-aided sample preparation protocol described elsewhere[15]. Liquid chromatography (LC) was performed on an Easy-nLC System (Thermo Fisher Scientific, MA, United States). Peptides were separated on a 15-cm fused silica emitter packed in-house with the reverse phase material ReproSil-Pur C18AQ, 3-μm resin (Dr. Maisch, Germany) with a 100-min gradient from 5% to 35% of 99.9% (v/v) CH_3CN, 0.1% (v/v) acetic acid. A quadrupole Orbitrap mass spectrometer (Q Exactive; Thermo Fisher Scientific, Germany) was operated in the positive-ion mode using a data-dependent "top 12" method. Survey scans and tandem mass spectrometry (MS/MS) scans were acquired at a resolution of 70000 and 17500, respectively, at 400 m/z. The top 12 most abundant isotope patterns with charges $\geqslant$ 2 from the survey scan were selected with an isolation window of 2 Thomson and fragmented by higher-energy collisional dissociation with a normalized collision energy of 25%. The maximum ion injection times for the survey scans and the MS/MS scans were 50 ms and 100 ms, respectively, and the ion target values were set to 1E6 and 1E5, respectively. Selected sequenced ions were dynamically excluded for 30 s.

Data analysis

Raw MS data were analyzed using MaxQuant software (version 1.5.2.8)[16]. A false discovery rate (FDR) of 0.01 and a minimum peptide length of seven amino acids for proteins and peptides were required. A time-dependent mass recalibration algorithm was used to improve the mass accuracy of precursor ions[17]. MS/MS spectra were queried using the Andromeda

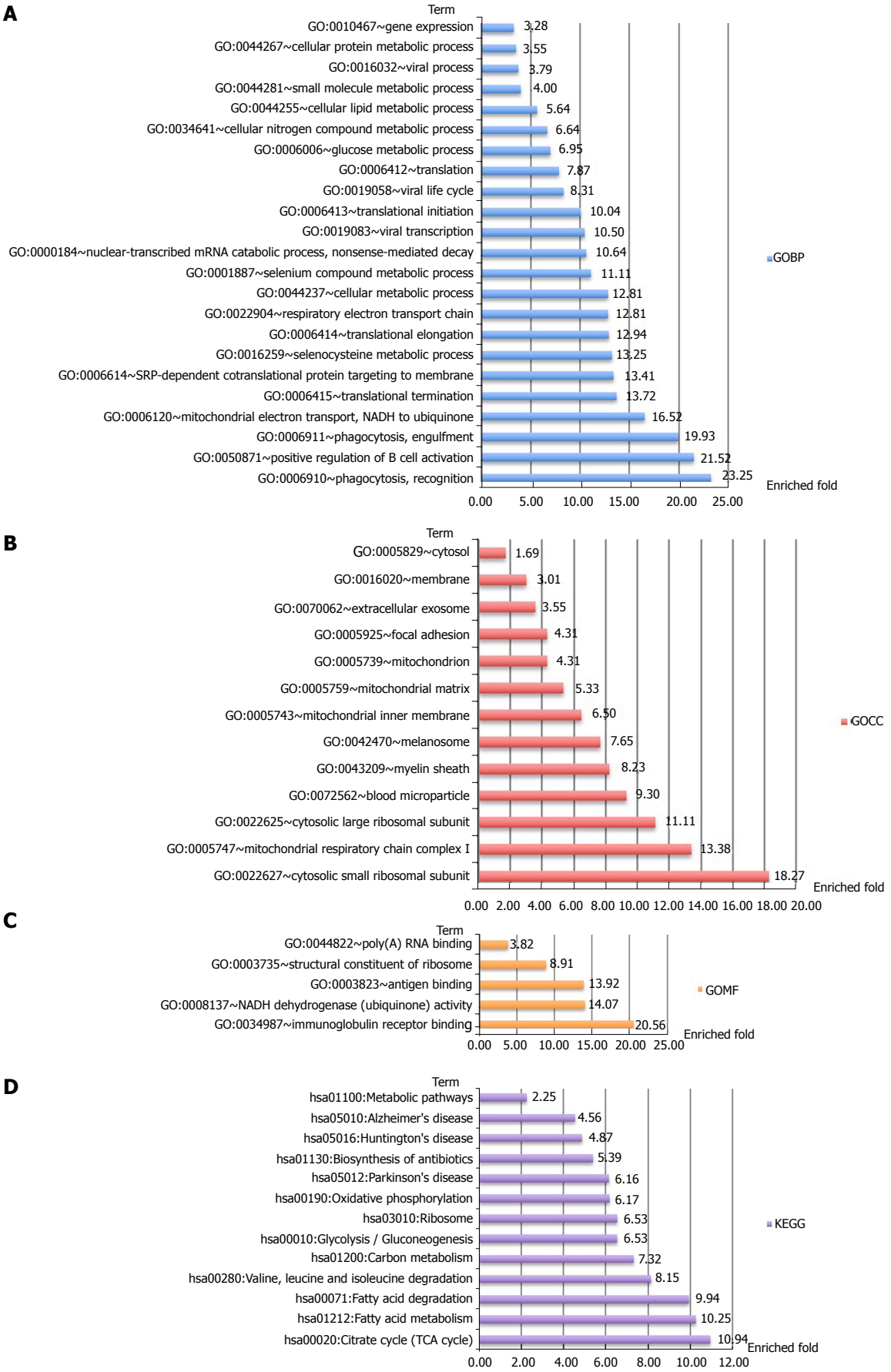

Figure 1 Enrichment analysis of 371 significantly differentially expressed proteins. A: Gene ontology biological processes; B: Gene ontology cellular components; C: Gene ontology molecular functions; D: Kyoto Encyclopedia of genes and genomes pathways.

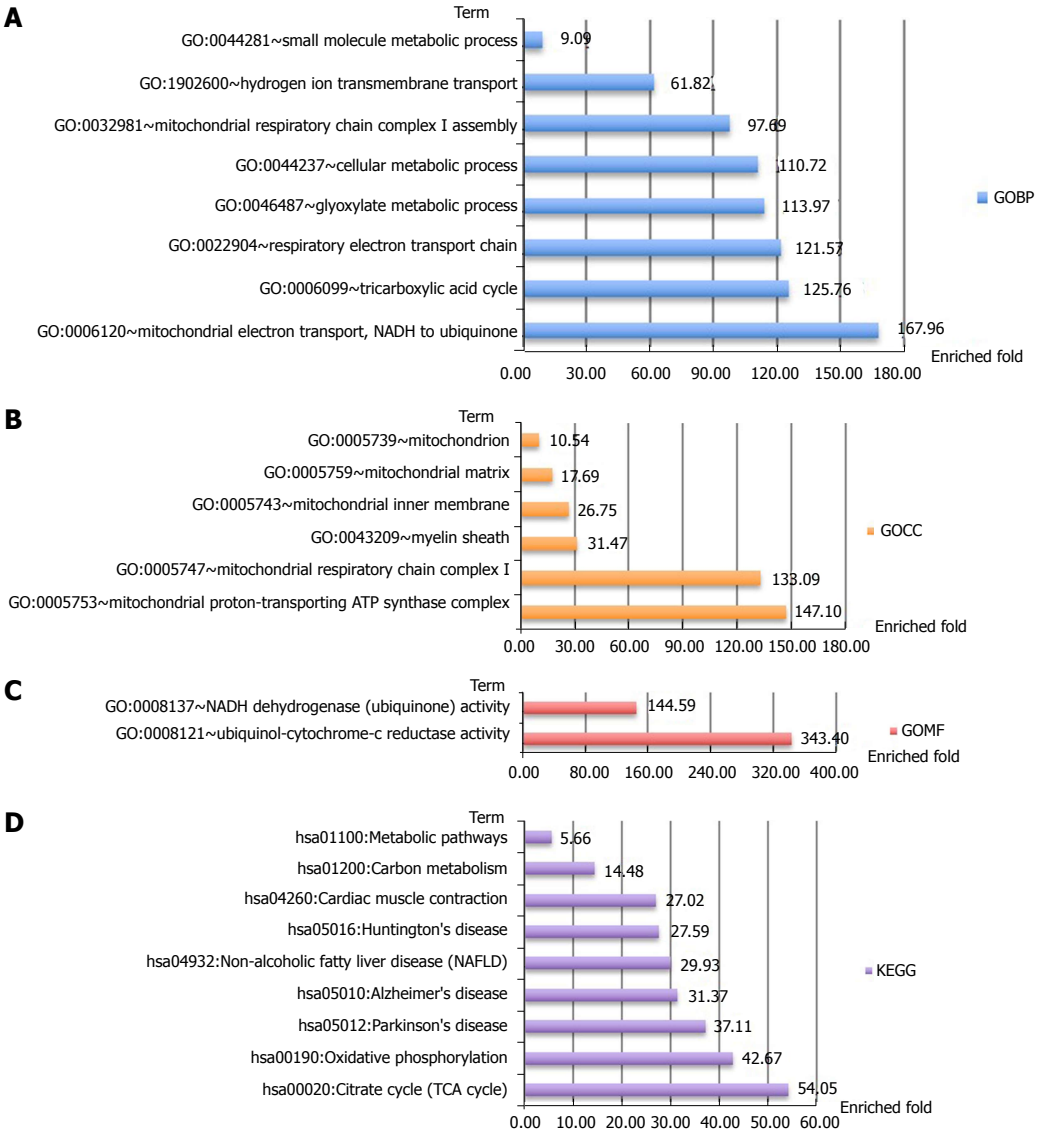

Figure 2 Enrichment analysis of significantly differentially expressed proteins of Hub-1. A: Gene ontology biological processes; B: Gene ontology cellular components; C: Gene ontology molecular functions; D: Kyoto Encyclopedia of genes and genomes pathways.

search engine, which is incorporated in the MaxQuant software suite, against the UniProt human database (201307, containing 88405 entries). For the search, trypsin was chosen for enzyme specificity[15], allowing for cleavage at the N-terminus of proline (trypsin/P). Cysteine carbamidomethylation was selected as a fixed modification, while protein N-terminal acetylation and methionine oxidation were selected as variable modifications. Maximally two missed cleavages were allowed. For MS and MS/MS, the tolerances of the main search for peptides were set at 7 ppm and 20 ppm, respectively. Quantification was performed in MaxQuant using the built-in label-free quantification algorithm MaxLFQ[18], enabling the "match between runs" option (time window, 2 min). Peptides that were shared between two proteins were combined and reported as one protein group. Proteins matching to the reverse database were filtered out.

For bioinformatics analysis, protein abundance information was collected to have at least two valid expression values in each group. Student's *t*-test was performed for comparison of differences between AMA-positive and -negative proteins, with $P < 0.05$ set as the significance cut-off. Enrichment analyses were conducted for the Gene ontology (GO) biological processes (GOBP), cellular components (GOCC), and molecular functions (GOMF) categories. Kyoto Encyclopedia of genes and genomes (KEGG) pathways were analyzed through the DAVID platform[19], using the complete UniProt human protein database as a background and FDR < 0.05 as a cut-off. Heatmap analyses and visualization of significantly different proteins were conducted using complete linkage hierarchical clustering. Significantly different proteins were used as the input for STRING[20] analysis and a network was built based on high-confidence (0.8)

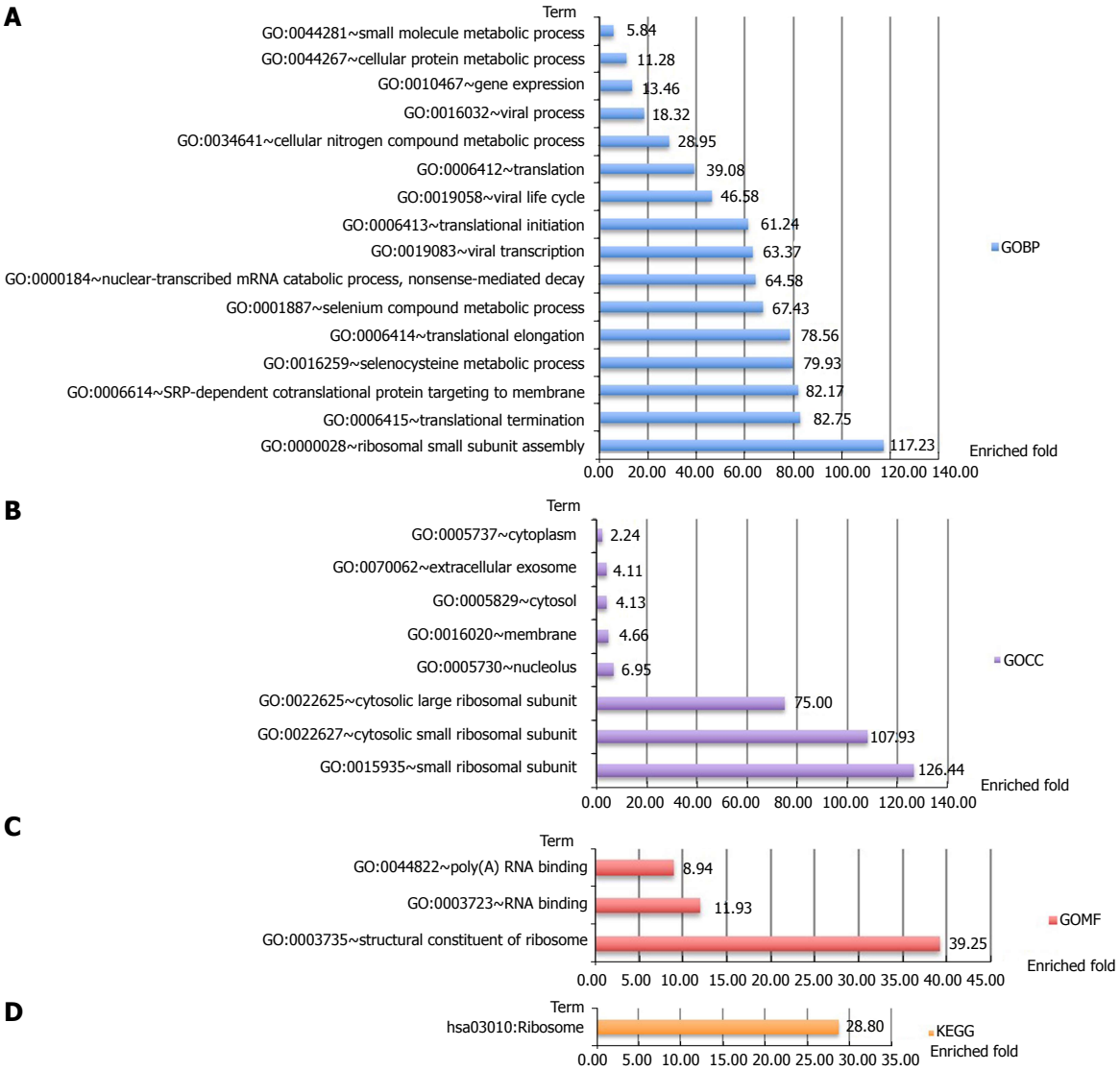

Figure 3 Enrichment analysis of significantly differentially expressed proteins of Hub-2. A: Gene ontology biological processes; B: Gene ontology cellular components; C: Gene ontology molecular functions; D: Kyoto Encyclopedia of genes and genomes pathways.

evidence of experimental protein-protein interactions provided by the STRING database. The network was visualized using Cytoscape v.3.0[21]. Jie Dai, from Shanghai Bioprofile Technology Company Ltd., reviewed the statistical methods of this study.

RESULTS

Identification of candidate autoantigens

In total, 1081 autoantigen candidates were identified from all PBC patients. Among these proteins, 371 were found to be significantly differentially expressed between AMA-positive and -negative groups. Fisher's exact test for the enrichment of GO protein annotations in the set of significantly different proteins revealed a range of protein categories (FDR < 0.05, Figure 1). As expected, the mitochondria-related biological process term was highly enriched. In addition, proteins that participated in the positive regulation of B-cell activation and phagocytic recognition and engulfment were also highly enriched.

Protein-protein interaction analysis

Analysis of protein-protein interactions revealed that the differential autoantigen candidates mainly interact *via* two hubs (Supplementary Figure 1). Enrichment analyses of GO terms and KEGG pathways highlighted differences between the two hubs (Figures 2 and 3). The candidate autoantigens of one hub mainly exist in the mitochondria, while those in the other hub reside in the cytosol. GOBP terms were also largely different between these two hubs.

Clustering and heatmap analysis

Clustering analysis was applied to examine the rationality and accuracy of the selected significant proteins (Supplementary Figure 2). Cluster 1 represents significantly upregulated proteins in AMA-negative PBC patients. They mainly participate in positive regulation of B-cell activation, recognition

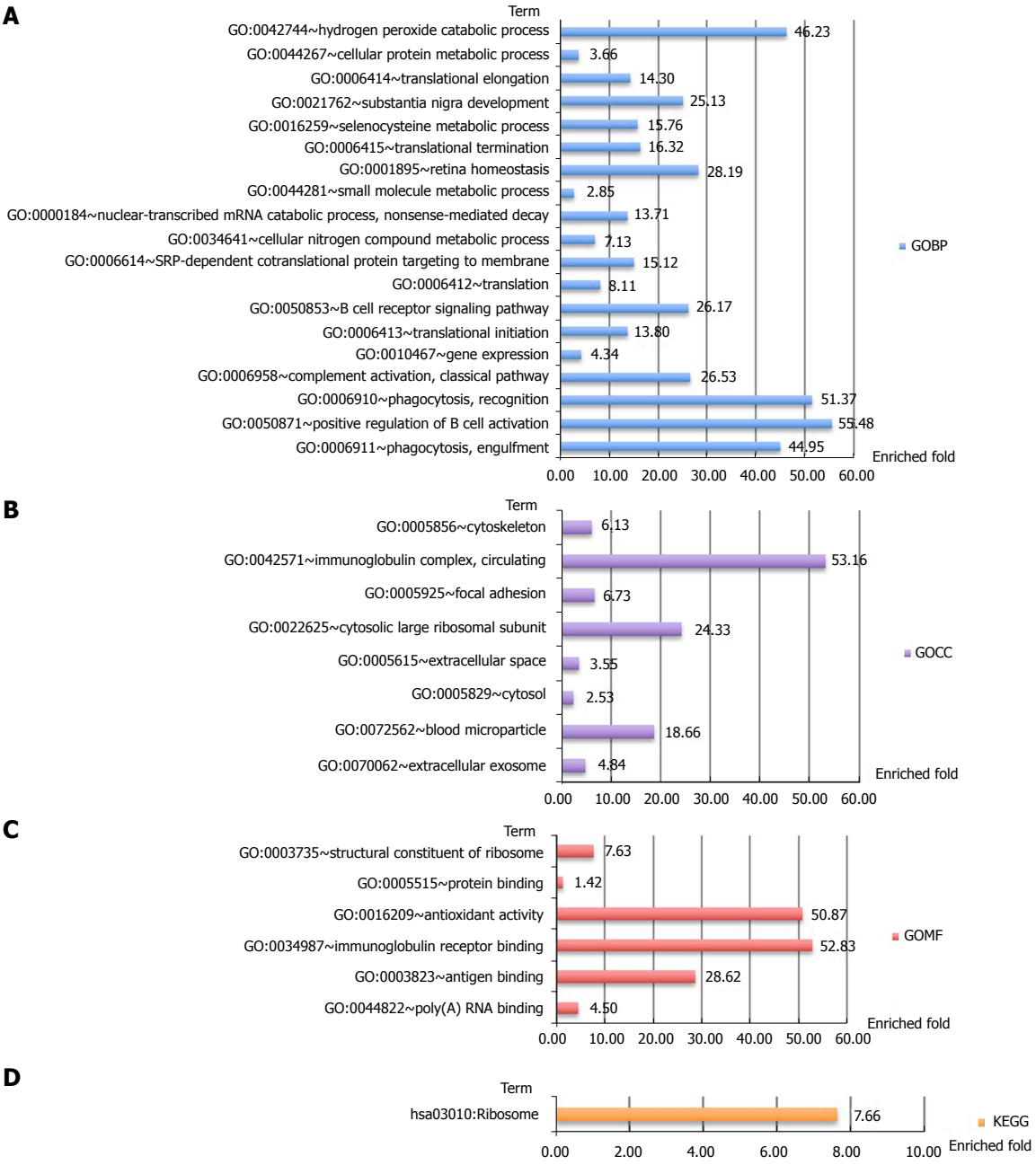

Figure 4 Enrichment analysis of significantly differentially expressed proteins of Cluster 1. A: Gene ontology biological processes; B: Gene ontology cellular components; C: Gene ontology molecular functions; D: Kyoto Encyclopedia of genes and genomes pathways.

of phagocytosis, and complement activation (Figure 4). Cluster 2 represents significantly downregulated proteins in AMA-negative PBC patients that are upregulated in AMA-positive PBC patients. These downregulated proteins mainly participate in electron transport and cellular metabolic processes, which are mostly restricted to mitochondria (Figure 5).

DISCUSSION

In this study, 1081 autoantigen candidates were identified from AMA-positive and -negative PBC patients. Among them, 371 were determined to be significantly differentially expressed between AMA-positive and -negative groups. Further analysis of these proteins revealed autoantibody responses to autoantigens in AMA-negative PBC patients. Of note, these autoantigens participate in the biological processes of HiBECs, including positive regulation of B-cell activation, phagocytic recognition and engulfment, and complement activation.

Enrichment analysis of GO protein annotations revealed that the autoantigens of mitochondria-related biological processes were highly enriched in AMA-positive PBC patients, which matches the expectation. More importantly, lower levels of these autoantigens were also detected in AMA-negative PBC patients.

Since the establishment of AMA as the diagnostic

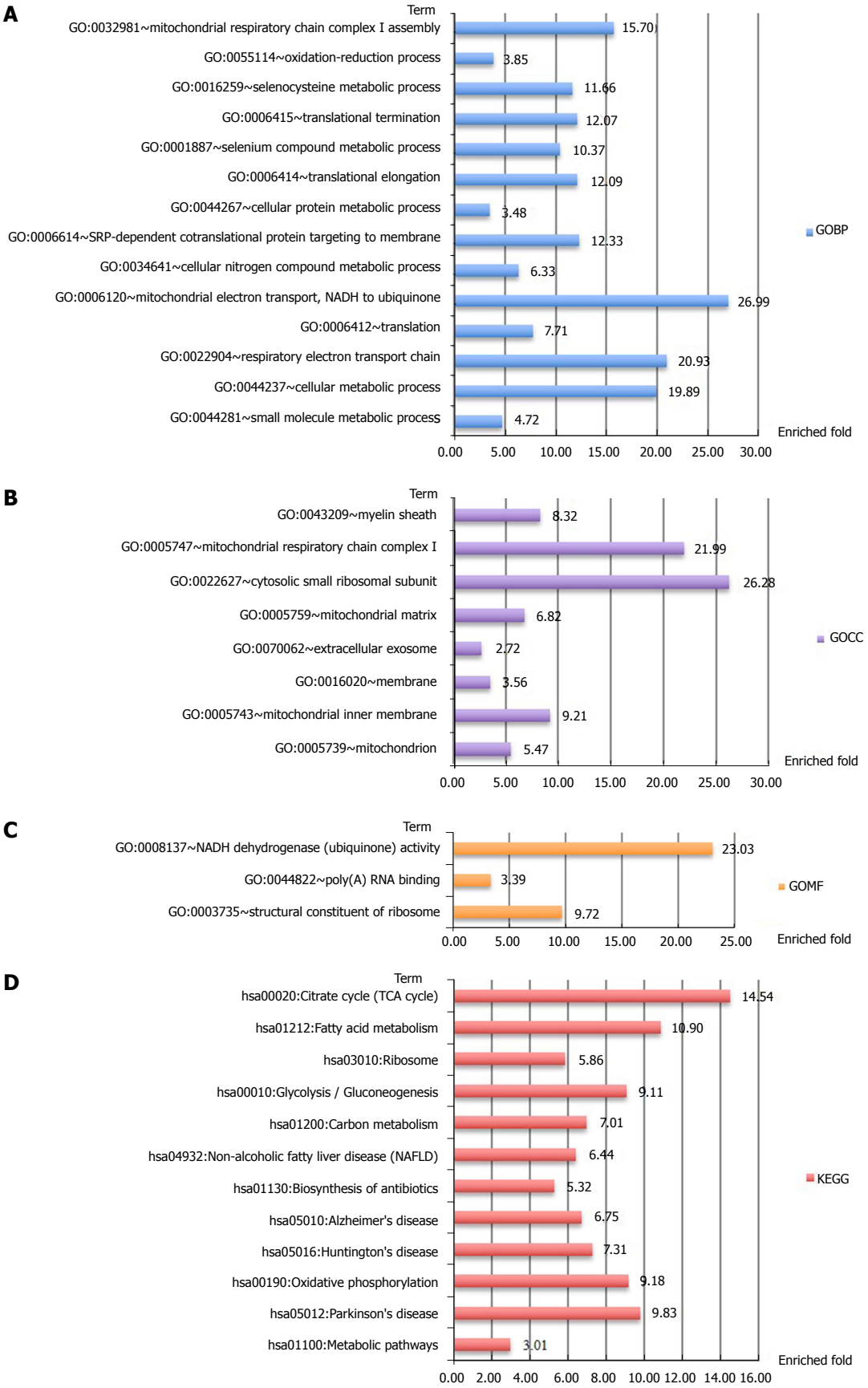

Figure 5 Enrichment analysis of significantly differentially expressed proteins of Cluster 2. A: Gene ontology biological processes; B: Gene ontology cellular components; C: Gene ontology molecular functions; D: Kyoto Encyclopedia of genes and genomes pathways.

biomarker of PBC, there has been controversy surrounding AMA-negative PBC[22]. Some studies have demonstrated that AMA cannot be detected in serum because the method applied in clinical practice is not sensitive enough[23]. Our results have confirmed this conclusion. Compared to traditional AMA detection methods such as indirect immunofluorescence and enzyme-linked immunosorbent assay, mass spectrometry was more sensitive, allowing the detection of lower levels of mitochondria-related autoantigens in AMA-negative PBC.

It seems that AMA-negative PBC technically does not exist, because AMA-related autoantibodies could be detected in these patients as well. However, some other studies did find differences between the two groups of PBC patients. It was reported that AMA-negative PBC patients tend to have lower serum levels of alkaline phosphatase and gamma-glutamyl transpeptidase, but higher levels of aspartate amino transferase[24,25]. In Japan, pruritus was observed less frequently among patients with AMA-negative PBC, but significantly higher number of patients were found to experience complications such as Sjögren's syndrome[24]. Upon scrutinizing these results, we suggest that AMA-negative PBC might exist as a unique clinical subset of PBC. Functional analysis of the antigens found in the current study may help explain this phenomenon.

Clustering analysis revealed that most of the autoantigens in AMA-negative PBC patients participate in the biological processes of HiBECs, including recognition of phagocytosis, positive regulation of B-cell activation, and complement activation. Epithelial cells are capable of phagocytosis and antigen presentation in the immune response system[26]. The destruction of phagocytosis recognition by the immune response might lead to exposure of more antigens from HiBECs. Second, a pathology study reported that AMA-negative PBC patients exhibit a significant decrease in the number of B-cell clusters infiltrating the bile ductal regions in the early stages of bile duct damage[27]. Interestingly, there were autoantibody responses to proteins that participated in the positive regulation of B-cell activation among AMA-negative PBC patients and that might be the cause of decreased B-cell infiltration. Last but not least, epithelial cells can secrete interleukins (*e.g.*, IL-8) to suppress inflammation[27]. If these cytokines are not activated (disturbed by an autoimmune response per the results of our study, for example), the inflammation in PBC patients will persist. In some way, the immune responses caused by the autoantigens we found may lead to dysfunction of the related pathways, which might result in the development of AMA-negative PBC, the unique clinical subtype.

This study had some limitations that should be addressed in future studies to confirm the interesting phenomena observed in this pilot study. First, a larger sample size should be analyzed. Second, simultaneous analysis of controls, a healthy cohort, and a cholestatic cohort would allow a better interpretation of the results. Last but not least, the significantly differentially expressed candidate autoantibodies identified in this study should be verified, for example, by enzyme-linked immunosorbent assay or Western blot analysis. Despite these limitations, this study provides important information for the ongoing discussion on the existence and pathogenesis of AMA-negative PBC.

In summary, the results of the current study are consistent with those in the current literature, which confirmed the feasibility and reliability of the design and technology we applied. This pilot study exploring PBC-related autoantigens demonstrated that the dysfunction of three pathways, recognition of phagocytosis, positive regulation of B-cell activation, and complement activation, in HiBECs might be causative in the pathogenesis of AMA-negative PBC. More interestingly, the existence of AMA-negative PBC was illustrated based on bioinformatics analysis. These data prompt us to launch an in-depth study using a larger sample size, including a control cohort, to verify the candidate autoantigens.

ARTICLE HIGHLIGHTS

Research background

Currently, the pathogenesis of primary biliary cholangitis (PBC) is still unclear. The appearance and fluctuation of autoantibodies not only represent the disease status but also further our understanding of the pathogenesis of the disease. The specific diagnostic biomarker of PBC, anti-mitochondrial antibody (AMA), was confirmed to participate in injuring intrahepatic biliary epithelial cells (iBECs) and perpetuating the destructive process. Exploring novel autoantibodies for PBC will help unravel the underlying mechanism of pathogenesis.

Research motivation

AMA cannot be detected in 10% of PBC cases, which prompted us to determine whether other pathogenesis-associated and AMA-like autoantibodies exist in AMA-negative PBC patients. This may aid in decrypting the pathogenesis of PBC.

Research objectives

We sought to carry out an exploratory study using a novel proteomics approach that combines the enrichment of antigens from human BECs (HiBECs), label-free mass spectrometry, and bioinformatics to identify candidate autoantibodies for AMA-negative PBC. In this pilot study, we aimed to ascertain the feasibility of this experimental approach and obtain preliminary results that will guide future larger-scale and in-depth studies. In addition, the novel experimental roadmap established here can be applied in related studies in future.

Research methods

Eighteen PBC patients were recruited from the Peking Union Medical College Hospital. They included nine AMA-positive and nine AMA-negative PBC cases, with pairs matched for age and sex. Sera were collected from patients and frozen at -80℃ until use. Human iBECs (HiBECs) were subcultured and lysed to provide sufficient target antigens for candidate autoantibodies enriched from the sera of PBC patients. Cell lysates and enriched autoantibodies were mixed and incubated under various conditions. Candidate autoantigens were eluted and subsequently identified by label-free mass spectrometry. Student's *t*-test was performed for comparison of differences between AMA-positive and -negative

proteins, with $P < 0.05$ set as the significance cut-off. Enrichment analyses were conducted for the Gene Ontology (GO) biological processes (GOBP), cellular components (GOCC), and molecular functions (GOMF) categories. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways were analyzed through the DAVID platform, using the complete UniProt human protein database as a background and an FDR < 0.05 as a cut-off. Heatmap analyses and visualization of significantly different proteins were conducted by complete linkage hierarchical clustering. Significantly differentially expressed proteins were used as the input for STRING analysis and a network was built based on high-confidence (0.8) evidence of experimental protein-protein interactions provided by the STRING database. The network was visualized using Cytoscape v.3.0. This is the first time that enrichment of antigens from HiBECs, label-free mass spectrometry, and bioinformatics were combined to explore candidate pathogenesis-associated autoantibodies for AMA-negative PBC.

Research results

In total, 1081 autoantigen candidates were identified from all PBC patients. Among these, 371 were significantly differentially expressed between AMA-positive and -negative groups. Fisher's exact test for the enrichment of GO protein annotations in the set of significantly different proteins revealed a range of protein categories (FDR < 0.05). As expected, the mitochondria-related biological process term was highly enriched. In addition, proteins that participate in the positive regulation of B-cell activation and phagocytic recognition and engulfment were highly enriched. Clustering analysis was applied to examine the rationality and accuracy of the selected proteins. Cluster 1 represents significantly upregulated proteins in AMA-negative PBC patients. They mainly participate in positive regulation of B-cell activation, recognition of phagocytosis, and complement activation. Cluster 2 represents significantly downregulated proteins in AMA-negative PBC patients that are upregulated in AMA-positive PBC patients. These downregulated proteins mainly participate in electron transport and cellular metabolic processes, which are mostly restricted to mitochondria.

Research conclusions

This pilot study exploring PBC-related autoantigens demonstrated that the dysfunction of three pathways in HiBECs might be causative in the pathogenesis of AMA-negative PBC, including phagocytosis recognition, positive regulation of B-cell activation, and complement activation. More interestingly, the controversy of the existence of AMA-negative PBC was illustrated based on bioinformatics analysis. AMA cannot be detected in sera of PBC patients because the method applied in clinical practice is not sensitive enough. However, AMA-negative PBC might exist as a unique clinical subset of PBC. This is the first study to combine enrichment of antigens from HiBECs, label-free mass spectrometry, and bioinformatics to explore candidate autoantibodies for AMA-negative PBC. Of note, the results of the current pilot study are consistent with those in research publications, confirming the feasibility and reliability of the design and technology we applied.

Research perspectives

The existence of serum autoantibody-negative autoimmune disease can be verified using proteomic technology, including enrichment of antigens from the HiBECs, label-free mass spectrometry, and bioinformatics. For future studies, the design needs to be optimized, including a larger sample size containing healthy and cholestatic cohorts, and the candidate autoantigens should be verified by Western blot analysis or enzyme-linked immunosorbent assay.

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ORIGINAL ARTICLE

Basic Study

Influence of TBX21 T-1993C variant on autoimmune hepatitis development by Yin-Yang 1 binding

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Abstract

AIM

To investigated the mechanism of the association between the TBX21 T-1993C promoter polymorphism and autoimmune hepatitis type 1 (AIH-1) development.

METHODS

In vivo, *In vivo*, and reporter analyses were performed to determine the function of transcription factors binding to the T-1993C element of the TBX21 promoter in human $CD4^+$ T and B cell lines. Flow cytometry and quantitative real-time PCR were used to analyze T-box transcription factor (T-bet) and interferon-γ (IFN-γ) expressions in $CD4^+$ T cells, B cells and monocytes from the peripheral blood of AIH-1 patients including 5-1993TC and 15-1993TT genotype carriers, and healthy controls including 10-1993TC and 25-1993TT genotype carriers. Furthermore, a range of biochemical indices was measured simultaneously in the blood of AIH-1 patients.

RESULTS

TBX21-1993C allele created a strong Yin-Yang 1 (YY1)-binding site and decreased transcriptional activity of TBX21 promoter in human $CD4^+$ T and B cells.

Higher levels of T-bet and IFN-γ were detected in the circulating $CD4^+$ T cells and B cells of AIH-1 patients carrying the TBX21-1993 TT genotype compared with the patients carrying the -1993 TC genotype and controls with the -1993 TC genotype. T-bet expression levels of circulating T cells and B cells were positively correlated with AIH-1 disease activity. Knockdown of YY1 with siRNA caused increased expression of T-bet and IFN-γ in peripheral blood mononuclear cells in AIH-1 patients.

CONCLUSION

The repression of TBX21 expression by high-affinity binding of YY1 to the -1993C allele may contribute to a decreased development of AIH-1 *via* suppression of type 1 immunity.

Key words: TBX21; Single nucleotide polymorphism; Yin-Yang 1; T helper cells; B cells; Autoimmune hepatitis

Core tip: The -1993C allele in the *TBX21* gene (encoding T-bet) promoter has been shown to associated with protection against autoimmune hepatitis type 1 (AIH-1), but the underlying mechanisms are unknown. We found that the TBX21-1993C allele created a strong Yin-Yang 1 (YY1)-binding site and decreased T-bet expression. Reduced T-bet and IFN-γ expression of circulating $CD4^+$ T cells and B cells existed in the individuals carrying the -1993C allele compared with those without the -1993C allele and played a protective role in AIH-1 development. The repression of T-bet expression by high-affinity binding of YY1 to the -1993C allele may contribute to a decreased development of AIH-1 *via* the suppression of type 1 immunity.

Sun W, Wu HY, Chen S. Influence of TBX21 T-1993C variant on autoimmune hepatitis development by Yin-Yang 1 binding. *World J Gastroenterol* 2017; 23(48): 8500-8511 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i48/8500.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i48.8500

INTRODUCTION

Autoimmune hepatitis (AIH) is a progressive inflammatory liver disorder characterized by hypergammaglobulinemia, circulating autoantibodies, and histological evidence of a florid mononuclear cell infiltration, which is referred to as interface hepatitis[1,2]. AIH type 1 (AIH-1) is distinguished by the presence of circulating antinuclear antibodies (ANAs) and/or smooth muscle antibodies (SMAs), and is the predominant form of AIH in adults. Although genetic factors, such as human leucocyte antigen HLA-DR3 and HLA-DR4 are associated with the development of AIH, Other factors contributing to the aetiology and the pathogenic process of AIH remains unclear. While most patients with AIH respond well to immunosuppressive therapy, about 10%-20% of the cases still progress to cirrhosis and require the liver transplantation[3,4]. Therefore, It is important to understand the pathogenesis of AIH in the management of patients with AIH.

Although the pathogenic initiating events are unknown, AIH-1 is believed to be mediated by CD4 T cells that recognize one or more liver-specific self antigenic peptides, and histological evidences show the presence of interferon-γ (IFN-γ)-producing T helper type 1 (Th1) cells in liver[5,6]. These imply the importance of Th1-like immune response in the pathogenesis of AIH. A recent study suggests that B cells also contribute to the development and progression of AIH[7]. Activated B cells can differentiate into $CD38^+$ plasma cells that secrete antibodies, such as ANA and SMA[8,9]. B effector 1 cells (Be1) can function as antigen presenting cells to present antigen determinants to trigger T-cell activation and secrete IFN-γ to regulate autoimmunity[10,11]. The T-box transcription factor, T-bet, is required for IFN-γ production and the generation of type 1 immunity[12]. Initial studies demonstrated that T-bet is expressed in developing the Th1 cells and mediates IFN-γ production, Th1 differentiation, and repression of the alternative Th2 program[13]. Recent studies have highlighted the importance of T-bet in other cellular subtypes implicated in the type 1 immune response, such as dendritic cells, natural killer (NK) cells, natural killer T (NKT) cells, and $CD8^+$ T cells. T-bet has also been shown to regulate IFN-γ production by Be1 cells[14,15]. The high level of T-bet production which is associated with increased numbers of IFN-γ-producing B cells have been identified in the blood and lymphoid tissues of mice and humans with autoimmune disorders, such as systemic lupus erythematosus (SLE) and rheumatoid arthritis[10,11]. Furthermore, high T-bet production is associated with the pathogenesis of T-cell-mediated liver injury in conA murine model[16]. T-bet-deficient mice immunized with myelin oligodendrocyte glycoprotein were resistant to the development of experimental autoimmune encephalomyelitis[17]. In a murine lupus model, the absence of T-bet strikingly reduces B cell-dependent manifestations, including autoantibody production, hypergammaglobulinemia, and immune-complex renal disease, and abrogates IFN-γ-mediated IgG2a production[18]. Although the role of T-bet has been studied in a variety of autoimmune diseases in mice, its relative importance in human AIH is not characterized.

Genetic variations in the nucleotide sequences of promoters can contribute to the altered expression of genes in complex inherited diseases[19,20]. In this regard, a genetically induced increase in T-bet production may result in the expansion of either polarized Th1 cells to promote the type 1 immune response or the Be1 cell compartment to gain a greatly enhanced T cell activation capability. Three single nucleotide polymorphisms (SNPs) at -1993(C/T), -1514(C/T), and

Table 1 The demographic and clinical characteristics of subjects

Parameters	AIH	HC
No	20	35
Age (yr)	47.2 ± 11.8	46.9 ± 9.9
Patient age at diagnosis(yr)	44.5 ± 11.8	-
Gender: female/male	16/4	27/8
IAIHG score	17.15 ± 2.23	-
ALT (< 40 IU/L)	301.7 ± 284.7[b]	21.9± 9.5
AST (< 40 IU/L)	306 ± 224.4[b]	21.8 ± 4.9
ALP (< 114 IU/L)	190 ± 130.4[b]	69 ± 21
Total Bilirubin (6-21 μmol/L)	112.5 ± 116.1[b]	13.6 ± 3.7
Albumin (38-51 g/L)	38.2 ± 6.9[a]	45.2 ± 8.4
IgG (7-16 g/L)	24.1 ± 11.8[b]	8.4 ± 2.8
Ant-ANA (+) (≥ 1:100)	15 (75)	-
Ant-SMA (+) (≥ 1:100)	3 (15)	-
Ant-ANA (+) + Ant-SMA(+)	2 (10)	-
Ant-LKM-1 (+) (≥ 1:40)	0	-
Cirrhosis at presentation	7 (35)	-

Data are expressed as *n* (%) or mean ± SD. [a]$P < 0.05$, [b]$P < 0.01$ *vs* HC. AIH: Autoimmune hepatitis; IAIHG: International autoimmune hepatitis group; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; ALP: Alkaline phosphate; IgG: Immunoglobulin G; Ant-ANA: anti-nuclear antibody; ant-SMA: Anti-smooth muscle antibody; HC: Healthy control; AIH: Autoimmune Hepatitis; –: Not available.

-999(G/A) sites of the *TBX21* gene (encoding T-bet) promoter have been identified[21]. We have previously found that the T-1993C variant, which increased the transcription factor YY1 binding to the TBX21 promoter in Jurkat T cell lines, subsequently caused lower transactivation of the promoter[22]. Furthermore, our case-control study (84 cases and 318 control subjects) demonstrated that TBX21 T-1993C SNP was associated with susceptibility to AIH-1[23]. Individuals carrying the -1993C allele had a decreased risk to AIH-1 compared with those without the -1993C allele (OR = 0.22; 95%CI: 0.09-0.56; $P = 0.0016$). Recently, the TBX21 T-1993C variant has been shown to be associated with a significant decrease in T-bet expression and IFN-γ production by stimulated peripheral blood $CD4^+$ T cells from healthy participants[24].

In the present study, we further determined the characterization of transcription factor binding to the T-1993C SNP site of the TBX21 promoter in CD4+ T and B cell lines. In addition, we measured the expression of T-bet and IFN-γ in peripheral blood $CD4^+$ T cells and B cells from active AIH-1 patients carrying -1993TC and -1993TT genotypes, compared with healthy controls carrying -1993TC and -1993TT genotypes, in an attempt to explore the mechanism of the association between the TBX21 T-1993C promoter polymorphism and AIH-1 development.

MATERIALS AND METHODS

Subjects

A total of 20 patients with new onset were enrolled in this study at Southwest Hospital (Chongqing, China). Of these 20 patients, 5 were carriers of the -1993TC genotype and 15 were carriers of the -1993TT genotype. All the patients fulfilled the criteria for AIH according to the 1999 revised scoring system of the International Autoimmune Hepatitis Group (IAIHG)[25] and were therefore found to be eligible for the study. Only pretreatment scores were analyzed. A definite diagnosis of AIH based on the IAIHG criteria requires a pretreatment score exceeding 15. Patients were excluded from the study if there was histological evidence of cholangitis or non-alcoholic steatohepatitis. In addition, patients who were positive for hepatitis B virus (HBV)-surface antigen (HBsAg) or hepatitis C virus (HCV)-RNA were also excluded. Patients with other causes of liver disease, such as excess alcohol or drug use, or those who had received immune-suppressive therapies or glucocorticoid therapies within the past 6 mo were excluded based on reviews of their appropriate history and investigations. The control population comprised 35 healthy Chinese Han adults, who were volunteer blood donors from Chongqing, China. They were matched with type 1 AIH patients for age, gender, and TBX21 genotyping (10-1993TC and 25-1993TT genotype carriers), and had no history of any chronic inflammatory disease. All subjects provided written consent to participate in the present study. This study was approved by the Ethics Committee of Southwest Hospital, Chongqing. The demographic and clinical characteristics of these subjects are shown in Table 1.

Clinical examination

The clinical data of each subject were collected from the hospital records. These data included age, sex and age at diagnosis, time of onset of symptoms or other evidence of liver disease, markers of infection with hepatitis viruses HBV and HCV, alcohol intake, coexisting autoimmune diseases, serum levels of alanine transaminase (ALT), aspartate aminotransferase (AST), alkaline phosphatase and bilirubin, and full blood cell counts. Autoantibodies were determined by indirect immunofluorescence on murine tissue sections (EuroImmun Medizinische Labordiagnostika AG, Lübeck, Germany), and a serum titer of 1:100 or greater was considered positive for all antibodies. All patients underwent liver biopsies to evaluate disease activity at the time of entry. The histological diagnosis of cirrhosis required a loss of the normal lobular architecture, reconstruction of hepatic nodules and presence of regenerative nodules.

Cell culture and peripheral blood mononuclear cell isolation

Jurkat cells ($CD4^+$ human T lymphoblasts) and Raji cells (human B lymphocytes) were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum, 2 mmol/L glutamine, 100 U/mL penicillin and 50 mg/mL streptomycin as described previously[23].

Human peripheral blood mononuclear cell (PBMC) were obtained by Ficoll-Paque density centrifugation (Amerhsam Pharmacia, Uppsala, Sweden) of whole blood from AIH-1 patients.

Electrophoretic mobility shift assay

The electrophoretic mobility shift assay (EMSA) was performed by the method described previously[22]. The synthesized oligonucleotides (5′-TACGGAGAAA TGG(T)GGGTAAGGTGTTG-3′ corresponding to the -2006/-1980 region of the human TBX21 gene with -1993T) and 5′-TACGGAGAAATGG(C)GGGTAAGGT GTTG-3′, -1993C), labeled with biotin at the 5′-end, were annealed to the complementary oligonucleotide labeled with biotin at the 5′-end. Nuclear extracts were prepared from Jurkat and Raji cells as described previously. For the competition assay, non-labeled double stranded DNA (dsDNA) fragments consisting of the following sequences were used: YY1 5′-CGCTCCCCGGCCATCTTGGCGGCTGGT-3′, and the sequences containing --1993T or -1993C. Supershifts were performed with antibodies to YY1, C/EBPβ (N-10) and c-Jun (N) that were purchased from Santa Cruz Biotechnology. All EMSA experiments were repeated more than three times, and a representative result for each experiment is shown.

Chromatin immunoprecipitation

The chromatin immunoprecipitation (ChIP) assay was performed using the ChIP-IT Express kit (Active Motif, Carlsbad, CA, United States) as described previously[22]. The following antibodies were used: a rabbit anti-YY1 (C-20; Santa Cruz Biotechnology), anti-C-Jun (N; Santa Cruz Biotechnology), ant-C/EBPβ (Santa Cruz Biotechnology) and a nonspecific rabbit IgG (Santa Cruz Biotechnology). PCR was performed with the following primer, which were designed to amplify the -2048 to -1912 region of the TBX21 promoter: 5′-CCCACTTTGAACATCAGGCAGA-3′ and 5′-CAGCACATCTTTTGAGGTTGGAG-3′.

Plasmid constructs

A reporter plasmid carrying luciferase gene under the control of a 2.2-kb TBX21 promoter fragment carrying the -1993T or -1993C allele was used as described previously[22]. The pCMV-YY1 vector expressing full-size YY1 and its control pCMV6-XL5 were obtained from OriGene.

Cell transfection and luciferase assay

Harvested cells were suspended in each culture medium supplemented with additional 10% fetal calf serum (FCS). Jurkat cells and Raji cells (2×10^5 cells in 0.5 mL) were cotransfected with 5 μg of the test construct and 25 ng of the pRL-TK Renilla reporter plasmid (Promega) using Nucleofector Kit V(LONZA) according to the manufacturer's protocol. On the coexpression analysis, 3 μg of pCMV-YY1 or control pCMV6-XL5 was added into the cell suspension. The luciferase activity was measured, as described previously[22].

siRNA-mediated suppression of YY1 expression in the PBMCs of AIH-1 patients

The isolated PBMCs were plated at a density of 10^6 cells/ml as described above. siRNA targeted against YY1 (sc-36864) and siRNA consisting of a scrambled sequence that would not lead to specific degradation of any cellular message (control) were purchased from Santa Cruz Biotechnology. The cells in 1 mL of medium were incubated in 5 mmol/L YY1-siRNA in the Nucleofector transfection reagent at room temperature with gentle agitation. Two hours after transfection, fresh medium was added to provide the cells with sufficient nutrients, and the transfected cells were harvested after 48 h for RNA extraction.

RNA isolation and real-time PCR analysis of the mRNA expression levels of YY1, T-bet and IFN-γ

The Trizol-purified (Sangon, China) total RNA (2 μg) from PBMCs was used for cDNA synthesis with the RevertAidTM First Strand cDNA Synthesis Kit (Fermentas, Canada) following the manufacturer's instructions. The mRNA expression levels of IFN-γ and of nuclear factors such as YY1 and T-bet were quantified by real-time PCR, and the level of β-actin mRNA was also detected as an internal control for each sample. The relative mRNA expression levels of genes were determined using the comparative (2-ΔΔCt) method. The following primer sequences were used for this analysis: IFN-γ 5′-TCAGATGTAGCGGATAATGGAAC-3′ and 5′-TTCCTTGATGGTCTCCACACTC-3′; T-bet 5′-CCCACTTTGAACATCAGGCAGA-3′ and 5′-CAGCACATCTTTTGAGGTTGGAG-3′; 5′-YY1 GGATAACTCGGCCATGAGAA-3′ and 5′-ATAGGGCCTGTCTCCGGTAT-3′.

T-bet and IFNγ expression by flow cytometry

The experiments were performed on whole blood using the BD FastImmune CD4 Intracellular Cytokine Detection Kit (cat# 340970, BDIS, San Jose, CA, United States) according to the manufacturer's instructions. In order to identify Th1 and B effector cells, 0.5 mL heparinized whole blood was stimulated with 2.5 μg/mL PMA and either the anti-CD28/ant-CD49d co-stimulatory monoclonal antibodies (final concentration of 1 μg/mL) or IFN-α2b (2000 U/mL, Schering-Plough, United States)[26]. For the five-color analysis of the $CD4^+$ and $CD20^+$ cytokine responses, the staining antibody cocktail consisted of anti-IFNγ-FITC/anti-CD4-PerCP(PE)-Cy5.5/anti-CD69-PE/anti-T-bet-Alexa-Fluor 647 (eBioscience, San Diego, United States) and anti-IFNγ-Cy5.5/anti-CD27-PE/anti-CD20-PE- Cy5.5/anti-T-bet-APC (eBioscience). Isotype-matched control antibodies were included to detect non-specific binding to the cells. Each analysis was

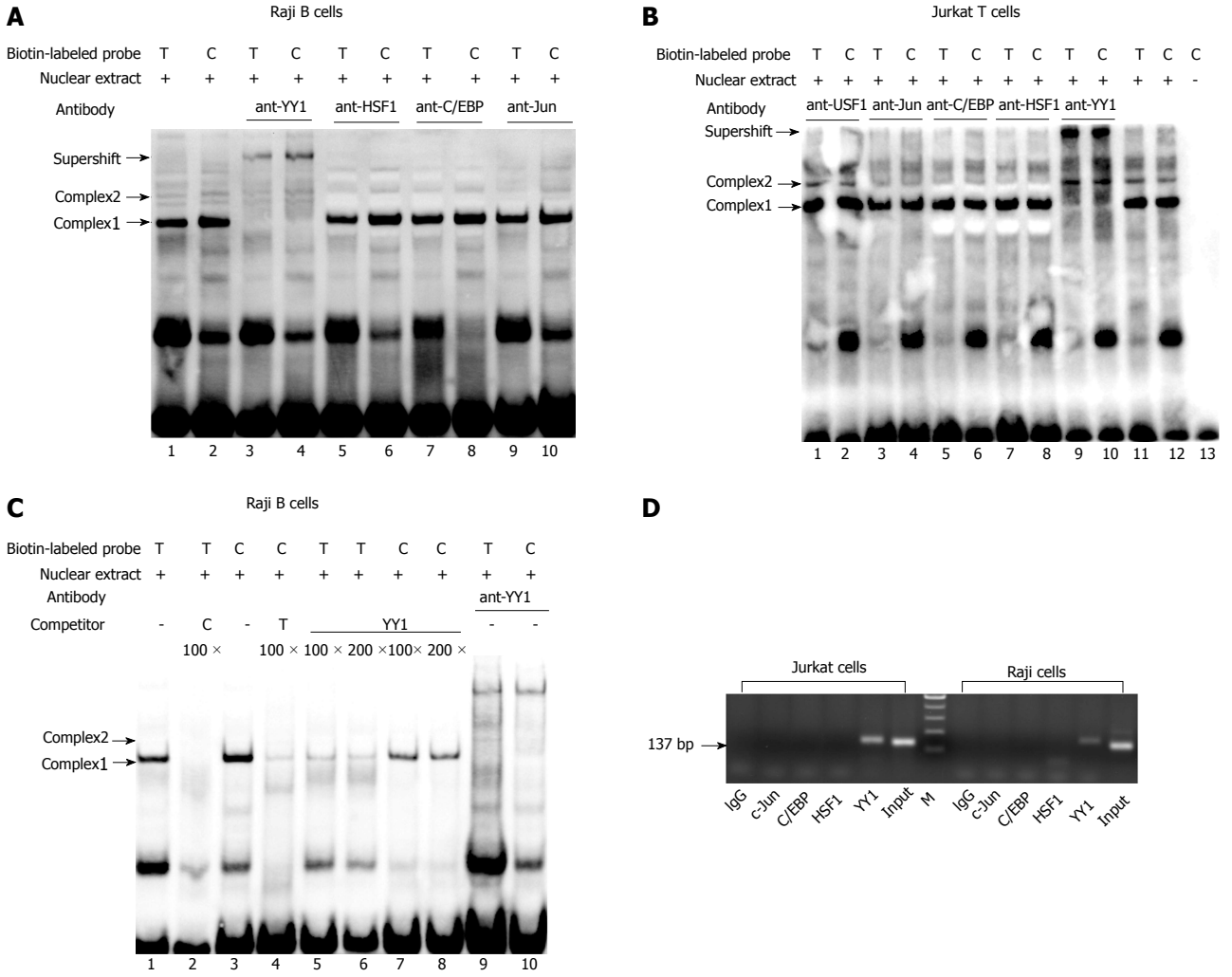

Figure 1 Identification of transcription factor binding to the T-1993C SNP site of TBX21 promoter in vitro and *in vivo*. A, B: EMSA analysis with biotin-labeled probes carrying the -1993T and -1993C allele and with nuclear extract from Jurkat cells and Raji cells was performed in the presence of anti-YY1 antibody; C: EMSA with biotin-labeled probes and with nuclear extract from Raji cells was performed in the presence of 100-200-fold excess of unlabeled self-oligonucleotide or YY1 probe; D: In vivo binding of YY1 to the T-1993C SNP site of the TBX21 promoter. ChIP assays with an anti-YY1, anti-C/EBPβ, ant-C-Jun, or control antibody (rabbit IgG) were performed on Jurkat cells or Raji cells. Input DNA or Immunoprecipitated DNA was used as template for PCR amplification of a 137-bp amplicon encompassing TBX21-1993. EMSA: Electrophoretic mobility shift assay; ChIP: Chromatin immunoprecipitation.

performed using at least 50000 cells that were gated in the region of the lymphocyte population, as determined by light-scatter properties (forwardscatter versus side-scatter). To analyze the expression of IFN-γ and T-bet in lymphocytes ($CD4^+$ T cells and $CD20^+$ B cells), cells were gated in both the lymphocyte and $CD4^+/CD20^+$ regions. The dot plots display the events as cytokine+ versus $CD69^+$ T or $CD27^+$ B cells. The specific responses of the lymphocytes to stimuli were obtained by subtracting the percentage of positive events in the unstimulated sample from that in the activated samples. The isotype-corrected responses of the unstimulated samples were subtracted from those of the activated samples.

Statistical analysis

Data are expressed as mean ± SD. All statistical analyses were performed in SPSS 19.0 software. The difference between the groups was analyzed by parametric Student's *t*-test or nonparametric Mann-Whitney *U* test. Correlations were examined by Pearson correlation. P values < 0.05 were considered significant.

RESULTS

General information

To determine the potential role of CD4+ T and B cells in the pathogenesis of AIH, 20 patients with active AIH and 35 gender- and age-matched healthy controls were recruited. There was no significant difference in the distribution of age and gender between the patients and healthy controls (Table 1). As expected, the concentrations of serum ALT and AST and total bilirubin in the patients were significantly higher than that in the healthy controls. Furthermore, 15 of 20 patients with active AIH were positive for anti-ANA antibodies, three were positive for anti-SMA

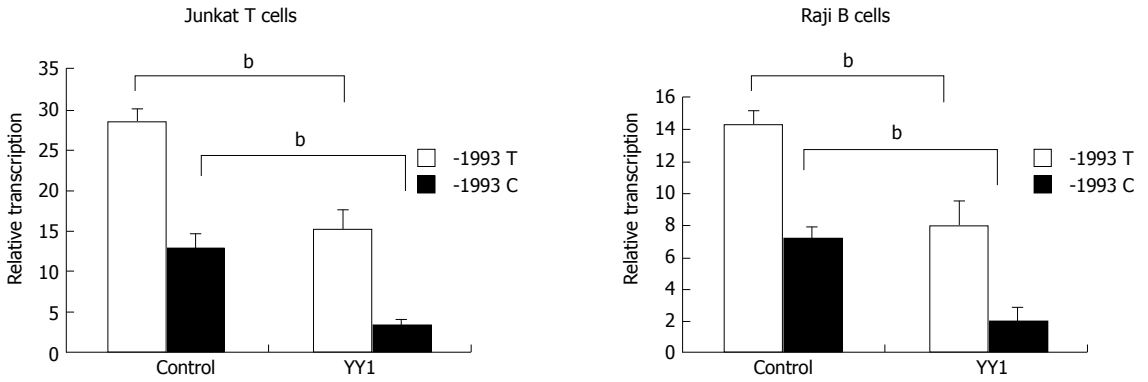

Figure 2 Effect of YY1 transcription factor on transcriptional activity of TBX21-1993T and -1993C promoter constructs. Luciferase reporter assay showing relative luciferase activity of the -1993T and -1993C promoter constructs following co-transfection of Raji cells or of Jurkat cells with pCMV-YY1 or pCMV6-XL5 (control). The results are expressed as mean ± SD for three independent experiments; $^{b}P < 0.01$.

antibodies, and two were positive for both. In addition, abnormally higher levels of serum IgG were detected in the patients, demonstrating the liver damage and hypergammaglobulinaemia in the AIH-1 patients.

Binding profile of transcription factor to the T-1993C polymorphism site in CD4$^+$ T and B cells

The results of EMSA showed the formation of two specific DNA-protein complexes (1 and 2) for both -1993T and -1993C allelic probes (Figure 1A and B). The addition of antibody against YY1 led to abrogation of only complex 2 in both the -1993T and -1993C probes (Figure 1A, lanes 3 and 4; Figure 1B, lanes 9 and 10), suggesting that YY1 participates in complex 2 formation. AP1, HSF1 and C/EBPβ did not seem to be involved in the formation of complexes 1 and 2 (Figure 1A, lanes 5-10; Figure 1B, lanes 3-8). A competition assay was performed to confirm whether the -1993C probe interacted more efficiently with the YY1 protein compared with the -1993T. It was found that the complexes formed with the -1993C probe were consistently more intense than these with -1993T (Figure 1C), which was consistent with the results of our study using Jurkat T cells[22]. In comparison with complex 1, we also observed that complex 2 required higher concentrations of competitors to diminish its band intensity. These data indicate that the complexes contain sequence-specific DNA-binding proteins and that the T-1993C SNP resides in a functionally important nucleotide, and is perhaps more relevant for complex 2.

ChIP assay was performed to further determine if YY1 binds to the T-1993C element of the TBX21 promoter *in vivo.* PCR amplification with the primers specific to the TBX21 promoter region, wherein the YY1-binding sites exist at the T-1993C locus, demonstrated a 137-bp product in both Jurkat and Raji chromatin after immunoprecipitation with the anti-YY1 antibody, but not with the ant-C/EBPβ and ant-C-Jun antibodies, or rabbit IgG (Figure 1D). These results demonstrate the direct interaction of YY1 with the TBX21 promoter in a non-cell-specific manner.

Transcriptional effect of YY1 at the T-1993C polymorphism site on the TBX21 promoter in CD4$^+$ T and B cells

Luciferase reporter assay was performed to study transcriptional regulation of the TBX21 promoter constructs carrying either the -1993T or -1993C allele in the Jurkat T cell and Raji B cell lines. The luciferase activity derived from the -1993T promoter was significantly higher than that from the -1993C promoter in both cell lines ($P < 0.01$) (Figure 2). These data indicate that TBX21-1993C is significantly less active than the -1993T allele in a non-cell-specific manner. We further examined whether the transfection of a YY1 expression vector induced changes in the promoter activity of the -1993T and -1993C constructs in both Jurkat T cells and Raji B cells. We found that the luciferase activity derived from the -1993T promoter was 4-fold lower in the presence of overproduced YY1, and that from the -1993C promoter decreased only by approximately 2-fold ($P < 0.01$) (Figure 2). These results demonstrated that the YY1 motif upstream of the T-1993C polymorphism plays a strong negative regulatory role in the activity of the TBX21 promoter, and the -1993C promoter responds to YY1 better than the -1993T promoter.

Association of IFN-γ and T-bet expression of circulating CD4$^+$ T cells and B cells with AIH-1 activity

Flow cytometric analysis revealed that the T-bet expression of circulating CD4$^+$ T cells and CD20$^+$ B cells in active AIH-1 patients was significantly greater than that in controls ($Z = -3.92$, $P < 0.01$, $Z = 2.43$, $P < 0.05$,) (Figure 3A and C). The levels of IFN-γ production in circulating CD4$^+$ T cells and CD20+ B cells from active AIH-1 patients were significantly higher than that from controls ($Z = -4.89$, $P < 0.01$; $Z = 2206$, $P < 0.05$) (Figure 3B and D). These data indicate an involvement of Th1 inflammation in Chinese patients with active AIH-1. The disease activity, represented by the IAIHG score, was positively correlated with T-bet expression in CD4$^+$ T cells and

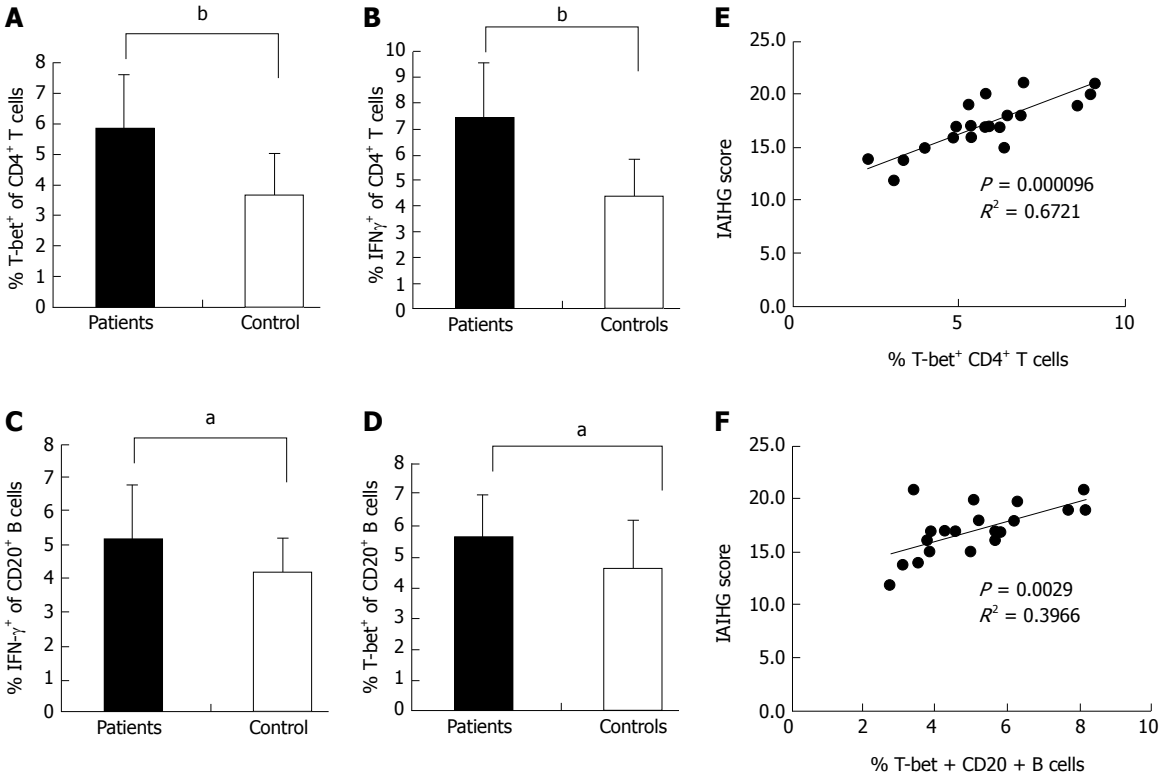

Figure 3 Expression of T-bet and IFN-γ in the peripheral blood of AIH-1 patients. A-D: Flow cytometry showing the percentage of cells positive for T-bet and IFN-γ expression among the stimulated $CD4^+$ T cells and $CD20^+$ B cells from the peripheral bloods of 20 active AIH-1 patients and 35 healthy subjects; E-F: The correlation between the percentage of T-bet-expressing B cells or T cells and the IAIHG score in active AIH-1 patients. IAIHG, the International Autoimmune Hepatitis Group. $^aP < 0.05$, $^bP < 0.01$ *vs* controls.

CD20+ B cells from active AIH-1 patients (Figure 3E and F). Together, these results suggest an association between AIH-1 development and T-bet expression of circulating T cells or B cells.

T-bet and IFN-γ expression of circulating $CD4^+$ T and B cells in -1993TT homozygous and -1993TC heterozygous patients with active AIH-1

Flow cytometry was performed to analyze T-bet expression and IFN-γ production in circulating T cells and B cells from AIH-1 patients carrying -1993TC and -1993TT genotypes, and from healthy controls carrying -1993TC and -1993TT genotypes (Figure 4). The T-bet expression in $CD4^+$ T cells and CD20+ B cells was significantly higher in the -1993TT patients than in the -1993TC patients and the -1993TT controls (Z = -2.75, $P < 0.01$; Z = -4.33, $P < 0.01$; Z = -4.21, $P < 0.01$; Z = -2.75, $P < 0.05$ respectively) (Figure 5A-D). No significant difference in T-bet expression was observed between in the -1993TC patients and -1993TC controls in both $CD4^+$ T cells and $CD20^+$ B cells. The levels of IFN-γ production was significantly higher in $CD4^+$ T cells and $CD20^+$ B cells from the -1993TT patients than from the -1993TC patients and the -1993TT controls (Z = -4.16, $P < 0.01$; Z = -5.07, $P < 0.01$; Z = -3.274, $P < 0.01$; Z = -2.529, $P < 0.05$ respectively) (Figure 5A-D). No significant difference in IFN-γ production was observed between in the -1993TC patients and -1993TC controls. The results demonstrated that increased IFN-γ and T-bet expression in circulating T and B cells from AIH-1 patients might result primarily from the -1993TT homozygotous patents with AIH-1.

Effect of YY1 knockdown on T-bet and IFN-γ expression in the peripheral blood of AIH-1 patients

Knockdown of YY1 with siRNA was performed to analyze whether YY1 affects T-bet and IFN-γ expression of circulating $CD4^+$ T and B cells in AIH-1 patients. Real-time PCR assay showed that YY1 siRNA significantly lowered the YY1 mRNA level in PBMCs from AIH patients (t = 13.83, $P < 0.01$) (Figure 6). The levels of T-bet and IFN-γ mRNA were up-regulated in the YY1-siRNA PBMCs compared with that in the control-siRNA PBMCs (t = -7.61; t = 12.11, $P < 0.01$ respectively) (Figure 6). These results suggested that YY1 affects type 1 immunity of AIH-1 patients by T-bet-mediated regulation.

DISCUSSION

Alterations in T-cell function and cytokines have been demonstrated to play a central role in AIH[2,27]. In human, liver tissue injury in AIH is also mediated by $CD4^+$ and $CD8^+$ T cells[28]. T-bet plays a critical role in both the development and maintenance of Th1 cells, and has been proved to be a positive transcriptional

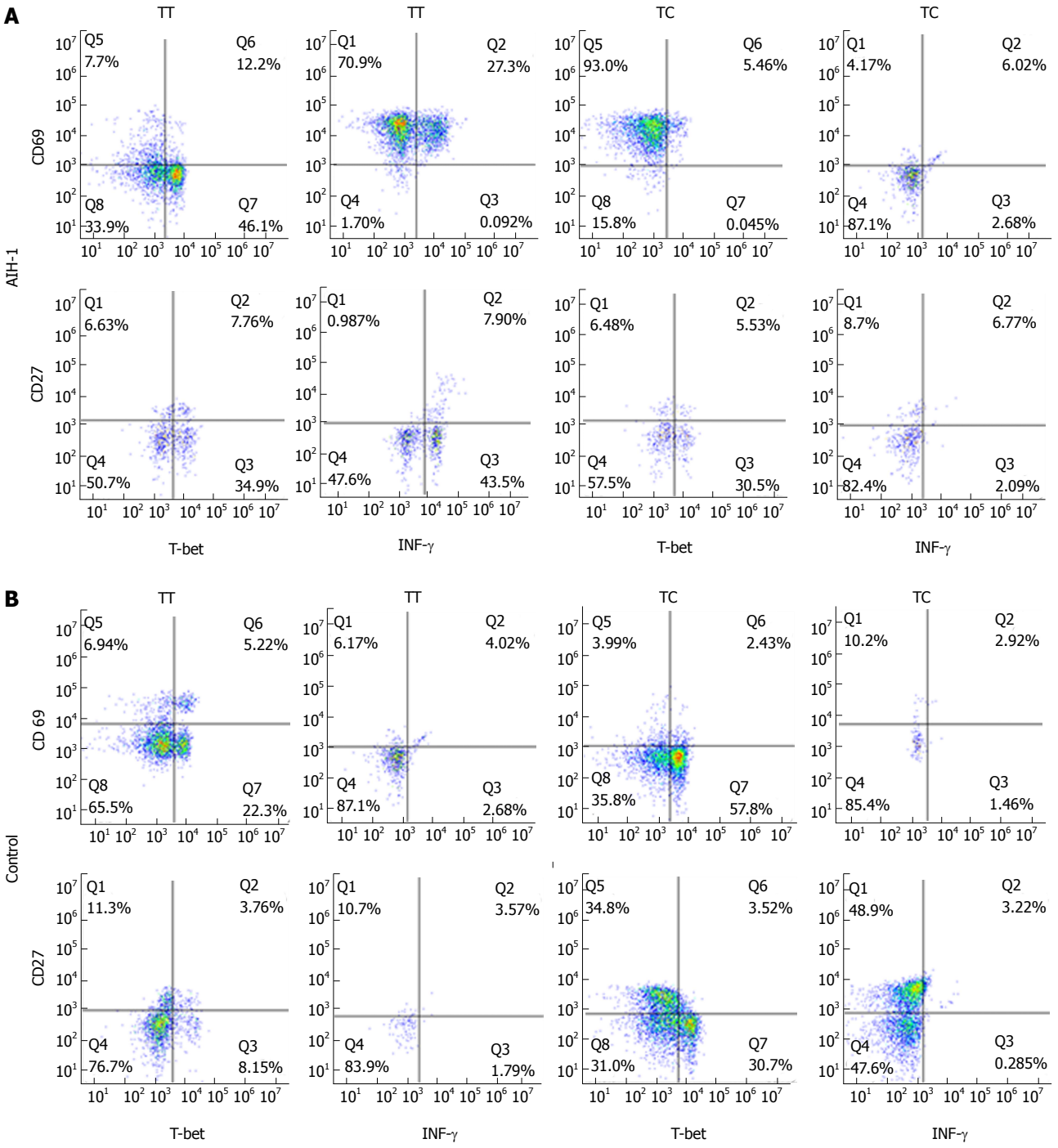

Figure 4 Representative two-parameter dot plots showing only the cells gated for CD4$^+$ T and CD20$^+$ B cells from the peripheral bloods of individuals carrying the TBX21-1993TC and -1993TT genotypes. The y-axis of each histogram represents the specific fluorescence of extracellular CD69-phycoerythrin (PE) (T lymphocytes) and CD27-PE (B lymphocytes). The x-axis represents the specific fluorescence of Alexa-Flour 647-T-bet, FITC-IFNγ, or Cy5.5-IFN-γ on four-decade logarithmic scales. The quadrants were assigned using appropriate isotype controls for each intra- and extracellular antibody. A: AIH-1 patients; B: Healthy controls.

regulator of IFN-γ production in CD4$^+$ T cells, CD8$^+$ T cells, and B effector cells[15]. Prominent T-bet-positive lymphocytes have been found in both the lobular and portal infiltrate of human AIH[29]. T-bet together with IFN-γ were highly expressed in the inflamed liver tissue of a mouse model of fatal AIH[30]. To date there are no data concerning the expression of T-bet in circulating lymphomononuclear cells from AIH patients. Previous studies have indicated that the Th1/Th2 balance in peripheral blood of AIH patients is shifted towards Th1 cytokines and circulating cytochrome P4502D6-specific CD4$^+$ T cells producing IFN-γ are present in AIH patients[31].

In this study, we first showed that active AIH-1 patients exhibited higher T-bet and IFN-γ expression in peripheral blood CD4$^+$ T cells than healthy controls. The increased expression of T-bet was associated with an increased production of IFN-γ by peripheral blood

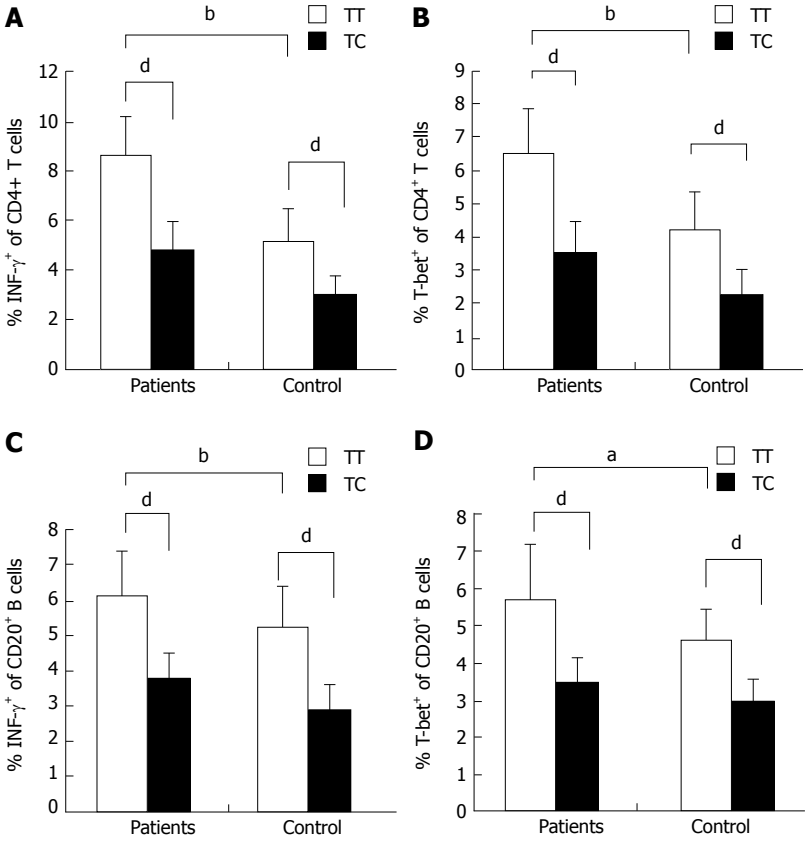

Figure 5 Expression of T-bet and IFN-γ in the peripheral blood of AIH-1 patients carrying -1993TC and -1993TT genotypes. A-D: Flow cytometry showing the percentage of cells positive for T-bet and IFN-γ expression among the stimulated $CD4^+$ T cells and $CD20^+$ B cells from the peripheral bloods of active AIH-1 patients including 5-1993TC and 15-1993TT genotype carriers, and healthy subjects including 10-1993TC and 25-1993TT genotype carriers. $^aP < 0.05$, $^bP < 0.01$, *vs* controls; $^dP < 0.01$, TT *vs* TC.

$CD4^+$ T cells, thus supporting the involvement of Th1 polarization in the pathogenesis of AIH-1. We found that the T-bet expression and IFN-γ production of circulating CD20+ B cells in AIH-1 patients increased significantly compared with that in the controls, which was consistent with other studies that demonstrated an elevation of T-bet and IFN-γ expression in circulating B cells from patients with other autoimmune diseases, such as coeliac disease and SLE[32,33]. Moreover, T-bet expression levels in peripheral blood T and B cells were directly correlated with the IAIHG score, suggesting an association between circulating T-bet-expressing T cells or B cells and disease activity. T-bet is expressed at low levels in naive B cells, but B cells in the presence of polarized Th1 cells and antigens were able to develop into high-IFN-γ-producing B cells that also express high levels of T-bet and ware capable of promoting the differentiation of naive T cells into Th1 effectors[14]. The cognate interactions that occur between T-bet-expressing and IFN-γ-producing T cells and B cells may result in the amplification of type 1 immune responses. Whether this mechanism contributes to AIH development requires further investigations.

Recent studies have shown that TBX21 T-1993C polymorphism is associated with susceptibility to autoimmune diseases including AIH[23,34]. To eliminate the possible effect of the T-1993C polymorphism on the regulation of the extensive activation of immune responses in AIH-1, we determined the expression of T-bet and production of IFN-γ in peripheral blood $CD4^+$ T cells and B cells from the -1993TC heterozygote and -1993TT homozygote AIH-1 patients, compared with -1993TC heterozygote and -1993TT homozygote controls. Increased T-bet and IFN-γ expression was detected in the activated $CD4^+$ T cells and B cells of AIH patients carrying the TBX21-1993 TT genotype compared with the patients carrying the -1993 TC genotype and controls with the -1993 TT genotype. The increased expression of T-bet correlated with an increased production of IFN-γ, demonstrating a higher biological effects of T-bet in Th1 and B cells from the patients with the TBX21-1993TT genotype. To verify whether the immunity of AIH patients is related to the regulation of TBX21 gene expression by YY1, we knocked down YY1 in PBMCs from AIH patients using siRNA. YY1 knockdown increased mRNA levels of T-bet and IFN-γ. This result highlights the increase of Th1 dominant response by repressing the production of YY1. These data suggested that the T-1993C variant in the TBX21 promoter, which represses T-bet expression and the size of the Th1 and B1 cell compartments by high-affinity binding of YY1 to the TBX21 promoter, may serve as a protective marker for AIH-1. To elucidate the relationship between the T-1993C polymorphism and

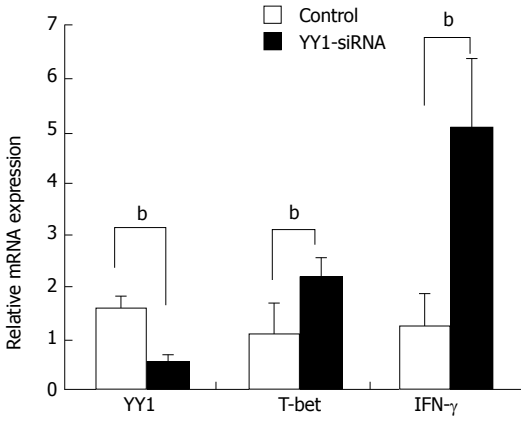

Figure 6 Effects of YY1 knockdown on T-bet and interferon-γ expression in peripheral blood mononuclear cells from autoimmune hepatitis type patients. PBMCs were isolated from 20 AIH-1 patients. PBMCs were transfected with YY1-siRNA or control-siRNA. Forty-eight hours after transfection, the mRNA expression levels of YY1, T-bet and IFN-γ were measured by real-time PCR. $^{b}P < 0.01$ *vs* control. PBMCs: Peripheral blood mononuclear cell.

the development of AIH, further detailed analysis of a large number of individuals including AIH patients carrying the CC genotype will be required.

YY1 is involved in repression and activation of several genes that play roles in various biological processes depending on the promoter sequence surrounding the YY1 binding site. Studies have shown YY1 positively regulated expression of several oncogenes, such as c-Myc and c-Fos[35]. In addition, YY1 can positively regulate some tumor suppressor genes, such as p21 and p16[36,37]. However, one study showed that YY1 was a negative cell growth regulator by inhibiting c-Myc function[38]. Interestingly, the promoter regions of genes that encode cytokines related to AIH, such as IL-6, IL-13 and IFN-γ, contain consensus YY1 binding sequences and some of them can be regulated by this transcription factor[30,39]. The transcriptional regulation of human TBX21 gene is complex, and several transcription factors such as SMAD4, SP-1, and NF-κB are involved[40,41]. One potential YY1 binding site has been previously identified in the -2211 to +1 region of the TBX21 promoter, wherein T/CCCATTTT was the critical base sequence for YY1 binding and the T-to-C mutation at the 5′ terminal of this sequence resulted in an increase of YY1 binding. These regions have also been associated with silencing of promoter activity in transient transfection assays of transformed cell lines. Our studies indicate that the silencing activity of this region of theTBX21 promoter is also operational in native T cells and B cells *in vivo* and is probably regulated by YY1. Thus, we propose that YY1 could be a crucial transcription factor for T-bet expression which play a critical role in the inflammation in AIH.

In summary, we showed that the TBX21-1993C allele created a strong YY1-binding site and decreased T-bet expression in $CD4^+$ T and B cells. Reduced T-bet and IFN-γ expression of circulating $CD4^+$ T cells and B cells existed in the individuals carrying the -1993C allele compared with those without the -1993C allele, and played a protective role in AIH-1 development. Furthermore, we propose a schematic model showing high-affinity binding of YY1 to the -1993C allele site leading to down-regulation of the TBX21 promoter activity, a mechanism for a promoter polymorphism affecting T-bet expression in AIH. We recognized that our study had limitations, such as a relative small sample size and the lack of functional study of T-bet-expressing T and B cells in the pathogenic process of AIH, as well as no information about T-bet-expressing T and B cells infiltrates in the liver. Therefore, further longitudinal studies are necessary to analyze the numbers and function of T-bet-expressing T and B cells in the pathogenic process of AIH -1 with a bigger population.

ARTICLE HIGHLIGHTS

Background background

Autoimmune hepatitis (AIH) is an autoimmune disease that involves aberrant B and T lymphocyte responses. T-box transcription factor (T-bet) is a key regulator for the lineage commitment in $CD4^+$ T hepler 1 and B effector 1 cells by activating the hallmark production of interferon-γ (IFN-γ). Although the role of T-bet has been studied in a variety of autoimmune diseases in mice, its relative importance in human AIH is not characterized. Detailed knowledge about T-bet-medicated immune responses will therefore enhance our understanding of the pathogenesis of AIH and might support the development of new immunomodulatory treatment approaches.

Research motivation

TBX21, which encodes T-bet, harbors many common polymorphisms at a strong linkage disequilibrium, and distinct TBX21 haplotypes are associated with autoimmune diseases in ethnically distinct populations. Our case-control study (84 cases and 318 control subjects) demonstrated a commone single nucleotide polymorphism (SNP) at the -1993 site of the TBX21 gene promoter that was associated with susceptibility to type 1 AIH (AIH-1) in a chinese population. Individuals carrying the -1993C allele had a decreased risk to AIH-1 compared with those without the -1993C allele (OR = 0.22; 95%CI: 0.09-0.56; $P = 0.0016$). Functional studies are necessary to dissect the mechanisms underlying the contribution of natural genetic variations to TBX21 dysregulation and the susceptibility to AIH-1 development.

Research objectives

In this study, the authors determined the characterization of transcription factor binding to the T-1993C SNP site of the TBX21 promoter in CD4+ T and B cell lines, and measured the expression of T-bet and IFN-γ in peripheral blood $CD4^+$ T cells and B cells from active AIH-1 patients carrying -1993TC and -1993TT genotypes, compared with healthy controls carrying -1993TC and -1993TT genotypes, in an attempt to provide functional evidence for the association of the TBX21 T-1993C promoter polymorphism and AIH-1 development.

Research methods

In vivo, *in vitro*, and reporter analyses were performed to determine the function of transcription factor Yin-Yang 1(YY1) binding to the T-1993C element of the TBX21 promoter in human $CD4^+$ T and B cell lines. Flow cytometry and quantitative real-time PCR were used to analyze T-bet and IFN-γ expressions in $CD4^+$ T cells, B cells and monocytes from the peripheral blood of AIH-1 patients including 5-1993TC and 15-1993TT genotype carriers, and healthy controls including 10-1993TC and 25-1993TT genotype carriers. Furthermore, knockdown of YY1 with siRNA was performed to investigate T-bet-medicated

regulation of immune response in peripheral blood of AIH-1 patients. The difference between the groups was analyzed by parametric Student's *t*-test or nonparametric Mann-Whitney *U* test. Correlations of T-bet expression in $CD4^+$ T cells and B cells from active AIH-1 patients with AIH disease activity, represented by the International Autoimmune Hepatitis Group score, were examined by Pearson correlation.

Research results

TBX21-1993C allele created a strong Yin-Yang 1 (YY1)-binding site and decreased TBX21 promoter activity in human $CD4^+$ T and B cells. Higher levels of T-bet and IFN-γ were detected in the circulating $CD4^+$ T cells and B cells of AIH-1 patients carrying the TBX21-1993 TT genotype compared with the patients carrying the -1993 TC genotype and controls with the -1993 TC genotype. T-bet expression levels of circulating T cells and B cells were also positively correlated with AIH-1 disease activity. Knockdown of YY1 with siRNA caused increased expression of T-bet and IFN-γ in peripheral blood mononuclear cells in AIH-1 patients. This study has limitations, such as a relative small sample size and the lack of functional study of T-bet-expressing T and B cells in the pathogenic process of AIH, as well as no information about T-bet-expressing T and B cells infiltrates in the liver.

Research conclusions

Reduced T-bet and IFN-γ expression of circulating $CD4^+$ T cells and B cells existed in the individuals carrying the -1993C allele compared with those without the -1993C allele, and played a protective role in AIH-1 development. Moreover, the authors propose a schematic model showing high-affinity binding of YY1 to the -1993C allele site leading to down-regulation of the TBX21 promoter activity, a mechanism for a promoter polymorphism affecting T-bet expression in AIH.

Research perspectives

Further studies are necessary to identify polymorphisms in other loci of the genome and to analyze their association with the T-1993C polymorphism for further elucidation of the genomic background of AIH. Furthermore, further longitudinal studies are necessary to analyze the numbers and function of T-bet-expressing T and B cells in the pathogenic process of AIH -1 with a bigger population.

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ORIGINAL ARTICLE

Basic Study

Astragaloside Ⅳ inhibits pathological functions of gastric cancer-associated fibroblasts

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Abstract

AIM

To investigate the inhibitory effect of astragaloside IV on the pathological functions of cancer-associated fibroblasts, and to explore the underlying mechanism.

METHODS

Paired gastric normal fibroblast (GNF) and gastric cancer-associated fibroblast (GCAF) cultures were established from resected tissues. GCAFs were treated with vehicle control or different concentrations of astragaloside Ⅳ. Conditioned media were prepared from GNFs, GCAFs, control-treated GCAFs, and astragaloside Ⅳ-treated GCAFs, and used to culture BGC-823 human gastric cancer cells. Proliferation, migration and invasion capacities of BGC-823 cells were determined by MTT, wound healing, and Transwell invasion assays, respectively. The action mechanism of astragaloside Ⅳ was investigated by detecting the expression of microRNAs and the expression and secretion of the oncogenic factor, macrophage colony-stimulating factor (M-CSF), and the tumor suppressive factor, tissue inhibitor of metalloproteinase 2 (TIMP2), in different groups of GCAFs. The expression of the oncogenic pluripotency factors SOX2 and NANOG in BGC-823 cells cultured with different conditioned media was also examined.

RESULTS

GCAFs displayed higher capacities to induce BGC-823 cell proliferation, migration, and invasion than GNFs ($P < 0.01$). Astragaloside Ⅳ treatment strongly inhibited the proliferation-, migration- and invasion-promoting capacities of GCAFs ($P < 0.05$ for 10 μmol/L, $P < 0.01$ for 20 μmol/L and 40 μmol/L). Compared with GNFs, GCAFs expressed a lower level of microRNA-214 ($P < 0.01$) and a higher level of microRNA-301a ($P < 0.01$). Astragaloside Ⅳ treatment significantly up-regulated microRNA-214 expression ($P < 0.01$) and down-regulated microRNA-301a expression ($P < 0.01$) in GCAFs. Reestablishing the microRNA expression balance subsequently suppressed M-CSF production ($P < 0.01$) and secretion ($P < 0.05$), and elevated TIMP2 production ($P < 0.01$) and secretion ($P < 0.05$). Consequently, the ability of GCAFs to increase SOX2 and NANOG expression in BGC-823 cells was abolished by astragaloside Ⅳ.

CONCLUSION

Astragaloside Ⅳ can inhibit the pathological functions of GCAFs by correcting their dysregulation of microRNA expression, and it is promisingly a potent therapeutic agent regulating tumor microenvironment.

Key words: Astragaloside Ⅳ; Gastric cancer-associated fibroblasts; Proliferation; Migration; Invasion; MicroRNA

Core tip: Most of chemotherapeutic agents directly act on cancer cells. However, direct drug attacks on cancer cells have the drawbacks of accelerating cancer evolution, chemoresistance, recurrence, and metastasis. Cancer-associated fibroblasts considerably contribute to cancer initiation and progression by providing nourishment and support for cancer cells. Blocking the pathological functions of cancer-associated fibroblasts can eliminate the conditions suitable for cancer cell survival and expansion, representing a novel effective anti-cancer strategy. Unfortunately, few effective drugs have been found against cancer-associated fibroblasts. Here, astragaloside Ⅳ significantly inhibited the pathological functions of gastric cancer-associated fibroblasts, suggesting its valuable therapeutic application in gastric carcinoma.

Wang ZF, Ma DG, Zhu Z, Mu YP, Yang YY, Feng L, Yang H, Liang JQ, Liu YY, Liu L, Lu HW. Astragaloside Ⅳ inhibits pathological functions of gastric cancer-associated fibroblasts. *World J Gastroenterol* 2017; 23(48): 8512-8525 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i48/8512.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i48.8512

INTRODUCTION

Cancer is a major burden of disease worldwide. Every year, approximately 14 million new cases are reported and 8.2 million cancer-related deaths occur[1]. Chemotherapy forms the mainstay of cancer treatment, particularly for patients who do not respond to local excision or radiation treatment[2]. Currently, most chemotherapeutic agents directly act on cancer cells. However, this approach has two serious drawbacks. First, because cancer cells have unstable genomes, repeated drug attacks aggravate their genomic instability and cause new mutations in their genomes, leading to acceleration of cancer evolution[3,4]. Second, some commonly used chemotherapeutic drugs induce the conversion of differentiated cancer cells to cancer stem cells, thus provoking treatment resistance and speeding up cancer metastasis[5,6]. Therefore, new strategies and drugs are urgently required to effectively treat cancer.

Cancer malignancy is caused not only by genetic and epigenetic abnormalities in cancer cells, but more profoundly, by the stimulation and support from the tumor microenvironment[7,8]. Within the tumor microenvironment, cancer-associated fibroblasts, which can accompany metastatic cancer cells to the invasive front and the tumor-interstitium interface, constitute the dominant cell population[8]. They secrete soluble factors, such as oncogenic cytokines, chemokines, and matrix metalloproteinases, into the microenvironment, thus creating suitable conditions for the activation

and maintenance of cancer cell survival, proliferation, migration, invasion, and treatment resistance[7,9]. Due to their stable genomes with few mutations, cancer-associated fibroblasts are proper drug targets. Inhibition of the nourishment and support provided by cancer-associated fibroblasts with proper drugs can destroy the "fertile soil" for cancer cell survival and expansion, representing a robust strategy to control cancer. However, few effective drugs have been found against cancer-associated fibroblasts.

Traditional Chinese medicine and pharmacology is a valuable source of knowledge warranting in-depth exploration. It has accumulated rich anti-cancer experience over thousands of years of clinical practice. Based on the unique concept of holism, traditional Chinese medicine investigates the mechanisms of cancer initiation and progression by considering the interactions among the tumor, human body, and surrounding environment instead of merely focusing on cancer cells[10]. Used under the guidance of traditional Chinese medicine theories, Chinese herbs can not only kill cancer cells, but more excellently, can regulate and improve patients' internal environment to restore various tissues and cells to their normal states and enhance their functions[11]. Consequently, the body's internal environment becomes unfavorable for tumor initiation and progression; the tumor microenvironment is unable to nourish cancer cells and support their growth; the cancer cells encounter conditions unsuitable for their survival. Thus, traditional Chinese herbs are a promising source of cancer-associated fibroblast inhibitors.

Radix astragali is a crucial Chinese herb prescribed for strengthening the constitutions of patients and eliminating toxins from their bodies. It is frequently present in anti-cancer formulas[12]. Astragaloside Ⅳ is a cycloartane-type triterpene glycoside isolated from Radix astragali. It has been reported to suppress matrix metalloproteinase-1 expression in dermal fibroblasts[13] and reduce collagen Ⅰ/Ⅲ secretion by skin fibroblasts[14], exhibiting an ability to affect fibroblast functions. Considering this property of astragaloside Ⅳ, we speculated that it can inhibit the pathological functions of cancer-associated fibroblasts. In the present study, for the first time, we treated gastric cancer-associated fibroblasts (GCAFs) with astragaloside Ⅳ, observed its effect on the malignancy-promoting functions of GCAFs, and explored the mechanism underlying the effect of astragaloside Ⅳ on GCAFs.

MATERIALS AND METHODS

Cells and reagents

Human gastric carcinoma BGC-823 cell line was purchased from the Cell Bank of Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China). Astragaloside Ⅳ was provided by Standard Technology Co., Ltd. (Shanghai, China) and dissolved in DMSO to a concentration of 20 mM as a stock solution. The stock solution was diluted with RPMI-1640 medium when used and the final DMSO concentration did not exceed 0.2% (v/v). A mouse monoclonal antibody against pan-cytokeratin (pan-CK) and rabbit polyclonal antibodies against E-cadherin, vimentin, PDGFR-β, and alpha smooth muscle actin (α-SMA) were obtained from Abcam (Cambridge, United Kingdom). Rabbit polyclonal antibodies against macrophage colony-stimulating factor (M-CSF), tissue inhibitor of metalloproteinase 2 (TIMP2), SRY-box 2 (SOX2), NANOG, GAPDH, and β-actin, as well as CY3-conjugated goat anti-mouse IgG, CY3-conjugated goat anti-rabbit IgG, and HRP-conjugated goat anti-rabbit IgG were obtained from Elabscience Biotechnology (Wuhan, Hubei, China). The 2'-O-methyl chemically modified oligonucleotides for microRNA-214 inhibitors (anti-miR-214), anti-miR-214 negative control (214-NC), microRNA-301a mimic (miR-301a mimic), and miR-301a mimic negative control (301a-NC) were produced by GenePharma (Suzhou, Jiangsu, China).

Isolation and culture of human gastric normal fibroblasts and GCAFs

Gastric tumor tissue and adjacent normal tissue (at least 2 cm from the outer tumor margin) were obtained from a patient with moderately differentiated gastric adenocarcinoma during gastric surgery and immediately transported to the laboratory. The fresh tissue samples were minced into 0.5-1 mm^3 fragments, seeded in 60-mm culture dishes in the presence of RPMI-1640 supplemented with 20% fetal bovine serum (FBS), and cultured at 37 ℃ in a humid atmosphere containing 5% CO_2. The culture medium was changed twice a week for 2-3 wk. Under these conditions, fibroblasts grew out from tissue fragments while other cells were mostly retained in the fragments. After reaching confluence, monolayers were trypsinized and passaged at a split ratio of 1:2 (passage 1). The fibroblasts were then sub-cultured for another 3-4 passages until the cultures were free of epithelial cell contamination and subsequently maintained in RPMI-1640 supplemented with 10% FBS, 2% penicillin, and 2% streptomycin (complete RPMI-1640 medium). GNFs and GCAFs were both used between passage 5 and 8.

Immunofluorescence

Gastric normal fibroblasts (GNFs) and GCAFs grown on glass coverslips were washed with cold phosphate-buffered saline (PBS), fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100, and then incubated with primary antibodies to pan-CK (1:100), E-cadherin (1:100), vimentin (1:150), PDGFR-β (1:100), and α-SMA (1:100) overnight at 4 ℃. The cells were subsequently incubated with fluorescence-conjugated secondary antibodies for 1 h at 37 ℃. After three PBS washes, cell nuclei were counterstained with DAPI for 5 min;_the cells were examined with a Nikon

Table 1 qPCR primers

Gene name	Forward primer (F) Reverse primer (R)
U6	F: 5′-TTCCTCCGCAAGGATGACACGC-3′ R: Universal miRNA qPCR primer
miR-214	F: 5′-CAGGCACAGACAGGCAGT-3′ R: Universal miRNA qPCR primer
miR-301a	F:5′-GGCAGTGCAATAGTATTGT-3′ R: Universal miRNA qPCR primer
miR-34b	F: 5′-TAGGCAGTGTCATTAGCTGATTG-3′ R: Universal miRNA qPCR primer
miR-31	F: GCCGCAGGCAAGATGCTGGC R: Universal miRNA qPCR primer
miR-155	F: TTAATGCTAATCGTGATAGGGGT R: Universal miRNA qPCR primer
miR-200b	F: GCGGCTAATACTGCCTGGT R: Universal miRNA qPCR primer
miR-16	F: GTAGCAGCACGTAAATATTGGCG R: Universal miRNA qPCR primer
miR-148a	F: AGCAGTTCAGTGCACTACAG R: Universal miRNA qPCR primer
GAPDH	F: 5′-TGTCCCCACTGCCAACGTGTCA-3′ R: 5′-GCGTCAAAGGTGGAGGAGTGGGT-3′
SOX2	F: 5′-TACAGCATGTCCTACTCGCAG-3′ R: 5′-GAGGAAGAGGTAACCACAGGG-3′
NANOG	F: 5′-CCAACATCCTGAACCTCAGCTAC-3′ R: 5′-GCCTTCTGCGTCACACATT-3′

Eclipse Ti-S Epifluorescence microscope equipped with a DS-L2 digital camera (Nikon).

Preparation of conditioned media

To prepare conditioned media from GNFs and GCAFs, GNFs and GCAFs were plated into 60-mm dishes (5 × 10^5 cells) and cultured with complete RPMI-1640 medium. Twelve hours later, the medium was replaced with 4 mL fresh RPMI-1640 medium for an additional 48 h culture. The supernatants were collected, centrifuged at 1000 rpm, filtered with 0.1 μm membranes, and supplemented with 3% FBS.

To prepare conditioned media from astragaloside Ⅳ-treated GCAFs, GCAFs were treated with 10, 20, or 40 μmol/L astragaloside Ⅳ or 0.2% DMSO (vehicle control) for 72 h. Then, the cells were collected and cultured in complete RPMI-1640 medium in 60-mm dishes (5 × 10^5 cells) for 12 h. Thereafter, the medium was replaced with 4 mL fresh RPMI-1640 medium for an additional 48 h culture. The supernatants were collected, centrifuged at 1000 rpm, filtered with 0.1 μm membranes, and supplemented with 3% FBS.

Culture of gastric cancer cells with conditioned media

BGC-823 cells were maintained in complete RPMI-1640 medium. At 80% confluence, the cells were collected, allocated into different groups, and cultured with appropriate conditioned media for 48 h.

MTT assay

Cells were seeded in each well of 96-well plates in 200 μL complete RPMI-1640 medium and cultured at 37 ℃. After 48 h of culture, MTT solution (5 mg/mL) was added and incubated at 37 ℃ for 4 h. Then, the medium was absorbed off and 100 μL of dimethyl sulfoxide was added to each well. Following vibrating on a shaker for 10 min, the plates were measured for absorbance at 490 nm wavelength.

Wound healing assay

Cell migration capacity was determined by wound healing assay. BGC-823 cells were seeded in 6-well plates at 5 × 10^5 cells per well to grow into a monolayer. The monolayers were wounded by scratching lines with a plastic tip. The wells were then washed twice with PBS to remove any debris, and photographed under a microscope. Thereafter, the plates were incubated at 37 ℃ under 5% CO_2 for 24 h with RPMI-1640, after which the cells were observed and photographed. The distance that the cells had migrated was measured on the photographs. The relative migration distance was calculated as follows: 1-(mean remained breadth/mean wounded breadth)[15].

Transwell invasion assay

Invasion assay was performed using 24-well Transwell chambers (polycarbonate membrane, 8 μm pore size; Costar, Cambridge, MA, United States). The upper surfaces of the Transwell membranes were pre-coated with Matrigel (BD Biosciences, San Jose, CA, United States) which was allowed to solidify at 37 ℃ for 4 h. Thereafter, 1.5 × 10^5 BGC-823 cells suspended in 150 μL RPMI-1640 were seeded into each upper chamber, and 600 μL of RPMI-1640 supplemented with 20% FBS were added into each lower chamber. The plates were incubated for 24 h at 37 ℃, and then the media were removed from the Transwell chambers and the cells on the upper surfaces of the Transwell membranes were wiped off. Cells that had migrated to the lower surfaces of the Transwell membranes were fixed and stained with crystal violet, and the number of cells in five randomly selected fields at × 200 magnification was counted.

Reverse transcription-quantitative PCR

Total RNA was isolated using RNAiso Reagent (TaKaRa, Dalian, Liaoning, China) according to the manufacturer's protocol. RNA concentrations were measured using a NanoDrop 2000 (Thermo Scientific, Wilmington, DE, United States). To evaluate the expression of microRNAs, reverse transcription and qPCR were performed with the TransScript Green miRNA Two-Step qRT-PCR SuperMix (Transgene, Beijing, China) using U6 as an endogenous control. The qPCR cycling condition was as follows: 94 ℃ for 30 s, and 40 cycles of 94 ℃ for 5 s and 60 ℃ for 34 s. To evaluate the expression of SOX2 and NANOG mRNAs, reverse transcription was performed with a PrimeScript RT reagent kit with gDNA Eraser (TaKaRa) using 1 μg of total RNA from each sample, followed by qPCR with the SYBR Premix Ex Taq (TaKaRa) using GAPDH as an endogenous control.

The qPCR cycling condition was as follows: 95 ℃ for 30 s, and 40 cycles of 95 ℃ for 5 s and 60 ℃ for 31 s. Relative expression levels of each gene were calculated using the ΔΔCt method. Primers used are listed in Table 1.

Transfection of microRNA oligonucleotides

Oligonucleotides for anti-miR-214, 214-NC, miR-301a mimic, and 301a-NC were transfected with siRNA-Mate at a final concentration of 50 nM. After 48 h of transfection, cells were harvested for further experiment.

Western blot analysis

Western blot procedures were conducted as previously reported[16]. Antibodies against M-CSF (1:3000), TIMP2 (1:500), SOX2 (1:2000), NANOG (1:2000), GAPDH (1:2000), and β-actin (1:2000) were used as primary antibodies. HRP-conjugated goat anti-rabbit IgG diluted in 0.5% non-fat milk was used as secondary antibody.

Enzyme-linked immunosorbent assay

The M-CSF and TIMP2 concentrations in the conditioned media were measured using ELISA kits (Baizhi, Beijing, China) according to the manufacturer's instructions.

Statistical analysis

Results are summarized as mean ± SD. Student's *t*-test and one-way analysis of variance were used to analyze the data and the significance level was set at $P < 0.05$. The statistical analyses were performed with SPSS 17.0 software (SPSS, Chicago, IL, United States).

RESULTS

Identification of GNFs and GCAFs

First, we assessed the cultured GNFs and GCAFs through immunofluorescence staining for the epithelial cell markers pan-CK and E-cadherin as well as the mesenchymal cell markers vimentin and PDGFR-β. The results revealed that GNFs and GCAFs were negative for pan-CK and E-cadherin and positive for vimentin and PDGFR-β (Figure 1). Furthermore, to identify GCAFs, we evaluated the cells for expression of α-SMA, a marker of activated fibroblasts typically expressed strongly in cancer-associated fibroblasts but weakly in quiescent fibroblasts[17]. Expression of α-SMA was considerably higher in GCAFs than in paracancerous GNFs (Figure 1). These results indicate that paired GNF and GCAF cultures were successfully established.

GCAFs enhance proliferation, migration, and invasion abilities of gastric cancer cells

To determine whether GCAFs influence the proliferation, migration, and invasion abilities of gastric cancer cells, BGC-823 cells were cultured with GNF- or GCAF-conditioned medium and then subjected to MTT, wound healing, and Transwell invasion assays. The results showed that BGC-823 cells cultured in the GCAF-conditioned medium exhibited significantly higher proliferation, migration, and invasion abilities than cells cultured in the GNF-conditioned medium ($P < 0.01$) (Figure 2), indicating that GCAFs promote the malignant behaviors of gastric cancer cells.

Astragaloside IV inhibits malignancy-promoting capacities of GCAFs

To determine whether astragaloside Ⅳ affects the malignancy-promoting capacities of GCAFs, GCAFs were treated with vehicle control or increasing concentrations of astragaloside Ⅳ (10, 20, or 40 μmol/L). Then, conditioned media were prepared to culture BGC-823 cells. BGC-823 cells cultured in the conditioned media from astragaloside Ⅳ-treated GCAFs showed lower proliferation, migration, and invasion abilities than BGC-823 cells cultured in the conditioned medium from control-treated GCAFs ($P < 0.05$ for 10 μmol/L group, $P < 0.01$ for 20 μmol/L and 40 μmol/L groups) (Figures 3 and 4), indicating that astragaloside Ⅳ inhibits the malignancy-promoting capacities of GCAFs.

Astragaloside IV up-regulates miR-214 and down-regulates miR-301a expression in GCAFs

Recent studies have shown that the dysregulation of microRNA expression substantially contributes to the malignancy-promoting capacities of cancer-associated fibroblasts[18]. In our study, we selected eight microRNAs that are related to the functions of cancer-associated fibroblasts, and evaluated their expression in GNFs and GCAFs using RT-qPCR. GCAFs exhibited lower expression of miR-214 and higher expression of miR-301a than GNFs ($P < 0.01$) (Figure 5). Treatment of GCAFs with astragaloside Ⅳ effectively up-regulated miR-214 and down-regulated miR-301a expressions ($P < 0.01$) (Figure 6).

miR-214 and miR-301a mediate astragaloside IV-induced inhibition of malignancy-promoting capacities of GCAFs

To determine whether miR-214 and miR-301a are involved in astragaloside Ⅳ-induced changes in GCAF functions, GCAFs were transfected with an anti-miR-214 or miR-301a mimic, or co-transfected with anti-miR-214 and miR-301a mimic. Then, the cells were treated with 40 μM astragaloside Ⅳ and conditioned media were prepared to culture BGC-823 cells.

MTT assay revealed that anti-miR-214 or miR-301a mimic transfection partially reversed astragaloside Ⅳ-induced inhibition of proliferation-promoting capacity of GCAFs ($P < 0.05$) (Figure 7A). Moreover, anti-miR-214 and miR-301a mimic co-transfection significantly reduced astragaloside Ⅳ-induced inhibition of proliferation-promoting capacity of GCAFs ($P < 0.01$) (Figure 7A). The results signify that miR-214 and miR-301a participate in astragaloside Ⅳ-induced inhibition

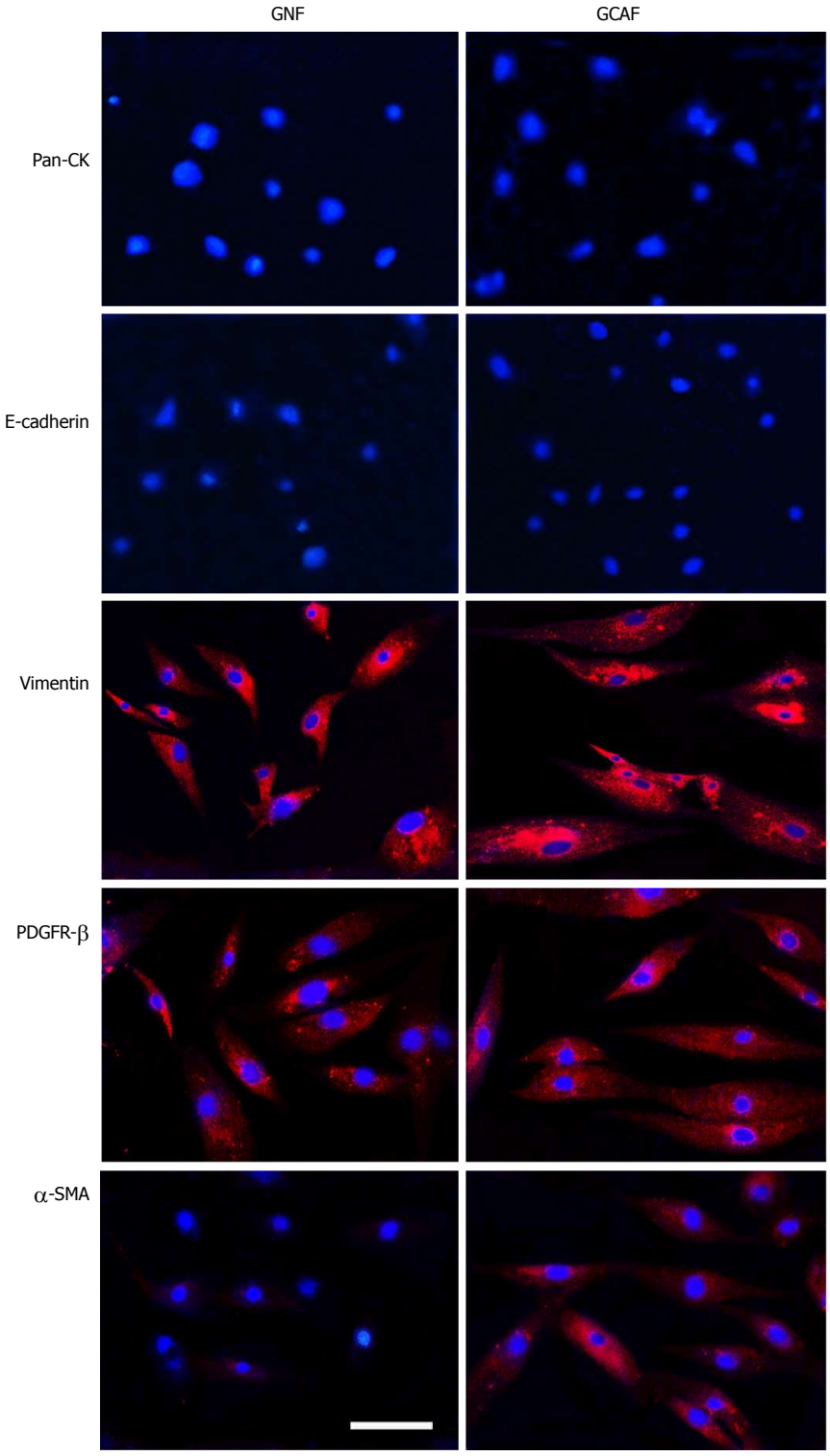

Figure 1 Identification of gastric normal fibroblast and gastric cancer-associated fibroblast. Fibroblasts were isolated from the tumor and adjacent normal tissues of a gastric adenocarcinoma patient. The expression of Pan-CK, E-cadherin, vimentin, PDGFR-β and α-SMA in the cultured fibroblast cells was detected by immunofluorescence. Scale bar: 100 μm.

of proliferation-promoting capacity of GCAFs.

Wound healing assay similarly showed that anti-miR-214 or miR-301a mimic transfection partially reversed astragaloside Ⅳ-induced inhibition of migration-promoting capacity of GCAFs ($P < 0.01$) (Figure 7B). Additionally, anti-miR-214 and miR-301a mimic co-transfection completely reversed astragaloside Ⅳ-induced inhibition of migration-promoting capacity of GCAFs ($P < 0.01$) (Figure 7B). These results reveal that miR-214 and miR-301a mediate astragaloside Ⅳ-induced inhibition of migration-promoting capacity of GCAFs.

Transwell invasion assay also revealed that anti-miR-214 or miR-301a mimic transfection partially

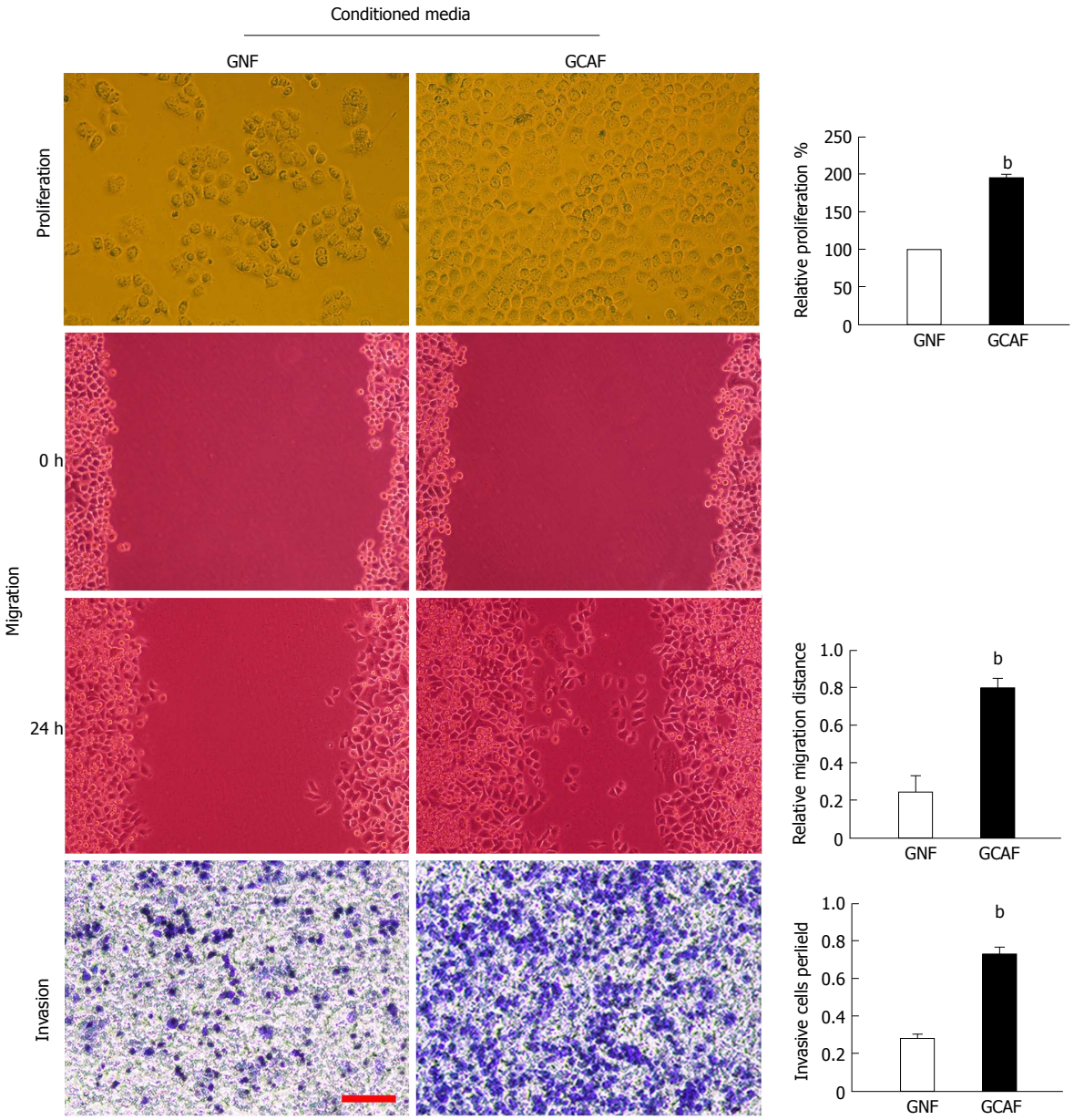

Figure 2 Gastric cancer-associated fibroblasts promote the proliferation, migration and invasion of gastric cancer cells. BGC-823 cells were cultured with GNF- or GCAF-conditioned medium for 48 h; then, proliferation, migration and invasion capacities were measured. Scale bar: 100 μm. Data are plotted as mean ± SD of three separate experiments. $^{b}P < 0.01$ *vs* the BGC-823 cells cultured with GNF-conditioned medium.

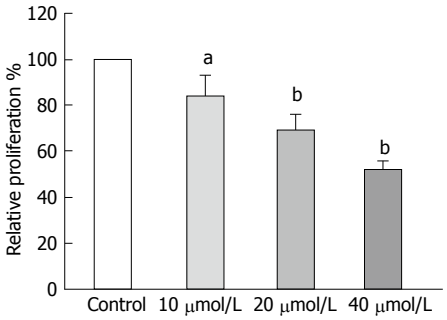

Figure 3 Astragaloside Ⅳ inhibited the proliferation-promoting capacity of gastric cancer-associated fibroblasts. Conditioned media were prepared from the GCAFs treated by vehicle control, 10 μmol/L astragaloside Ⅳ, 20 μmol/L astragaloside Ⅳ or 40 μmol/L astragaloside Ⅳ, and used to culture BGC-823 cells. After culture for 48 h, the proliferation capacity of the BGC-823 cells was measured. Data are plotted as mean ± SD of three separate experiments. $^{a}P < 0.05$ and $^{b}P < 0.01$ *vs* the BGC-823 cells cultured with the conditioned medium from control-treated GCAFs.

reversed astragaloside Ⅳ-induced inhibition of invasion-promoting capacity of GCAFs ($P < 0.05$) (Figure 7C). Moreover, anti-miR-214 and miR-301a mimic co-transfection completely reversed astragaloside Ⅳ-induced inhibition of invasion-promoting capacity of GCAFs ($P < 0.01$) (Figure 7C). These results indicate that miR-214 and miR-301a mediate astragaloside Ⅳ-induced inhibition of invasion-promoting capacity of GCAFs.

Astragaloside Ⅳ reduces M-CSF expression and increases TIMP2 expression in GCAFs

M-CSF and TIMP2 are validated targets of miR-214 and miR-301a, respectively. We investigated whether astragaloside Ⅳ-induced changes in microRNA expression affected M-CSF and TIMP2 expression in GCAFs. Western blot analysis showed that astragaloside

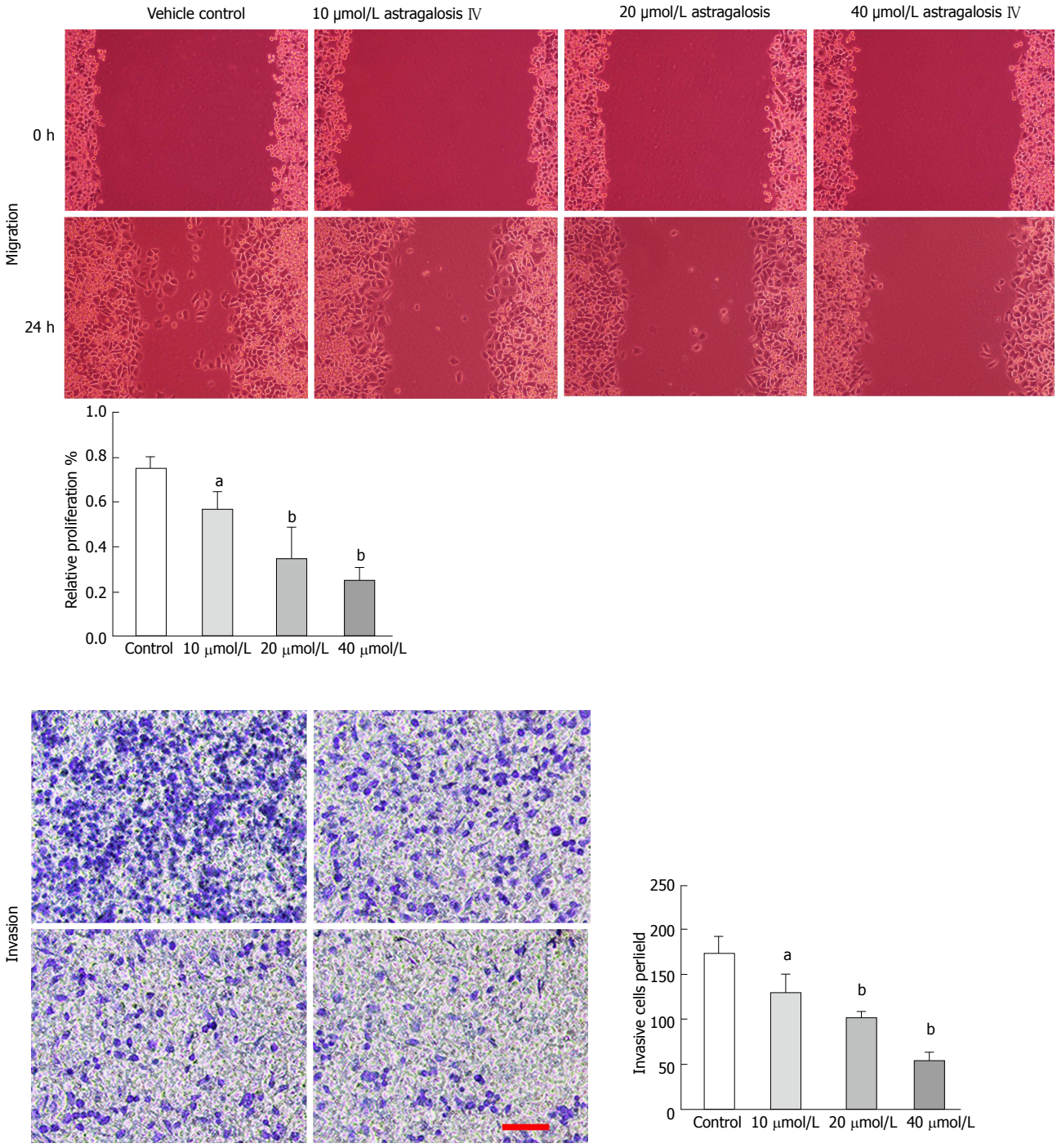

Figure 4 Astragaloside Ⅳ inhibited the migration- and invasion-promoting capacities of gastric cancer-associated fibroblasts. Conditioned media were prepared from the GCAFs treated by vehicle control, 10 μmol/L astragaloside Ⅳ, 20 μmol/L astragaloside IV or 40 μmol/L astragaloside Ⅳ, and used to culture BGC-823 cells. After culture for 48 h, the migration and invasion capacities of the BGC-823 cells were measured. Scale bar: 100 μm. Data are plotted as mean ± SD of three separate experiments. [a]$P < 0.05$ and [b]$P < 0.01$ *vs* the BGC-823 cells cultured with the conditioned medium from control-treated GCAFs.

Ⅳ treatment reduced M-CSF and increased TIMP2 protein expression in GCAFs ($P < 0.01$), and that pre-transfection of GCAFs with anti-miR-214 and miR-301a mimic prevented astragaloside Ⅳ from altering M-CSF and TIMP2 expression, respectively (Figure 8A). ELISA also confirmed that astragaloside Ⅳ treatment suppressed M-CSF and elevated TIMP2 protein secretion by GCAFs ($P < 0.05$) (Figure 8B).

Astragaloside Ⅳ prevents GCAFs from up-regulating SOX2 and NANOG in BGC-823 cells

M-CSF and TIMP2 both participate in regulating cancer stem cell phenotype in the tumor microenvironment. We next explored whether treatment of GCAFs with astragaloside Ⅳ influenced the expression of the oncogenic pluripotency factors SOX2 and NANOG in gastric cancer cells. RT-qPCR and Western blot

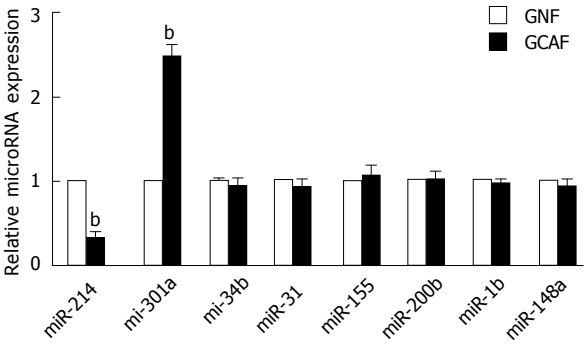

Figure 5 The expression of miR-214 and miR-301a was abnormally decreased and increased in the gastric cancer-associated fibroblasts, respectively. $^{b}P < 0.01$ *vs* the GNFs.

analysis revealed that BGC-823 cells cultured in the GCAF-conditioned medium expressed higher levels of SOX2 and NANOG than those cultured in the GNF-conditioned medium ($P < 0.01$), and that BGC-823 cells cultured in the conditioned medium from astragaloside Ⅳ-treated GCAFs expressed similar levels of SOX2 and NANOG to those cultured in the GNF-conditioned medium (Figure 9). These results indicate that astragaloside Ⅳ prevents GCAFs from up-regulating the oncogenic pluripotency factors SOX2 and NANOG in gastric cancer cells.

DISCUSSION

Cancer-associated fibroblasts, the most abundant cells in the tumor stroma, differ from normal fibroblasts in expression profiles and functions. They produce and secrete high levels of factors that promote cancer cell proliferation, migration, and invasion, simultaneously synthesizing and releasing low levels of factors that maintain tissue homeostasis. Thus, they create an environment favorable for tumor survival and progression[7,9]. Blocking the pathological functions of cancer-associated fibroblasts can diminish the nourishment and support they provide for cancer cells. Some researchers have attempted to inhibit the pathological functions of cancer-associated fibroblasts by targeting fibroblast activation proteins on the membranes of cancer-associated fibroblasts[19,20]. However, the efficacy of this strategy is unsatisfactorily low. For example, neither sibrotuzumab (a monoclonal antibody) nor talabostat (a small-molecule inhibitor of fibroblast activation protein) generated tumor responses in patients with metastatic colorectal cancer during phase Ⅱ clinical trials[19,20]. One likely reason for the unsatisfactory outcome is that cancer-associated fibroblasts have complex pathological mechanisms, and thus it is difficult to completely inhibit their pathological functions by targeting only one molecule. Notably, in the present study, astragaloside Ⅳ strongly inhibited GCAFs from supporting gastric cancer cells by correcting the dysregulated expression of multiple

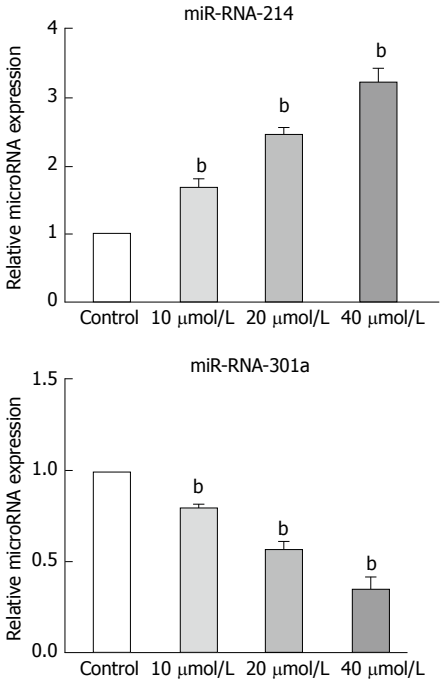

Figure 6 Astragaloside Ⅳ treatment up-regulated miR-214 and down-regulated miR-301a expression in the gastric cancer-associated fibroblasts. Data are plotted as mean ± SD of three separate experiments; $^{b}P < 0.01$ *vs* control-treated GCAFs.

oncogenic and tumor suppressive molecules, exhibiting potential as an effective new drug against cancer-associated fibroblassts.

Astragaloside Ⅳ is a main bioactive component of Radix astragali, a commonly used nontoxic Chinese herb. Apart from its anti-inflammatory and antiviral activities, astragaloside Ⅳ also has chemoprotective effects on human somatic cells. For example, at 100 μmol/L, it can protect dopaminergic neurons against 6-hydroxydopamine-induced degeneration[21]; at 250 μmol/L, it can prevent glucose-induced podocyte apoptosis[22]; at 100 μmol/L, it can protect ventricular myocytes from isoproterenol-induced hypertrophy without causing cytotoxicity[23]. In the current study, astragaloside Ⅳ strongly inhibited the proliferation-, migration- and invasion-promoting capacities of GCAFs at a concentration of only 40 μmol/L, suggesting that astragaloside Ⅳ could be considered a safe therapeutic agent for gastric malignancy. This property may provide astragaloside Ⅳ with an advantage over cytotoxic chemotherapy drugs, which form the traditional mainstay of cancer drug treatment despite their substantial injuries to multiple tissues and organs of patients[24].

MicroRNAs, a class of small non-coding RNAs that typically suppress the translation and reduce the stability of mRNAs, are involved in cancer initiation and progression[25]. Down-regulation of miR-214 has been reported in cervical[26], hepatocellular[27], and gastric[28] carcinomas. In particular, decreased miR-214 expression in gastric carcinoma tissues is associated with lymph node metastasis and tumor size[28].

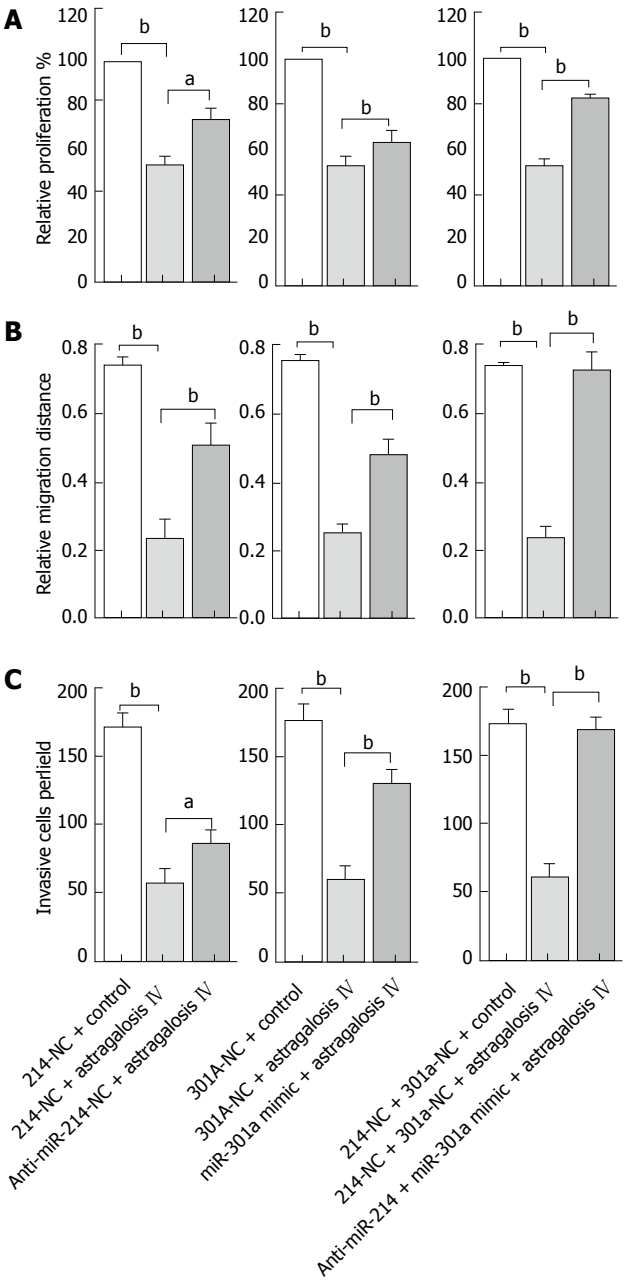

Figure 7 Effects of anti-miR-214 and miR-301a mimic on the astragaloside Ⅳ-induced inhibition of proliferation-, migration- and invasion-promoting capacities of the gastric cancer-associated fibroblasts. The GCAFs were transfected with 50 nmol/L 214-NC, anti-miR-214, 301a-NC or miR-301a mimic, or co-transfected with 50 nmol/L 214-NC and 50 nmol/L 301a-NC or 50 nmol/L anti-miR-214 and 50 nmol/L miR-301a mimic. After 48 h of transfection, the cells were treated with vehicle control or 40 μmol/L astragaloside Ⅳ; conditioned media were prepared to culture BGC-823 cells, whose proliferation (A), migration (B) and invasion (C) capacities were then measured. Data are plotted as mean ± SD of three separate experiments. $^{a}P < 0.05$ and $^{b}P < 0.01$.

Overexpression of miR-301a has also been observed in gastric cancer tissues, and it is positively associated with tumor size, invasion depth, and lymph node metastasis[29]. However, the expression and functions of the two microRNAs in GCAFs have rarely been reported. In the current study, we found that miR-214 expression was decreased and miR-301a expression increased in GCAFs as compared with GNFs, and that astragaloside Ⅳ inhibited the migration- and invasion-promoting capacities of GCAFs by enhancing miR-214 and reducing miR-301a expression. Thus, the dysregulated expression of the two microRNAs is a critical cause of the pathological functions of GCAFs; correcting microRNA expression abnormalities in GCAFs is an important anti-cancer mechanism of astragaloside Ⅳ.

M-CSF has been experimentally proved to be a direct target of miR-214 by a recent study[28]. In addition to regulating macrophage cell proliferation and differentiation, M-CSF also plays a vital role in promoting tumor progression. In the colon tumor microenvironment, M-CSF released by macrophages activates the tumorigenicity of non-cancer stem cell subpopulations by triggering their cancer stem cell properties[30]. In Lewis lung carcinoma, M-CSF secreted by lung cancer cells induces the pro-tumoral function of stromal myeloid cells to facilitate tumor invasion[31]. In mice bearing breast cancer xenografts, administration of M-CSF neutralizing antibody reduced primary tumor growth[32]. In the present study, we found that GCAFs produced and secreted M-CSF protein, suggesting that M-CSF is also an oncogenic factor in the gastric cancer microenvironment. Treatment of GCAFs with astragaloside Ⅳ effectively suppressed M-CSF production and secretion. This effect can reduce the oncogenic signals that gastric cancer cells receive from the surrounding microenvironment, thereby inhibiting the malignant progression of gastric cancer.

Pre-transfection of GCAFs with anti-miR-214 prevented astragaloside Ⅳ from suppressing M-CSF production and secretion, indicating that astragaloside Ⅳ suppresses M-CSF protein expression in GCAFs by increasing miR-214 expression. This result is consistent with the study of Wang *et al*[28] who observed a decrease in M-CSF protein expression after transfection of miR-214 precursor into gastric cancer cells, and it provides another evidence for the role of miR-214 in targeting M-CSF to regulate cell behaviors.

Matrix metallaproteinases in the tumor microenvironment are crucial oncogenic factors. They can degrade the protein components of extracellular matrices to destroy the physical barriers to metastasis, thus promoting cancer cell invasion and entry into blood or lymphatic vessels[33]. TIMP2 is a critical tumor suppressive factor that can inhibit the activity of matrix metallopeptidases by binding with a 1:1 stoichiometry to their active sites[34]. We found that astragaloside Ⅳ significantly elevated the production and secretion of TIMP2 by GCAFs. TIMP2 released from GCAFs by astragaloside Ⅳ stimulation could effectively inhibit the activity of matrix metallaproteinases and disrupt gastric cancer cell migration and invasion. Furthermore, purified TIMP2 protein showed an anti-proliferation activity against human cancer cells in a latest study[35]. Thus, astragaloside Ⅳ may be able to repress gastric cancer cell proliferation by increasing the concentration of TIMP2 in the tumor microenvironment.

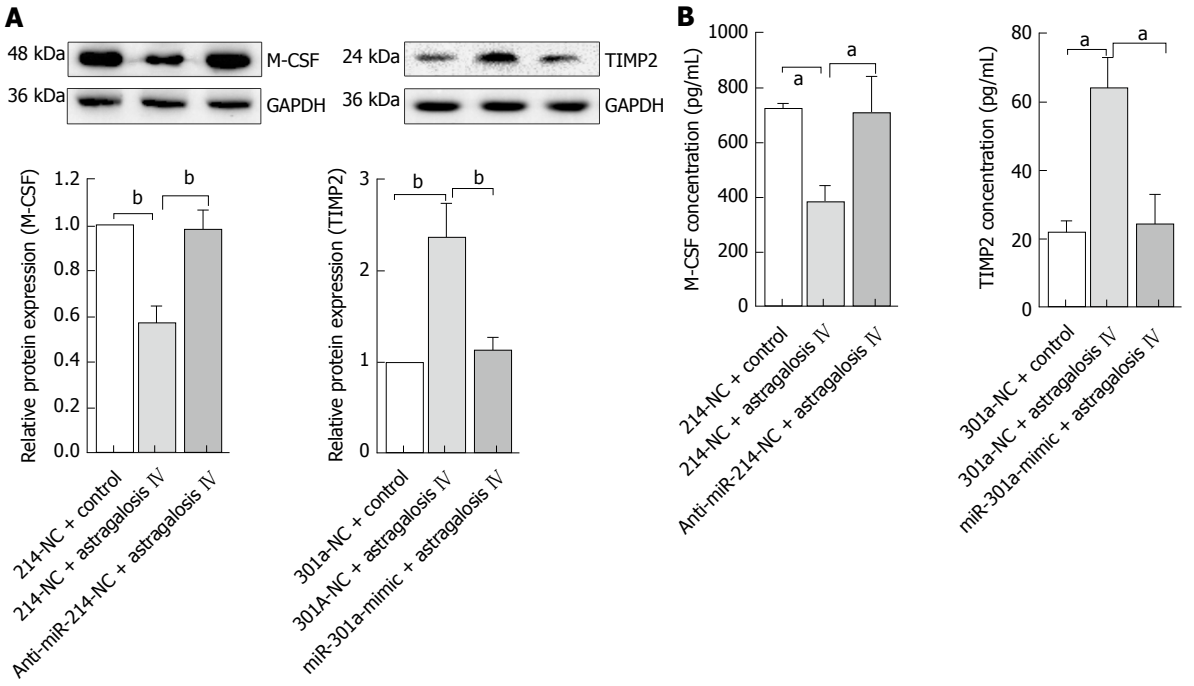

Figure 8 Astragaloside Ⅳ suppressed M-CSF and elevated TIMP2 protein expression (A) and secretion (B) in the gastric cancer-associated fibroblasts. Data are plotted as mean ± SD of three separate experiments. $^{a}P < 0.05$ and $^{b}P < 0.01$.

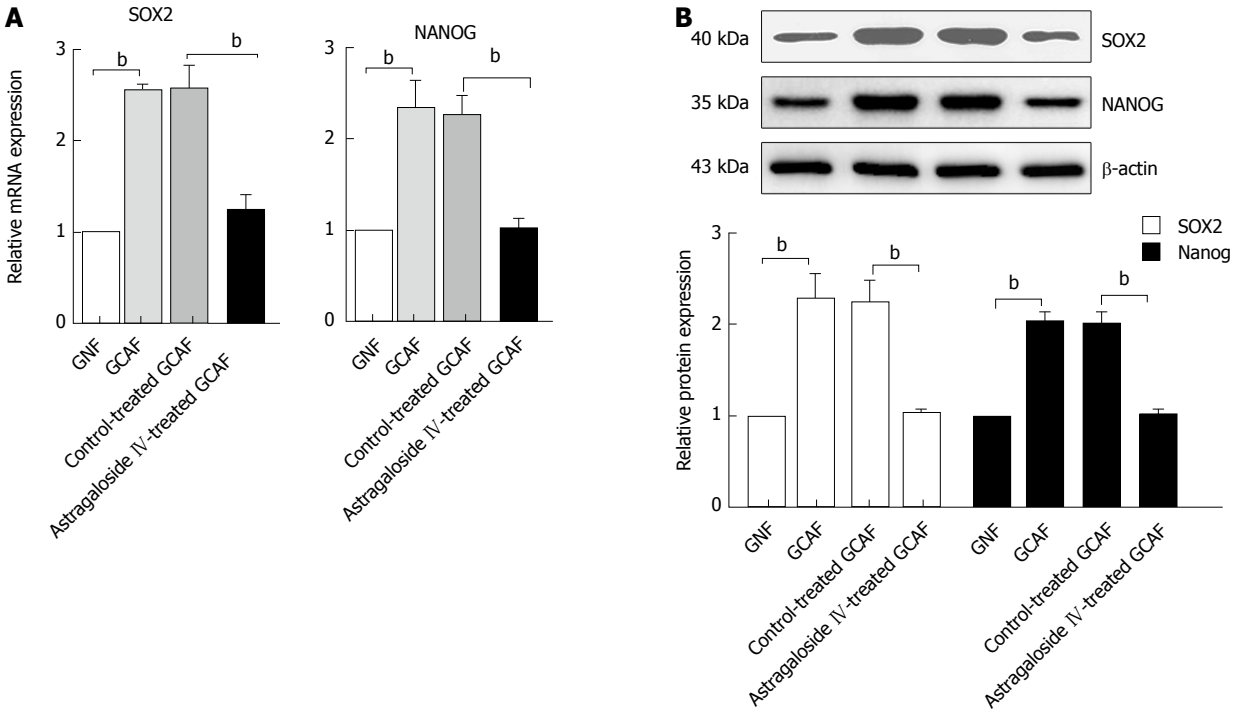

Figure 9 Astragaloside Ⅳ abolished the GCAF-induced up-regulation of SOX2 and NANOG in BGC-823 cells. BGC-823 cells were cultured with conditioned media from the GNFs, GCAFs, control-treated GCAFs or 40 μmol/L astragaloside Ⅳ-treated GCAFs. Then, the mRNA (A) and protein (B) expression of SOX2 and NANOG were determined. Data are plotted as mean ± SD of three separate experiments. $^{b}P < 0.01$.

We found that astragaloside Ⅳ failed to elevate TIMP2 protein expression and secretion levels when GCAFs were pre-transfected with miR-301a mimic. A study by Liang *et al*[36] has shown that TIMP2 is a direct target of miR-301a, which can bind to the 3′-untranslated region of TIMP2 mRNA to inhibit its translation. Therefore, astragaloside Ⅳ did not directly increase TIMP2 production and secretion by GCAFs; instead, it acted by down-regulating miR-301a expression to relieve miR-301a-induced inhibition of TIMP2 translation. This result suggests a potential use of astragaloside Ⅳ as an epigenetic therapeutic agent for gastric cancer.

SOX2 and NANOG are two critical stemness

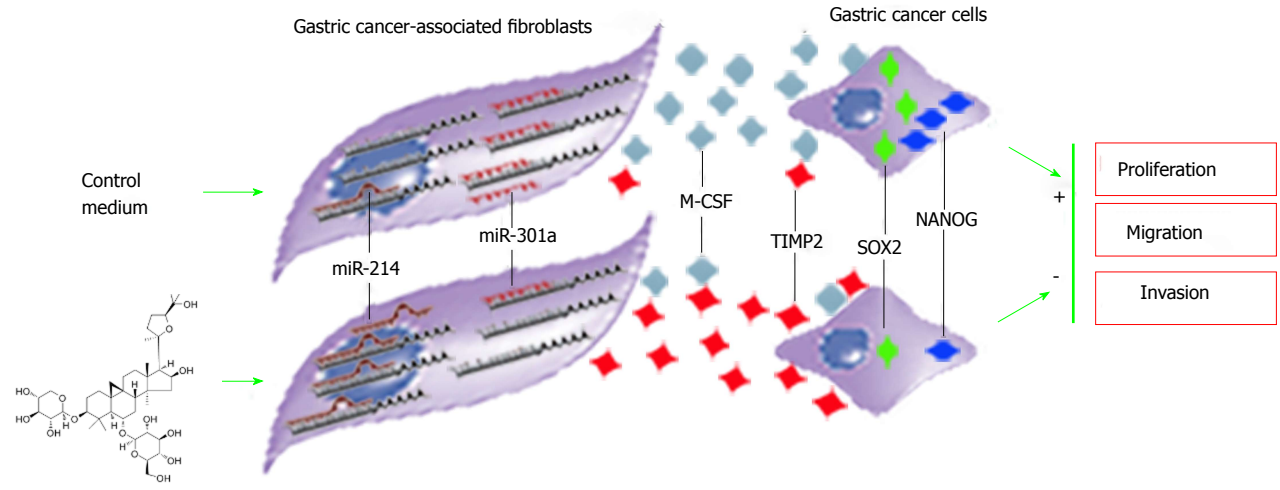

Figure 10 Schematic of the mechanism for astragaloside IV-induced inhibition on the proliferation-, migration- and invasion-promoting capacities of gastric cancer-associated fibroblasts.

markers. Their overexpression contributes greatly to the maintenance of cancer stem cell phenotypes in multiple types of tumors[37,38]. In this study, GCAFs significantly up-regulated SOX2 and NANOG expression in gastric cancer cells, suggesting that promoting the generation of gastric cancer stem cells and supporting their malignant phenotypes is an important oncogenic mechanism of GCAFs. Treatment with astragaloside Ⅳ prevented GCAFs from inducing SOX2 and NANOG up-regulation in gastric cancer cells, suggesting that astragaloside Ⅳ could impair the niche of gastric cancer stem cells by acting on GCAFs, and thus may radically inhibit gastric cancer progression.

The activity of astragaloside Ⅳ to prevent GCAFs from up-regulating SOX2 and NANOG in gastric cancer cells is largely due to its ability to change M-CSF and TIMP2 secretion levels by GCAFs. M-CSF present in the tumor microenvironment acts as a dedifferentiating factor. It can enhance the stemness of cancer cells by increasing their expression of the pluripotent factors SOX2 and NANOG[39,40]. The tumor suppressor TIMP2 acts as a differentiating factor in multiple cancers. It can inhibit the malignant behaviors of cancer stem cells through two pathways: direct down-regulation of the genes associated with cancer stem cell characteristics[41], and indirectly through inhibiting matrix metalloproteinases from activating pluripotent factor expression[42]. Astragaloside Ⅳ treatment significantly reduced M-CSF secretion and increased TIMP2 secretion by GCAFs, making gastric cancer cells receive less dedifferentiating signals and more differentiating signals. Thus, the expression of SOX2 and NANOG in gastric cancer cells was decreased.

Astragaloside Ⅳ regulated the microRNA expression of GCAFs in a bidirectional pattern. It increased the expression of abnormally down-regulated miR-214 and simultaneously reduced the expression of abnormally up-regulated miR-301a. Reestablishment of the microRNA expression balance subsequently suppressed the production and secretion of the oncogenic factor M-CSF as well as elevated the production and secretion of the tumor suppressive factor TIMP2. Consequently, the nourishment and support for gastric cancer cells from GCAFs diminished considerably, and gastric cancer cells lost the environment suitable for proliferation, migration, and invasion (Figure 10). These effects are consistent with the properties of the traditional Chinese herb Radix astragali of "eliminating toxins" and "strengthening constitutions".

Our results confirm the scientific validity of traditional Chinese medicine and highlight the feasibility, effectiveness, and uniqueness of the methods guided by the traditional Chinese medicine concept of holism. Traditional Chinese medicine and pharmacology is truly a valuable source of knowledge, warranting in-depth exploration for the improvement of cancer treatment strategies.

ARTICLE HIGHLIGHTS

Research background

Currently, most chemotherapeutic agents perform by directly acting on cancer cells. They have limited efficacy and generate adverse reactions. Cancer-associated fibroblasts, the dominant cell population within cancer stroma, create favorable conditions for cancer initiation and progression. Preventing cancer-associated fibroblasts from nourishing and supporting cancer cells is a novel and effective anti-cancer pathway.

Research motivation

So far, few effective drugs have been found against cancer-associated fibroblasts. Developing effective inhibitors of cancer-associated fibroblasts is an urgent issue for cancer therapy.

Research objectives

Chinese herbs can regulate and improve patients' internal environment to restore various tissues and cells to their normal states and enhance their functions. It is promising to find effective inhibitors of cancer-associated fibroblasts from Chinese herbs. Astragaloside IV is a cycloartane-type triterpene

glycoside isolated from Radix astragali. Here, for the first time, we studied whether astragaloside Ⅳ can inhibit the pathological functions of cancer-associated fibroblasts, and explored the mechanism underlying the inhibitory effect. The research will provide valuable information on the feasibility of developing cancer-associated fibroblast inhibitors from Chinese herbs.

Research methods

Paired gastric normal fibroblast (GNF) and gastric cancer-associated fibroblast (GCAF) cultures were established from resected tissues. GCAFs were treated with vehicle control or different concentrations of astragaloside Ⅳ. Conditioned media were prepared from GNFs, GCAFs, control-treated GCAFs, and astragaloside Ⅳ-treated GCAFs, and used to culture BGC-823 human gastric cancer cells. Proliferation, migration, and invasion capacities of BGC-823 cells were determined with MTT, wound healing, and Transwell invasion assays, respectively. The expression of microRNAs in the GCAFs was detected by RT-qPCR. The expression and secretion of the oncogenic factor M-CSF and the tumor suppressive factor TIMP2 in different groups of GCAFs were determined by Western blot and ELISA analysis, respectively. The expression of the oncogenic pluripotency factors SOX2 and NANOG in BGC-823 cells cultured with different conditioned media was also examined by RT-qPCR and Western blot analysis.

Research results

GCAFs displayed higher capacities to induce BGC-823 cell proliferation, migration, and invasion than GNFs. Astragaloside Ⅳ treatment strongly inhibited the proliferation-, migration- and invasion-promoting capacities of GCAFs. Compared with GNFs, GCAFs expressed a lower level of mir-RNA-214 and a higher level of microRNA-301a. Astragaloside Ⅳ treatment significantly up-regulated microRNA-214 expression and down-regulated microRNA-301a expression in GCAFs. Reestablishing the microRNA expression balance subsequently suppressed M-CSF production and secretion, and elevated TIMP2 production and secretion. Consequently, the ability of GCAFs to increase SOX2 and NANOG expression in BGC-823 cells was abolished by astragaloside Ⅳ. These results demonstrate that astragaloside Ⅳ is promisingly a potent therapeutic agent regulating tumor microenvironment.

Research conclusions

This study shows for the first time that astragaloside Ⅳ can effectively inhibit the pathological functions of cancer-associated fibroblasts, and that astragaloside IV is useful for cancer therapy by regulating tumor microenvironment.

Previous studies focused on investigating the abnormal mir-RNA expression in cancer cells. Our results showed that the dysregulation in mir-RNA expression is an important cause of pathological functions of cancer-associated fibroblasts. Correcting the mir-RNA expression dysregulation in cancer associated fibroblasts is a new anti-cancer mechanism of Chinese herbs. Chinese herbs are a valuable source for developing new drugs regulating tumor microenvironment.

The method used in this study was to culture gastric cancer cells with the conditioned medium from GCAFs. In the culture system, GCAFs promoted the malignant behaviors of gastric cancer cells. Astragaloside Ⅳ inhibited the malignancy-promoting capacity of GCAFs through down-regulating the expression of oncogenic factors and up-regulating the expression of tumor suppressive factors. Also, we found that promoting the generation of gastric cancer stem cells and supporting their malignant phenotypes are an important oncogenic mechanism of GCAFs. Importantly, some Chinese herbs can destroy the niche of gastric cancer stem cells by acting on GCAFs.

Research perspectives

The concept of holism of traditional Chinese medicine is valuable for guidance of developing new anti-cancer drugs. Non-toxic Chinese herbs may have great values in cancer therapy by regulating the tumor microenvironment. Chinese herbs that can regulate the tumor microenvironment are a promising resource for the development of novel anti-cancer drugs.

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ORIGINAL ARTICLE

Retrospective Cohort Study

Prevalence and outcomes of pancreatic cystic neoplasms in liver transplant recipients

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Informed consent statement: As this is a large retrospective study of over 800 patients, it was not feasible to obtain informed consent. All patient data are anonymized and therefore risk of identification is low.

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Abstract

AIM

To determine the prevalence, characteristics and clinical course of pancreatic cystic neoplasms (PCNs) in liver transplantation (LT) recipients.

METHODS

We retrospectively studied consecutive patients

who underwent LT between January 1998 to April 2016. Clinical and laboratory data were obtained from patient medical records. Imaging findings on computed tomography and magnetic resonance cholangiopancreatography were reviewed by two radiologists.

RESULTS

During the study period, 872 patients underwent cadaveric LT. Pancreatic cysts were identified in 53/872 (6.1%) and 31/53 (58.5%) were PCNs [28 intraductal papillary mucinous neoplasm (IPMN), 2 mucinous cystic neoplasm (MCN), 1 serous cystadenoma]. Patients with PCNs exhibited less male predominance (55% *vs* 73%, $P = 0.03$) compared to patients without pancreatic cysts. Thirteen patients (42%) were diagnosed with PCN pre-LT while 18 patients (58%) developed PCN post-LT. The median size of PCNs was 13mm [interquartile range (IQR) 10-20 mm]. All IPMNs were side-branch type. Most PCNs were found in the head and body of pancreas (37% each), followed by the tail (25%). Five patients underwent further evaluation with endoscopic ultrasound. Progress imaging was performed on 81% of patients. PCNs remained stable in size and number in all but 2 patients. During a median follow up of 39 mo (IQR 26-58 mo), the 2 (6%) patients with MCN underwent pancreatectomy. No PCN patient developed pancreatic adenocarcinoma, while 5 died from illnesses unrelated to the PCN. Among patients without PCN, 1/841 (0.1%) developed pancreatic adenocarcinoma.

CONCLUSION

The prevalence of PCNs in LT recipients was similar to the general population (3.6%, 31/872). Side-branch IPMNs do not appear to have accelerated malignant potential in post-LT patients, indicating the current surveillance guidelines are applicable to this group.

Key words: Pancreatic cystic neoplasm; Intraductal papillary mucinous neoplasm; Mucinous cystic neoplasm; Liver transplantation; Pancreatic adenocarcinoma; Immunosuppression

Core tip: The prevalence of pancreatic cystic neoplasms (PCNs) in liver transplantation (LT) recipients is similar to that of the general population and PCNs do not appear to behave more aggressively in this setting. Our results suggest that current surveillance guidelines can be safely applied in the immunosuppressed LT population.

Liu K, Joshi V, van Camp L, Yang QW, Baars JE, Strasser SI, McCaughan GW, Majumdar A, Saxena P, Kaffes AJ. Prevalence and outcomes of pancreatic cystic neoplasms in liver transplant recipients. *World J Gastroenterol* 2017; 23(48): 8526-8532 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i48/8526.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i48.8526

INTRODUCTION

Pancreatic cystic lesions are a common incidental finding in asymptomatic adults undergoing abdominal imaging, with a quoted prevalence of 2%-13%[1,2]. While 80% of pancreatic cysts are non-neoplastic (simple cysts, pseudocysts, retention cysts), some are considered to be premalignant lesions and require surveillance[3]. These cysts with neoplastic potential are classified as pancreatic cystic neoplasms (PCNs) of which the two most common types are intraductal papillary mucinous neoplasm (IPMN) and mucinous cystic neoplasm (MCNs). Currently, the assessment of malignant progression risk in PCNs mainly focuses on cyst type and characteristics including size, presence of a solid component and main pancreatic duct dilatation[4,5].

The prevalence of PCNs has been reported to be twice as common in cirrhotic patients compared to the general population while their risk of pancreatic cancer is increased five-fold[6,7]. Liver transplantation (LT) is the treatment of choice for appropriately selected patients with end-stage liver cirrhosis. Limited data exist regarding whether LT and its ensuing immunosuppression influences the incidence and risk of malignant progression of PCNs. Furthermore, patient populations and immunosuppression protocols differ between LT centers worldwide, which may influence the prevalence and natural history of PCNs. LT recipients undergo regular cross-sectional abdominal imaging [computed tomography (CT) and/or magnetic resonance imaging (MRI)] as a part of hepatocellular carcinoma (HCC) screening and LT work up. The aim of this study was to determine the prevalence, characteristics and clinical course of PCNs in LT recipients in a large Australian LT center.

MATERIALS AND METHODS

Patients

A retrospective review of medical records for all LT recipients from January 1998 to April 2016 at Royal Prince Alfred Hospital, a quaternary state-wide LT referral center in Sydney, Australia was performed. Patient demographics, etiology of liver disease, and radiology reports of CT or MRI scans were reviewed by electronic medical records. Those with pancreatic lesions reported on abdominal imaging had their scans reviewed and further data were collected including: clinical findings including history of pancreatitis, laboratory values, histology reports, and results of fluid analysis, if available. Clinical pancreatitis was defined by the presence of two of the following three criteria: (1) characteristic abdominal pain; (2) elevated ($\geqslant$ 3 times the upper limit of normal) levels of serum amylase and/or lipase; or (3) characteristic findings on a CT scan[8]. Pancreatitis was attributed to the PCN if no other etiology was present *e.g.* alcohol abuse (alcoholic pancreatitis) or choledocholithiasis (gallstone pancreatitis). Patients with known chronic pancreatic disease were excluded. Outcomes measures included

growth of the PCN, the development of pancreatic adenocarcinoma, and requirement for surgical resection and mortality. Patients were otherwise followed until the date of their last abdominal CT or MRI scan. The study protocol was approved by the Sydney Local Health District Human Research Ethics Committee (X15-0426 and LNR/15/RPAH/570).

Imaging analysis of pancreatic lesions

Abdominal imaging (either CT, MRI or both), for all patients with a pancreatic cystic lesion was reviewed by two independent radiologists. CT was performed on either GE 16 or 64 slice or Siemens SOMATOM Plus 4 spiral scanners depending on year. Either single portal venous phase (70 s after injection) or multiphase images were obtained with pre-contrast, arterial (40 s after injection), portal-venous (70 s after injection) and delayed (five minutes after injection). Magnetic resonance cholangiopancreatography (MRCP) was performed on either Siemens (3T) or GE (1.5T) magnets depending on inpatient or outpatient status. The institution's MRCP standard protocol for both magnets includes axial and coronal T2-weighted single shot fast spin echo breath-hold, respiratory triggered axial T2-weighted fast recovery fast spine echo (FRFSE), heavily-weighted axial T2 with fat suppression, axial T1-weighted pancreas in and out of phase, axial T1 pancreas fat suppressed, three-dimensional and thick slab MRCP respiratory triggered. Where indicated, gadolinium (Gadovist, Bayer Group, Germany) was administered at a rate of 1.5mL/s with pancreatic (20-40 s after injection), portal venous (65-85 s after injection) and delayed phase (three minutes and five minutes after injection) sequences obtained. Review of images was performed on a PACS-integrated workstation (Centricity RA 1000, GE Healthcare, Little Chalfont, United Kingdom).

Characterization of pancreatic cysts on imaging was performed using generally accepted imaging criteria[9,10]. A presumed imaging diagnosis of a mucinous cystadenoma was made if the cyst was unilocular with or without mild septations, located in the body or tail and with the signal intensity of simple fluid on MRI. If a unilocular or multilocular cyst demonstrated communication with a non-dilated main pancreatic duct, then a side-branch IPMN was diagnosed. If the cyst was comprised of numerous small cysts, with or without central calcification and no ductal communication on imaging, then a favored diagnosis of serous cystadenoma was given. A lesion was classified as a pseudocyst if a unilocular cyst was seen with layering debris and/or features of acute or chronic pancreatitis. In the absence of the above imaging features, a diagnosis of an undifferentiated pancreatic cyst was made. Final cyst diagnoses were also governed by results of cyst fluid analysis, cytology or histology in patients who underwent endoscopic ultrasound (EUS) with cyst aspiration or resection.

PCNs were evaluated for cyst size, multiplicity, location in the pancreas (head, body and tail). For patients with multiple PCNs, data from the largest cyst were recorded. The presence of worrisome features was also assessed including: size > 30 mm, thickened or enhancing cyst walls, dilated pancreatic duct, or solid component to the cyst[5]. The evolution of PCNs was assessed for changes in cyst size and characteristics in patients who underwent subsequent imaging.

Statistical analysis

Categorical data was analyzed by the Pearson χ^2 test or Fisher's Exact test where appropriate. The Mann Whitney *U* test was used to analyze continuous data with non-normal distribution. Statistical analyses were performed using SPSS v.22.0 software (IBM, Armonk, NY, United States). A result was considered statistically significant if $P < 0.05$.

RESULTS

Patient characteristics

During the study period, 872 patients underwent cadaveric LT. Median age at time of LT was 60 years [interquartile range (IQR) 55-66 years]. The majority of patients were male (630/872, 72%). The most common cause of liver disease was hepatitis C (35%), followed by alcohol (15%), hepatitis B (13%), primary sclerosing cholangitis (9%), and non-alcoholic fatty liver disease (4%). HCC complicated the liver disease in 14% (126/872) of patients pre-LT.

Pancreatic cysts were identified in 53/872 (6.1%) of which 31 (3.6%) were PCNs (28 IPMNs, two MCNs, one serous cystadenoma). The other cystic lesions included: undifferentiated cyst (13/53), simple cysts (6/53) and pseudocysts (2/53). All PCNs were discovered incidentally (71% on CT, 29% on MRCP), with no prior history of pancreatitis. All undifferentiated cysts were < 30 mm in size, well-defined, round and exhibited a homogenous fluid signal. Most PCNs (22/31, 71%) were diagnosed in the last five years of the study period while 27/31 (87%) were diagnosed in the last ten years. Thirteen patients (42%) were diagnosed with PCN pre-LT while 18 patients (58%) developed PCN post-LT (Figure 1). The median age at cyst discovery was 59 years (IQR 56-62 years). Patients with PCNs exhibited less male predominance (55% *vs* 73%, $P = 0.03$) and were comparable in terms of age at time of LT (median 62 years *vs* 60 years, $P = 0.18$) compared to those without PCNs. There were no significant differences in etiology of liver disease in patients with and without PCN ($P = 0.65$). Presence of HCC also did not differ between the two groups (10% PCN group *vs* 15% no PCN, $P = 0.44$). The median white cell count in patients at time of PCN discovery was 5.0×10^9/L (IQR 4.1-7.1 $\times 10^9$/L, normal laboratory range 4.0-10.0 $\times 10^9$/L). Patient

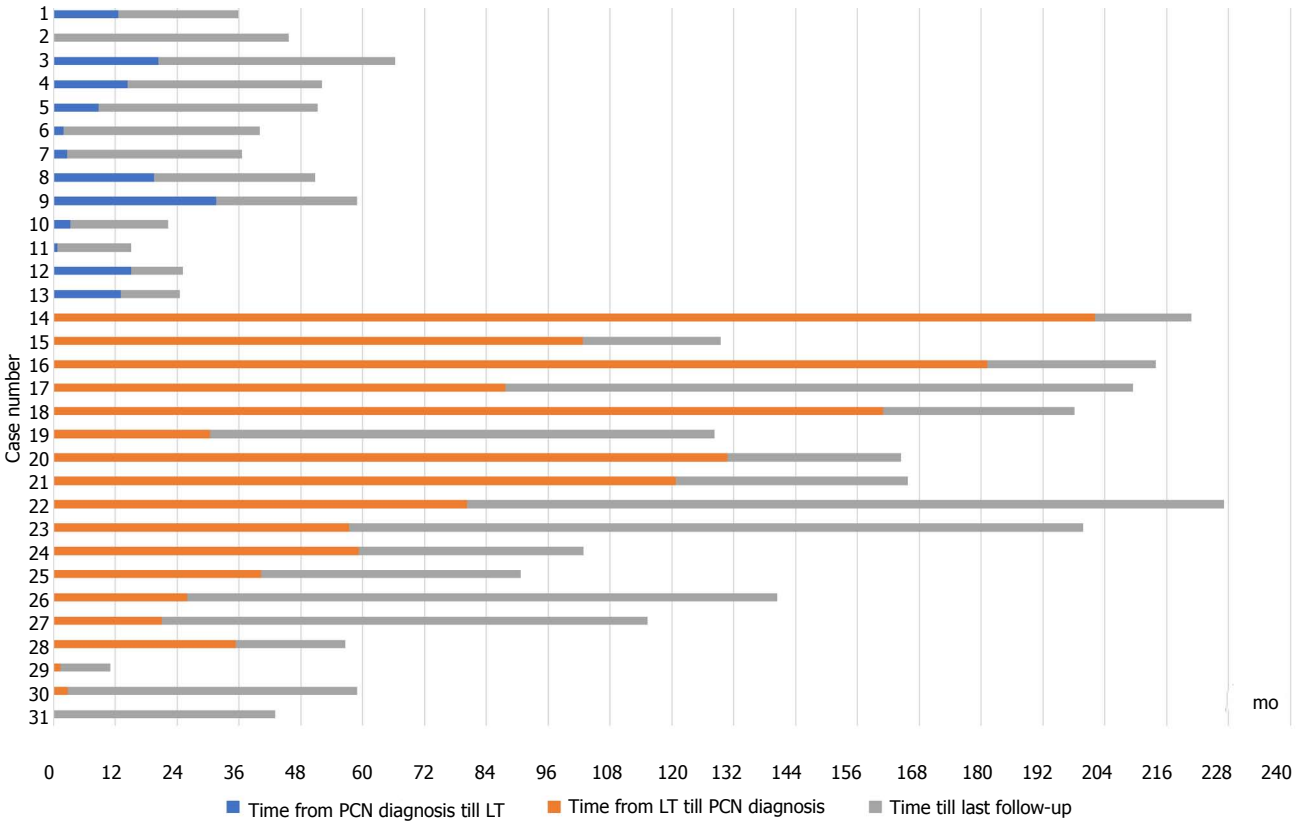

Figure 1 Time points for pancreatic cystic neoplasms diagnosis, liver transplantation and follow-up in the 31 patients with pancreatic cystic neoplasms.

characteristics comparing patients with and without PCNs are shown in Table 1.

Pancreatic cystic neoplasm characteristics

The majority of patients with PCNs had multiple cystic lesions (20/31, 65%). Most PCNs occurred in the head of pancreas (37%) and body (37%) followed by the tail (25%). The median size of PCNs at initial diagnosis was 13 mm (IQR 10-20 mm). All 28 IPMNs were side-branch type. No PCNs exhibited worrisome features in the form of having a solid component, thickened or enhancing wall or pancreatic duct dilatation. However, three PCNs were > 30 mm in size: an IPMN at 35 mm, a MCN at 40 mm and a serous cystadenoma at 35 mm. Five patients (16%) underwent further evaluation with EUS evaluation which confirmed the suspected lesion seen on imaging (three side-branch IPMNs, two MCNs). Only one patient underwent cyst aspiration with cytology and fluid analysis. At time of cyst discovery, the median serum Ca 19.9 was 48 U/mL (IQR 35.5-112.5 U/mL, reference range < 37 U/mL) and the median serum lipase was 48 U/L (IQR 44-75 U/L, reference range 13-60 U/L).

Outcomes

Patients with PCN were followed up for a median duration of 40 mo from date of cyst discovery (IQR 27-59 mo). Surveillance with CT, MRCP and/or EUS was performed on 25/31 (81%) patients. Two of the remaining six patients who did not have surveillance had only recently been diagnosed with PCNs and were not yet due for repeat imaging according to guidelines[5]. Of the patients with subsequent imaging, PCNs remained stable in size in 20/25 (80%) patients, reduced in size in 3/25 (12%) patients and increased in size in 2/25 (8%) patients. No patients developed clinical pancreatitis during follow-up.

No patient with a PCN developed pancreatic adenocarcinoma. Two patients (6%) underwent surgical resection of their PCN. Both patients had a MCN in the tail of the pancreas and underwent distal pancreatectomy and splenectomy without operative complications. The first patient was a 38-year-old woman whose MCN was discovered on routine LT assessment (for primary biliary cholangitis) initially measuring 9 mm in diameter. This subsequently grew to 17 mm and 26 mm at two and five years post-LT, respectively, which prompted the decision to resect. Histopathology revealed a MCN with intermediate dysplasia. The second patient, a 61-year-old woman, had her MCN discovered on LT assessment (for primary sclerosing cholangitis) initially measuring 40mm in diameter. The lesion grew further to 47 mm and was subsequently resected post-LT with histopathology showing a MCN with low grade dysplasia (Figures 2 and 3). Surgical resection was not considered for the other two aforementioned patients with PCNs > 30 mm in size (one 35 mm IPMN, one 35 mm serous cystadenoma)

Table 1 Demographics of liver transplant recipients with and without pancreatic cystic neoplasms

Characteristic	All Patients, *n* = 872	Patients with PCN, *n* = 31	Patients without PCN, *n* = 841	*P* value
Age at LT (yr)	60 (55-66)	62 (59-66)	60 (54-66)	0.18
Age at cyst PCN discovery (yr)	N/A	59 (56-62)	N/A	N/A
Males	630 (72)	17 (56)	613 (73)	0.03[a]
Cause of liver disease				0.65
Hepatitis C	304 (35)	8 (26)	296 (35)	
Alcoholic liver disease	134 (15)	2 (7)	132 (16)	
Hepatitis B	109 (13)	3 (10)	106 (13)	
Primary sclerosing cholangitis	79 (9)	4 (13)	75 (9)	
Non-alcoholic fatty liver disease	39 (4)	3 (10)	36 (4)	
Primary biliary cholangitis	38 (4)	2 (7)	36 (4)	
Autoimmune hepatitis	31 (4)	2 (7)	29 (3)	
Cryptogenic	19 (2)	1 (3)	18 (2)	
Other	118 (14)	6 (19)	112 (13)	
Concomitant HCC	126 (14)	3 (10)	123 (14)	0.44

The data are shown in *n* (%) and median (interquartile range), [a]$P < 0.05$. HCC: hepatocellular carcinoma; PCN: pancreatic cystic neoplasm; LT: liver transplantation.

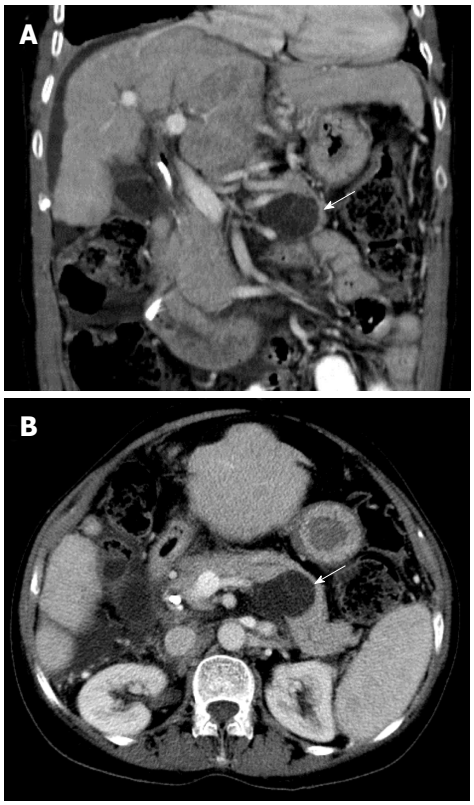

Figure 2 Coronal (A) and axial (B) computed tomography images in portal venous phase demonstrate a well-defined low density lesion in the body/tail of the pancreas measuring 40 mm in maximum transverse dimensions (white arrows). Thin septa are demonstrated within the cyst.

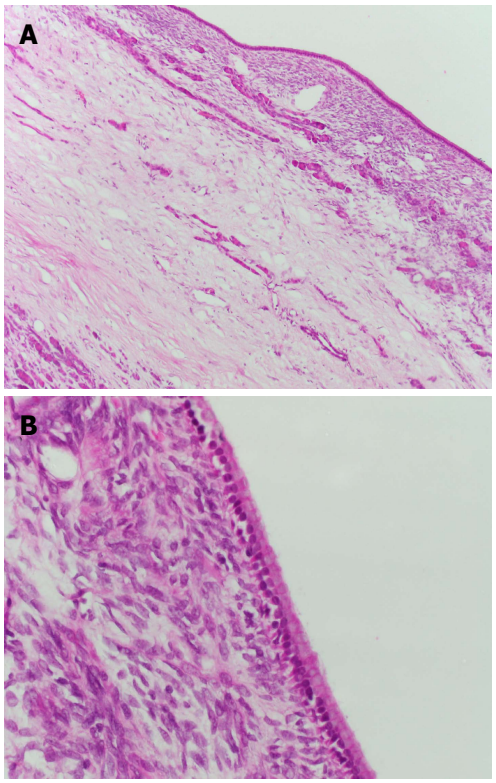

Figure 3 Lower power 100 × magnification view (A) and higher power 400 × magnification view (B) of a mucinous cystic neoplasm with low grade dysplasia. The cyst is lined by columnar mucinous epithelium with underlying ovarian-type stroma (hematoxylin-eosin stain).

due to significant medical co-morbidities.

During the follow-up period, five patients died due to acute pathologies or post-LT complications unrelated to their PCNs. Incidentally, among the patients without PCN, 1/841 (0.1%) developed metastatic pancreatic adenocarcinoma eight years after LT for hepatitis C and HCC.

DISCUSSION

In this large cohort study of 872 LT patients, the prevalence of PCNs was found to be 3.6%. This is similar to the rate of incidental PCNs in the general population found on abdominal imaging for other indications[1,2]. Although PCNs were commonly encountered, cyst size > 30 mm (10%) and cyst enlargement on surveillance

imaging (6%) were uncommon, while the presence of other worrisome features was not seen. Accordingly, the rate of pancreatic adenocarcinoma in our study was low with no cases seen among patients with PCNs.

Patients with cirrhosis have been shown to be immune suppressed[11,12]. Furthermore, patients who undergo LT remain on life-long immunosuppressive drugs. Based on our results, the immunosuppressive effect of cirrhosis pre-LT and anti-rejection medications post-LT does not seem to alter the natural course of PCNs nor increase the risk of malignant transformation. Hence current guidelines for surveillance of PCNs can likely be safely applied in the LT population.

Our findings are consistent with other studies of PCNs in the LT setting. Vidhyarkorn *et al*[13] reported a prevalence of 3.9% (70/1778 patients) for pancreatic cystic lesions demonstrated on CT or MRI among LT recipients. Despite showing an increase in the average size and number of PCNs by 4.5mm and 1.4, respectively during the follow-up period, only one patient developed a mixed acinar-neuroendocrine carcinoma 9 years post-LT. In a French study of LT waitlist patients, IPMN was identified in 6.6% (14/212 patients) using MRI. No worrisome features, pancreatic resections or pancreatic adenocarcinomas were observed[14]. In contrast, Lennon *et al*[15] followed 297 patients with side-branch IPMN and found high-risk features in up to 17%, potentially related to a higher proportion of patients undergoing EUS. There was no significant difference in risk of progression when comparing 23 patients who had undergone LT versus 274 patients without a history of immunosuppression. All the above authors concluded that LT does not appear to increase the risk of malignancy in patients with PCNs despite immunosuppression.

The strength of our study is the large cohort of LT patients (872 patients over 18 years) with a relatively large number of PCNs (28 IPMNs versus 14-23 in other studies). While previous studies in the LT population have focused on IPMNs[13-15], our study is the first to describe MCNs in this setting. The two patients with MCNs were middle-aged females with single lesions located in the tail of the pancreas - hallmark characteristics of MCNs in the general population[3,4]. Surgical resection of MCNs is recommended for all surgically fit patients[5]. Both patients with MCN in our study underwent pancreatectomy post-LT with significant dysplasia seen on histopathology, however it remains unknown whether the behavior of MCNs is altered by immunosuppression. Clearly, further studies with a focus on MCNs in immunosuppressed patients are needed.

A limitation of the present study is its retrospective nature which impacts on the accuracy and completeness of the data, as well as the potential influence of length time bias. However, retrospective data collection provided the advantage of capturing patients with PCNs over an 18-year period of LT. Furthermore, our study outcomes of PCN growth, pancreatic adenocarcinoma, surgical resection and mortality are hard endpoints which minimizes other potential biases. Although the imaging of patients without PCNs described on their initial radiological reports was not re-reviewed, all scans at our institution are reported formally by at least one experienced radiologist. Our data also suggest that awareness and identification of PCNs is increasing with most (72%) of our PCNs diagnosed in the last five years of the study period. However, the recency of these diagnoses has meant that our follow-up period in this study was relatively short (median 40 mo) which limited our ability to capture events such as cyst growth and malignancy transformation.

In conclusion, the prevalence of PCNs was 3.6% in our cohort of Australian LT patients. This is similar to that of the general population. All PCNs were discovered incidentally and over half of PCNs developed post-LT. All patients with IPMN exhibited a benign course in this study while 2/2 MCN (6% of PCNs) patients underwent surgical resection. No cases of pancreatic adenocarcinoma occurred in patients with pre-existing PCNs. These results suggest that current surveillance guidelines can likely be safely applied in the LT population.

ARTICLE HIGHLIGHTS

Research background

Pancreatic cystic neoplasms (PCNs) are a common incidental finding and their behaviour in liver transplantation (LT) recipients has not been well studied.

Research motivation

By studying PCNs in the LT setting, we can observe whether the immunosuppressive effects of cirrhosis (pre-LT) and anti-rejection medications (post-LT) have any impact on PCN development and progression. This would provide important information on whether current screening and surveillance for the general population can be safely applied to these patients.

Research objectives

We aimed to determine the prevalence, characteristics and clinical course of PCNs in LT recipients.

Research methods

Consecutive patients who underwent LT between January 1998 to April 2016 were retrospectively studied. Clinical and laboratory data and imaging findings on computed tomography and/or magnetic resonance cholangiopancreatography were assessed.

Research results

The prevalence of PCNs was 3.6% in our cohort of Australian LT patients which is similar to that of the general population. All PCNs were discovered incidentally and over half of PCNs developed post-LT. Only 3/31 (10%) PCNs exhibited worrisome features. All patients with intraductal papillary mucinous neoplasm (IPMN) exhibited a benign course in this study while 2/2 mucinous cystic neoplasm (MCN) (6% of PCNs) patients underwent surgical resection. No cases of pancreatic adenocarcinoma occurred in patients with pre-existing PCNs.

Research conclusions

This is the first cohort study of PCNs in Australian LT recipients. It is also the first study to describe MCNs in the LT setting. Our results suggest that current

surveillance guidelines can likely be safely applied in the LT population. Our data also suggest that awareness and identification of PCNs is increasing with most being identified in the last five years of our study period.

Research perspectives

It would be important to pool experience from multiple centers to improve our knowledge of the behavior of non-IPMN PCNs in the context of immunosuppression.

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ORIGINAL ARTICLE

Retrospective Study

Analysis of 12 variants in the development of gastric and colorectal cancers

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Author contributions: Cavalcante GC and Amador MAT performed the laboratory experiments; Cavalcante GC, Carvalho DC and Andrade RB drafted the manuscript; Pereira EEB, Fernandes MR and Costa DF provided the samples for the study; Cavalcante GC, Ribeiro dos Santos AM and Santos S performed the data analysis; Santos S reviewed the statistical methods of the study; Santos NPC, Assumpção PP and Ribeiro dos Santos Â made substantial contributions to the study design and the manuscript; Cavalcante GC and Santos S designed the study and wrote the final version of the paper.

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Abstract

AIM

To evaluate the relation between 12 polymorphisms and the development of gastric cancer (GC) and colorectal cancer (CRC).

METHODS

In this study, we included 125 individuals with GC diagnosis, 66 individuals with CRC diagnosis and 475 cancer-free individuals. All participants resided in the North region of Brazil and authorized the use of their samples. The 12 polymorphisms (in *CASP8*, *CYP2E1*, *CYP19A1*, *IL1A*, *IL4*, *MDM2*, *NFKB1*, *PAR1*, *TP53*, *TYMS*, *UGT1A1* and *XRCC1* genes) were genotyped in a single PCR for each individual, followed by fragment analysis. To avoid misinterpretation due to population substructure, we applied a previously developed set of 61 ancestry-informative markers that can also be genotyped by multiplex PCR. The statistical analyses were performed in Structure v.2.3.4, R environment and SPSS v.20.

RESULTS

After statistical analyses with the control of confounding factors, such as genetic ancestry, three markers (rs79071878 in *IL4*, *rs3730485* in *MDM2* and *rs28362491* in *NFKB1*) were positively associated with the development of GC. One of these markers (rs28362491) and the marker in the *UGT1A1* gene (rs8175347) were positively associated with the development of CRC. Therefore, we investigated whether the joint presence of the deleterious alleles of each marker could affect the development of cancer and we obtained positive results in all analyses. Carriers of the combination of alleles RP1 + DEL (rs79071878 and rs28361491, respectively) are at 10-times greater risk of developing GC than carriers of other combinations. Similarly, carriers of the combination of DEL + RARE (rs283628 and rs8175347) are at about 12-times greater risk of developing CRC than carriers of other combinations.

CONCLUSION

These findings are important for the comprehension of gastric and CRC development, particularly in highly admixed populations, such as the Brazilian population.

Key words: Inflammatory processes; Immune response; Genomic and cellular stability; Gastric cancer; Colorectal cancer; Amazon

Core tip: Gastric cancer and colorectal cancer (CRC) are among the most incident and aggressive types of cancer in Brazil, especially in the Amazon region. Alterations in genes involved in pathways of immune responses, inflammatory processes or genomic and cellular stability may generate cellular imbalances and lead to tumorigenesis. Therefore, it is vital to understand the effect of different alleles in the development of gastric and CRC, which could contribute to the early detection of these types of cancer, increasing the survival chances of the patient.

Cavalcante GC, Amador MAT, Ribeiro dos Santos AM, Carvalho DC, Andrade RB, Pereira EEB, Fernandes MR, Costa DF, Santos NPC, Assumpção PP, Ribeiro dos Santos Â, Santos S. Analysis of 12 variants in the development of gastric and colorectal cancers. *World J Gastroenterol* 2017; 23(48): 8533-8543 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i48/8533.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i48.8533

INTRODUCTION

Cancer is one of the main causes of death worldwide[1]. In Brazil, it is considered a severe problem of public health, and in the North region of this country gastric cancer (GC) and colorectal cancer (CRC) are among the three most incident and aggressive types of cancer[2].

Carcinogenesis is a multifactorial process. Gastritis and colitis have been related to the development of GC[3,4] and CRC[5,6], respectively, but they are not determinant. Infection by *Helicobacter pylori*, one of the most common human infectious agents, is also very important for the development of gastritis and GC[7]. However, it should not be considered the only cause for development of this type of cancer[8]. Genetics also play a major role in the carcinogenesis, and there is much to be discovered regarding this subject.

Genes involved in important pathways, such as inflammatory processes, metabolism of carcinogens, cell stability and hormonal pathways, are possible susceptibility factors to cancer[9-14]. Alterations in these genes may generate imbalances in such pathways and trigger tumor development. In this study, we investigated the following 12 polymorphisms of important genes of these pathways: *CASP8* (rs3834129), *CYP2E1* (96 bp-deletion), *CYP19A1* (rs11575899), *IL1A* (rs3783553), *IL4* (rs79071878), *MDM2* (rs3730485), *NFKB1* (rs28362491), *PAR1* (rs11267092), *TP53* (rs17878362), *TYMS* (rs16430), *UGT1A1* (rs8175347) and *XRCC1* (rs3213239).

These genes and polymorphisms have been studied in association with various types of cancer in different populations, *e.g*, breast cancer[15-19], bladder cancer[20], endometrial cancer[21], acute lymphoblastic leukemia[22], chronic lymphoblastic leukemia[23], oral carcinoma[24,25], lung cancer[26], nasopharyngeal cancer[27], thyroid cancer[28], hepatocellular carcinoma[29], GC[30-39] and CRC[40-50]. Therefore, these markers were chosen based on the importance of each gene as a potential influencing factor in the susceptibility of tumor development. All are functional polymorphisms that correspond to insertion/deletion (INDEL) of small DNA fragments and can be analyzed in a single multiplex PCR, which makes it a cheap and accessible methodology that could be used in different laboratories worldwide.

Thus, the aim of this work was to investigate the association between 12 polymorphisms in genes related to pathways of immune/inflammatory response (*CYP2E1*, *CYP19A1*, *IL1A*, *IL4*, *NFKB1* and *PAR1*) and cellular or genomic stability (*CASP8*, *MDM2*, *TP53*, *TYMS*, *UGT1A1* and *XRCC1*) and the development of GC

Table 1 Technical characteristics of the studied markers

Gene	ID	Type	Length, bp	Primers	Amplicon, bp
CASP8	rs3834129	INDEL	6	F-5′CTCTTCAATGCTTCCTTGAGGT3′ R-5′CTGCATGCCAGGAGCTAAGTAT3′	249-255
CYP2E1	-	INDEL	96	F-5′TGTCCCAATACAGTCACCTCTTT3′ R-5′GGCTTTTATTTGTTTTGCATCTG3′	303-399
CYP19A1	rs11575899	INDEL	3	F-5′TGCATGAGAAAGGCATCATATT3′ R-5′AAAAGGCACATTCATAGACAAAAA3′	122-125
IL1A	rs3783553	INDEL	4	F-5′TGGTCCAAGTTGTGCTTATCC3′ R-5′ACAGTGGTCTCATGGTTGTCA3′	230-234
IL4	rs79071878	VNTR	70	F-5′AGGGTCAGTCTGGCTACTGTGT3′ R-5′CAAATCTGTTCACCTCAACTGC3′	147/217/287
MDM2	rs3730485	INDEL	40	F-5′GGAAGTTTCCTTTCTGGTAGGC3′ R-5′TTTGATGCGGTCTCATAAATTG3′	192-232
NFKB1	rs28362491	INDEL	4	F-5′TATGGACCGCATGACTCTATCA3′ R-5′GGCTCTGGCATCCTAGCAG3′	366-370
PAR1	rs11267092	INDEL	13	F-5′AAAACTGAACTTTGCCGGTGT3′ R-5′GGGCCTAGAAGTCCAAATGAG3′	265-277
TP53	rs17878362	INDEL	16	F-5′GGGACTGACTTTCTGCTCTTGT3′ R-5′GGGACTGTAGATGGGTGAAAAG3′	148-164
TYMS	rs16430	INDEL	6	F-5′ATCCAAACCAGAATACAGCACA3′ R-5′CTCAAATCTGAGGGAGCTGAGT3′	213-219
UGT1A1	rs8175347	VNTR	2	F-5′CTCTGAAAGTGAACTCCCTGCT3′ R-5′AGAGGTTCGCCCTCTCCTAT3′	133/135/137/139
XRCC1	rs3213239	INDEL	4	F-5′GAACCAGAATCCAAAAGTGACC3′ R-5′AGGGGAAGAGAGAGAAGGAGAG3′	243-247

F: Forward; INDEL: Insertion/deletion; R: Reverse; VNTR: Variable number tandem repeat.

and CRC in a population in Northern Brazil. In addition, we investigated the influence of genetic ancestry in the development of these types of cancer in the studied population.

MATERIALS AND METHODS

Samples

In this study, we included three groups: (1) 125 individuals with GC diagnosis; (2) 66 individuals with CRC diagnosis; and (3) 475 cancer-free individuals that were considered the control group. The cancer-free individuals did not have personal or familial histories of any kind of cancer and they did not show any symptoms or signs of cancer. All participants resided in Belém, which is a city located in the Northern region of Brazil, and signed an informed consent, with approval by the Committee for Research Ethics of Hospital João de Barros Barreto under Protocol No. CAAE 25865714.6.0000.0017.

DNA Extraction and Quantification

Samples of peripheral blood were collected from all individuals of the study and the DNA extraction was performed accordingly[51]. DNA quantification was performed with NanoDrop 1000 spectrophotometer (Thermo Scientific, Wilmington, DE, United States).

Genotyping

Samples were then submitted to multiplex PCR and fragment analysis in an ABI PRISM 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA, United States) according to the protocol described[22]. Technical characteristics of the studied markers are presented in Table 1. Due to the high level of genetic admixture in the studied population, we applied a panel of 61 ancestry-informative markers to avoid misinterpretations caused by population substructure, as described[52,53].

Statistical Analyses

Statistical analyses were conducted with different programs. Ancestry analyses were performed in Structure v.2.3.4[54], and tests concerning the genotyping analyses (Student's *t*-test, Pearson's χ^2 test, Mann-Whitney test and logistic regression) were performed in R[55] and in SPSS v.20.0 (IBM Corp., Armonk, NY, United States).

The genotype distribution was assessed as established by Hardy-Weinberg equilibrium (HWE), with post-test correction by the Bonferroni method for multiple tests. *P*-value $\leqslant$ 0.05 was considered statistically significant.

RESULTS

All population distributions were according to HWE ($P > 0.004$) for the analyzed polymorphisms, with the exception of the *IL4* marker in the control group. The observed deviation seems to be due to a significant increase of heterozygotes in this population ($P = 0.0003$).

We also investigated the possible confounding factors of age, sex and genetic ancestry. Table 2 shows these results. When considered statistically significant in the comparison between groups (GC patients *vs*

Table 2 Demographic data for patient and control groups

Variable	GC	CRC	Control	*P*-value	
				GC *vs* Control	CRC *vs* Control
n	120	64	475	-	-
Age, yr[1]	57.02 ± 1.29	52.84 ± 1.90	55.59 ± 0.91	0.522	0.294
Sex, % of male/female	55.0/45.0	45.3/54.70	34.7/65.3	0.000[3]	0.098
European ancestry[2]	0.42 ± 0.01	0.53 ± 0.02	0.47 ± 0.01	0.002[3]	0.003[3]
African ancestry[2]	0.26 ± 0.01	0.20 ± 0.01	0.23 ± 0.01	0.071	0.016[3]
Amerindian ancestry[2]	0.32 ± 0.01	0.27 ± 0.02	0.30 ± 0.01	0.114	0.100

[1]Values are expressed as mean ± SD. Significance was obtained by Student's *t*-test; [2]Values are expressed as mean ± SD. Significance was obtained by Mann-Whitney test; [3]Statistically significant. CRC: Colorectal cancer; GC: Gastric cancer.

cancer-free individuals, and CRC patients *vs* cancer-free individuals; $P \leq 0.05$), such characteristics were controlled in the logistic regression that assessed whether there are significant differences in the following tests: (1) carriers of INS/INS genotype *vs* carriers of other genotypes (INS/DEL + DEL/DEL); (2) carriers of DEL/DEL genotype *vs* carriers of other genotypes (INS/DEL + INS/INS); and (3) additive effect of the alleles (joint presence of the significant alleles from tests Ⅰ and Ⅱ).

In the analyses with GC patients, positive associations were observed for the markers rs79071878 (*IL4* gene), rs3730485 (*MDM2* gene) and rs28362491 (*NFKB1* gene) after correction of confounding factors for this group (sex and European ancestry) (Table 3). For rs79071878, carriers of the RP1/RP1 genotype have approximately 3-fold increased chances of developing GC than carriers of other genotypes (RP1/RP1 + RP1/RP2) [$P = 0.002$; odds ratio (OR) = 2.857; 95% confidence interval (CI) = 1.490-5.479]. For rs3730485, INS/INS genotype shows a protection effect for the development of GC in comparison with different genotypes ($P = 0.021$; OR = 0.409; 95%CI: 0.192-0.872). For rs28362491, carriers of the DEL/DEL genotype have more chances of developing GC than carriers of the other genotypes ($P = 0.006$; OR = 2.918; 95%CI: 1.352-6.298).

In the analyses with CRC patients, markers rs28362491 (*NFKB1* gene) and rs8175347 (*UGT1A1* gene) showed positive association after the correction of confounding factors (European and African ancestries) (Table 4). Similar to the result for GC, carriers of the DEL/DEL genotype for rs28362491 should present more chances of developing CRC in comparison to carriers of other genotypes ($P = 0.006$; OR = 3.732; 95%CI: 1.451-9.599). For rs8175347, which has multiple alleles (*1, *28, *36 and *37), our results show that 8% of the CRC patients and 0.6% of the cancer-free individuals carry at least one of the rare alleles (*36 and *37). Comparing both groups, we observed that such allele presence could lead to almost 13-fold increased chances of developing CRC ($P = 0.001$; OR = 12.849; 95%CI: 2.906-56.817).

In addition, we analyzed whether the joint presence of the alleles that were statistically significant when in homozygosis (RP1 allele of rs79071878, INS allele of rs3730485, DEL allele of rs28362491 and *36 and *37 alleles in rs8175347) may affect the development of GC and CRC. After controlling for the confounding factors, we obtained statistically significant results for both GC ($P = 0.004311$) and CRC ($P = 3.52 \times 10^{-6}$) analyses. These findings are shown in Figure 1 for GC and in Figure 2 for CRC.

We highlight some positive associations of these alleles due to the absence of neutral effect (logOR = 0 or OR = 1) in the 95%CI for GC [IL4(RP1): OR = 3.068, 95%CI: 1.036-9.088; NFKB1(DEL): OR = 3.414, 95%CI: 1.347-8.654; IL4(RP1) + NFKB1(DEL): OR = 10.475, 95%CI: 4.845-22.624); IL4(RP1) + NFKB1(DEL) + MDM2(INS): OR = 4.437, 95%CI: 2.948-6.686] and CRC [NFKB1(DEL): OR = 2.552, 95%CI: 2.014-3.238; NFKB1(DEL) + UGT1A1(RARE): OR = 11.929, 95%CI: 1.732-82.187)].

DISCUSSION

In the HWE analysis for the *IL4* marker in the control group, the large amount of heterozygotes could be explained either by selective advantage of the heterozygote or by an intense or continuous process of admixture between populations with different genetic backgrounds. Allele frequencies for this marker vary greatly between the three main populations that contributed to the formation of the Brazilian population; the frequency of the RP2 allele has been described as 0.74 among Europeans, 0.23 among Amerindians and 0.42 among Africans[56]. Due to the recent formation of the Brazilian population, we believe that the admixture process is more fitted to explain the observed disequilibrium.

In the analysis for GC, we observed a positive association between the *IL4* marker (rs79071878) and the development of this type of cancer. This polymorphism is a 70-bp variable number tandem repeat located in an intron of *IL4*, which is an interleukin involved in inflammatory pathways. We did not find other studies relating to this polymorphism and GC, but the increased risk of the development of bladder cancer among the carriers of RP1 allele has been previously described[14,57]. Recently, we reported

Table 3 Genotypic and allelic distributions of the investigated polymorphisms for patients with gastric cancer in comparison to control group

Genotype	GC	Control	*P* value[1]	OR (95%CI)[1]	Genotype	GC	Control	*P* value[1]	OR (95%CI)[1]
CASP8	120	475			RP2/RP2	18 (15.1)	154 (32.5)	0.189	0.673 (0.372-1.216)
DEL/DEL	11 (9.2)	90 (19.0)	0.650	0.892 (0.545-1.461)	Allele RP1	0.54	0.41		
INS/DEL	70 (58.3)	230 (48.4)			Allele RP2	0.46	0.59		
INS/INS	39 (32.5)	155 (32.6)	0.080	1.936 (0.924-4.058)	*NFKB1*	120	473		
Allele DEL	0.38	0.43			DEL/DEL	34 (28.3)	117 (24.7)	0.006[2]	2.918 (1.352-6.298)
Allele INS	0.62	0.57			INS/DEL	71 (59.2)	246 (52.0)		
MDM2	120	475			INS/INS	15 (12.5)	110 (23.3)	0.88	0.959 (0.5662-1.610)
DEL/DEL	13 (10.8)	33 (6.9)	0.199	1.365 (0.849-2.192)	Allele DEL	0.58	0.51		
INS/DEL	46 (38.3)	168 (35.4)			Allele INS	0.42	0.49		
INS/INS	61 (50.9)	274 (57.7)	0.021[2]	0.409 (0.192-0.872)	*PAR1*	113	473		
Allele DEL	0.30	0.25			DEL/DEL	66 (58.4)	273 (57.7)	0.068	0.482 (0.221-1.054)
Allele INS	0.70	0.75			INS/DEL	36 (31.9)	169 (35.7)		
TP53	120	475			INS/INS	11 (9.7)	31 (6.6)	0.949	0.984 (0.601-1.610)
DEL/DEL	91 (75.8)	350 (73.7)	0.999	138214253.0 (0.000)	Allele DEL	0.74	0.76		
INS/DEL	27 (22.5)	116 (24.4)			Allele INS	0.26	0.24		
INS/INS	2 (1.7)	9 (1.9)	0.247	0.708 (0.395-1.270)	*CYP2E1*	116	475		
Allele DEL	0.87	0.86			DEL/DEL	94 (81.0)	398 (83.8)	0.999	276187721.0 (0.000)
Allele INS	0.13	0.14			INS/DEL	21 (18.1)	73 (15.4)		
TYMS	120	475			INS/INS	1 (0.9)	4 (0.8)	0.574	1.193 (0.644-2.212)
DEL/DEL	16 (13.3)	65 (13.7)	0.409	1.231 (0.752-2.015)	Allele DEL	0.90	0.91		
INS/DEL	53 (44.2)	224 (47.2)			Allele INS	0.10	0.09		
INS/INS	51 (42.5)	186 (39.2)	0.867	1.060 (0.536-2.096)	*CYP19A1*	120	475		
Allele DEL	0.35	0.37			DEL/DEL	18 (15.0)	76 (16.0)	0.654	1.127 (0.669-1.897)
Allele INS	0.65	0.63			INS/DEL	67 (55.8)	248 (52.2)		
XRCC1	119	474			INS/INS	35 (29.2)	151 (31.8)	0.415	1.334 (0.667-2.671)
DEL/DEL	10 (8.4)	35 (7.4)	0.346	1.257 (0.781-2.021)	Allele DEL	0.43	0.42		
INS/DEL	48 (40.3)	179 (37.8)			Allele INS	0.57	0.58		
INS/INS	61 (51.3)	260 (54.8)	0.396	0.697 (0.303-1.604)	*UGT1A1*	120	464		
Allele DEL	0.29	0.26			*1/*1	49 (40.8)	206 (44.5)	0.792	1.109 (0.515-2.386)
Allele INS	0.71	0.74			*1/*28	57 (47.5)	209 (45.0)		
IL1A	120	475			*28/*28	12 (10.0)	46 (9.9)	0.445	1.205 (0.746-1.946)
DEL/DEL	17 (14.2)	86 (18.1)	0.626	0.882 (0.522-1.460)	*36/*1	2 (1.7)	3 (0.6)		
INS/DEL	63 (52.5)	246 (51.8)			*36/*37	0 (0.0)	0 (0.0)	0.585	1.941 (0.180-20.973)
INS/INS	40 (33.3)	143 (30.1)	0.143	1.705 (0.835-3.482)	*1/*37	0 (0.0)	0 (0.0)		
Allele DEL	0.40	0.44			Allele *36	0.01	0.01		
Allele INS	0.60	0.56			Allele *1	0.65	0.67		
IL4	119	474			Allele *28	0.34	0.32		
RP1/RP1	28 (23.6)	69 (14.5)	0.002[2]	2.857 (1.490-5.479)	Allele *37	0.00	0.00		
RP1/RP2	73 (61.3)	251 (53.0)							

Data for GC and Control columns are presented as *n* or *n* (%). [1]Analysis of combined genotypes (INS/INS *vs* others, or DEL/DEL *vs* others) with adjusted values for confounding factors (sex and European ancestry) in logistic regression; [2]Statistically significant. GC: Gastric cancer.

that the frequency of the RP1 allele of rs79071878 is higher in the North of Brazil (0.414) than in the other regions of the country (mean = 0.233), probably due to the elevated frequency of this marker in Amerindian populations[56]. Data have revealed that the highest incidence of GC in Brazil occurs in the North region. The apparent overlap between the greater incidence of GC and the elevated frequency of RP1 (rs78071878) in the North region of Brazil seems to corroborate the results that indicate that the carriers of homozygous RP1 allele have greater chances of developing GC than the carriers of other genotypes, possibly due to the close relation of this type of cancer with increased inflammation. More studies involving this polymorphism in different admixed populations in this country are recommended.

As for the polymorphism in the *MDM2* gene (rs3730485), we observed that the carriers of INS/INS genotype have less chances of developing GC than carriers of the other genotypes of this marker. To the best of our knowledge, there are no other studies reporting the positive association of this polymorphism and GC development, but the DEL allele has been shown to be associated with increased risk of developing various types of cancer, *e.g*, hepatocellular carcinoma[29], breast cancer[58], prostate cancer[59] and colon cancer[60] in different populations. *MDM2* is an oncogene responsible for the regulation of *TP53* expression[61]. The INS allele of rs3730485 may reduce the activity of *MDM2*, possibly increasing the activity of the tumor suppressor *TP53* and then reducing the chances of developing cancer.

In the current study, we observed an association of the DEL/DEL genotype of the polymorphism in *NFKB1* (rs28362491) with increased chances of developing both GC and CRC. This is an INDEL polymorphism that is

Table 4 Genotypic and allelic distributions of the investigated polymorphisms for patients with colorectal cancer in comparison to control group

Genotype	CRC	Control	*P* value[1]	OR (95%CI)[1]	Genotype	CRC	Control	*P*-value[1]	OR (95%CI)[1]
CASP8	63	475			RP2/RP2	16 (25.4)	154 (32.5)	0.871	1.068 (0.482-2.368)
DEL/DEL	13 (20.6)	90 (19.0)	0.676	0.888 (0.508-1.552)	Allele RP1	0.44	0.41		
INS/DEL	28 (44.4)	230 (48.4)			Allele RP2	0.56	0.59		
INS/INS	22 (35.0)	155 (32.6)	0.939	0.974 (0.503-1.887)	*NFKB1*	63	473		
Allele DEL	0.43	0.43			DEL/DEL	16 (25.4)	117 (24.7)	0.006[2]	3.732 (1.451-9.599)
Allele INS	0.57	0.57			INS/DEL	42 (66.7)	246 (52.0)		
MDM2	64	475			INS/INS	5 (7.9)	110 (23.3)	0.829	0.935 (0.508-1.723)
DEL/DEL	7 (10.9)	33 (6.9)	0.412	1.166 (0.143-9.487)	Allele DEL	0.60	0.51		
INS/DEL	25 (39.1)	168 (35.4)			Allele INS	0.40	0.49		
INS/INS	32 (50.0)	274 (57.7)	0.986	0.995 (0.546-1.811)	*PAR1*	63	473		
Allele DEL	0.30	0.25			DEL/DEL	37 (58.7)	273 (57.7)	0.464	0.704 (0.275-1.801)
Allele INS	0.70	0.75			INS/DEL	20 (31.8)	169 (35.7)		
TP53	64	475			INS/INS	6 (9.5)	31 (6.6)	0.813	0.937 (0.546-1.608)
DEL/DEL	47 (73.4)	350 (73.7)	0.886	1.166 (0.143-9.487)	Allele DEL	0.75	0.76		
INS/DEL	16 (25.0)	116 (24.4)			Allele INS	0.25	0.24		
INS/INS	1 (1.6)	9 (1.9)	0.986	0.995 (0.546-1.811)	*CYP2E1*	62	475		
Allele DEL	0.86	0.86			DEL/DEL	56 (90.3)	398 (83.8)	0.999	189364591.0 (0.000)
Allele INS	0.14	0.14			INS/DEL	6 (9.7)	73 (15.4)		
TYMS	63	475			INS/INS	0 (0.0)	4 (0.8)	0.351	0.655 (0.269-1.593)
DEL/DEL	11 (17.5)	65 (13.7)	0.304	1.342 (0.765-2.354)	Allele DEL	0.95	0.91		
INS/DEL	31 (49.2)	224 (47.2)			Allele INS	0.05	0.09		
INS/INS	21 (33.3)	186 (39.2)	0.429	0.751 (0.369-1.526)	*CYP19A1*	64	475		
Allele DEL	0.42	0.37			DEL/DEL	7 (10.9)	76 (16.0)	0.297	0.747 (0.431-1.293)
Allele INS	0.58	0.63			INS/DEL	33 (51.6)	248 (52.2)		
XRCC1	64	474			INS/INS	24 (37.5)	151 (31.8)	0.313	1.532 (0.669-3.508)
DEL/DEL	4 (6.2)	35 (7.4)	0.771	1.082 (0.637-1.838)	Allele DEL	0.37	0.42		
INS/DEL	27 (42.2)	179 (37.8)			Allele INS	0.63	0.58		
INS/INS	33 (51.6)	260 (54.8)	0.445	1.528 (0.515-4.535)	*UGT1A1*	63	464		
Allele DEL	0.27	0.26			*1/*1	20 (31.7)	206 (44.5)	0.098	0.541 (0.262-1.120)
Allele INS	0.73	0.74			*1/*28	32 (50.8)	209 (45.0)		
IL1A	64	475			*28/*28	6 (9.5)	46 (9.9)	0.370	1.282 (0.745-2.205)
DEL/DEL	10 (15.6)	86 (18.1)	0.657	0.880 (0.500-1.548)	*36/*1	3 (4.8)	3 (0.6)		
INS/DEL	33 (51.6)	246 (51.8)			*36/*37	1 (1.6)	0 (0.0)	0.001[2]	12.849 (2.906-56.817)
INS/INS	21 (32.8)	143 (30.1)	0.610	1.208 (0.584-2.368)	*1/*37	1 (1.6)	0 (0.0)		
Allele DEL	0.41	0.44			Allele *36	0.03	0.01		
Allele INS	0.59	0.56			Allele *1	0.60	0.67		
IL4	63	474			Allele *28	0.35	0.32		
RP1/RP1	8 (12.7)	69 (14.5)	0.195	1.493 (0.814-2.740)	Allele *37	0.02	0.00		
RP1/RP2	39 (61.9)	251 (53.0)							

Data for CRC and Control columns are presented as *n* or *n* (%). [1]Analysis of combined genotypes (INS/INS *vs* others, or DEL/DEL *vs* others) with adjusted values for confounding factors (European and African ancestries) in logistic regression; [2]Statistically significant. CRC: Colorectal cancer; INDEL: Insertion/deletion.

located in the promoter region of the gene, which is highly involved in inflammatory pathways. The DEL/DEL genotype has been previously associated with an increased risk of developing GC in a Japanese population[37] and bladder cancer in a Chinese population[62]. In addition, the DEL allele of this polymorphism has been related to the development of ulcerative colitis and *H. pylori* infection[63,64], which can increase the risk of CRC and GC. Regarding the INS/INS genotype, it has been associated with decreased development risk of ovarian cancer[65] and with increased risk of developing melanoma[66], while the DEL/DEL genotype has also been associated with reduced risk of developing other types of cancer[67]. Previous studies have suggested that the effects of rs28362491 on the risk of carcinogenesis may be ethnic- and cancer type-specific, as described by two meta-analyses involving Asian and Caucasian populations[68,69].

The *UGT1A1* gene is involved in hepatic detoxification and metabolization of different substances. The studied marker in this gene (rs8175347) has four possible alleles [*36 (5 repeats), *1 (6 repeats), *28 (7 repeats) and *37 (8 repeats)]. Allele *1 is considered the wild-type and the most common allele, *28 is the second most common allele and *36 and *37 are considered rare alleles. In this study, we observed that the presence of at least one of the rare alleles of this polymorphism appears to increase the chances of developing CRC by 13-times. In the literature, some studies show that alleles *36 and *37 are absent or extremely rare in different populations[70,71], but there are no studies relating the association of these alleles with the development of CRC. Although little is known about *36 and *37 alleles, it

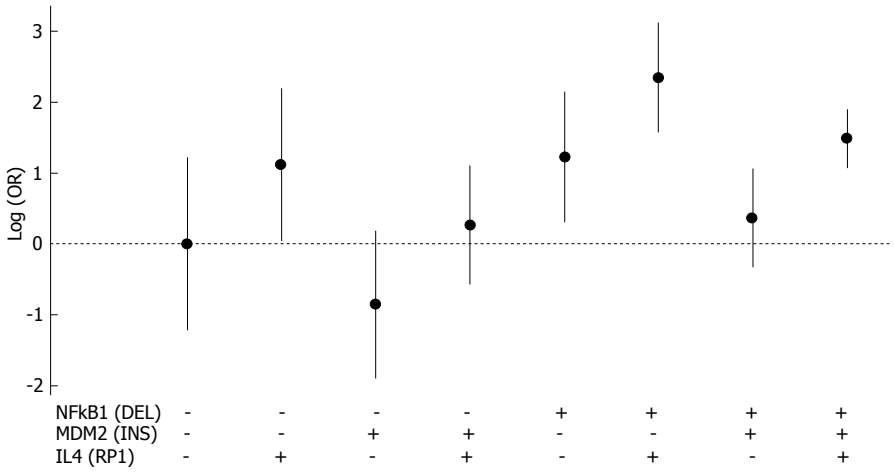

Figure 1 Analysis of the joint presence of three alleles regarding gastric cancer development. DEL allele of rs28362491 is represented by NFKB1 (DEL), INS allele of rs3730485 is represented by MDM2 (INS) and RP1 allele of rs79071878 is represented by IL4(RP1). All possible combinations were considered. Allele presence is represented by (+) and allele absence is represented by (-). DEL: Deletion; GC: Gastric cancer; INS: Insertion.

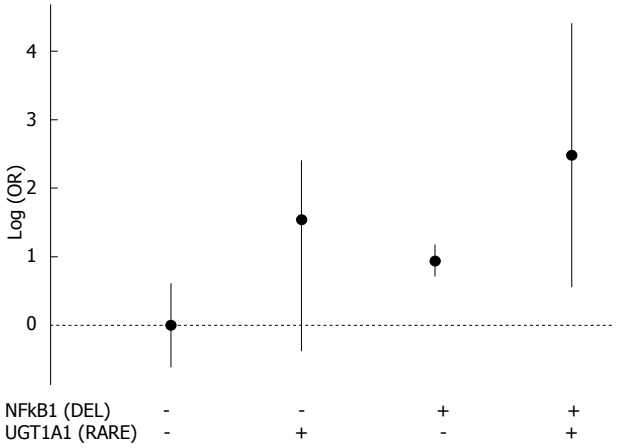

Figure 2 Analysis of the joint presence of two alleles regarding colorectal cancer development. DEL allele of rs28362491 is represented by NFKB1 (DEL) and *36 and *37 alleles in rs8175347 are represented by UGT1A1 (RARE). All possible combinations were considered. Allele presence is represented by (+) and allele absence is represented by (-). CRC: Colorectal cancer; DEL: Deletion.

is possible that the presence of such alleles could lead to a decreased activity of the *UGT1A1* gene, inducing the carcinogenesis process. We understand that the sample size of CRC patients may have influenced the observed result in this study, but we believe that our findings indicate the need to expand the investigation to a great number of patients from other Brazilian admixed populations, considering the important increase rate we observed.

In addition, we investigated the joint presence of the alleles that were statistically significant in homozygosis in the analyses discussed above. This is important because the interaction of alleles in different loci could lead to an increased effect in the carcinogenesis. Recently, this kind of additive effect has been reported for multiple types of cancer in different populations[72,74], but there is a lack of this type of study involving GC and CRC in the Brazilian population. To the best of our knowledge, this is the first study using this approach for these types of cancer in a Brazilian population.

The analyses of combined effect showed statistical significance for both types of cancer, presenting some interesting results. Among these, it is notable that: (1) individuals carrying both RP1 (*IL4* marker) and DEL (*NFKB1* marker) alleles have more than 10-fold increased chances of developing GC than carriers of the other alleles; and (2) individuals carrying the DEL allele (*NFKB1* marker) and at least one of the rare alleles *36 and *37 (*UGT1A1* marker) have almost 12-fold increased chances of developing CRC than carriers of other alleles of these markers. These results reinforce the importance of knowing which markers may play a role in cancer development.

In conclusion, we investigated 12 polymorphisms in genes with functions in inflammatory pathways, immune response or cellular and genomic stability (*i.e. CASP8*, *CYP2E1*, *CYP19A1*, *IL1A*, *IL4*, *MDM2*, *NFKB1*, *PAR1*, *TP53*, *TYMS*, *UGT1A1* and *XRCC1*) regarding the development of GC and CRC. Our findings indicate that some of these markers may be related to the development of GC and CRC. Moreover, the interaction between such polymorphisms may increase the risk of developing these types of cancer. These results contribute to a greater knowledge of possible risk factors in the development of GC and CRC.

ARTICLE HIGHLIGHTS

Research background

Our research group, located in the North region of Brazil, has been working with population genetics for many years. More recently, we have designed a set of 12 markers that are able to be genotyped in a single multiplex PCR and capillary electrophoresis, which is faster than Sanger sequencing and cheaper than real-time PCR. All markers in this set are in genes related to different pathways (*e.g.* inflammatory and immune response, and cellular and genomic stability). We have previously investigated not only the association of this set with the development of different diseases (*i.e.* acute lymphoblastic leukemia and leprosy), but also the distribution of these markers in individuals from the five regions of Brazil (North, Northeast, Midwest, Southeast and South) and in individuals representative of the main parental populations of this country

(Europeans, Africans and Native Americans). However, we believe it also is important to investigate the association of this set with the development of other types of cancer, such as gastric cancer (GC) and colorectal cancer (CRC).

Research motivation

GC and CRC are two of the most incident and aggressive types of malignant neoplasms in Brazil. A notable aspect of the Brazilian population is that it is highly admixed and, then, it is important not to extrapolate results from one region to another. For instance, these types of cancer are particularly frequent in the North region of Brazil. In general, most cases of GC and CRC are diagnosed in advanced stages and the death rate related to these types of cancer is high. To help early diagnosis, many research groups worldwide have been working to identify biomarkers able to detect increased risk of developing such types of cancer. Considering the high incidence of GC and CRC in the North region, we believe that it is important to study such neoplasms in this region.

Research objectives

In this study, we analyzed the association of 12 polymorphisms in genes involved in inflammatory pathways, immune response or cellular and genomic stability (namely, *CASP8*, *CYP2E1*, *CYP19A1*, *IL1A*, *IL4*, *MDM2*, *NFKB1*, *PAR1*, *TP53*, *TYMS*, *UGT1A1* and *XRCC1*) regarding GC and CRC development in a population from the North region of Brazil. Understanding the distribution of these markers in the studied population helps to improve the knowledge of the different factors that lead to cancer development.

Research methods

We collected blood samples from the participants (125 GC patients, 66 CRC patients and 475 cancer-free individuals), from which we extracted the DNA using a phenol-chloroform-based method. The studied 12-polymorphism set can be genotyped through amplification in a single multiplex PCR, followed by capillary electrophoresis. The different statistical analyses were performed in Structure v.2.3.4 and SPSS v.20 programs, and the R language. We analyzed the allelic and genotypic distribution of these markers, as well as the combined effect of the statistically significant alleles. The latter approach is not a common approach for studying GC and CRC. In fact, to the best of our knowledge, this is the first study using this kind of approach for these types of cancer in the Brazilian population. It gave us interesting results.

Research results

After performing the statistical analyses with correction of confounding factors, we observed positive associations between the markers rs79071878 (*IL4* gene), rs3730485 (*MDM2* gene) and rs28362491 (*NFKB1* gene) and GC development, as well as between the markers rs28362491 (*NFKB1* gene) and rs8175347 (*UGT1A1* gene) and CRC development. When we analyzed the combined effect of the alleles of the statistically significant genotypes of each marker (RP1 allele of rs79071878, INS allele of rs3730485, DEL allele of rs28362491 and *36 and *37 alleles in rs8175347), we obtained statistically significant results for both types of cancer. From these results, we highlight that: (1) individuals carrying both RP1 (IL4 marker) and DEL (NFKB1 marker) alleles have more than 10-fold increased chances of developing GC than carriers of the other alleles; and (2) individuals carrying the DEL allele (NFKB1 marker) and at least one of the rare alleles *36 and *37 (UGT1A1 marker) have almost 12-fold increased chances of developing CRC than carriers of other alleles of these markers. Our results reinforce the importance of knowing the role that different markers play in the development of cancer, which may contribute to the early detection of GC and CRC.

Research conclusions

In this study, we observed that the individual or joint presence of some alleles of the 12 polymorphisms of the set may affect the development of GC (RP1 allele of rs79071878, INS allele of rs3730485 and DEL allele of rs28362491) and/or CRC (DEL allele of rs28362491 and *36 and *37 alleles in rs8175347) in a population from the North region of Brazil. To the best of our knowledge, this is the first time it has been reported, and it supports the notion that more attention should be given to these polymorphisms in relation to the development of GC and CRC. Considering the results we obtained, we recommend that the individual and the joint presence of these markers should be further investigated in the other regions of Brazil, due to the high levels of admixture in this country, and in other types of cancer.

Research perspectives

Although there have been many advances in the complex field of oncogenetics, there is still a lot remaining to be discovered. The present study investigated 12 polymorphisms, some of them not frequently studied, and showed statistically significant association between four of these markers and the development of GC and CRC in a population from the North region of Brazil. It shows the importance of studying different polymorphisms in important genes, some of which may be involved not only in the development of GC and CRC but also of other types of malignant neoplasms. In addition, our study reinforces the notion of investigating different types of cancer in genetically admixed populations, such as the Brazilian population.

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Retrospective Study

Childhood-onset inflammatory bowel diseases associated with mutation of Wiskott-Aldrich syndrome protein gene

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Abstract

AIM

To screen primary immunodeficiency, Wiskott-Aldrich syndrome (WAS), and chronic granulomatous disease (CGD) among children with inflammatory bowel disease (IBD).

METHODS

This was a single-center retrospective study. Eighteen children with IBD were investigated. We analyzed their expression of Wiskott-Aldrich syndrome protein (WASP) in lymphocytes and superoxide generation in phagocytes using flow cytometry. When the expression of WASP or superoxide generation was low or absent,

we performed genetic analysis to determine the cause of this.

RESULTS

Eighteen patients were classified as having ulcerative colitis (n = 10), Crohn's disease (n = 5), or IBD-unclassified (n = 3). In total, three patients revealed low expression of WASP associated with a *WAS* gene c.1378 C>T p.Pro460Ser mutation, which has previously been reported as a pathogenic mutation in WAS and X-linked thrombocytopenia. However, with respect to the major symptoms of WAS, none of these three patients showed either thrombocytopenia or increased susceptibility to infection, but one patient showed generalized eczema. No CGD patients were discovered in this study.

CONCLUSION

Despite the lack of typical clinical manifestations of WAS, low expression of WASP could be associated with the pathogenesis of a subtype of IBD patients.

Key words: Inflammatory bowel disease; Wiskott-Aldrich syndrome; Primary immunodeficiency; Children; Screening

Core tip: Inflammatory bowel disease (IBD) has multiple etiologies, including genetic and environmental factors. Recent reports have described how some children with Wiskott-Aldrich syndrome (WAS) present IBD or IBD-like gastroenterocolitis. In this study, we found a *WAS* c.1378C>T, p.Pro460Ser mutation in three children with IBD. These patients did not present typical symptoms of WAS, such as thrombocytopenia and recurrent infection. However, WAS is known to be associated with an increased risk of malignancies including lymphoma, as well as autoimmune diseases. Therefore, in any long-term follow-up, the analysis of WASP expression in children with IBD should be considered even if major symptoms of WAS are absent.

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INTRODUCTION

Inflammatory bowel disease (IBD), including ulcerative colitis (UC) and Crohn's disease (CD), is a chronic inflammatory disorder of the gastrointestinal tract. IBD is caused by multiple factors; genetics, epigenetics, environment, microbiota and immune responses[1-3]. Recently, it was discovered that some patients with primary immunodeficiencies initially develop IBD or IBD-like gastroenterocolitis, especially in childhood. IBD that occur in primary immunodeficiencies is likely to be refractory to conventional treatments and is often more prominent than the susceptibility to infection[4]. It has been reported that children with Wiskott-Aldrich syndrome (WAS) and chronic granulomatous disease (CGD) could develop IBD or IBD-like gastroenterocolitis[5]. WAS is an X-linked disorder characterized by the triad of thrombocytopenia with small platelets, eczema, and recurrent infection. X-linked thrombocytopenia (XLT) is a milder form of WAS characterized by isolated thrombocytopenia. In WAS cases, gastrointestinal inflammation mimicking UC has occasionally been documented[5-7]. CGD is caused by defective phagocyte superoxide generation leading to impaired microbial killing, in which gastrointestinal inflammation mimicking CD has also occasionally been documented[5,8-10]. In this study, we analyzed WAS and CGD in children with IBD and described their clinical features. WAS and CGD are among the more common monogenic primary immunodeficiencies, for the diagnosis of which rapid methods using flow cytometry have been established[11,12]. Therefore, these two diseases were selected for this study. The diagnosis of underlying primary immunodeficiencies is important for investigating the pathogenesis of IBD, selecting appropriate treatment, taking precautions regarding malignancies and autoimmune diseases, and performing genetic counseling.

MATERIALS AND METHODS

Patients and methods

Patients with childhood-onset IBD, which developed earlier than at 17 years old and was consistent with the Paris classification A1a + A1b[13], were recruited from Saiseikai Yokohama-shi Tobu Hospital, Yokohama, Japan, between July 2015 and July 2016. All patients had already been diagnosed with IBD prior to recruitment into this study. The diagnosis and classification of IBD were made based on clinical, endoscopic, radiological, and histological findings, in accordance with the Revised Porto Criteria[14]. IBD was classified into three disease entities: CD, UC, and IBD-unclassified (IBD-U). Blood samples were collected from patients after obtaining written informed consent from their parents or guardians, and also collected from healthy young adults as a control. This study was performed in accordance with the Declaration of Helsinki and approved by the institutional ethics committees of Yokohama City University School of Medicine and Saiseikai Yokohama-shi Tobu Hospital (number: A140724004).

For initial screening, flow cytometric analysis was performed to evaluate the expression of Wiskott-Aldrich syndrome protein (WASP) in lymphocytes

and superoxide generation in phagocytes. Patients' white blood cells were analyzed using an EC800 flow cytometry analyzer (Sony Biotechnology, Tokyo, Japan). Forward scatter and side scatter were collected in linear mode to gate lymphocytes and neutrophils. Genetic analysis of the *WAS* gene was performed upon the discovery of low or absent expression of WASP.

WASP analysis

Intracellular staining of WASP was performed in accordance with a previously described method[12,15]. Whole blood was separated into peripheral blood mononuclear cells (PBMCs) by Lymphoprep® (Axis-Shield PoC AS, Oslo, Norway). The PBMCs were fixed in a fixation buffer (BD Biosciences Pharmingen, San Diego, CA, United States) for 15 min at room temperature, and then permeabilized in Perm/Wash buffer (BD Biosciences Pharmingen). They were then incubated with a rabbit anti-WASP monoclonal antibody (Abcam, Cambridge, United Kingdom) for 30 min at 4 ℃. After washing, they were incubated with an Alexa Fluor 488-conjugated anti-rabbit IgG Fab2 fragment (Cell Signaling Technology, Danvers, MA, United States) for 30 min at 4 ℃. The PBMCs were then washed again and centrifuged at 1500 × *g* for 1 min, twice. The obtained pellets were then resuspended in buffer and immediately analyzed by flow cytometry.

DHR123 assay

The DHR123 assay was performed in accordance with a previously described method[16]. Whole blood (100 μL) and 1 mL of 0.1 mmol/L DHR123 (Lambda Fluoreszenz Technologie GmbH, Vienna, Austria) were added to each tube. The tubes were incubated at 37 ℃ for 15 min to stain the phagocytes with DHR123. After incubation, 25 mmol/L ethylenediaminetetraacetic acid and 25 μg/mL phorbol myristate acetate (Sigma-Aldrich, St. Louis, MO, United States) were added to each tube. The tubes were incubated again at 37 ℃ for 20 min. They were then centrifuged at 400 × *g* for 5 min and the supernatant was discarded. Lysis buffer was added to the tubes. After 15 min, the tubes were centrifuged at 400 × *g* for 5 min and the supernatant was discarded. Subsequently, the pellets were suspended in buffer and immediately analyzed by flow cytometry.

Gene mutation analysis

WAS gene analysis was performed for all patients who showed normal and low expression of WASP. Genomic DNA was extracted from peripheral blood leukocytes using a QIAamp® DNA Blood Mini Kit (QIAGEN, Hilden, Germany). Polymerase chain reaction (PCR) primer sequences were derived from previous reports[17,18]. PCR was performed with a thermal cycler under the following conditions: initial denaturation at 94 ℃ for 5 min; then 32 cycles with denaturation at 94 ℃ for 1 min, annealing at 55-60 ℃ for 1 min, and extension at 72 ℃ for 1 min; and then final extension at 72 ℃ for 10 min. Each PCR product was electrophoresed on an agarose gel to confirm its size. To determine its DNA sequence, direct sequencing was performed using an Applied Biosystems 3730xl DNA Analyzer and Sequence Scanner version 1.0 software (Applied Biosystems, Waltham, MA, United States), under the conditions recommended by the manufacturer. *In silico* analysis of the mutated *WAS* sequence was performed using PolyPhen-2 (Polymorphism Phenotyping V.2, http://genetics.bwh.harvard.edu/pph2/dbsearch.shtml) and SIFT (http://sift.jcvi.org/) in addition to a literature review of the mutated gene sequence.

Table 1 Characteristics of patients with childhood-onset inflammatory bowel disease in this study (*n* = 18)

Disease	UC	CD	IBD-U
n	10	5	3
Sex (male, female)	8, 2	4, 1	1, 2
Median age at onset in yr (range)	9.5 (1-13)	12.0 (6-13)	6.0 (1-9)
Symptoms (number of positive patients)			
Diarrhea	10	5	1
Mucosal aphthae	1	2	0
Eczema	0	1	0
Skin abscesses	0	0	0
Thrombocytopenia	0	0	0
Recurrent infection	0	0	0

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

RESULTS

Patient characteristics

Eighteen patients were enrolled in this study, the characteristics of whom are summarized in Table 1. Ten patients were classified as having UC, five as CD, and three as IBD-U. The median ages at first presentation of clinical symptoms for these three groups were 9.5, 12.0, and 6.0 years old, respectively. In five patients, age at the onset was 6 years or younger and they classified into very early onset IBD (VEOIBD)[19]. One patient with CD had refractory eczema, but no patients developed thrombocytopenia or susceptibility to infection suggestive of WAS or CGD.

WASP analysis, DHR123 assay, and genetic analysis

WASP expressions of healthy controls were normal. Three patients (UC, *n* = 2; CD, *n* = 1) showed low expression of WASP compared with the healthy controls (Figure 1A), but no patients showed a complete lack of WASP expression. Subsequent *WAS* gene analysis revealed the same mutation (c.1378C>T, p.Pro460Ser) in all three patients (Figure 1B). This mutation is located in the verprolin, cofilin, and acidic domain in exon 11 of the *WAS* gene. Additionally, this mutation was not found in any of 15 patients with normal expression of WASP. We performed *in silico* analysis of the mutation using PolyPhen-2 and SIFT, in

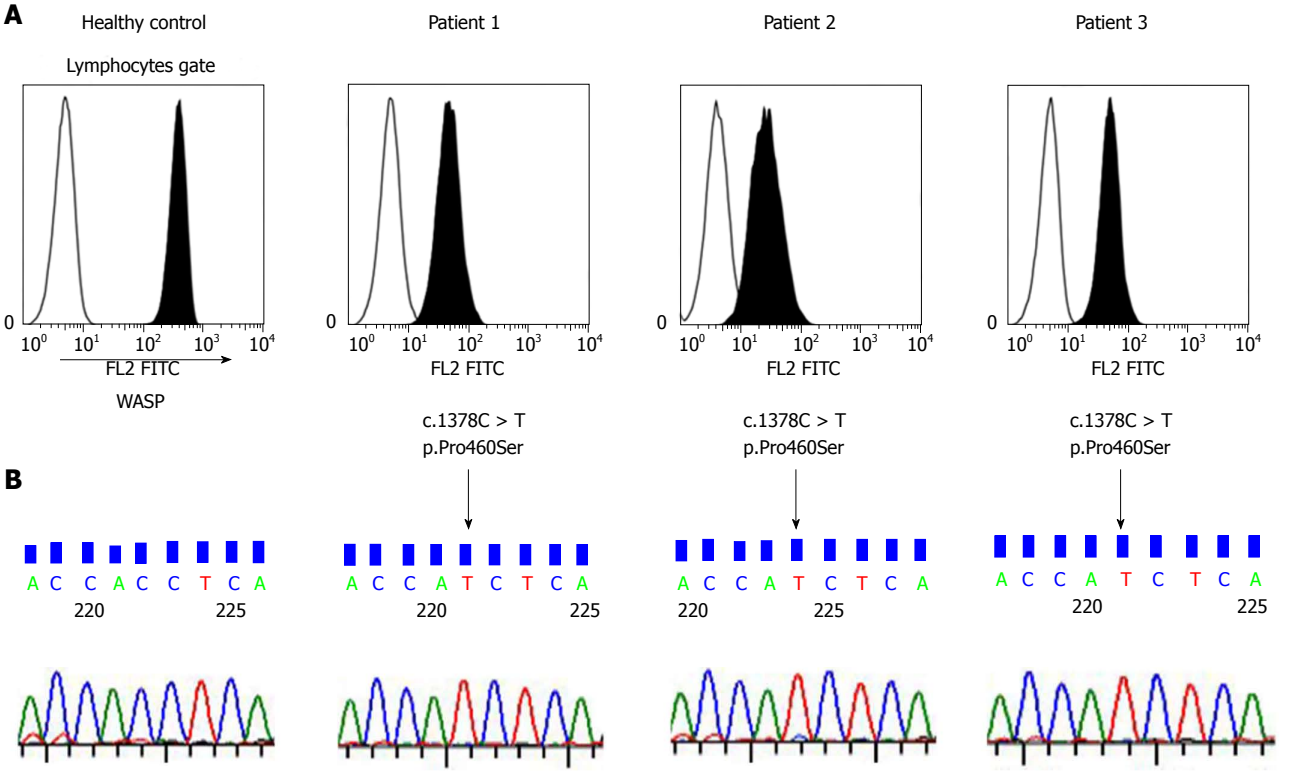

Figure 1 Analysis of Wiskott-Aldrich syndrome protein in patients' lymphocytes (A). Histograms represent intracellular Wiskott-Aldrich syndrome protein (WASP) staining (shaded area) and negative staining (unshaded area). Three patients with childhood-onset IBD presented low expression of WASP compared with that in the healthy control. B: *WAS* gene analysis. The three patients who showed low expression of WASP had the same mutation, c.1378C>T p.Pro460Ser.

addition to a literature review. PolyPhen-2 suggested that this is a benign mutation, while SIFT suggested that it is a tolerated one. However, in the literature review, we found four patients with typical WAS (*n* = 1) or XLT (*n* = 3) sharing the same c.1378C>T, p.Pro460Ser mutation of the *WAS* gene, who exhibited low expression of WASP (Table 2)[20-23]. DHR123 assays revealed no patients with abnormal superoxide generation. Therefore, no patients were diagnosed with CGD.

Clinical course of three patients with WAS mutation

The clinical features of the three patients with the *WAS* mutation are summarized in Table 3. Patient 3 was classified into VEOIBD. Although all three patients had diarrhea, none of them showed either thrombocytopenia or increased susceptibility to infection, two of the major symptoms of WAS and XLT. Their mean platelet volumes were within the normal range. Only Patient 1 showed eczema, one of the major symptoms of WAS. Interestingly, this eczema was markedly exacerbated after the initiation of tumor necrosis factor alpha (TNFα) blockade treatment (Figure 2), but immediately improved upon its discontinuation. At the time of writing, none of these patients has developed other autoimmune diseases or malignancies. Additionally, no patients have a family history suggestive of WAS.

Endoscopic findings in these three patients were consistent with those of typical CD or UC. Patient 1 had long linear ulcerations and cobble stone appearance in the ileum to the colon (Figure 3A). Patient 2 showed edematous and friable mucosa with superficial bleeding in the descending colon and sigmoid colon (Figure 3B and C). Patient 3 had edematous mucosa with granularity and erythema in the rectum from the sigmoid colon to the rectum (Figure 3D) and inflammatory polyps in the sigmoid colon. All three patients had successfully achieved remission with the medications shown in Table 3.

DISCUSSION

In infants and children, primary immunodeficiencies such as common variable immunodeficiency, CGD, IL-10 signaling defects, X-linked lymphoproliferative syndrome type 2, immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome and WAS could present as IBD or IBD-like colitis[24-29]. Recently, genome-wide association studies of IBD have identified 163 genetic loci[30] and 50 monogenic disorders including primary immunodeficiency associated with IBD-like immunopathology[19]. Underlying primary immunodeficiency may easily be missed by clinicians. Cannioto *et al*[5] reported one CGD and three WAS patients among 16 children with IBD diagnosed before 2 years of age. However, their clinical symptoms were typical of WAS and CGD.

In this study, we investigated children with IBD

Table 2 Previous reports of the c.1378C>T, p.Pro460Ser mutation of *WAS*

Phenotype	Sex	Age at onset	Platelet ($\times 10^3/\mu L$)	WASP expression	Ref.
XLT	M	8 mo	65-100	low	Lutskiy *et al*[20]
XLT	M	4 d	low	low	Lee *et al*[21]
WAS	M	N.D.	low	N.D.	Gulácsy *et al*[22][1]
XLT	M	6 yr	5	low	Ouchi-Uchiyama *et al*[23]

[1]This patent has double mutation (p.Pro460Ser and p.Met474Thr). ND: No data; XLT: X-linked thrombocytopenia; WAS: Wiskott-Aldrich syndrome; WASP: Wiskott-Aldrich syndrome protein.

Table 3 Clinical features of three patients with *WAS* c.1378C>T, p.Pro460Ser mutation

Patient	Sex	Diagnosis	Age at onset	Clinical symptoms	Platelet count ($\times 10^3/\mu L$) /mean platelet volume (fl)	Present status and treatment
Patient 1	M	CD	12 yr	Fever, Eczema, Watery diarrhea	431/8.9	Remission mesalazine, azathioprine and infliximab
Patient 2	M	UC	11 yr	Mucous-bloody diarrhea	220/10.4	Remission mesalazine and azathioprine
Patient 3	M	UC	2 yr	Mucous-bloody diarrhea	339/9.4	Remission mesalazine and prednisolone enema

UC: Ulcerative colitis; CD: Crohn's disease; mean platelet volume (fl), normal 8.9-12.6.

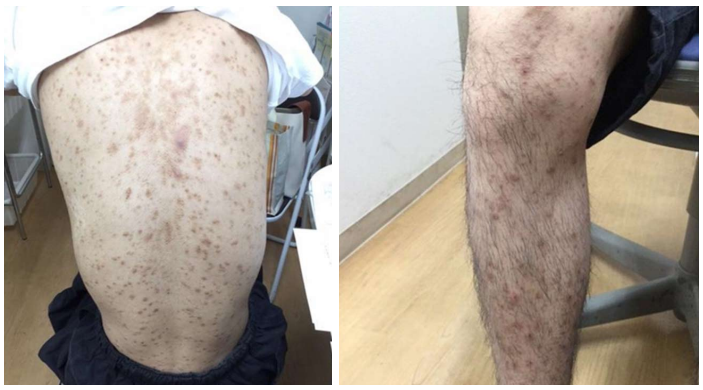

Figure 2 Cutaneous manifestations of Patient 1 (scaling eczema and pigmentation).

to screen underlying WAS and CGD. As a result, we found three patients with a *WAS* c.1378C>T, p.Pro460Ser mutation, but found none with CGD using flow cytometry. WAS is an X-linked disorder characterized by the triad of thrombocytopenia with small platelets, eczema, and recurrent infection. *WAS* gene mutations are associated with a wide spectrum of disease, from typical WAS to XLT characterized by isolated thrombocytopenia[31,32]. Generally, clinical manifestations correlate with the level of WASP expression. Classical WAS tends to be associated with the complete absence of WASP, whereas incomplete WAS and XLT are likely to be associated with low or absent expression. However, the phenotype does not always reflect the genotype of *WAS* mutations. Although *in silico* analysis suggested that the *WAS* c.1378C>T, p.Pro460Ser mutation would not be pathogenic, the mutation detected in our patients was previously reported in four patients with typical WAS or XLT[29-32] (Table 2). One of them had double mutations (p.Pro460Ser and p.Met474Thr). Three of them showed low WASP expression, but none developed IBD or IBD-like colitis. In contrast, our patients did not show thrombocytopenia or recurrent infection despite low WASP expression in their lymphocytes. Only one patient showed refractory eczema. Eczema in WAS is known as an atopic dermatitis-like manifestation. This patient's cutaneous manifestation was atopic dermatitis-like eczema at onset, which then shifted to scaling eczema and pigmentation (Figure 2). His eczema was exacerbated by TNFα blockade treatment, but improved rapidly upon its discontinuation. TNFα blockade frequently causes cutaneous complications such as vasculitis and eczema in patients with IBD. Scaling eczema is the most common cutaneous complication in adults, while psoriasis-like manifestations are most frequently seen in children[33,34]. Our patient's eczema differed from the typical TNFα blockade-related cutaneous complications in children, but resembled those in adults. There may be possibility that *WAS* mutation is associated with TNFα blockade-cutaneous complication and prediction for the complication. Endoscopic findings in three patients were typical of CD or UC, and were not distinguishable between patients with the mutation and without it. Only one of five VEOIBD patients in our study showed low expression

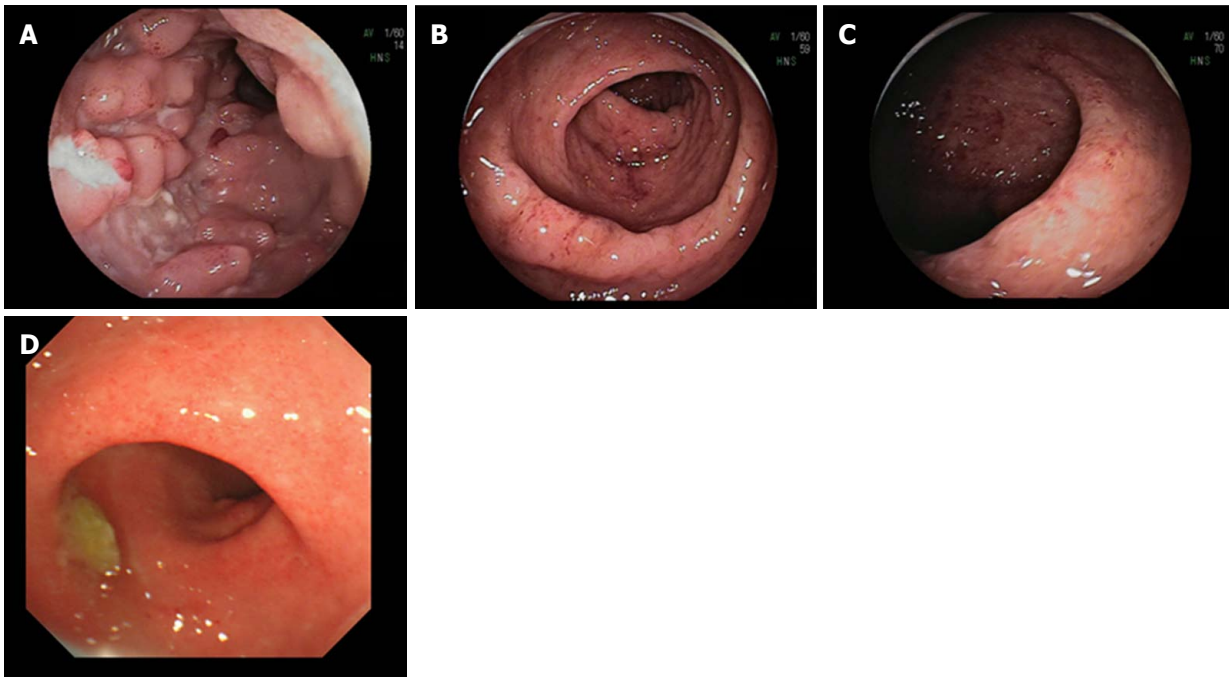

Figure 3 Endoscopic findings in the patients with WAS c.1378C>T, p.Pro460Ser mutation. A: Patient 1: long linear ulcerations and cobble stone appearance in the ileum. B and C: Patient 2: edematous and friable mucosa with superficial bleeding in the descending colon and sigmoid colon. D: Patient 3: edematous mucosa with granularity and erythema in the rectum.

of WASP and *WAS* mutation. VEOIBD patients often have different symptoms from older children and adults with IBD[25]. In general, genetics is suggested to be an important factor in VEOIBD[35]. *WAS* mutation might be associated with pathogenesis of VEOIBD. In the ExAC database (exac.broadinstitute.org), the frequency of *WAS* c.1378C>T, p.Pro460Ser mutation is 0.03817 in East Asians, and it appears to be more common in East Asians than in other ethnic groups. The frequency of this mutation in this study is 0.1667 (3/18), which is much higher than in East Asians. Therefore, *WAS* c.1378C>T, p.Pro460Ser mutation could be a risk factor for IBD development.

Patients with a *WAS* mutation are likely to develop autoimmune diseases, with up to 40% developing hemolytic anemia, neutropenia, vasculitis, IBD/IBD-like colitis, or renal disease[5,9,10,36]. The incidence of autoimmune diseases in XLT is lower than in typical WAS[37]. However, Imai *et al*[38]. reported that autoimmune diseases are equally common in patients with absent versus low expression of WASP[38]. Precaution for new-onset autoimmune diseases is important in our patients.

Snapper *et al*[39] reported that WASP-deficient mice developed chronic colitis. The colons of these mice were diffusely dilatated and had mucosal thickening due to crypt hyperplasia and the presence of mixed lymphocytic and neutrophilic infiltrate within the lamina propria[39]. WASP is expressed in the cytoplasm of hematopoietic cells. It acts as a signal transducer from cell surface receptors, and also plays essential roles in cell-cell interactions, cell movement, and cell division[40,41]. WASP dysfunction, leading to impaired regulatory T cells and expansion of autoreactive B cells[42,43], may provoke autoimmune diseases including IBD/IBD-like colitis[44]. The impaired regulatory T cells caused by WASP dysfunction also affect microbiota, which may lead to IBD/IBD-like colitis. Above all, WASP analysis may reveal the possible risk of new-onset autoimmune diseases.

Patients with typical WAS also have an increased risk of malignancies: 12%-30% of patients suffer from them, among which B-cell lymphoma is particularly common. Because there have been few reports of malignancies in patients with XLT, their incidence is presumably lower in XLT than in WAS[37,45,46]. However, TNFα blockade and azathioprine, which are the main treatments for refractory IBD, significantly increase the risk of lymphoma[47-49]. Thus, careful monitoring in patients with *WAS* mutation is necessary, especially under these two treatments.

This study has several limitations, including the small number of patients and the fact that it is a single-center study. Additional study with a greater number of patients is thus now underway. Further functional analysis to examine whether the *WAS* c.1378C>T, p.Pro460Ser mutation affects thrombocytosis and lymphocyte function is needed, although this mutation has previously been reported in some patients with WAS or XLT. In addition, there is a need for the screening of other primary immunodeficiencies known to be associated with the presentation of IBD or IBD-

like gastroenterocolitis.

In conclusion, we found a *WAS* c.1378C>T, p.Pro460Ser mutation in three children with IBD, the lymphocytes of whom exhibited low WASP expression. We suggest that low WASP expression has an association with the development of IBD/IBD-like colitis. Therefore, the analysis of WASP expression in children with IBD should be considered even if the triad of WAS symptoms is absent. Screening for underlying immunodeficiencies including WAS and CGD may contribute to improving patient management and outcome. Especially, physicians can pay more attention to the increased future risk of malignancy and autoimmune disease in IBD patients with WAS mutation.

ARTICLE HIGHLIGHTS

Research background

Inflammatory bowel disease (IBD), including ulcerative colitis (UC) and Crohn's disease (CD), is a chronic inflammatory disorder of the gastrointestinal tract. Recently, it was discovered that some patients with primary immunodeficiencies initially develop IBD or IBD-like gastroenterocolitis, especially in childhood.

Research motivation

Children with Wiskott-Aldrich syndrome (WAS) and chronic granulomatous disease (CGD) could develop IBD or IBD-like gastroenterocolitis. The diagnosis of underlying primary immunodeficiencies such as WAS and CGD is important for investigating the pathogenesis of IBD, selecting appropriate treatment, taking precautions regarding malignancies and autoimmune diseases, and performing genetic counseling.

Research objectives

To screen primary immunodeficiency, WAS and CGD, among children with inflammatory bowel disease (IBD), and to investigate their clinical features.

Research methods

This was a single-center retrospective study. Eighteen children with IBD were investigated. We performed intracellular staining of Wiskott-Aldrich syndrome protein (WASP) to analyzed their expression in lymphocytes and DHR123 assay to analyze superoxide generation in phagocytes using flow cytometry. When the expression of WASP or superoxide generation was low or absent, we performed direct DNA sequence to determine the cause of this. *In silico* analysis was performed using PolyPhen-2 (Polymorphism Phenotyping V.2, http://genetics.bwh.harvard.edu/pph2/dbsearch.shtml) and SIFT (http://sift.jcvi.org/), in addition to a literature review of the mutated gene sequence.

Research results

DHR123 assays revealed no patients with abnormal superoxide generation. Three patients (UC, n = 2; CD, n = 1) showed low expression of WASP compared with the healthy controls. *WAS* gene analysis revealed the same mutation (c.1378C>T, p.Pro460Ser) in all three patients. The mutation was previously reported in four patients with typical WAS or XLT. But, *in silico* analysis suggested that the mutation would not be pathogenic. Our patients with the mutation did not show thrombocytopenia or recurrent infection despite low WASP expression in their lymphocytes. Only one patient showed refractory eczema.

Research conclusions

We found a *WAS* c.1378C>T, p.Pro460Ser mutation in three children with IBD, the lymphocytes of whom exhibited low WASP expression. We suggest that low WASP expression has an association with the development of IBD/IBD-like colitis. Therefore, the analysis of WASP expression in children with IBD should be considered even if the triad of WAS symptoms is absent.

Research perspectives

In this study, we found a *WAS* c.1378C>T, p.Pro460Ser mutation in three children with IBD. These patients did not present typical symptoms of WAS, such as thrombocytopenia and recurrent infection. However, WAS is known to be associated with an increased risk of malignancies including lymphoma, as well as autoimmune diseases. Therefore, in any long-term follow-up, the analysis of WASP expression in children with IBD should be considered even if major symptoms of WAS are absent.

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ORIGINAL ARTICLE

Retrospective Study

Comparison of totally laparoscopic total gastrectomy using an endoscopic linear stapler with laparoscopic-assisted total gastrectomy using a circular stapler in patients with gastric cancer: A single-center experience

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Abstract

AIM

To evaluate the safety and efficacy of totally laparoscopic total gastrectomy (TLTG) with esophagojejunostomy using a linear stapler compared with laparoscopic-assisted total gastrectomy (LATG) using a circular stapler in gastric cancer patients.

METHODS

We retrospectively reviewed 687 patients who underwent laparoscopic total gastrectomy for gastric cancer at a single institution from August 2008 to August 2014. The patients were divided into two groups according to the type of operation: 421 patients underwent TLTG and 266 underwent LATG. Clinicopathologic characteristics and surgical outcomes in the two groups were compared and analyzed.

RESULTS

The TLTG group had higher mean ages at the time

of operation (57.78 ± 11.20 years and 55.69 ± 11.96 years, P = 0.020) and more histories of abdominal surgery (20.2% and 12.4%, P = 0.008) compared with the LATG group. Surgical outcomes such as intraoperative and postoperative transfusions, combined operations, pain scores and administration of analgesics, and complications were similar between the two groups. However, compared with the LATG group, the TLTG group required a shorter operation time (149 min *vs* 170 min, P < 0.001), had lower postoperative hematocrit change (3.49% *vs* 4.04%, P = 0.002), less intraoperative events (3.1% *vs* 10.2%, P < 0.001), less intraoperative anastomosis events (2.4% *vs* 7.1%, P = 0.003), faster postoperative recovery such as median time to first flatus (3.30 d *vs* 3.60 d, P < 0.001), faster median commencement of soft diet (4.30 d *vs* 4.60 d, P < 0.001) and shorter length of postoperative hospital stay (6.75 d *vs* 7.02 d, P = 0.005).

CONCLUSION

The intracorporeal method for reconstruction of esophagojejunostomy using a linear stapler may be considered a feasible procedure comparing with extracorporeal anastomosis using circular stapler because TLTG is simpler and more straightforward than LATG. Therefore, TLTG can be recommended as an appropriate procedure for gastric cancer.

Key words: Totally laparoscopic total gastrectomy; Laparoscopic-assisted total gastrectomy; Gastric cancer

Core tip: There are many studies that compared totally laparoscopic total gastrectomy (TLTG) with laparoscopic-assisted total gastrectomy (LATG). Moreover, various modified methods of intracorporeal esophagojejunostomy have been presented, but standardized methods have not been established. Our results show that TLTG by esophagojejunostomy intracorporeal anastomosis using linear stapler is an easier and more straightforward procedure compared with LATG by extracorporeal anastomosis using circular stapler.

Gong CS, Kim BS, Kim HS. Comparison of totally laparoscopic total gastrectomy using an endoscopic linear stapler with laparoscopic-assisted total gastrectomy using a circular stapler in patients with gastric cancer: A single-center experience. *World J Gastroenterol* 2017; 23(48): 8553-8561 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i48/8553.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i48.8553

INTRODUCTION

Asian countries, especially South Korea and Japan, have the highest incidences of gastric cancer in the world[1]. Gastric cancer is the second most common cause of cancer-related deaths worldwide[2,3], and surgery is the only curative modality for primary treatment of resectable gastric cancer[4,5]. The proportion of early gastric cancer patients has increased in Korea and Japan as a result of improved nationwide surveillance[6,7], and now accounts for nearly 60% of all cases. The incidence of upper and middle body gastric cancer has also increased. Consequently, the demand for minimally invasive treatments for upper body early gastric cancer has grown, and there is more need for new therapeutic methods and modalities. Laparoscopic gastrectomy has become one of the most popular modalities because of less postoperative pain, rapid postoperative recovery, lower blood loss, better cosmetic outcomes, and fewer complications compared with open gastrectomy[5,8-10]. In addition, the oncologic outcomes of laparoscopic gastrectomy for gastric cancer are acceptable as well[11,12].

Intracorporeal anastomosis has advantages over extracorporeal anastomosis because the former creates a smaller wound, provides a larger workspace and is less invasive[13-19]. Totally laparoscopic total gastrectomy (TLTG) using various types of intracorporeal anastomosis methods has been developed due to improvements in surgical devices and the accumulation of operative experience, but an optimal method for TLTG is yet to be established because of the technical challenges, especially for reconstruction of the esophagojejunostomy (EJ). Since 2008, TLTG using endoscopic linear staplers has been performed in our institute on more than 400 patients by expert surgeons with much experience of laparoscopic surgery, and we have developed a secure and effective technique for reconstructing the EJ[15-17].

In this study, we aimed to evaluate the surgical safety and efficacy of TLTG for treating gastric cancer of the upper third of the stomach by comparing its outcomes with those of laparoscopic-assisted total gastrectomy (LATG) using a circular stapler.

MATERIALS AND METHODS

Patients

We reviewed the retrospectively collected data of 687 consecutive patients who underwent total gastrectomy by LATG (266 patients) and TLTG (421 patients), for gastric cancer in the upper and middle stomach, between August 2008 and August 2014 at Asan Medical Center in Seoul, South Korea. The diagnosis was based on preoperative examinations including esophagogastroduodenoscopy, endoscopic ultrasound (EUS), and computed tomography (CT). Patients with gastric cancer were selected by preoperative diagnostic test under T3N2M0 according to the American Joint Committee on Cancer (AJCC) - International Union for Cancer Control (UICC) 7th edition[20]. Based on

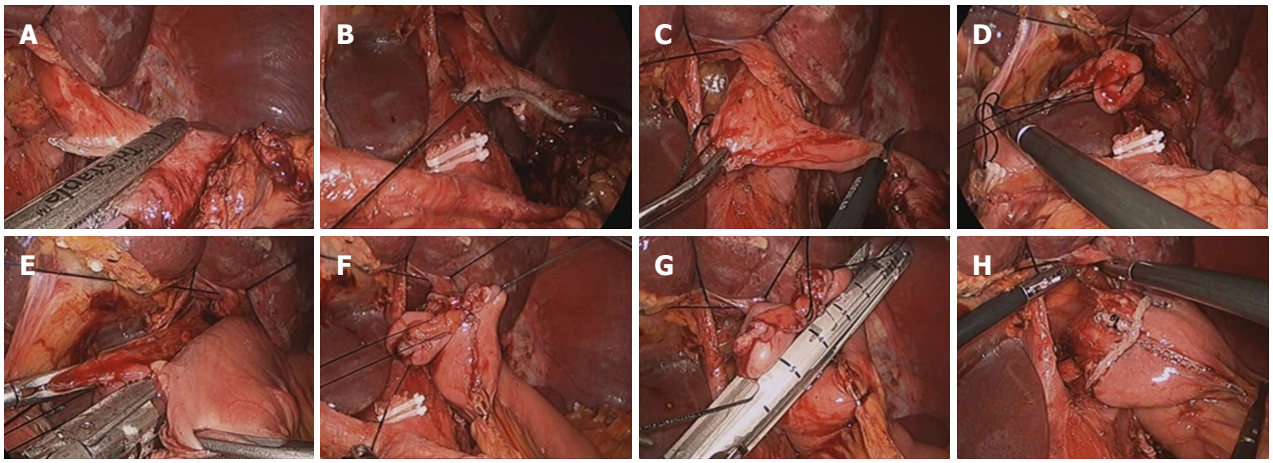

Figure 1 Forming an esophagojejunostomy. A: Nearly two-thirds of the esophagus diameter is transected 2 cm above the gastroesophageal junction using an endoscopic linear stapler; B: The first intracorporeal suture is made at the end of the staple line of the esophageal stump; C: The unstapled esophagus is transected with laparoscopic scissors after the remnant stomach has been clipped with manual titanium clips to avoid spillage of cancer cells; D: The second and third intracorporeal sutures are made at the esophagostomy site of the esophageal stump; E: To create an esophagojejunostomy, an endoscopic linear stapler is inserted by the operator between the esophagostomy and enterostomy of the jejunum. At this time the first assistant retracts the first thread towards the operator's direction inside the abdominal cavity, and the second assistant retracts the second thread through the right lower trocar from the outside of the abdomen; F: After an esophagojejunostomy has been constructed, the entry hole is held with tress suturing to approximate the tissue; G: The remnant entry hole is closed by the operator with an endoscopic linear stapler; H: An esophagojejunal anastomosis after completion of the reconstruction.

operative findings, patients with serosa-exposed advanced gastric cancer were converted to open surgery and were not included in this study. All patients were managed by clinical pathway after surgery[21]. The study was approved by the Institutional Review Board of Asan Medical Center.

Surgical techniques

Partial omentectomy with either D1+ or D2 lymphadenectomy for early gastric cancer and total omentectomy with either D2 lymphadenectomy for advanced gastric cancer were performed using an ultrasonic scalpel according to the treatment guidelines published by the Japanese Gastric Cancer Association[22]. EJ was performed with a circular stapler (DST EEA™ 25 single use stapler with 3.5 staples; Covidien, North Haven, CT, United States) via mini-laparotomy in LATG and with an endoscopic linear stapler (Endo GIA™ 60 mm and 45 mm Articulating Medium/Thick Reload with Tri-Staple™ Technology; Covidien) in TLTG (Figure 1). Finally, defects in the transmesentery and transcolon were closed *via* suturing. Details of the technique of TLTG have been described previously[16,17]. In case of advanced gastric cancer or with spleen hilar lymph node swelling, hilar lymph node was harvested and intraoperative frozen biopsy was carried out. If the frozen biopsy result was positive, then splenectomy was also carried out.

Clinical analysis of surgical outcomes

Data obtained from medical records included patient age, sex, body mass index (BMI), American Society of Anesthesiologist (ASA) score, history of previous abdominal surgery, operative time, pre- and postoperative hematocrit, time to first flatus, day of commencement of soft diet, pain score by visual analogue scale, number of analgesics administered, intra- and postoperative transfusion, intraoperative events, postoperative hospital stay, tumor size, number of retrieved lymph nodes, resection margins and cancer stage according to the AJCC/UICC 7th edition. Intraoperative events included jejunojejunostomy site kicking or narrowing, emphysema, and injury to organs such as pancreas, spleen, colon, small bowel, liver and major vessels. Intraoperative anastomosis events-related EJ refers to all unexpected events related to the EJ anastomosis, such as leakage after anastomosis, small bowel or esophagus injury caused by small diameter, pseudo-lumen stapling, sticking together of the crus muscle, *etc*.

Postoperative pain control consisted of intravenous, patient-controlled analgesia (fentanyl 2500 μg, ketorolac tromethamine 180 mg, and ondansetron hydrochloride 16 mg) and intermittent analgesic infusions. The amount of postoperative pain was assessed by visual analogue scale and by the number of additional doses of analgesics required until hospital discharge. A postoperative complication was defined as any event that required conservative or surgical treatment after surgery. Early complications were defined as events occurring within 30 d, and late complications as those occurring after 30 d. These complications were examined and classified by the Clavien-Dindo system[23].

Statistical analysis

Statistical analyses were performed with SPSS v18.0. Categorical variables were compared using the chi-square test or Fisher's exact test. All continuous variables were analyzed using the Mann-Whitney

Table 1 Clinical characteristics of patients who underwent laparoscopic-assisted total gastrectomy and totally laparoscopic total gastrectomy

Variable	LATG, *n* = 266	TLTG, *n* = 421	*P* value
Age in years, mean ± SD	55.69 ± 11.96	57.78 ± 11.20	0.020
Sex			0.583
Male	167 (62.8)	273 (64.8)	
Female	99 (37.2)	148 (35.2)	
ASA score			0.064
Ⅰ	181 (68.0)	249 (59.1)	
Ⅱ	68 (25.6)	145 (34.4)	
Ⅲ	17 (6.4)	27 (6.4)	
BMI in kg/m^2			0.883
< 23	119 (44.7)	198 (47.0)	
⩾ 23, < 25	70 (26.3)	103 (24.5)	
⩾ 25, < 30	69 (25.9)	110 (26.1)	
⩾ 30	8 (3.0)	10 (2.4)	
History of abdominal surgery	33 (12.4)	85 (20.2)	0.008

Values are expressed as mean ± SD or *n* (%). ASA: American Society of Anesthesiologists physical status classification; BMI: Body mass index; LATG: Laparoscopic-assisted total gastrectomy; TLTG: Totally laparoscopic total gastrectomy.

test, the *t*-test or the chi-square test, depending on the data. A *P*-value of less than 0.05 was considered statistically significant.

RESULTS

Clinical features and pathological characteristics

The clinical characteristics of the LATG and TLTG groups are presented in Table 1. The LATG and TLTG groups consisted of 266 and 421 patients, respectively. Their mean ages at the time of operation were 55.69 ± 11.96 years and 57.78 ± 11.20 years, respectively (P = 0.020). There were no significant differences between the two groups for sex (P = 0.583), ASA score (P = 0.064) or BMI (P = 0.883). Frequencies of abdominal surgery were 12.4% and 20.2% (P = 0.008) in LATG and TLTG groups, respectively. In summary, the TLTG group was slightly older and had more histories of abdominal surgery than LATG group.

Table 2 presents the pathologic results for the LATG and TLTG groups. The mean numbers of retrieved lymph nodes were 34.91 ± 13.92 and 40.04 ± 15.59 in the LATG and TLTG groups, respectively, indicating that lymph node dissection was adequate in both groups. The remaining pathological characteristics did not differ significantly between the groups, except for proximal resection margin length (LATG: 3.85 ± 3.11 cm and TLTG: 2.68 ± 2.62 cm, P < 0.001).

Surgical outcomes and postoperative clinical course

Table 3 shows the early surgical outcomes. There were significant differences in operation time (LATG: 170 (range, 65-453) min and TLTG: 149 (range, 75-342) min, P < 0.001), postoperative hematocrit change (LATG: 4.05% (range, -3.8%-15.2%) and TLTG: 3.50% (range, -4.9%-18.6%), P = 0.002), intraoperative events (LATG: 27 cases (10.2%) and TLTG: 13 cases (3.1%), P < 0.001), and intraoperative anastomosis events related to EJ (LATG: 19 cases (7.1%) and TLTG: 10 cases (2.4%), P < 0.001). There were no significant differences in postoperative transfusions, combined operations, pain scores or administration of analgesics. Combined operations were appendectomy, cholecystectomy, distal pancreatectomy and splenectomy, *etc*. There were three splenectomy cases in the TLTG group. Splenectomy was carried out in two cases in order to control splenic bleeding and in one case because of metastasis found in splenic hilar lymph node biopsy. However, the median time to first flatus [LATG: 3.60 (range, 1-7) d and TLTG: 3.30 (range, 1-7) d, P < 0.001] and to median commencement of soft diet (LATG: 4.61 (range, 2-68) d and TLTG: 4.30 (range, 3-36) d, P < 0.001), as well as length of post-operative hospital stay [LATG: 7.02 (range, 5-1117) and TLTG: 6.75 (range, 4-82), P = 0.005] were significantly longer in LATG than in TLTG.

Postoperative complications

Early and late postoperative complications are presented in Table 4. There was no significant difference in Clavien-Dindo classification between the groups. Overall, early postoperative complications were observed in 53 (19.9%) patients in the LATG group and 87 (20.7%) in the TLTG group (P = 0.447). There were 21 (7.9%) and 37 (8.8%) overall late postoperative complications in the LATG and TLTG groups, respectively (P = 0.681). In addition, the occurrence rate of EJ-related early complications, such as leakage, did not significantly differ between the two groups (LATG: 14 cases (5.3%) and TLTG: 14 cases (3.3%), P = 0.211). Late complications related to EJ were also similar in the two groups (LATG: 4 cases (0.9%) and TLTG: cases (0.7%), P = 0.439). The classes of the postoperative complications are given in Table 4, and the types of complications, including bleeding, leakage, stricture, intraabdominal fluid collection, internal hernia, ileus

Table 2 Pathologic results of the laparoscopic-assisted total gastrectomy and totally laparoscopic total gastrectomy groups

Variable	LATG, *n* = 266	TLTG, *n* = 421	*P* value
Tumor size in cm	3.72 ± 2.47	3.95 ± 2.90	0.302
Retrieved lymph nodes, *n*	34.91 ± 13.92	40.04 ± 15.59	< 0.001
metastatic lymph nodes, *n*	0.63 ± 2.59	0.82 ± 3.20	0.421
Proximal margin in cm	3.85 ± 3.11	2.68 ± 2.62	< 0.001
Distal margin in cm	12.72 ± 4.86	12.79 ± 4.67	0.870
TNM (AJCC/UICC) staging			0.395
Ⅰ A	202 (75.9)	285 (67.7)	
Ⅰ B	26 (9.8)	52 (12.4)	
Ⅱ A	19 (7.1)	40 (9.5)	
Ⅱ B	8 (3.0)	22 (5.2)	
Ⅲ A	5 (1.9)	8 (1.9)	
Ⅲ B	4 (1.5)	11 (2.6)	
Ⅲ C	2 (0.8)	3 (0.7)	

Values are expressed as mean ± SD or *n* (%). AJCC/UICC: 7th edition of the American Joint Committee on Cancer staging - Union for International Cancer Control; LATG: Laparoscopic-assisted total gastrectomy; TLTG: Totally laparoscopic total gastrectomy.

Table 3 Early surgical outcomes in patients undergoing laparoscopic-assisted total gastrectomy and totally laparoscopic total gastrectomy

Variables	LATG, *n* = 266	TLTG, *n* = 421	*P* value
Operation time in min	170 (65-453)	149 (75-342)	< 0.001
Hematocrit change in (%)	4.04 (-3.8-15.2)	3.49 (-4.9-18.6)	0.002
Intra-operative transfusion	1 (0.4)	1 (0.2)	1.000
Post operative transfusion	28 (10.5)	55 (13.1)	0.32
Intra-operative event	27 (10.2)	13 (3.1)	< 0.001
Intra-operative anastomosis event	19 (7.1)	10 (2.4)	0.003
Combined operation	17 (6.4)	27 (6.4)	1.000
Time to first flatus in d (range)	3.60 (1-7)	3.30 (1-7)	< 0.001
Time to soft diet in d (range)	4.61 (2-68)	4.30 (3-36)	< 0.001
Pick of pain score, score (range)	7.11 (2-10)	6.96 (3-10)	0.912
8AM Pain socre of POD #1, score (range)	3.45 (0-10)	3.49 (0-10)	0.841
8AM Pain socre of POD #3, score (range)	2.44 (0-9)	2.54 (0-7)	0.529
8AM Pain socre of POD #5, score (range)	1.75 (0-10)	1.51 (0-8)	0.055
Number of administration of analgesics, *n* (range)	2.49 (0-69)	2.86 (0-67)	0.131
Post-operative hospital stay in d (range)	7.02 (5-1117)	6.75 (4-82)	0.005

Values are expressed as median (range) or *n* (%). Hematocrit change means the difference between preoperative hematocrit and postoperative hematocrit. LATG: Laparoscopic-assisted total gastrectomy; POD: Postoperative days; TLTG: Totally laparoscopic total gastrectomy.

and wound infection, are presented in Table 5. Early complications following TLTG classified as Clavien-Dindo classification grade ≥ Ⅲ were observed in 85 (8.3%) patients, and late complications classified as Clavien-Dindo classification grade ≥ Ⅲ were observed in 18 (4.3%) patients.

DISCUSSION

Various modified methods of TLTG have been developed, but no standard method for upper and middle gastric cancer has been established because the reconstruction of intracorporeal EJs requires a high level of technical proficiency and is difficult, even for experienced surgeons[24-28]. We have recently reported a TLTG method developed for intracorporeal EJ using an endoscopic linear stapler, and we believe that it could become a standard method for these patients[16,17]. Although extracorporeal EJ anastomosis using a circular stapler is the generally accepted method for laparoscopic total gastrectomy, the anastomosis is often difficult to complete because of the limited working space formed by the mini-laparotomy[24]. Furthermore, an extended laparotomy incision is sometimes required, but this may reduce the benefits of the laparoscopic approach.

In a study on distal gastrectomy, TLTG without a mini-laparotomy was unaffected by obesity and could, thus, be a safe procedure for avoiding the impact of obesity[18,19]. Similarly, TLTG helps the surgeon easily resect and reconstruct the anastomosis without limiting the surgeon's view. In a previous study, TLTG produced similar early surgical outcomes to LATG, although the BMI was higher in the TLTG group[29]. In the present retrospective study, the TLTG patients had similar BMIs and tended to be slightly older, with more histories of abdominal surgery compared with LATG patients. Nevertheless, TLTG was superior to LATG

Table 4 Early and late postoperative complications *n* (%)

	Early complications			Late complications		
	LATG, *n* = 266	TLTG, *n* = 421	*P* value	LATG, *n* = 266	TLTG, *n* = 421	*P* value
CDC			0.447			0.681
0	213 (80.1)	334 (79.3)		245 (92.1)	384 (91.2)	
1	24 (9.0)	26 (6.2)		10 (3.8)	15 (3.6)	
2	13 (4.9)	26 (6.2)		0 (0)	4 (1.0)	
3	12 (4.5)	33 (7.8)		11 (4.1)	18 (4.3)	
4	4 (1.5)	2 (0.5)		0 (0)	0 (0)	
Cx of EJ			0.211			0.439
None	252 (94.7)	407 (96.7)		262 (98.5)	418 (99.3)	
Leakage	14 (5.3)	14 (3.3)		1 (0.4)	1 (0.2)	
Stricture	0 (0.0)	0 (0.0)		3 (1.1)	2 (0.5)	

Values are expressed as *n* (%). CDC: Clavien-Dindo classification; Cx: Complications; EJ: Esophagojejunostomy; LATG: Laparoscopic-assisted total gastrectomy; TLTG: Totally laparoscopic total gastrectomy.

Table 5 Postoperative complications in patients who underwent laparoscopic-assisted total gastrectomy and totally laparoscopic total gastrectomy *n* (%)

	LATG, *n* = 266	TLTG, *n* = 421
Bleeding	4 (1.50)	8 (1.90)
EJ leakage	15 (5.64)	15 (3.56)
EJ stricture	3 (1.13)	2 (0.48)
Intraabdominal fluid collection	8 (3.01)	26 (6.18)
Internal hernia	5 (1.88)	12 (2.85)
Mechanical ileus	10 (3.76)	28 (6.65)
Paralytic ileus	3 (1.13)	7 (1.66)
Wound infection	18 (6.77)	9 (2.14)
Other surgical complications	4 (1.50)	8 (1.90)
Medical complications	4 (1.50)	2 (0.48)

EJ: Esophagojejunostomy; LATG: Laparoscopic-assisted total gastrectomy; TLTG: Totally laparoscopic total gastrectomy.

in terms of operation time, postoperative hematocrit change, intraoperative events, bowel movements, and postoperative hospital stays. Although TLTG is less invasive than LATG, there was no significant difference in pain score, which was probably due to the use of active pain control, such as patient-controlled analgesia.

Chen *et al*[5] found in their meta-analysis that the number of lymph nodes harvested in TLTG was marginally higher than in LATG (P = 0.06). In our study, lymphadenectomy seems to have been adequate in both groups, despite the significant difference in the number of retrieved lymph nodes in the LATG and TLTG group (34.91 ± 13.92 and 40.04 ± 15.59, respectively, P < 0.001). The reason for this difference is unclear since the lymphadenectomy procedure is the same in both LATG and TLTG. There was a significant difference between the LATG and TLTG groups with regard to the length of the resection margin (LATG: 3.85 ± 3.11 cm and TLTG: 2.68 ± 2.62 cm, P < 0.001). This could be attributed to the fact that linear staplers are often placed on either side of the resection line, and might hinder accurate histopathologic evaluation of the surgical margin of the resected specimen. Linear staplers generally have four or six rows of staples and form two or three staple lines on a margin of length approximately 4-5 mm as exempted staples on the resection line, in contrast to conventional circular staplers[30]. Moreover, the linear staplers used in TLTG require a substantial length of esophagus for anastomosis. On the other hand, the circular stapler used in LATG allows the esophagus to be transected more proximally and does not need a long esophageal stump. EJ anastomosis using a circular stapler, thus, allows higher anastomosis in patients with tumors at the gastroesophageal junction, or in the upper stomach and invading the esophagus[31,32].

In the current study, the TLTG group was older and had more histories of abdominal surgery. However, the operation time for TLTG was shorter than for LATG. Our TLTG experience suggests several factors that may contribute to this shorter operation time. First, TLTG provides a wider view than LATG. Second, reconstruction in TLTG carried out with a linear stapler is easy, rapid and requires no hand-sewn reinforcement procedure. Finally, opening and closing a mini-laparotomy is not required. Incision for a mini-laparotomy may take especially long additional incision in obese patients. Moreover, our data show that TLTG has superior surgical outcomes in terms of postoperative hematocrit change, intraoperative events, time to first flatus, soft diet and postoperative hospital stay because the intracorporeal method has a wider view and causes less surgical trauma.

Postoperative morbidity after LATG has been reported to range from 17% to 27%[33-38]. In our study, early complication occurring within 30 d following LATG and TLTG classified as Clavien-Dindo classification grade ≥ Ⅲ were observed in 16 (6.0%) and 35 (8.3%) patients, respectively; late complications developing after 30 d following LATG and TLTG were observed in 11 (4.1%) and 18 (4.3%) patients, respectively. These results show that there were no significant differences between LATG and TLTG in terms of postoperative complications.

Patient characteristics such as age and obesity are risk factors for postoperative complications in laparoscopic gastrectomy. ASA scores may be influenced by age and comorbidity because these factors reinforce each other. Most of all, being overweight is a potent risk factor for poor surgical outcomes[39,40]. Delayed bowel movement, increased postoperative pain, and prolonged hospital stay can occur in obese patients, as we have suggested in a previous report on laparoscopic distal gastrectomy[40]. We found no significant differences in early and late complications between LATG and TLTG, even though the TLTG group patients were much older and had more histories of abdominal surgery. In addition, there was no significant difference between the two groups in complications related to EJ. However, TLTG makes a wide operating space and carries out EJ construction safely, and several investigators have insisted that the anastomotic site should be further secured and have a wider diameter when using a linear stapler than when using a circular stapler[5,16,41].

The procedure of LATG and TLTG differ in many ways. First, TLTG is less invasive, and requires a smaller incision than does LATG. Second, the wider working space in TLTG ensures safe reconstruction of the EJ. Therefore, laparoscopic surgeons are more comfortable with the intracorporeal than the extracorporeal one. Furthermore, using a linear stapler in TLTG has another advantage in that whereas circular staplers have only two staggered rows, endoscopic linear staplers have three staggered rows and provide better staple line security.

We conclude that TLTG requires a shorter operation time and permits faster postoperative recovery than LATG, while having similar surgical outcomes and complications. Therefore, TLTG using a linear stapler may be considered a more appropriate procedure than LATG using a circular stapler, and may be recommended for the treatment of gastric cancer of the upper third of the stomach.

This study has certain limitations. It is a retrospective study from a single institution and the baseline clinical characteristic of the two groups were different. Although the pathologic results for the patients in the LATG and TLTG groups were similar, the LATG and TLTG operations were performed at different periods of time. In addition, cancer recurrence and long-term survival rates were not analyzed because approximately half the patients underwent surgery, and 5 years had not yet passed. Therefore, data on long-term outcomes are still needed in order to compare the oncological adequacy of these two methods.

ARTICLE HIGHLIGHTS

Research background

In Korea and Japan, the incidence of upper and middle body gastric cancer has increased as a result of improved nationwide surveillance. Furthermore, the indication of laparoscopic gastrectomy has also extended. Therefore, the demand for minimally invasive surgery for upper body gastric cancer has grown, and there is more need for new therapeutic methods and modalities.

Research motivation

Intracorporeal anastomosis and extracorporeal anastomosis in laparoscopic total gastrectomy has developed due to improvements in surgical devices and the accumulation of operative experience, but an optimal method for laparoscopic total gastrectomy has yet to be established due to the difficulties of esophagojejunostomy.

Research objectives

We aimed to evaluate the surgical safety and efficacy of intracorporeal anastomosis using linear stapler for treating gastric cancer of the upper third of the stomach by comparing its outcomes with those of extracorporeal anastomosis using circular stapler.

Research methods

From August 2008 to August 2014, 687 consecutive patients who underwent total gastrectomy (266 laparoscopic-assisted total gastrectomy (LATG) patients and 421 totally laparoscopic total gastrectomy (TLTG) patients) were reviewed retrospectively. Data obtained from medical records included patient age, sex, body mass index, American Society of Anesthesiologist score, history of abdominal surgery, operative time, pre- and postoperative hematocrit, time to first flatus, day of commencement of soft diet, pain score by visual analogue scale, number of analgesics administered, intra- and postoperative transfusion, intraoperative events, postoperative hospital stay, tumor size, number of retrieved lymph nodes, resection margins and cancer stage according to the American Joint Committee on Cancer - International Union for Cancer Control 7th edition.

Research results

The TLTG group had higher mean age at time of operation, and more histories of abdominal surgery. However, the TLTG group required a shorter operation time, lower postoperative hematocrit change, less intraoperative events, less intraoperative anastomosis events, and permitted faster postoperative recovery, such as median time to first flatus, median commencement of soft diet and length of postoperative hospital stay.

Research conclusions

TLTG may be considered a feasible procedure, as compared to LATG. Because TLTG provides a wider view than TLTG, reconstruction in TLTG carried out with a linear stapler is easy, rapid and requires no hand-sewn reinforcement procedure, and TLTG does not need additional mini-laparotomy. Furthermore, TLTG had superior surgical outcomes in terms of operation time, postoperative hematocrit change, intraoperative events and postoperative recovery.

Research perspectives

Based on our results, we can consider TLTG as a feasible and straightforward procedure. But this study has certain limitations. It is a retrospective study from a single institution, and although the pathologic results in the LATG and TLTG groups were similar, data on long-term outcomes are still needed to compare the oncological adequacy of these two methods.

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ORIGINAL ARTICLE

Retrospective Study

Prognostic significance of pretreatment serum carcinoembryonic antigen levels in gastric cancer with pathological lymph node-negative: A large sample single-center retrospective study

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Abstract

AIM

To assess whether elevated serum carcinoembryonic antigen (CEA) is in the inferior prognosis for pathological lymph node-negative (pN_0) gastric cancer (GC) patients who underwent D_2 gastrectomy.

METHODS

About 469 pN_0 GC patients, who received D_2 radical gastrectomy were retrospectively analyzed. The X-tile plots cut-off point for CEA were 30.02 ng/mL using minimum *P*-value from log-rank χ^2 statistics, and pN_0 GC patients were assigned to two groups: those more than 30.02 ng/mL (n = 48; CEA-high group) and those less than 30.02 ng/mL (n = 421; CEA-low group). Clinicopathologic characteristics were compared using

Pearson's χ^2 or Fisher's exact tests, and survival curves were so manufactured using the Kaplan-Meier method. Univariate and multivariate analysis were carried out using the logistic regression method.

RESULTS

The percentage of vessel carcinoma embolus (31.35% *vs* 17.1%) and advanced GC (T_{2-4b}) (81.25% *vs* 65.32%) were higher in CEA-high group than CEA-low group. The CEA-positive patients had a significantly poorer prognosis than the CEA-nagetive patients in terms of overall survival (57.74% *vs* 90.69%, $P < 0.05$), and no different was found between subgroup of T category, differentiation, nerve invasion, and vessel carcinoma embolus (all $P > 0.05$). Multivariate survival analysis showed that CEA (OR = 4.924), and T category (OR = 2.214) were significant prognostic factors for stage pN_0 GC (all $P < 0.05$). Besides, only T category (OR = 1.962) was an independent hazard factor in the CEA-high group ($P < 0.05$).

CONCLUSION

Those pretreatment serum CEA levels over 30.02 ng/mL on behalf of worse characteristics and unfavourable tumor behavior, and a poor prognosis for a nearly doubled risk of mortality in GC patients.

Key words: Carcinoembryonic antigen; Gastric cancer; Pathological lymph node-negative; X-tile plots; 5-year survial rate

Core tip: Currently, the survival rate for gastric cancer (GC) is still unsatisfactory. Reliable biomarker such as carcinoembryonic antigen (CEA) is necessary to improve the management of GC and pathological lymph node-negative (pN_0) represents a group of reliable biological status. About 469 pN_0 GC patients, who received D_2 radical gastrectomy were retrospectively analyzed, and an optimal cut-off value of CEA was reset, and we found that pretreatment serum CEA levels over 30.02 ng/mL on behalf of worse characteristics and unfavourable tumor behavior, and a poor prognosis for a nearly doubled risk of mortality in staging pN_0 GC patients.

Xiao J, Ye ZS, Wei SH, Zeng Y, Lin ZM, Wang Y, Teng WH, Chen LC. Prognostic significance of pretreatment serum carcinoembryonic antigen levels in gastric cancer with pathological lymph node-negative: A large sample single-center retrospective study. *World J Gastroenterol* 2017; 23(48): 8562-8569 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i48/8562.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i48.8562

INTRODUCTION

Currently, the therapeutic effect for gastric cancer (GC) is still dispiriting[1], especially in China. This reason may be partly ascribed to the delayed diagnosis of GC. In addition to tumor-nodes-metastasis (TNM) stage and selection of treatment, the survival rate of GC patients may be hit by other factors such as differentiation, behavior and genetic mutation[2].

Pathological lymph node-negative (pN_0) represents a group of reliable biological status, however, the survival for patients can unending changes, even when they share the same clinical stage[3]. Therefore, clinicians and researchers keep looking for other survival factors that might be able to help in the selection of a suitable treatment strategy.

Carcinoembryonic antigen (CEA), an acknowledged as an intracellular adhesion molecule, is one of the most common markers used in GC[2]. Up to now, many studies showed that extremely elevated serum CEA, which is closely related to an awful prognosis[4]. Numerous studies have been in favor of preoperative CEA levels as predictive marker for the survival situation of GC[5-8]. However, other studies have reported the opposite results[9-13]. Inconsistent views can be partly explained by different cutoff values of CEA, limited number of eligible cases and study endpoints, and the inadequate statistical power.

To solve the above-mentioned problem, we performed a large sample retrospective study and reset an optimal cut-off value of CEA and to explore the relationship between preoperative serum CEA and clinicopathological traits and prognostic information.

MATERIALS AND METHODS

Patients

From January 2000 to December 2010, a retrospective analysis was conducted of 1801 consecutive patients with GC who underwent D_2 lymphadenectomy, at the Department of gastrointestinal surgery, Fujian tumor hospital. Among them, 469 pN_0 resectable GC patients suffered from stage $pTxN_0M_0$ GC according to the 7th edition of the TNM classification. Data from these patients were enrolled into a prospectively maintained database.

The inclusion criteria were as follows: (1) pN_0 resectable GC; (2) Adenocarcinoma confirmed by histopathology; (3) Physical fitness suitable for surgery; (4) D_2 lymphadenectomy; and (5) no prior history of any type of adjunctive therapy.

The exclusion criteria were as follows: (1) older than 85 years of age; (2) previous or concomitant other cancer; (3) previous or concomitant gastrectomy for benign disease; (4) previous chemotherapy or

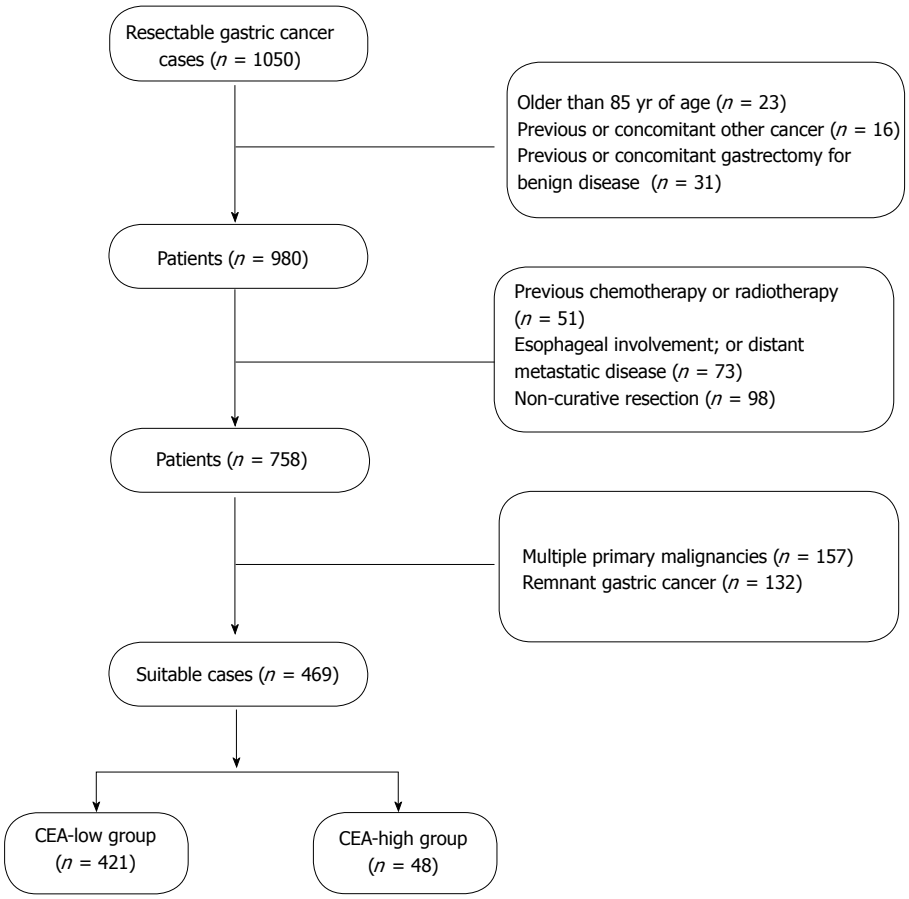

Figure 1 Inclusion and exclusion criteria. CEA: Carcinoembryonic antigen.

radiotherapy; (5) esophageal involvement; or (6) distant metastatic disease; (7) non-curative resection; (8) multiple primary malignancies; (9) remnant GC; and (10) mortality within 30 d after surgery (Figure 1).

All of the above patients were followed up by posting letters or by telephone interviews. The last follow-up was 1 January 2017. The cardiopathy logical and follow-up findings were collected and recorded in the database. All subjects gave written informed consent to the study protocol, which was approved by the Ethical Committees of Fujian Provincial Tumor Hospital.

Surgery

According to the 7th edition NCCN guidelines[2], surgery with lymph node (LN) dissection is the primary treatment option for medically fit patients with resectable T_{1b}, any N tumors. All patients in the study underwent standard total or distal gastrectomy, depending on the location and macroscopic appearance of the primary tumor (Table 1). The strategy for LN dissections was determined using a standardized technique according to the guidelines of the 2010 Japanese Classification of Gastric Cancer and Gastric Cancer Treatment Guidelines edited by the Japanese Gastric Cancer Association[14].

Clinicopathological characteristics

The clinicopathological findings, including depth of tumor invasion and LN metastases, were utilized to stage tumors according to the 7th edition NCCN guidelines[2]. LNs were dissected and described according to the Japanese Classification of Gastric Carcinoma[14], which was also used to classify the location, histological type, and lymphatic invasion of tumors.

Statistical analysis

Statistical analyses were conducted using Statistical Product for Social Sciences (SPSS) 19.0 software (SPSS, Inc, Chicago, IL, United States). The distribution of baseline characteristics was compared by using either Fisher's exact test or the chi-square test. The CEA cut-off points were produced and analyzed using the X-tile program which identified the cut-off with the minimum *P* values from log-rank χ^2 statistics for the categorical CEA in terms of survival. Meaningful factors were extracted for further analysis, which was conducted by using the logistic regression method. The overall cumulative probability of survival was calculated by the Kaplan-Meier method, and differences were evaluated by using the log-rank test. A *P* value less than 0.05 was regarded as statistically significant.

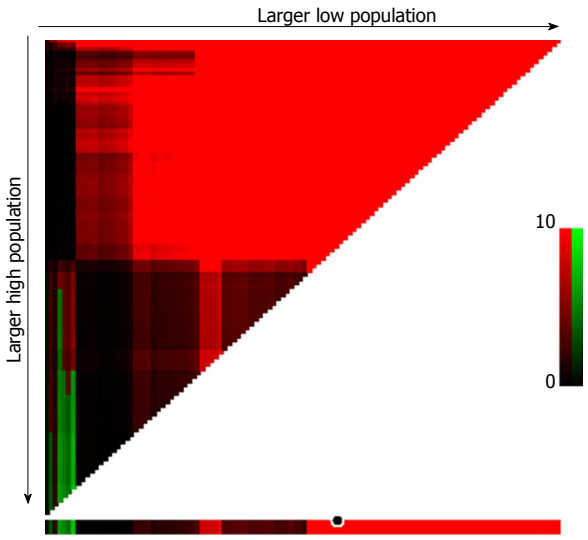

Figure 2 Division of patients by the cut-off points produced by X-tile plot. X-tile plots for CEA. The produced log-rank χ^2 value stratifies the pTxN0M0 GC patients into two groups by a cut-off value 30.02 ng/mL, showing a strong discriminatory capacity, with a χ^2 value of 85.15 and a relative risk ratio of 1:2.15. CEA: Carcinoembryonic antigen.

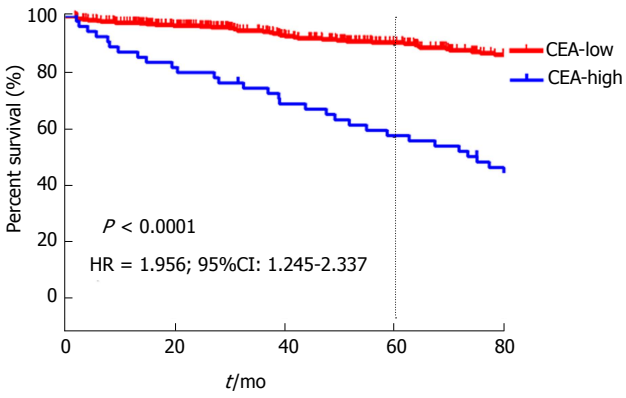

Figure 3 Survival analysis of pN0 patients with gastric cancer undergoing curative intent surgery. The *P* values for the survival comparison was determined by the log-rank test. CEA: Carcinoembryonic antigen.

RESULTS

Correlation analysis between the clinicopathologic factors and CEA

X-tile plots, constructed in Figure 2, illustrated that the optimal cut-off point for CEA was 30.02 ng/mL, and GC patients with in stage pN_0 were assigned to two groups: those more than 30.02 ng/mL (n = 48; CEA-high group) and those less than 30.02 ng/mL (n = 421; CEA-low group), with the strongest discriminatory capacity, with a χ^2 value of 85.15 and a relative risk ratio of 1:2.15.

Clinicopathological characteristics

Depending on the 7th editions of the TNM system, a total of 469 pN_0 GC patients were recruited in this study. Patient demographic data are summarized in Table 1. Overall, no observably difference was found in these characteristics, including gender, age, family history (FH), HP infection status, BMI, location, and lauren classification (all $P > 0.05$).

A slightly higher proportion of male patients constituted in the CEA-high patients (76% *vs* 64.04%), and male-to-female ratio was 3:1 among the CEA-high compare to 2.81:1 with CEA-low patients. In the CEA-high group, the proportion of was slightly higher than the negative group in poor differentiation (54.2% *vs* 46.3%), and nerve invasion (22.92% *vs* 16.6%). What is more, percentage was dramatically higher in CEA-high group than CEA-low counterparts in stage of $T_{2\text{-}4b}$ (81.25% *vs* 65.32%, P = 0.026), vessel carcinoma

Table 1 Demographic data of the 469 patients with gastric cancer, *n* (%)

Characteristics	CEA-Low group (n = 421)	CEA-High group (n = 48)	*P* value
Age (yr), mean ± SD	58.74 ± 10.98, 60 (20-83)	60.4 ± 11.55, 61 (31-78)	
Gender			
Female	118 (28)	12 (25)	0.657
Male	303 (72)	36 (75)	
Male-to-female ratio	2.81:1	3:01	
Family history			
Positive	8 (1.9)	1 (2.01)	0.930
Negative	413 (98.1)	47 (97.99)	
HP infection status			
Positive	37 (8.8)	5 (10.4)	0.708
Negative	384 (91.2)	43 (89.6)	
BMI (kg/m^2)			
Less than 18.5	28 (6.65)	4 (8.33)	0.358
18.5-24.99	304 (72.21)	38 (79.17)	
More than 25	89 (21.14)	6 (12.5)	
Differentiation degree			
Well	226 (53.7)	22 (45.8)	0.302
Poor	195 (46.3)	26 (54.2)	
Location			
Upper	113 (26.8)	16 (33.33)	0.779
Middle	129 (30.64)	10 (20.83)	
Lower	168 (39.9)	21 (43.75)	
Mixed	11 (2.61)	1 (2.08)	
Lauren classification			
Intestinal type	105 (24.94)	10 (20.83)	0.668
Diffuse type	270 (64.13)	31 (64.59)	
Mixed type	46 (10.93)	7 (14.58)	
T category			
T1a	68 (16.2)	4 (8.3)	0.033[a]
T1b	78 (18.5)	5 (10.4)	
T2	89 (21.1)	13 (27.1)	
T3	65 (15.4)	14 (29.2)	
T4a	116 (27.6)	10 (20.8)	
T4b	5 (1.2)	2 (4.2)	
T1	146 (34.68)	9 (18.75)	0.026[a]
T2-4b	275 (65.32)	39 (81.25)	
Nerve invasion			
Positive	70 (16.6)	11 (22.92)	0.275
Negative	351 (83.4)	37 (77.08)	
Vessel carcinoma embolus			
Positive	72 (17.1)	15 (31.35)	0.017[a]
Negative	349 (82.9)	33 (68.75)	

[a]$P < 0.05$. HP: *Helicobacter pylori*; BMI: Body mass index; CEA: Carcinoembryonic antigen.

Table 2 Multivariate analysis for stage pTxN0M0 gastric cancer patients with D2 resection

	Univariate analysis				Multivariate analysis			
	P value	Exp(B)	95%CI used for Exp (B)		*P* value	Exp (B)	95%CI used for Exp (B)	
			Lower	Upper			Lower	Upper
Family history	0.912	0.923	0.765	1.311	0.069	1.017	0.72	1.896
HP infection status	0.209	0.832	0.781	1.226	0.754	1.088	0.643	1.840
Gender	0.000[a]	1.716	1.316	2.553	0.590	0.898	0.608	1.327
CEA	0.000[a]	1.919	1.319	2.352	0.000[a]	1.924	1.353	2.232
Location	0.245	0.841	0.792	1.234	0.749	1.012	0.861	1.531
Lauren classification	0.241	0.851	0.814	1.091	0.711	1.109	0.891	1.154
T category	0.000[a]	1.906	1.659	2.271	0.009[a]	1.714	1.050	2.403
Differentiation degree	0.279	0.932	0.881	1.126	0.784	1.188	0.663	1.640
Nerve invasion	0.971	0.801	0.731	1.145	0.097	0.951	0.7768	1.655
Vessel carcinoma embolus	0.000[a]	1.764	1.321	2.562	0.983	0.994	0.895	1.660
BMI	0.732	0.812	0.729	1.234	0.356	1.228	0.912	2.229

[a]*P* < 0.05. HP: *Helicobacter pylori*; BMI: Body mass index; CEA: Carcinoembryonic antigen.

Table 3 Multivariate analysis of overall survival in pTxN0M0 gastric cancer patients

	CEA-Low group (*n* = 421)				CEA-High group (*n* = 48)			
	P value	Exp(B)	95%CI		*P* value	Exp(B)	95%CI	
			Lower	Upper			Lower	Upper
Family history	0.077	2.978	0.888	3.986	0.512	1.191	0.501	2.019
HP infection status	0.140	1.590	0.858	2.947	0.247	0.522	0.174	1.570
Gender	0.478	0.834	0.504	1.378	0.919	1.036	0.527	2.037
Location	0.482	0.831	0.764	1.124	0.897	1.012	0.752	2.102
Lauren classification	0.831	0.911	0.891	1.103	0.843	1.245	0.984	1.435
T category	0.647	0.941	0.725	1.222	0.001[a]	1.962	1.139	2.629
Differentiation degree	0.879	0.931	0.811	1.176	0.884	1.148	0.673	1.641
Nerves invaded	0.811	1.090	0.539	2.205	0.987	0.993	0.438	2.251
Vessel carcinoma embolus	0.064	0.315	0.093	1.068	0.883	0.889	0.685	2.281
BMI	0.392	0.424	0.851	1.124	0.356	1.228	0.912	2.229

[a]*P* < 0.05. HP: *Helicobacter pylori*; BMI: Body mass index; CEA: Carcinoembryonic antigen.

embolus (31.35% *vs* 17.1%, *P* = 0.017) among the CEA-positive goup.

Survival analysis

The 5-year OS of stage pN0 GC patients with high level of CEA was significantly inferior than CEA-low groups (57.74% *vs* 90.69%, *P* < 0.05, Figure 3).

Univariate and multivariate analysis

Univariate analysis exhibited that FH of GC, HP infection status, gender, CEA, T category, differentiation degree, location, and lauren classification, nerve invasion, vessel carcinoma embolus, and BMI; among which T category (OR = 1.906), CEA (OR = 1.919), vessel carcinoma embolus (OR = 1.764), and gender (OR = 1.716) were independent hazard prognostic factors(all *P* < 0.05, Table 2, Figure 4A).

Further multivariate analysis showed that CEA (OR = 1.924), T category (OR = 1.714) were significant prognostic factors for pN0 GC (all *P* < 0.05, Table 2, Figure 4B). In the CEA-high sub-group, T category (OR = 1.962) was an independent hazard factor in CEA-high group by multivariate analysis (*P* < 0.05, Table 3, Figure 4C).

DISCUSSION

As we known, CEA is part of the most familiarly used cancer biomarkers, and high preoperative CEA are closely associated with tumor load[10,15-19]. However, there had been few literatures regarding the treatment outcome of evaluating the prognostic significance of CEA, in particularly to those pN0 GC patients. Previous studies have offered ambivalent testimony on the survival value of pretreatment CEA levels in resectable GC.

At the present stage, there existed no unified and well-recognized cut-off points[2]. Tied to various objective factors such as the sample size, different follow-up periods, ethnicities and different tumor stage, it leaded to inconsistent bias. To strengthen the statistical power, we collected a large sample analysis, and the number of eligible patients on the basis of similar endpoints. In the present study, the cut-off point was applied to 30.02 ng/mL.

In addition, study characteristics that miscellaneous large span studies might have influenced the effect size in GC patients. To confirm this synergistic effect, we performed subgroup analyses by clinicopathologic baseline. Firstly, in the CEA-high group, the proportion

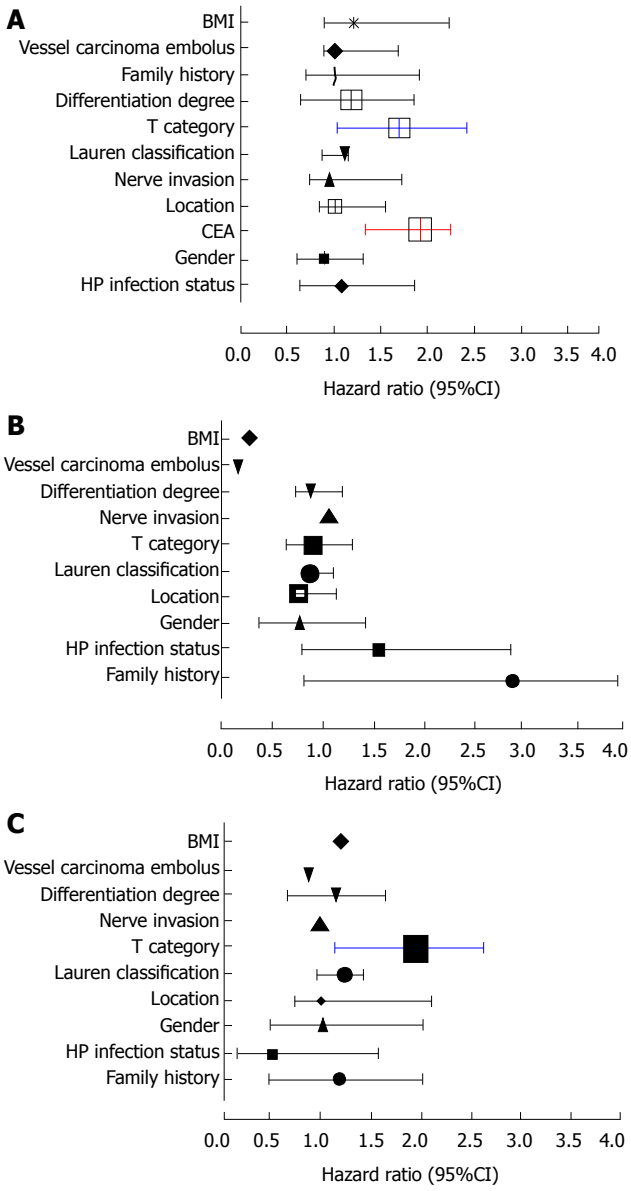

Figure 4 Univariate and multivariate analyses for pN0 gastric cancer patients using the Cox regression model. A: HR was calculated in multivariate analyses; B: HR was calculated in CEA-low group; C: HR was calculated for CEA-high group. CEA: Carcinoembryonic antigen; HR: Hazard ratio.

of was slightly higher than the negative group in poor differentiation (54.2% *vs* 46.3%), and nerve invasion (22.92% in *vs* 16.6%), showing that CEA-high GC patients with stage pN0 may be at higher risk, and it should be remunerated meticulous attention to the crowd.

Although the biological actions of CEA are not fully understood, the close link of preoperative CEA to cancer aggressiveness has been known for many years[20]. Specifically, the patients with a high level of CEA were consulted more frequently in the presence of a advanced stage (T_{2-4b}: 81.25% *vs* 65.32%, $P = 0.026$), vessel carcinoma embolus (31.35% *vs* 17.1%, $P = 0.017$). The baseline data supported the view that a high level of CEA in stage pN0 patients were identified as having worse biological behavior and more aggressive baseline conditions, which might be fastened to a potential genetic susceptibility and infaust living habits[21-24].

In consideration of worse characteristics and unfavourable tumor behavior, CEA-high patients' survival rate was poor. In the data, the 5-year OS of patients with high expression of CEA was strikingly inferior (57.74% *vs* 90.69%, $P < 0.05$). The data added weight to show that preoperative prominent CEA correlates with more aggressive and poor survival, and the point above had to be in conformity with a former research[25].

Further verification was tested by multivariate analysis, the findings highlighted that CEA (OR = 1.924), T category (OR = 1.714) were significant prognostic factors for GC cases with stage of pN0, suggesting that these two factors were closely associated with the survival and multicollinearity might exist between them. The view was in accordance with many scholars[2] who had found that elevated serum CEA was involved in tumor depth (T category), lymphatic metastasis, and TNM stage, and liver metastasis[26,27], and it was an independent prognostic risk factor.

Further analysis show that T category (OR = 1.962) was an objective hazard factor in the CEA-high group for pN0 GC patients. It was found consistently in the aforementioned studies, which were at the root of the CEA was substituted for T category in the current TNM staging system to come up with a modified staging system.

To our knowledge, this analysis is one of the relatively few that have been reported. However, there were several limitations inherent in this study. First, it was intended to serve as a retrospective study and a clinical bias could potentially occur. Also, follow-ups were achieved through phone calls and a recall bias existed.

In spite of the assistance brought by the optimal cut-off value for serum CEA level in clinical practice, there exists limitations. Firstly, the possibility of patient selection introducing bias was inherent, which can affect surgical outcomes. Secondly, the number of CEA-high patients was relatively small, which reducing the intensity of statistics. What's more, the data come from a single hospital, so the results may not represent the Chinese population well.

In conclusion, the CEA, categorized by cut-off points of 30.02 ng/mL, could produce the best prognostic discriminatory ability, and increased pretreatment CEA levels nearly doubled the risk of mortality in pN0 GC patients.

COMMENTS

Background

The survival value of carcinoembryonic antigen (CEA) for gastric cancer patients remains obscure. This study aims at assessing whether elevated serum CEA is a partner in the inferior prognosis for pathological lymph node-negative (pN0) patients.

Research frontiers

CEA, an acknowledged as an intracellular adhesion molecule, is one of the most common markers used in GC. Numerous studies have been in favour of preoperative CEA levels as biomarker for the survival of GC. However, other studies have reported the opposite results. The X-tile plot has been recently elaborated to establish cut-off point for biomarkers in cancer. we performed a large sample retrospective study and reset an optimal cut-off value.

Innovations and breakthrough

The authors found that the CEA, categorized by cut-off points of 30.02 ng/mL could develop the best prognostic discriminatory ability and predictive accuracy for staging pN_0 GC patients. Increased pretreatment serum CEA levels (> 30.02 ng/mL) nearly doubled the risk of mortality in in pN_0 GC patients.

Applications

This study results suggest that those pretreatment serum CEA levels over 30.02 ng/mL on behalf of worse characteristics and unfavourable tumor behavior.

Peer-review

The authors examined subjects with pretreatment serum CEA > 30.02 ng/mL have a poor prognosis in terms of survival, vascular invasion and transmural invasion. Clinicopathologic factors affecting outcome were evaluated. Their results show that those pretreatment serum CEA levels over 30.02 ng/mL on behalf of worse characteristics and unfavourable tumor behavior, and a poor prognosis for a nearly doubled risk of mortality in GC patients.

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ORIGINAL ARTICLE

Retrospective Study

Fecal microbiota transplantation induces remission of infantile allergic colitis through gut microbiota re-establishment

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Author contributions: Liu SX and Li YH contributed equally to this work; Shu SN and Huang ZH designed the research; Dai WK designed the follow-up plans and collected patients' information with Li XS, Ruan ML, Zou B and Dong C after FMT; Qiu CZ, Liu YH and He JY performed bioinformatics analysis; Ruan ML and Zou B recorded patients' and donors' clinical data; Shu SN and Huang ZH conducted FMT.

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Abstract

AIM

To investigate the impact of fecal microbiota trans-

plantation (FMT) treatment on allergic colitis (AC) and gut microbiota (GM).

METHODS

We selected a total of 19 AC infants, who suffered from severe diarrhea/hematochezia, did not relieve completely after routine therapy or cannot adhere to the therapy, and were free from organ congenital malformations and other contraindications for FMT. Qualified donor-derived stools were collected and injected to the AC infants *via* a rectal tube. Clinical outcomes and follow-up observations were noted. Stools were collected from ten AC infants before and after FMT, and GM composition was assessed for infants and donors using 16S rDNA sequencing analysis.

RESULTS

After FMT treatment, AC symptoms in 17 infants were relieved within 2 d, and no relapse was observed in the next 15 mo. Clinical improvement was also detected in the other two AC infants who were lost to follow-up. During follow-up, one AC infant suffered from mild eczema and recovered shortly after hormone therapy. Based on the 16S rDNA analysis in ten AC infants, most of them (n = 6) had greater GM diversity after FMT. As a result, Proteobacteria decreased (n = 6) and Firmicutes increased (n = 10) in post-FMT AC infants. Moreover, Firmicutes accounted for the greatest proportion of GM in the patients. At the genus level, *Bacteroides* (n = 6), *Escherichia* (n = 8), and *Lactobacillus* (n = 4) were enriched in some AC infants after FMT treatment, but the relative abundances of *Clostridium* (n = 5), *Veillonella* (n = 7), *Streptococcus* (n = 6), and *Klebsiella* (n = 8) decreased dramatically.

CONCLUSION

FMT is a safe and effective method for treating pediatric patients with AC and restoring GM balance.

Key words: Pediatric; Infantile allergic colitis; Fecal microbiota transplantation; Gut microbiota; Immune reaction

Core tip: This retrospective study explored the therapeutic effects and safety of fecal microbiota transplantation (FMT) treatment in 19 allergic colitis (AC) infants who were younger than 1 year old. After FMT treatment, AC symptoms were relieved in the patients rapidly, and no patient relapsed within 15 mo. With gut microbiota (GM) analysis, six of ten patients exhibited higher microbial diversity after FMT treatment. Moreover, decreased Proteobacteria and increased Firmicutes supplied the hints of GM re-establishment in the patients after FMT treatment. Therefore, this work showed the curative effects of FMT in AC infants and its possible mechanism.

Liu SX, Li YH, Dai WK, Li XS, Qiu CZ, Ruan ML, Zou B, Dong C, Liu YH, He JY, Huang ZH, Shu SN. Fecal microbiota transplantation induces remission of infantile allergic colitis through gut microbiota re-establishment. *World J Gastroenterol* 2017; 23(48): 8570-8581 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i48/8570.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i48.8570

INTRODUCTION

Allergic colitis (AC) is a common infantile rectal bleeding disorder which is caused by severe allergic reactions within the digestive system[1,2]. AC is normally identified in infants younger than one year of age and its representative clinical features are hematochezia and diarrhea[3]. Bloody purulent stools, abdominal pain, and vomiting are also used to diagnose AC[3]. Various factors such as food allergens, aberrant immune system, and imbalanced gut microbiota (GM) are thought to contribute to AC[4-6].

Conventional therapies for AC are reducing exposure to suspicious allergens and applying hypoallergenic milk powder[7]. Baldassarre *et al*[8] used probiotics to treat AC infants and found that *Lactobacillus GG* may relieve symptoms of AC by altering GM composition[8]. Fecal microbiota transplantation (FMT) can change gut micro-ecology more robustly in comparison to food or probiotics. Several reports suggested that FMT was therapeutically efficacious for treating diseases associated with GM dysbiosis, such as *Clostridium difficile* infection (CDI)[9,10], inflammatory bowel disease (IBD)[11,12], and irritable bowel syndrome (IBS)[13,14]. However, to our knowledge, FMT has not been used to treat AC infants.

Thus, we assessed 19 AC infants with severe hematochezia and/or diarrhea, who had not acquired complete remission after 2 wk of routine therapy or because the guardians cannot adhere to the routine therapy thoroughly. Our intention was to confirm the safety and efficacy of FMT in AC treatment, and detect the sustained GM changes after FMT.

MATERIALS AND METHODS

Ethics

This study was approved by the Medical Ethics Committee of Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology (TJ-C20140712). All subjects and donors gave signed informed consent. Principles of patients care and all experimental procedures followed the guidelines established by the Institutional Review Board of Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology.

Patient selection

AC was diagnosed based on the following clinical symptoms: (1) rectal bleeding with/without mucus and diarrhea; (2) exclusion of infectious colitis, anal fissure, lymphoid nodular hyperplasia, and uncommon conditions such as necrotizing enterocolitis, Hirschsprung's enterocolitis, IBD[15], and IBS[16]; (3) clinical remission after milk exclusion and recurrence after milk re-challenge[3,17]; and (4) histological examination indicated that the intestinal mucosa exhibited chronic inflammation with eosinophils infiltration and colonic lesions (Supplementary File 1). Pediatric AC patients meeting the following criteria were selected as FMT candidates: (1) the patients had no complete remission after routine therapy, the patients cannot adhere to the therapy thoroughly, or their parents had strong intention to receive the treatment of FMT; (2) free from contraindications for FMT, such as intestinal obstructions, perforations, and bleeding, or severe immunodeficiency diseases; and (3) colonoscopic inspection indicated no mucosal congestion, edema, multiple spot-like erosion, or lymphoid granular nodes (four cases included in Figure 1). As a result, 19 AC patients were enrolled in the study between September 2015 and December 2015 (Table 1).

Donor screening

Patients' mothers were considered to be donors of the highest priority, followed by fathers and healthy peers. Adult donors were screened as follows[18-20]: (1) no infectious diseases history (*e.g.*, tuberculosis and hepatopathy); (2) no metabolic diseases history (*e.g.*, obesity and diabetes); (3) no gastrointestinal diseases (*e.g.*, diarrhea, constipation, IBD, IBS, colorectal polyps, and gastrointestinal tumors); (4) no allergic diseases (*e.g.*, food allergy, eczema, and allergic gastroenteritis); (5) no antibiotic exposure in the last 3 months; (6) no mental disorders or autoimmune diseases; and (7) no drug abuse history, amenorrhea (for mother donors), or psychological imbalance.

Candidate donors of the same age were selected with the following criteria[18-20]: (1) preferred relatives with breast milk fed and same gender; (2) no antibiotic treatment in the last 3 months; (3) no allergic disease (*e.g.*, food allergy, eczema, and allergic gastroenteritis); (4) no gastrointestinal disease (*e.g.*, diarrhea, constipation, IBD, IBS, colorectal polyps, and gastrointestinal tumors); (5) no metabolic disease history (*e.g.*, obesity and diabetes); and (6) no infectious disease history (*e.g.*, tuberculosis, hepatopathy, and measles), and normal health and development. Tests for serum biochemistry and stool were performed for donors to ensure subject safety (Table 2).

FMT procedure

The application of parenteral nutrition and probiotics was stopped as soon as FMT began in the patients. No bowel preparation (cleanout or antibiotic pretreatment) was used prior to FMT, but pre-FMT clinical tests were performed as described in Table 3. Donor stool, collected 2 h before FMT, was diluted and mixed with sterile saline (1 mg of stool was diluted with 3 mL of saline). Samples were filtered through sterile gauze and 30-50 mL fecal suspension was prepared for FMT. FMT was administered over 5-10 min *via* a rectal tube into the left colon. The rectal tube was removed 15 min after administration and the fecal suspension was retained in the recipients' gut for 4-6 h. Multi-FMT was given for patients with severe symptoms (Table 1).

Follow-up

Clinical symptoms, stool frequency, symptom remission time, and adverse events (*e.g.,* abdominal pain, gastrointestinal infection, constipation, fever, and allergic disease) were recorded at the end of FMT (Table 1). Follow-up was conducted at $\geqslant$ 15 mo after FMT, except for two cases with 0.3 and 0.5 mo follow-up (AC17 and AC19), to evaluate FMT efficacy and safety (Table 1). The remission of AC was defined as the cease of rectal bleeding and decreased stool frequency (no more than two times/d) in the patients. The primary endpoint was the improved AC symptoms and sustained clinical remission at 12 mo. Secondary endpoint was the safety of FMT which was implied by the occurrence of adverse events.

Microbiota analysis and statistics

Fecal microbiota was analyzed for ten patients before FMT and during follow-ups. Donor feces were also assayed for GM. Microbial DNA was extracted using a PowerSoil DNA Isolation Kit (Mo Bio Laboratories, Carlsbad, CA) according to the manufacturer's protocol and the hyper-variable V3-V4 region was amplified using 338F (5'-ACTCCTACGGGAGGCAGCA-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3') primers. Library construction and sequencing were conducted on an Illumina MiSeq platform (Illumina, San Diego, United States). Data filtration and analysis were performed as a prior report with the RDP database as an annotation reference[21]. A Wilcoxon signed-rank test was used to compare samples of one patient, which were collected at different time points, and a Wilcoxon rank-sum test was used to compare donor and patient samples. Graphs were produced with R

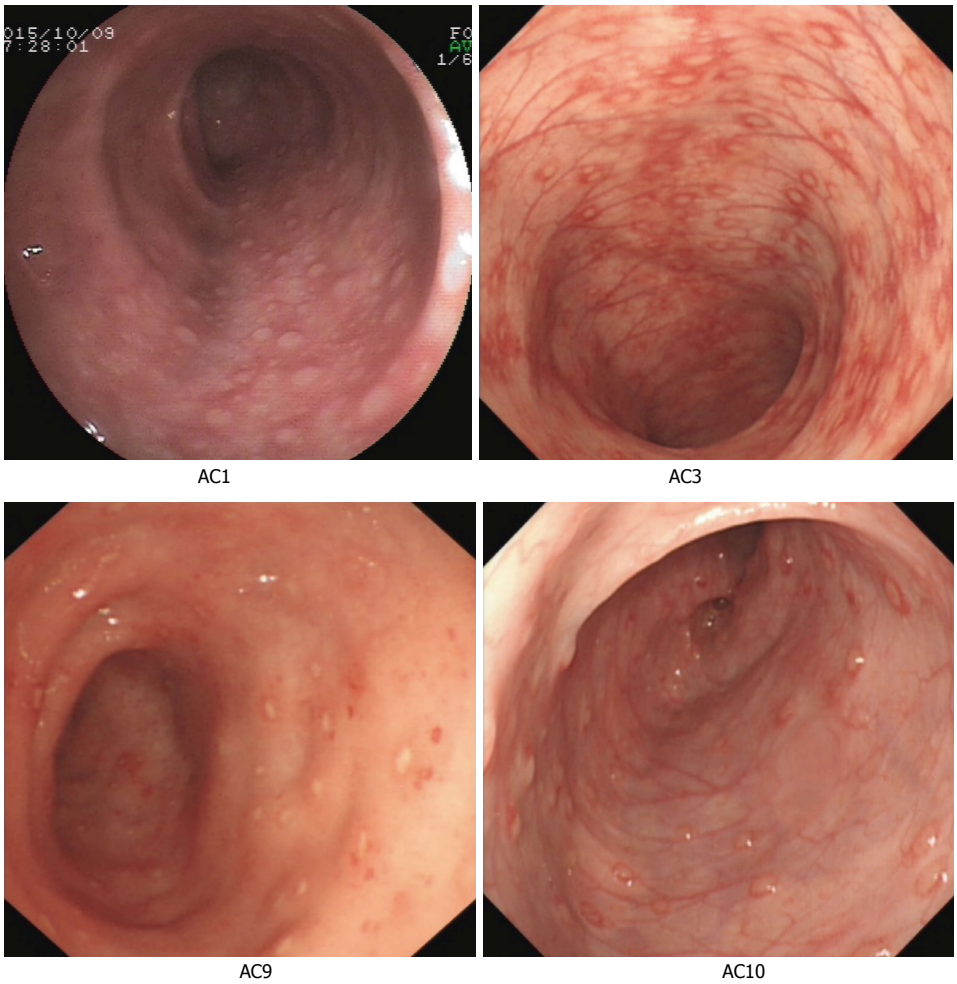


Figure 1 Colonoscopic inspection of four allergic colitis patients prior to fecal microbiota transplantation. Colonoscopic images of patients (AC1, AC3, AC9, and AC10) were obtained prior to FMT. AC: Allergic colitis; FMT: Fecal microbiota transplantation.

package (version 3.2.3).

RESULTS

Recipient characteristics

FMT recipients were aged from 4 to 11 mo (11 boys and 8 girls) and had hematochezia or severe diarrhea. Disease duration of AC patients was 0.5-3 mo for 16 cases and 3-6 mo for three cases (Table 1). Formula was replaced with hypoallergenic milk powder in all patients' dietary, and 11 of them were exposed to probiotics before FMT treatment (Table 1). The colonoscopic inspection of four AC infants was included in Figure 1.

FMT safety and efficacy

Infants experienced low-quality sleep and weight loss since the onset of AC, but no one was malnourished. They had significant clinical remission within 2 d after the first FMT treatment (Table 1). After FMT, hematochezia or diarrhea rapidly improved in AC patients, and decreased defecation frequency with improved stool consistency was also observed (Table 1). Within more than 15 mo of follow-up, the symptoms of AC had not relapsed except two patients who were lost to follow-up (AC17 and AC19). Only one patient suffered from eczema, which appeared 2 mo after FMT and was resolved with hormone therapy. Beyond this, no other adverse event was recorded during FMT or the follow-up.

FMT treatment associated microbiota changes

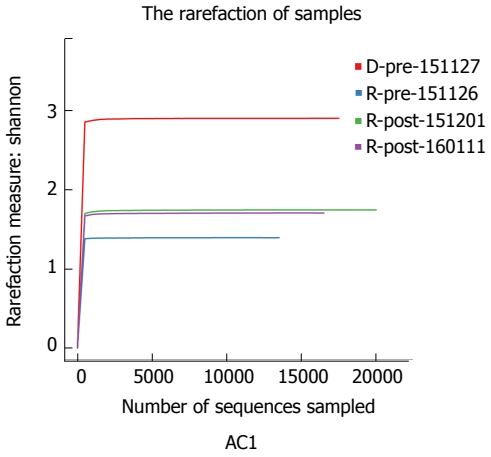
Figures 2 and 3 depict microbiota changes of ten patients before and after FMT compared to donors. Microbiota diversity increased dramatically in five patients while it decreased in three patients after FMT (Figure 2). When sampled 1 or 2 mon after FMT, microbiota of six patients was more similar to donors' microflora by comparison with pre-FMT samples (Figure 3).

After FMT treatment, Firmicutes accounted for the greatest proportion of GM in the AC infants, followed

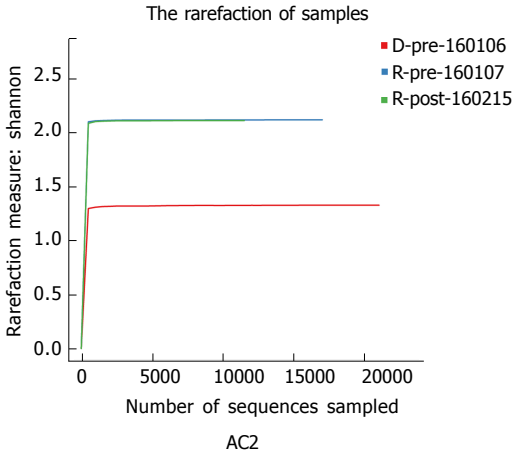
Table 1 Clinical information for 19 allergic colitis infants

ID	Gender	Age (mo)	Symptom(s)	Duration of disease (mo)	Treatment(s) before FMT	Donor source	FMT times	Symptom remission after first FMT (d)	Stool frequency before and after FMT (times/d)	Follow-up (mo)	Availability of gut microbiota data
AC1	Female	7	Diarrhea, hematochezia sometimes; anemia; hypohepatia	> 3	Applying amino acid formula and probiotics (*C. butyricum*)	Mother	2	1	3-4, 1	19	Yes
AC2	Male	10	Hematochezia	> 0.5	Applying amino acid formula and probiotics (*Bifidobacteria*)	Healthy infants aged 10 mo old	2	1	2-3, 1	18	Yes
AC3	Female	11	Hematochezia	> 3	Applying amino acid formula and probiotics (*S. boulardii*)	Healthy infants aged 8 mo old	3	1	5-6, 2	19	Yes
AC4	Male	9	Hematochezia	> 3	Applying amino acid formula and probiotics (*S. boulardii*)	Mother's cousin sister	3	1	6-7, 1-2	18	Yes
AC5	Male	5	Diarrhea and hematochezia sometimes	> 3	Applying amino acid formula	Healthy infants aged 8 mo old	3	1	3-4, 2	19	Yes
AC6	Male	5	Hematochezia	> 3	Applying amino acid formula and probiotics (*C. butyricum*)	Mother	1	2	5-6, 1	18	Yes
AC7	Male	4	Hematochezia and cough sometimes	> 2	Applying amino acid formula and nebulization	Mother	2	2	4-7, 1	15	Yes
AC8	Female	3	Diarrhea and mucoid feces sometimes	> 2	Applying amino acid formula	Mother	2	1	3-4, 1-2	19	Yes
AC9	Male	11	Interval hematochezia	> 6	Appling amino acid formula and probiotics (*S. boulardii*)	Mother	2	1	3-4, 2	23	Yes
AC10	Female	3	Hematochezia	> 1.5	Applying amino acid formula	Healthy infants aged 10 mo old	4	2	5-6, 1	21	Yes
AC11	Male	7	Diarrhea	> 2	Applying amino acid formula, probiotics (*Bifidobacteria*), Smecta and Oral Rehydration Salts (ORS)	Healthy infants aged 10 mo old	5	1	5-6, 1	23	No
AC12	Female	10	Diarrhea and hematochezia sometimes	> 1	Applying amino acid formula and probiotics (*Bifidobacteria*)	Mother	3	1	5-6, 1-2	22	No
AC13	Male	5	Hematochezia and diahhrea sometimes	> 3	Applying amino acid formula	Mother	1	1	3-4, 1	15	No
AC14	Female	5	Hematochezia and then peptone shaped feces	> 1	Applying amino acid formula and probiotics (*Bifidobacteria*)	Mother	1	1	7-8, 2	15	No
AC15	Male	7	Diarrhea	> 2	Applying amino acid formula, ORS, and probiotics (*C. butyricum*)	Mother	5	1	5-6, 1	21	No
AC16	Female	5	Interval hematochezia	> 2	Appling amino acid formula	Healthy infants aged 8 mo old	2	1	4-5, 1	21	No
AC17	Male	7	Diarrhea and hematochezia sometimes	> 3	Applying amino acid formula, probiotics (*Bifidobacteria* and *C. butyricum*)	Healthy infants aged 11 mo old	1	2	3-4, 1-2	0.5	No
AC18	Female	8	Diarrhea and cough sometimes	> 4	Applying amino acid formula and nebulization	Healthy infants aged 8 mo old	2	1	3-4, 2	17	No
AC19	Male	5	Interval diarrhea	> 4	Applying amino acid formula	Mother	4	1	3-4, 1	0.3	No

AC: Allergic colitis; FMT: Fecal microbiota transplantation.



AC1



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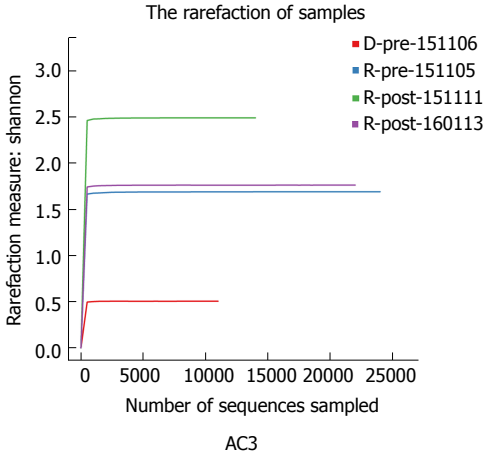

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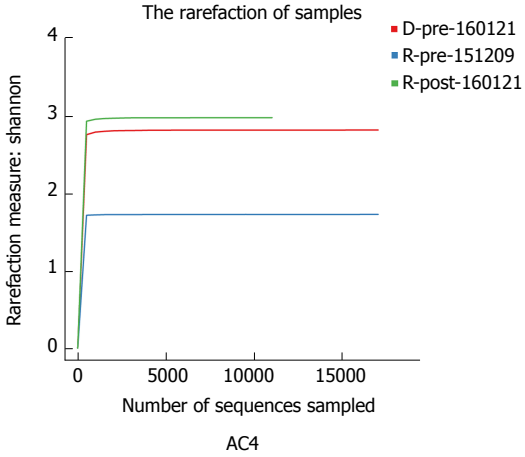
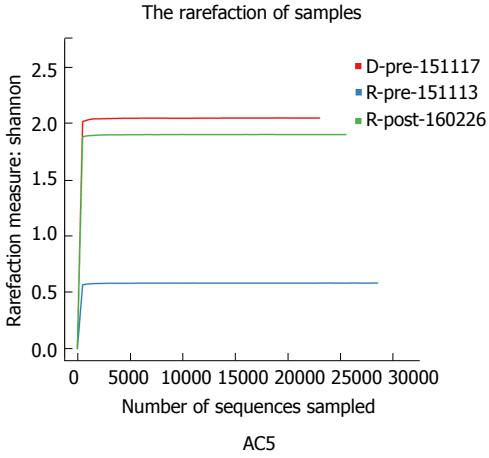
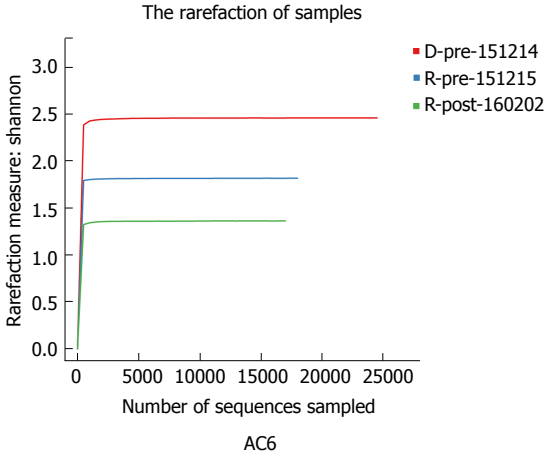


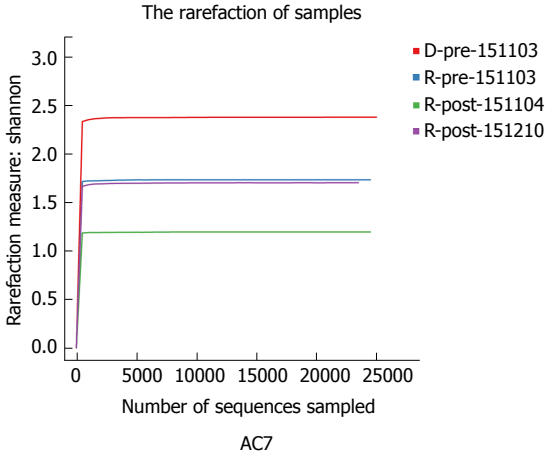
AC4



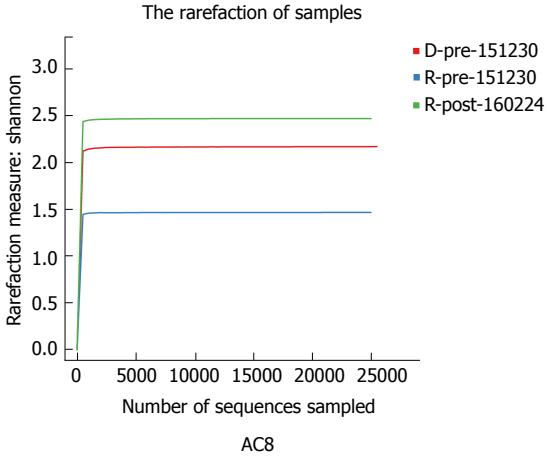
AC5



AC6



AC7



AC8

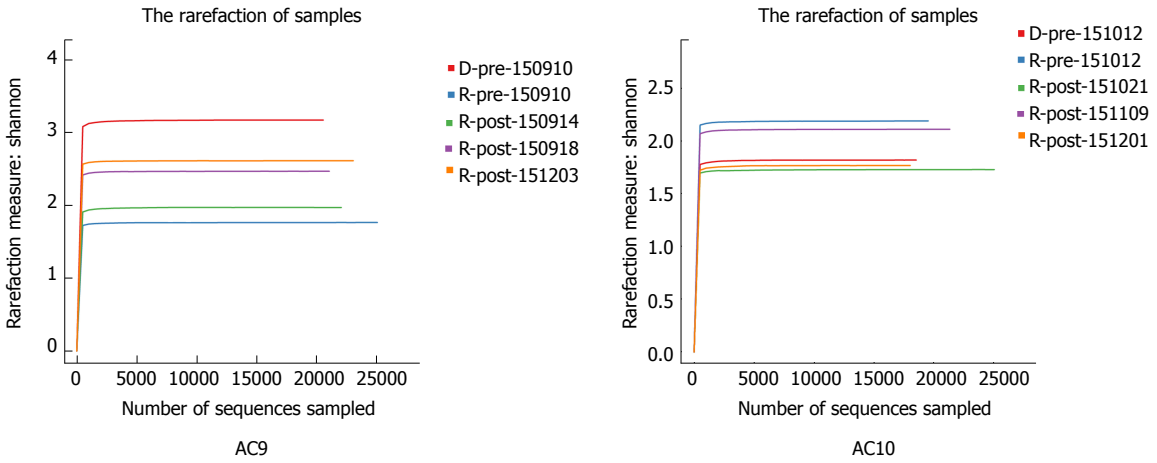


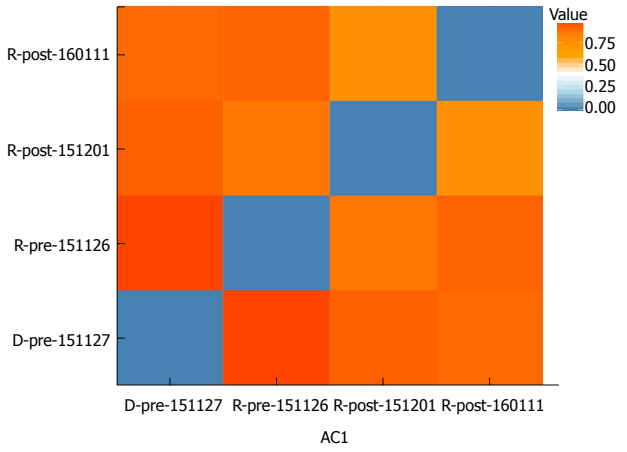
Figure 2 Shannon rarefaction curves of gut microbiota from ten allergic colitis infants and their donors. Each image represents one AC infant, and each curve represents one fecal sample from a patient or the corresponding donor. Sample ID has three parts: 'R' or 'D' indicates AC infants or donors, 'pre' or 'post' represents the stools collected before or after FMT, and fecal collection date. Microbiota diversity in six patients (AC1, AC4, AC5, AC7, AC8, and AC9) increased after FMT treatment. AC: Allergic colitis; FMT: Fecal microbiota transplantation.

Table 2 Laboratory testing on donors

Blood testing
Blood transfusion examinations: Quantifications of hepatitis B surface antigen, hepatitis B surface antibody, hepatitis B E antigen, hepatitis B E antibody, hepatitis B core IgM antibody, hepatitis C antibody, human immunodeficiency virus antibody, and treponema pallidum antibody.
TORCH examinations: Detections on toxoplasmosis IgG, toxoplasmosis IgM, rubella virus IgG, rubella virus IgM, cytomegalovirus IgG, cytomegalovirus IgM, herpes simplex virus 1/2 IgG, and herpes simplex virus 1/2 IgM.
Detection on parvovirus B19.
Epstein-Barr virus examinations: Detections on Epstein-Barr virus capsid antigen IgA, Epstein-Barr virus capsid antigen IgG, Epstein-Barr virus capsid antigen IgM, Epstein-Barr virus early antigen IgG, and Epstein-Barr virus nuclear antigen IgG.
Blood type examination.
Lymphocyte subpopulation examination.
Food allergen examination (sIgE).
Hepatic and renal function examinations: Glutamic-pyruvic transaminase, glutamic oxalacetic transaminase, total protein, albumin, globulin, prealbumin, total bilirubin, direct bilirubin, indirect bilirubin, alkaline phosphatase, gamma glutamyltranspeptidase, total cholesterol , triglycerides, high-density lipoprotein, low density lipoprotein, apolipoprotein A1, apolipoprotein B, lactic dehydrogenase, calcium, corrected calcium, phosphorus, magnesium, urea, creatinine, trioxypurine, bicarbonate radical, total bile acid, 5-nucleotidase, α-L-fucosidase, cholinesterase, cystatin C, and lipase andamylopsin.
Mycobacterium tuberculosis antibody examination (or the enzyme-linked immuno-spot assay test for tuberculosis).
Immune system examinations: Quantifications of immune globulin A, immune globulin G, immune globulin M, alexin C3, and alexin C4.
Detection on hepatitis A-IgM.
Qualifications of C-reaction protein and erythrocyte sedimentation rate.
Stool testing
Fecal routine examinations: Detections on fecal color, character, red blood cells, white blood cells, occult blood, parasite eggs, protozoon, fat ball, rotavirus antigen, and fungus.
Bacterial culture tests: Detections on Vibrio cholera, Salmonella, Shigella, Aeromonas, Plesiomonas, and pathogenic Escherichia coli.
Other testing
Chest X-ray.
Urea[C13] Capsule Breath Test.
Abdominal ultrasound scan.
Electrocardiographic examination.

by Bacteroidetes and Proteobacteria. Proteobacteria decreased dramatically to < 10% for most patients except four patients (Supplementary File 2). Whilst, Firmicutes increased in all patients (Supplementary File 2).

The relative abundance of *Escherichia* significantly increased in eight AC infants (Supplementary File 3). *Bacteroides* increased in five AC infants including three who had no *Bacteroides* pre-FMT (Supplementary File 3). For four patients, *Lactobacillus* was enriched after FMT, but for three subjects, it was absent even after FMT. Possible pathogens including *Clostridium* and *Klebsiella* generally decreased after FMT. *Clostridium* and *Klebsiella* decreased in five and eight AC infants respectively (Supplementary File 3). The relative abundance of *Streptococcus* was lowered in six patients. Whilst, *Veillonella* was found decreased in seven patients and its relative abundance was no more than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Value
0.75
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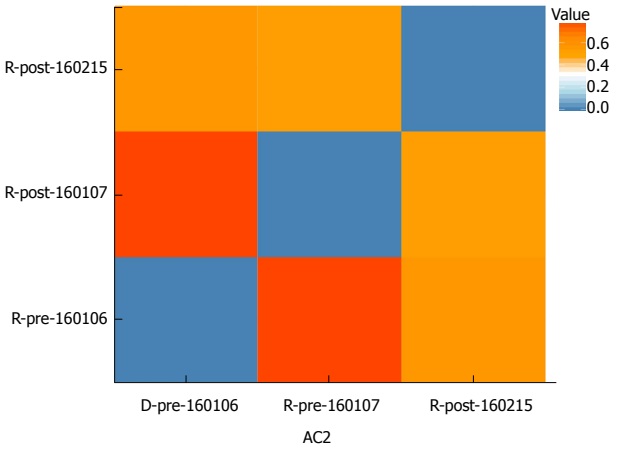
AC1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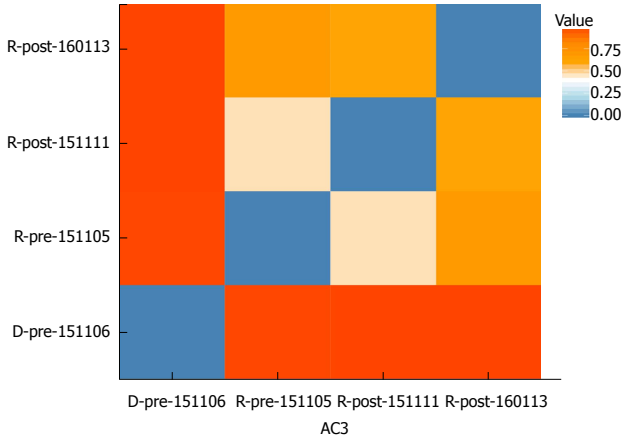
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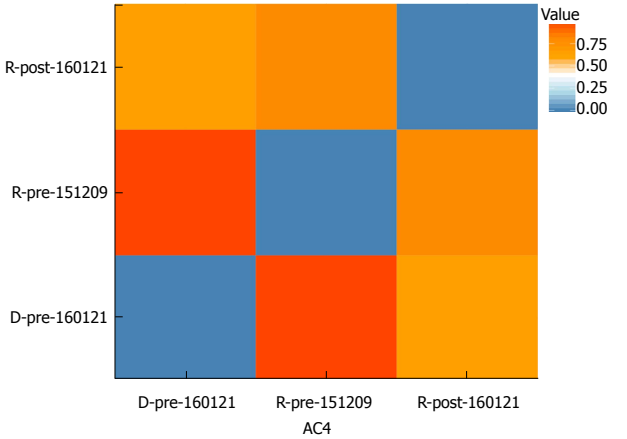
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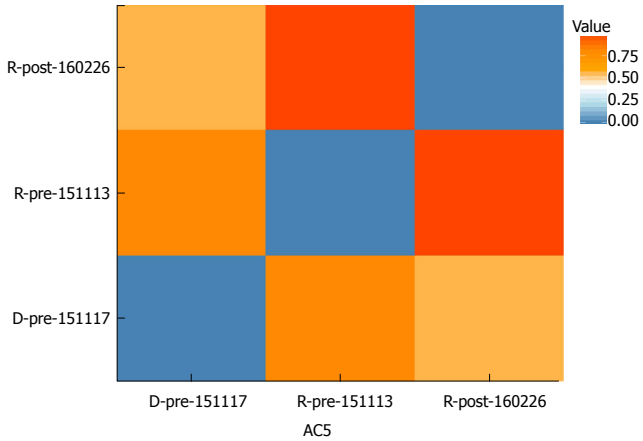
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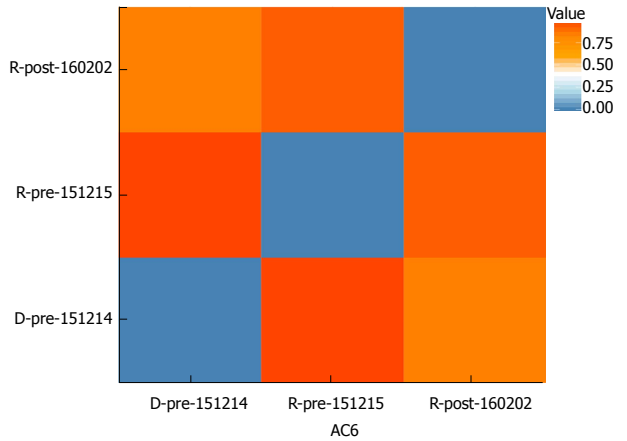
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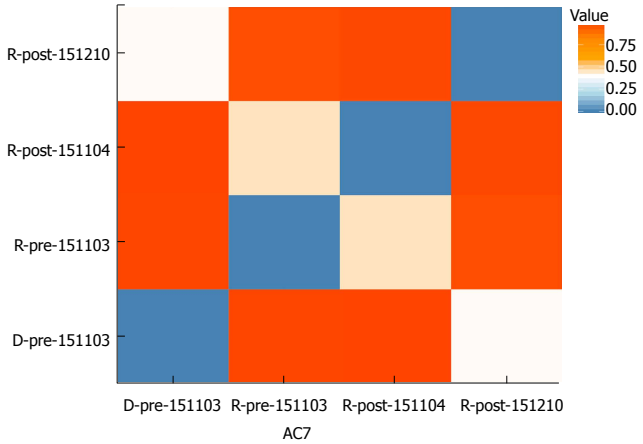
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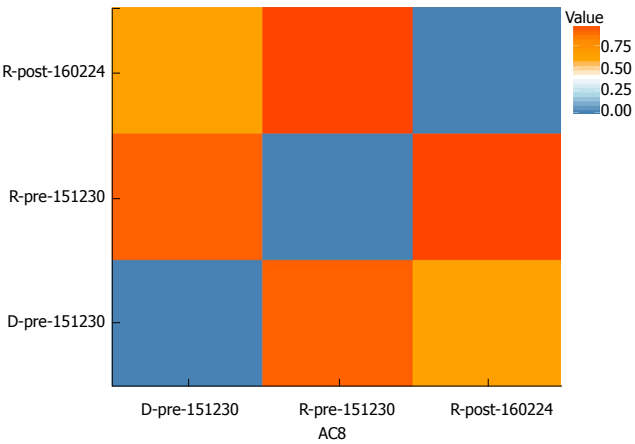
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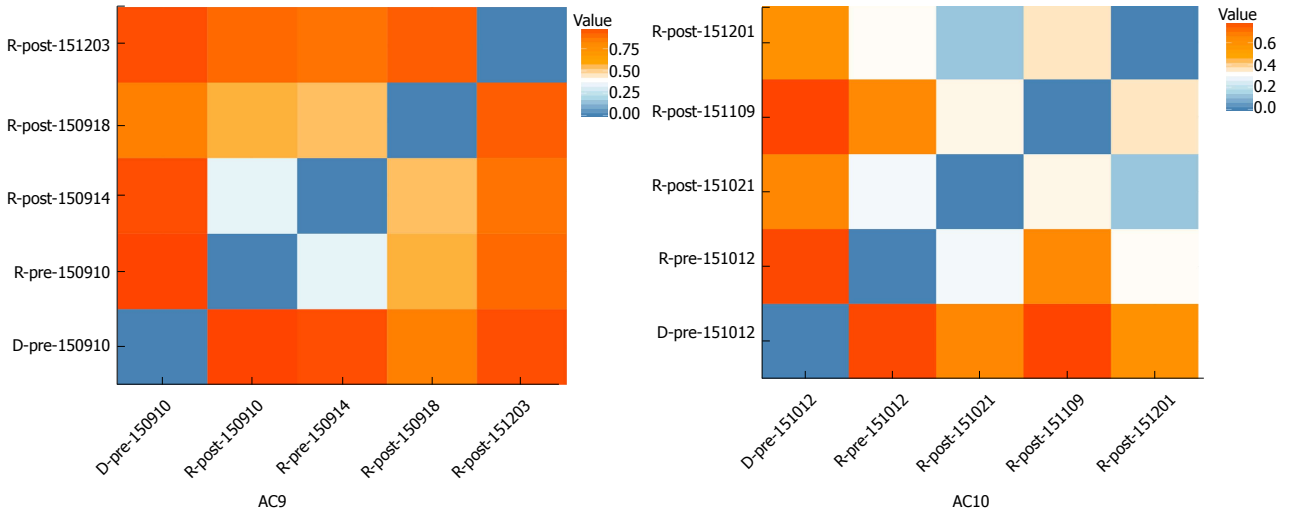

Figure 3 Microbiota similarity between allergic colitis infants and their donors. Values in red indicate low microbiota similarity between two samples. Blue represents high microbiota similarity. The microbiota compositions of patients (AC1, AC2, AC4, AC5, AC6, AC7, AC8, and AC10) were more similar to their donors' composition after FMT treatment. One patient (AC9) had more and then less microbiota similarity and AC3 did not change in this regard. AC: Allergic colitis.

Table 3 Laboratory testing of the patients before fecal microbiota transplantation

Blood testing
Hepatic and renal function examinations: Glutamic-pyruvic transaminas, glutamic oxalacetic transaminase, total protein, albumin, globulin, prealbumin, total bilirubin, direct bilirubin, indirect bilirubin, alkaline phosphatase, gamma glutamyltranspeptidase, total cholesterol, triglycerides, high-density lipoprotein, low density lipoprotein, apolipoprotein A1, apolipoprotein B, lactic dehydrogenase, calcium, corrected calcium, phosphorus, magnesium, urea, creatinine, trioxypurine, bicarbonate radical, total bile acid, 5-nucleotidase, α-L-ducosidase, cholinesterase, cystatin C, lipase, and amylopsin.
Food allergen examination (sIgE).
Lymphocyte subpopulation examination.
Detection on hepatitis A-IgM.
Blood transfusion examinations: Quantifications of hepatitis B surface antigen, hepatitis B surface antibody, hepatitis B E antigen, hepatitis B E antibody, hepatitis B core IgM antibody, hepatitis C antibody, human immunodeficiency virus antibody, and treponema pallidum antibody.
TORCH examinations: Detections on toxoplasmosis IgG, toxoplasmosis IgM, rubella virus IgG, rubella virus IgM, cytomegalovirus IgG, cytomegalovirus IgM, herpes simplex virus 1/2 IgG, and herpes simplex virus 1/2 IgM.
Detection on parvovirus B19.
Blood coagulation examinations: Detections on prothrombin time, prothrombin activity, international normalized ratio, fibrinogen, activated partial thromboplastin time, thrombin time, and D-dimer.
Blood type examination.
Mycobacterium tuberculosis antibody examination (or the enzyme-linked immuno-spot assay test for tuberculosis).
Stool testing
Fecal routine examinations: Detections on fecal color, character, red blood cells, white blood cells, occult blood, parasite eggs, protozoon, fat ball, rotavirus antigen, and fungus.
Bacterial culture tests: Detections on Vibrio cholera, Salmonella, Shigella, Aeromonas, Plesiomonas, and pathogenic Escherichia coli.
Other testing
Enteroscopic examination.
Abdominal ultrasound scan (intestinal adhesion).
Electrocardiographic examination.

8% after FMT. *Bifidobacterium* kept decreased in seven AC infants after FMT, and increased in two cases.

DISCUSSION

We chiefly considered the curative effects of FMT therapy in 19 AC infants and microbiota changes during treatment. Stools from both infant and adult donors suggested the same efficacy, and it was noted that all subjects had relieved symptoms of hematochezia and/or diarrhea in 2 d after the first FMT treatment. Due to the longer illness time or sever clinical symptoms, 15 patients experienced multi-FMT for the sustained clinical remission. And the multiple FMT in these patients gave us the idea that artificially modified microbiota for the specified patient might elevate the efficiency of FMT and attenuate transplantation times in the future. After being discharged from hospital, the patients were advised to take hypoallergenic milk powder instead of formula, and most patients had no relapse of colitis within more than 15 mo of follow-up. The recurrence of eczema

in one infant might be caused by the inflammatory reactions which were triggered by discontinuous intake of hypoallergenic milk powder.

Fecal microbiota was analyzed in ten patients and their donors. We noted that the microbiota diversity increased in six patients after FMT. For three other subjects, GM diversity decreased after an initial increase while all the patients demonstrated clinical improvement. Individual-specific GM changes suggested the effect of donor's GM complexity and patient's gut micro-ecology imbalance. Khoruts *et al*[10] also suggested that bacteria can be eliminated due to nutrient competition, antimicrobial peptide suppression, and immune-mediated colonization resistance during GM re-establishment.

Proteobacteria, which contain opportunistic pathogens[22], decreased in six AC infants, and their relative abundance was less than 10% after FMT. In contrast, Firmicutes increased in all AC infants. Previous work implied that Firmicutes decreased in patients with Cohn's disease[23], and the proportion of Firmicutes was negatively associated with gastrointestinal inflammation[24].

After FMT, the relative abundances of *Bacteroides* and *Lactobacillus* increased. Prior reports showed that species in *Bacteroides* could secrete polysaccharide A, which promoted the number of T regulatory (Treg) cells[25,26]. Interleukin (IL)-10 produced by Treg cells also eliminated inflammatory reactions and protected against infectious pathogens[25,26]. *Lactobacillus* can secrete lactic acid, increase the proportion of Treg cells, and relieve symptoms of AC[8,27]. Generally, the relative abundance of opportunistic pathogens decreased, including *Veillonella*, *Streptococcus*, *Clostridium,* and *Klebsiella*. Prior reports suggested that the combination of *Veillonella* and *Streptococcus* had been found in various GM systems and can augment IL-8, IL-6, and tumor necrosis factor-α (TNF-α) responses, which were associated with inflammatory reactions[28]. *Clostridium* can cause diarrhea *via* enterotoxin secretion, and *Klebsiella* was positively associated with macrophage migration-inhibitory factor (MIF) and affected host immunity[29]. However, GM imbalance and post-FMT improvement need more analysis to understand the mechanisms underlying AC improvements.

This study pioneered the application of FMT in AC treatment and provided important reference to understand microbiota changes before and after FMT. Although the results favor the application of FMT for AC treatment, it is still important to clarify whether AC symptoms can be improved in our future studies with larger sample size. Also, we will explore the microbiota changes at the gene or functional level before and after FMT, to further the understanding of GM imbalance and re-configuration during FMT treatment of infantile AC.

ARTICLE HIGHLIGHTS

Research background

Allergic colitis (AC), which is characterized as hematochezia and severe diarrhea, is caused by an intense allergic reaction of the digestive system. Currently, first-line therapies for AC patients are reducing exposure to suspicious allergens and applying hypoallergenic milk powder. However, some pediatric patients could not relieve from AC symptoms completely with routine treatment, and long-term illness causes adverse impact on nutrition absorption and physical development in the children.

Research motivation

Previous studies indicated that gut microbiota (GM) was closely related with the digestive system, neural system, and immune system in human. Meanwhile, the positive effects of fecal microbiota transplantation (FMT) have been confirmed in various gastrointestinal diseases, including *Clostridium difficile* infection (CDI), inflammatory bowel disease (IBD), and irritable bowel syndrome (IBS). However, FMT had not been applied to treat AC infants before. This research could provide important references for the treatment and research of infantile AC with FMT therapy.

Research objectives

The research aimed to detect the safety and efficiency of FMT treatment in AC, and compare GM composition before and after FMT treatment in the patients.

Research methods

The procedures of FMT, including selection of AC patients and donors, were conducted according to the guidelines established by the Institutional Review Board of Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology. Wilcoxon tests were adopted in the research.

Research results

In this study, the safety and efficacy of FMT treatment were investigated in 19 AC infants with GM analysis. The results indicated that the AC symptoms, which included rectal bleeding, diarrhea and hematochezia, were relieved rapidly by FMT treatment. During the 15 mo follow-up, no relapse was recorded except that eczema happened in one patient. After FMT treatment, the elevation of microbial diversity was detected in six of ten patients. Meanwhile, the relative abundances of Proteobacteria and Firmicutes were decreased (6/10) and increased (10/10), respectively, in the AC infants.

Research conclusions

This study documents the positive effect of FMT treatment on infantile AC remission, suggesting the potential of FMT in gastrointestinal allergic diseases. Individual-specific GM re-configuration also extended our understanding of FMT efficacy and associated mechanisms.

Research perspectives

Despite the aspiring results of FMT in pediatric AC, verified improvements with larger cohorts and longer follow-up are necessary. In parallel, GM analysis should be performed before and after FMT, to unravel keystone microbial components in the specific disease.

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ORIGINAL ARTICLE

Retrospective Study

Prognostic value of lymph node metastasis in patients with T1-stage colorectal cancer from multiple centers in China

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Author contributions: Sun ZQ, Ma S and Zhou QB contributed equally to this work; Sun ZQ and Zhou QB designed the study and analyzed the data; Ma S, Yang SX and Chang Y performed the experiments; Ma S wrote the paper; Zeng XY, Ren WG, Han FH, Xie X, Zeng FY, Sun XT, Wang GX, Li Z, Zhang ZY, and Song JM collected the data; Liu JB and Yuan WT designed the study and revised the paper; all authors have read and approved the final version to be published.

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Informed consent statement: All patients who provided clinical material signed an informed consent form.

Conflict-of-interest statement: All authors have declared that there is no conflict of interest.

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Abstract

AIM

To explore the features and prognostic value of lymph node metastasis in patients with T1-stage colorectal cancer (CRC).

METHODS

In all, 321 cases of T1-stage CRC were selected from 10132 patients with CRC who received surgical therapy in six large-scale hospitals in China and were retrospectively analyzed. Univariate and multivariate analyses were performed to analyze the risk factors for lymphatic metastasis. A survival analysis was then performed to analyze the prognostic value of lymph node metastasis.

RESULTS

The occurrence rate of T1 stage was 3.17% (321/10132); of these patients, the lymph node metastasis rate was 8.41% (27/321), and the non-lymph node metastasis rate was 91.59% (294/321). Univariate analysis showed that preoperative serum CEA, preoperative serum CA199, preoperative serum CA724, vascular invasion, and degree of differentiation were associated with lymph node metastasis in T1-stage CRC ($P < 0.05$ for all). Multivariate analysis indicated that preoperative serum CA724, vascular invasion, and degree of differentiation were closely related to lymph node metastasis ($P < 0.05$ for all). Log-rank survival analysis showed that age, preoperative serum CEA, preoperative serum CA199, vascular invasion, degree of differentiation, and lymph node metastasis ($\chi^2 = 24.180$, $P < 0.001$) were predictors of 5-year overall survival (OS) ($P < 0.05$ for all). COX regression analysis demonstrated that preoperative serum CA199 and lymph node metastasis (HR = 5.117; $P < 0.05$; 95%CI: 0.058-0.815) were independent prognostic indicators of 5-year OS in patients with T1-stage CRC ($P < 0.05$ for both).

CONCLUSION

The morbidity of T1-stage CRC was 3.17% for all CRC cases. Preoperative serum CA724, vascular invasion, and degree of differentiation are independent risk factors for lymph node metastasis. Lymph node metastasis is an independent prognostic factor for OS in patients with T1-stage CRC.

Key words: Colorectal cancer; Lymph node metastasis; T1 stage; Prognosis

Core tip: The high morbidity of patients with colorectal cancer (CRC) is caused by the likelihood of recurrence and metastasis. This study focused on the features and prognostic value of lymph node metastasis in patients with T1-stage CRC. According to the statistical analysis, we found a very low morbidity in patients with T1-stage CRC. Moreover, our findings confirm that preoperative serum CA724, vascular invasion, and degree of differentiation were independent risk factors for lymph node metastasis, which was demonstrated to be an independent prognostic factor for 5-year OS in patients with T1-stage CRC.

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INTRODUCTION

Colorectal cancer (CRC) is one of the most common malignancies worldwide[1]. With economic development and changes in dietary history, CRC has shown a steadily increasing incidence and is now the fifth leading cause of cancer-related death in China[2,3]. Due to adverse treatment-related side effects and the poor prognosis of this disease, which results from easy recurrence and metastasis, oncotherapy for CRC has posed a dilemma[4]. In addition, lymph node metastasis is the main type of metastasis in advanced CRC. The occurrence rate of T1-stage CRC has been reported to be approximately 3.51%[5,6]. When the tumor is completely removed, patients with T1-stage CRC generally have a good prognosis. However, because metastasis does not often occur in lymph nodes in T1-stage CRC, lymph node metastasis is often overlooked during the process of diagnosis and treatment. Nevertheless, lymph node metastasis is one of the most essential prognostic risk factors. Chock *et al*[7] reported that the incidence of lymph node metastasis was 5.6% in T1-stage CRC, whereas Gao *et al*[8] demonstrated that the occurrence of lymph node metastasis was 5.5% in T1-stage CRC. Zheng *et al*[9] reported that elevated serum levels of tumor markers indicated a high risk of cancer recurrence and poor survival, yet the relationship between tumor

markers and lymph node metastasis in T1-stage CRC remains unknown. Our study found that the incidence of lymph node metastasis was 8.41% in T1-stage CRC. What is the detailed prognostic value of lymph node metastasis in T1-stage CRC? This question has received increased attention in clinical practice, but as of now, no definite answer has been provided.

In this study, 321 cases of T1-stage CRC were selected from 10132 patients with CRC who received surgical therapy in six large-scale hospitals in China and were retrospectively analyzed. A statistical analysis was performed to analyze the features of lymph node metastasis and to evaluate its risk factors and prognostic value in patients with T1-stage CRC. These data will provide a theoretical basis for more effective treatment of patients with T1-stage CRC.

MATERIALS AND METHODS

Research subjects

In all, 321 cases of T1-stage CRC were screened from 10132 patients with CRC who received surgical therapy in six large-scale hospitals in China (the First Affiliated Hospital of Zhengzhou University, the Affiliated Tumor Hospital of Xinjiang Medical University, Sun Yat-sen Memorial Hospital of Sun Yat-sen University, the First Affiliated Hospital of Xinjiang Medical University, the Third Xiangya Hospital of Central South University, and the Affiliated Hospital of Traditional Chinese Medicine of Xinjiang Medical University) from June 2001 to June 2011. These cases consisted of 172 males and 149 females. The mean patient age was 61.37 ± 13.41 years. Prior to participation, a diagnosis of CRC was confirmed by histopathology for all patients. The tumor-node-metastasis (TNM) stage was determined according to the American Joint Committee on Cancer/International Union Against Cancer TNM staging system for colorectal cancer (2010, 7th edition). No patient received preoperative chemotherapy, radiotherapy, or immunotherapy. The following exclusion criteria were used: cases with incomplete clinical data, those who were inappropriate for statistical analysis, cases who had other malignant tumors, and cases who were treated by endoscopic resection.

All tissues were approved by the Ethics Review Committees of the First Affiliated Hospital of Zhengzhou University before they were used for research purposes. All the patients who provided clinical material signed an informed consent form.

Patient follow-up

After surgery, the patients were assessed once a month for the first 6 mo, once every 3 mo from 6 mo to 2 years, once every 6 mo from 2 years to 5 years, and finally, once a year after 5 years. Follow-ups were conducted either by outpatient or inpatient review or by telephone. Forty patients did not participate in the follow-up analyses because they did not communicate with the physicians after surgery. In addition, 16 patients developed dysthymia and were unable to cooperate for the remainder of the study, two patients committed suicide, and 21 patients did not participate in the follow-up for unknown reasons. Therefore, the total follow-up rate in the study was 75.39%.

Chemotherapy and radical surgery

According to the NCCN Guidelines, CRC with lymph node metastasis is defined as stage Ⅲ disease, but postoperative chemotherapy should be performed in CRC patients with lymph node metastasis, regardless of T stage. FOLFOX6 was used as the first-line adjuvant or neoadjuvant therapy regimen for CRC patients with stage Ⅲ disease. CapeOX was used as either a first- or second-line adjuvant or neoadjuvant chemotherapy regimen for patients with stage Ⅲ CRC, those with drug resistance, or those with postoperative recurrence. FOLFIRI was used as the chemotherapy regimen for CRC patients with postoperative recurrence, metastasis, or drug resistance.

Radical surgery was performed according to complete mesocolic excision for patients with colon cancer and total mesorectal excision for patients with rectal cancer. All the patients received scheduled surgery (*i.e.*, not emergency surgery). More than 12 lymph nodes were removed during surgery.

Statistical analysis

All statistical analyses were performed with SPSS version 18.0. Graphs were constructed with GraphPad Prism software. Univariate analysis was performed using the χ^2 test to analyze the association between lymph node metastasis and clinicopathological parameters. Kaplan-Meier survival curves and the log-rank test were used to compare the group with lymph node metastasis and the group without lymph node metastasis. The multivariate survival analysis was performed using the Cox regression model to determine the relative risk (RR) and 95% CI. Statistical significance was defined as $P < 0.05$.

RESULTS

Univariate analysis of correlation between lymph node metastasis and clinicopathological parameters of patients with T1-stage CRC

In all, 321 patients with T1-stage CRC were divided into a lymph node metastasis group (27 cases) and a non-lymph node metastasis group (294 cases). The occurrence rate of lymph node metastasis was 8.41%. The univariate analysis showed that lymph node metastasis was associated with preoperative CEA, preoperative CA199, preoperative CA724, vascular invasion, and degree of differentiation ($P < 0.05$, for all parameters; Table 1). Lymph node metastasis was not associated with gender, age, smoking status, absolute

Table 1 Univariate analysis of correlation between lymph node metastasis and clinicopathological parameters of T1-stage colorectal cancer patients

Clinicopathological characteristic	*n*	Lymph node metastasis		χ^2	*P* value
		Yes	No		
Gender				1.955	0.162
Male	172	11	161		
Female	149	16	133		
Age (yr)				0.436	0.509
⩾ 60	174	13	161		
< 60	147	14	133		
Smoking				0.766	0.382
No	279	22	257		
Yes	42	5	37		
Preoperative CEA (ng/mL)				5.994	0.014
< 5	284	20	264		
⩾ 5	37	7	30		
Preoperative CA199 (ng/mL)				5.015	0.025
< 9	173	9	164		
⩾ 9	148	18	130		
Preoperative CA724 (ng/mL)				12.275	0.000
< 2	163	5	158		
⩾ 2	158	22	136		
Granulocyte absolute value				0.771	0.380
< 2.2	32	4	28		
⩾ 2.2	289	23	266		
D-dimer				2.227	0.136
< 0.21	158	17	141		
⩾ 0.21	163	10	153		
Preoperative hemoglobin				1.504	0.220
< 132	154	16	138		
⩾ 132	167	11	156		
Vascular invasion				18.421	0.000
No	313	23	290		
Yes	8	4	4		
Tumor location				1.184	0.277
Rectum	170	17	153		
Colon	151	10	141		
Tumor size (cm)				1.526	0.217
< 3	190	19	171		
⩾ 3	131	8	123		
Differentiation degree				7.723	0.005
High/moderate	307	23	284		
Low	14	4	10		
Tumor general type				0.219	0.640
Ulcer	238	19	219		
Non-ulcer	83	8	75		
Tumor tissue type				2.293	0.130
Non-adenocarcinoma	9	2	7		
Adenocarcinoma	312	25	287		

granulocyte count, D-dimer value, preoperative hemoglobin level, tumor location, tumor size, general tumor type, or tumor tissue type ($P > 0.05$ for all).

Multivariate analysis of correlation between lymph node metastasis and clinicopathological parameters of patients with T1-stage CRC

The multivariate analysis showed that lymph node metastasis was associated with preoperative CA724, vascular invasion, and degree of differentiation ($P < 0.05$ for all parameters; Table 2). Lymph node metastasis was not associated with gender, age, smoking status, preoperative CEA, preoperative CA199, absolute granulocyte count, D-dimer value, preoperative hemoglobin level, tumor location, tumor size, general tumor type, or tumor tissue type ($P > 0.05$ for all).

Univariate analysis of correlation between clinicopathological parameters and 5-year OS

As shown in Table 3, the univariate survival analysis demonstrated that age, preoperative CEA, preoperative CA199, vascular invasion, degree of differentiation, and lymph node metastasis ($\chi^2 = 24.180$, $P < 0.001$) were associated with 5-year OS ($P < 0.05$ for all). Gender, smoking status, preoperative CA724, absolute granulocyte count, D-dimer value, preoperative hemoglobin level, tumor location, tumor size, general tumor type, and tissue type were not associated with 5-year OS ($P > 0.05$ for all). The Kaplan-Meier curve showed that the 5-year OS of patients in the lymph

Table 2 Multivariate analysis of correlation between lymph node metastasis and clinicopathological parameters of T1-stage colorectal cancer patients

Clinicopathological characteristic	HR	*P* value	95%CI	
			Lower bound	Upper bound
Gender, Male *vs* Female	1.636	0.201	0.698	5.518
Age (yr), < 60 *vs* ⩾ 60	0.063	0.801	0.357	2.218
Smoking No *vs* Yes	0.880	0.348	0.529	6.077
Preoperative CEA (ng/mL) < 5 *vs* ⩾ 5	1.188	0.276	0.603	5.904
Preoperative CA199 (ng/mL) < 9 *vs* ⩾ 9	2.765	0.096	0.869	5.541
Preoperative CA724 (ng/mL) < 2 *vs* ⩾ 2	5.461	0.019	1.232	10.783
Granulocyte absolute value, < 2.2 *vs* ⩾ 2.2	0.004	0.951	0.285	3.797
D-dimer < 0.21 *vs* ⩾ 0.21	1.398	0.237	0.651	5.652
Preoperative hemoglobin < 132 *vs* ⩾ 132	0.437	0.508	0.249	1.992
Tumor size (cm), < 3 *vs* ⩾ 3	2.090	0.148	0.185	1.290
Vascular invasion, No *vs* Yes	8.461	0.004	2.358	81.596
Tumor location, Rectum *vs* Colon	0.264	0.607	0.485	3.444
Differentiation degree, High/moderate *vs* Low	4.204	0.040	1.057	11.956
Tumor general type, Non-ulcer *vs* Ulcer	0.686	0.407	0.556	4.239
Tissue type, Non-adenocarcinoma *vs* Adenocarcinoma	0.897	0.344	0.043	2.989

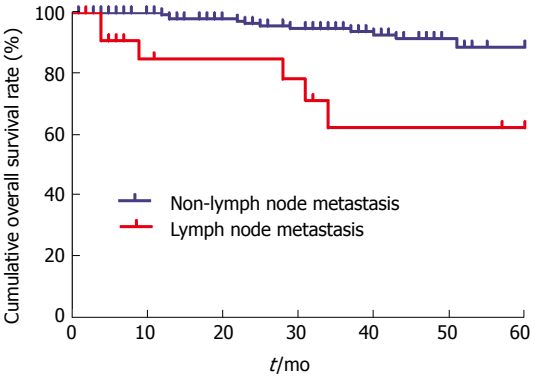

Figure 1 Kaplan-Meier curve of lymph node metastasis in T1-stage colorectal cancer patients. OS of T1-stage CRC patients without lymph node metastasis (blue) and those with lymph node metastasis (red) was compared based on Kaplan-Meier curves and log-rank test.

node metastasis group was lower than that of patients in the non-lymph node metastasis group (Figure 1).

Cox regression analysis of correlation between clinicopathological parameters and 5-year OS

The multivariate survival analysis showed that preoperative CA199 level and lymph node metastasis (RR = 5.117, $P < 0.05$, 95%CI: 0.058-0.815) were associated with 5-year OS ($P < 0.05$ for all, Table 4). In contrast, gender, age, smoking status, preoperative CEA level, preoperative CA724 level, absolute granulocyte count, D-dimer value, preoperative hemoglobin level, tumor location, tumor size, general tumor type, tissue type, vascular invasion, and degree of differentiation were not associated with 5-year OS ($P > 0.05$ for all).

DISCUSSION

CRC is the third most common cancer and the third leading cause of cancer-related death worldwide[10]. The 5-year OS rate of patients with colon cancer is 64.9% and is 66.5% for those with rectal cancer. T1 stage is generally early-stage CRC and has a good prognosis, but if lymph node metastasis occurs, the prognosis is usually poor. Therefore, lymph node metastasis has garnered increased attention in recent years. According to previous studies, the proportion of lymph node metastasis in patients with T1-stage CRC was reported to be 5.6% and 5.5%, which is relatively low[11,12]. In this study, the proportion of lymph node metastasis in patients with T1-stage CRC was 8.41%. The factors that affect lymph node metastasis of T1-stage CRC are multifaceted and are also interrelated with each other. Lymph node metastasis is the main basis for clinic pathological staging of CRC for the prediction of prognosis of patients and for the determination of the appropriate regimen of postoperative adjuvant therapy. Therefore, it is necessary to study the risk factors that are correlated with lymph node metastasis of CRC, as well as their influence on the prognosis of T1-stage CRC[13-15].

Some studies have reported that tumor markers had certain clinical value in the detection of postoperative recurrence and metastasis of CRC as well as in the judgment of prognosis[16-18]. In our study, univariate analysis showed that preoperative CEA, preoperative CA199, and preoperative CA724 were associated with lymph node metastasis, while multivariate analysis showed that preoperative CA724 was an independent risk factor for lymph node metastasis. CA724 has been reported to be a marker for gastrointestinal and ovarian cancers and was shown to be a better indicator for the diagnosis of gastric cancer compared with the levels of CA199 and CEA. Our study found that the CA724 level was a good indicator of lymph node metastasis, which has not been previously reported in T1-stage CRC.

Previous studies have reported that the degree of differentiation of tumor cells in rectal cancer was

Table 3 Univariate analysis of correlation between clinicopathological parameters and 5-year overall survival

Clinicopathological characteristic	*n*	OS rate	χ^2	*P* value
Gender			1.225	0.268
Male	172	90.90%		
Female	149	90.10%		
Age (yr)			6.103	0.013
⩾ 60	174	86.50%		
< 60	147	95.50%		
Smoking			0.429	0.513
No	279	91.00%		
Yes	42	87.50%		
Preoperative CEA (ng/mL)			13.594	0.000
< 5	284	92.70%		
⩾ 5	37	78.40%		
Preoperative CA199 (ng/mL)			7.229	0.007
< 9	173	94.70%		
⩾ 9	148	85.70%		
Preoperative CA724 (ng/mL)			0.012	0.912
< 2	163	87.40%		
⩾ 2	158	93.20%		
Granulocyte absolute value			1.613	0.204
< 2.2	32	85.00%		
⩾ 2.2	289	91.00%		
D-dimer			0.001	0.979
< 0.21	158	88.80%		
⩾ 0.21	163	91.90%		
Preoperative hemoglobin			0.907	0.341
< 132	154	89.10%		
⩾ 132	167	91.70%		
Vascular invasion			12.955	0.000
No	313	91.60%		
Yes	8	40.00%		
Tumor location			1.121	0.290
Rectum	170	89.80%		
Colon	151	91.90%		
Tumor size (cm)			0.210	0.647
< 3	190	90.00%		
⩾ 3	131	91.20%		
Differentiation degree			6.825	0.009
High/moderate	307	91.30%		
Low	14	78.60%		
Tumor general type			3.149	0.207
Protruded	228	91.80%		
Infiltration	10	100.00%		
Ulcer	83	86.20%		
Tumor tissue type			1.828	0.176
Non-adenocarcinoma	9	75.00%		
Adenocarcinoma	312	91.30%		
Lymph node metastasis			24.180	0.000
No	294	93.20%		
Yes	27	65.20%		

closely related to lymph node metastasis[19,20]. Our study demonstrated that the degree of differentiation was an independent risk factor for lymph node metastasis of T1-stage CRC. The reason for this may be that when the degree of differentiation is relatively high, the tumor cells are still in a more primitive stage, and the possibility that they may invade the lymph nodes is much lower. Poorly differentiated or undifferentiated carcinomas have a strong ability to invade the surrounding tissues, especially the lymphatic vessels. Derwinger *et al*[21] showed that the degree of differentiation of CRC was significantly associated with lymph node metastasis. In our study, the rates of lymph node metastasis in patients with highly/moderately and poorly differentiated T1-stage CRC were 7.5% and 28.6%, respectively. In addition, univariate survival analysis showed that the degree of tumor differentiation was a prognostic factor in patients with T1-stage CRC.

Most studies have reported that vascular invasion was an essential risk factor for lymph node metastasis in CRC[22,23]. Similarly, our study verified that vascular invasion was a positive independent risk factor for lymph node metastasis in T1-stage CRC[23]. Univariate survival analysis showed that vascular invasion was associated with the 5-year OS of patients with T1-

Table 4 COX regression analysis of correlation between clinicopathological parameters and 5-year overall survival

	RR	*P* value	95%CI	
			Lower bound	Upper bound
Gender, Male *vs* Female	1.784	0.182	0.695	6.816
Age (yr), < 60 *vs* ≥ 60	3.805	0.051	0.995	9.034
Smoking, No *vs* Yes	0.590	0.442	0.413	7.565
Preoperative CEA, (ng/mL), < 5 *vs* ≥ 5	1.121	0.290	0.580	6.206
Preoperative CA199, (ng/mL), < 9 *vs* ≥ 9	6.452	0.011	1.411	14.481
Preoperative CA724, (ng/mL), < 2 *vs* ≥ 2	0.935	0.333	0.201	1.725
Granulocyte absolute value, < 2.2 *vs* ≥ 2.2	0.040	0.841	0.171	8.761
D-dimer, < 0.21 *vs* ≥ 0.21	2.373	0.123	0.777	8.217
Preoperative hemoglobin, < 132 *vs* ≥ 132	0.577	0.448	0.198	2.042
Tumor size (cm), < 3 *vs* ≥ 3	0.581	0.446	0.261	21.193
Vascular invasion, No *vs* Yes	0.343	0.558	0.468	4.076
Tumor location, Rectum *vs* Colon	0.210	0.647	0.310	2.068
Differentiation degree, High/moderate *vs* Low	1.031	0.310	0.435	13.792
Tumor general type, Ulcer *vs* Non-ulcer	2.338	0.126	0.811	5.471
Tumor tissue type, Non-adenocarcinoma *vs* Adenocarcinoma	0.783	0.376	0.068	2.755
Lymph node metastasis, No *vs* Yes	5.328	0.021	1.264	17.592

stage CRC. However, according to the multivariate analysis, no correlation was observed between lymph node metastasis and the 5-year OS in T1-stage CRC, which may have been due to the limited case number.

In clinical practice, lymph node metastasis is a significant indicator of clinical evaluation of rectal cancer recurrence and the survival of patients, and is also the primary method used to determine the therapeutic schedule in patients with rectal cancer[24-27]. When the tumor is confined to the mucosal layer, no lymph node metastasis occurs because the layer has no lymphatic vessels. When lymphatic vessels are distributed in the submucosa, lymph node metastasis is likely to occur when the tumor invades the submucosa. When the tumor invades the deep intestinal wall, the lymph node metastasis rate will increase significantly[28,29]. In our study, the lymph node metastasis rate of this group of patients with T1-stage CRC was 8.41%, which is mostly consistent with previous reports[7,8]. In this study, the survival analysis of T1-stage CRC patients showed that patients without lymph node metastasis had a significantly higher 5-year survival rate than those with lymph node metastasis. Furthermore, our study verified that lymph node metastasis was an independent prognostic factor in patients with T1-stage CRC, which is also consistent with previous reports[30]. With the development of several new technologies, such as endoscopic mucosal resection, endoscopic submucosal dissection, and transanal endoscopic microsurgery, studies on local resection for the treatment of early rectal cancer have gradually increased[31-33]. The biggest drawback of local resection is its failure to dissect the lymph nodes in relevant drainage areas. Left metastatic lymph nodes are an important reason for postoperative recurrence, which is also the reason why caution should be taken if local resection is selected[34-36]. Consequently, if it is not clear whether preoperative lymph node metastasis is present in T1-stage CRC, radical surgery may be the most suitable choice. Moreover, the intraoperative dissection of lymph nodes should be standardized.

In conclusion, through statistical analysis, we verified that the occurrence rate of T1 stage out of all the cases of CRC was 3.17%; the lymph node metastasis rate was 8.41%, and the non-lymph node metastasis rate was 91.59%. Preoperative serum CA724 level, vascular invasion, and degree of differentiation were independent risk factors for lymph node metastasis in patients with T1-stage CRC. Lymph node metastasis was an essential prognostic factor in patients with T1-stage CRC. An accurate assessment of lymph node metastasis status is essential for decision-making regarding effective intraoperative therapeutic strategies for T1-stage CRC.

ARTICLE HIGHLIGHTS

Research background

Lymph node metastasis is the primary type of metastasis seen in advanced colorectal cancer (CRC). The occurrence rate of T1-stage CRC has been reported to be approximately 3.51%[5,6]. When the tumor is completely removed, patients with T1-stage CRC generally have a good prognosis. However, since lymph node metastasis rarely occurs in T1-stage CRC, lymph node metastasis is often overlooked during the process of diagnosis and treatment. Nevertheless, lymph node metastasis is one of the most essential prognostic factors. In this study, we explored the features and prognostic value of lymph node metastasis, which will provide a theoretical basis for more effective treatment of patients with T1-stage CRC.

Research motivation

The main topic of this study is the exploration of whether lymph node metastasis in patients with T1-stage CRC is valuable for patient survival in multiple centers in China. The key is to find the risk factors for lymph node metastasis of CRC. The significance is the confirmation of the prognostic value of lymph node metastasis in patients with T1-stage CRC.

Research objectives

Studies have reported that lymph node metastasis is an essential prognostic factor for patients with CRC and that lymph node metastasis seldom occurs in T1-stage CRC. However, the definitive prognostic value of lymph node metastasis of T1-stage CRC remains elusive. The main objective of this study was to explore the features and prognostic value of lymph node metastasis in patients with T1-stage CRC.

Research methods
The current research was a case-control study.

Research results
The occurrence rate of T1 stage CRC was 3.17% (321/10,132); of these cases, the lymph node metastasis rate was 8.41% (27/321), and the non-lymph node metastasis rate was 91.59% (294/321). Univariate analysis showed that preoperative serum CEA, preoperative serum CA199, preoperative serum CA724, vascular invasion, and degree of differentiation were associated with lymph node metastasis in T1-stage CRC. Multivariate analysis indicated that preoperative serum CA724, vascular invasion, and degree of differentiation were closely related to lymph node metastasis. Log-rank survival analysis showed that age, preoperative serum CEA, preoperative serum CA199, vascular invasion, degree of differentiation, and lymph node metastasis were prognostic factors for 5-year OS. COX regression analysis demonstrated that preoperative serum CA199 and lymph node metastasis were independent prognostic factors for 5-year OS of patients with T1-stage CRC.

Research conclusions
The morbidity of T1-stage CRC was 3.17% out of all cases of CRC. Preoperative serum CA724, vascular invasion, and degree of differentiation were independent risk factors for lymph node metastasis of T1-stage CRC. Lymph node metastasis was an independent prognostic factor of OS in patients with T1-stage CRC.

Research perspectives
T1-stage CRC is generally regarded as the early stage, easily leading to the neglect of metastasis, especially lymph node metastasis. However, the prognosis of a little part of these cases (8.41%) with lymph node metastasis will be much poorer than those without. We also analysed high risk factors of lymph node metastasis of T1-stage CRC patients. Therefore, we must pay enough attention to lymph node metastasis status of T1-stage CRC patients to guide clinic therapy. Future studies should be focused on greater verifying study to expand further clinical samples. In addition, the mechanistic study of lymph node metastasis in T1-stage CRC patients should be further explored. Multi-center prospective cohort clinical studies will be needed to further validate the conclusion. Moreover, high-throughput transcriptome or proteome screening technology will be necessary for analysing the regulators of lymph node metastasis in T1-stage CRC patients in the future.

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ORIGINAL ARTICLE

Clinical Trials Study

Association between acute pancreatitis and small intestinal bacterial overgrowth assessed by hydrogen breath test

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Abstract

AIM

To elucidate the effects of small intestinal bacterial overgrowth (SIBO) on the severity and complications of acute pancreatitis (AP).

METHODS

In total, 208 patients with AP as defined by the revised Atlanta classification were admitted to Xuanwu Hospital of Capital Medical University from 2013 to 2016. All patients were admitted within 72 h of AP onset. The hydrogen breath test was performed 7 d after AP onset to detect hydrogen production and evaluate the development of SIBO. The incidence of SIBO was analyzed in patients with AP of three different severity grades. The association between SIBO and complications of AP was also assessed.

RESULTS

Of the 27 patients with severe AP (SAP), seven (25.92%) developed SIBO. Of the 86 patients with moderately severe AP (MSAP), 22 (25.58%) developed SIBO. Of the 95 patients with mild AP (MAP), eight (8.42%) developed SIBO. There were significant differences in the rates of SIBO among patients with AP of different severities. Additionally, more severe AP

was associated with higher rates of SIBO positivity (P < 0.05). SIBO in patients with AP mainly occurred within 72 h of the onset of AP. The incidence of organ failure was significantly higher in patients with SIBO than in those without (P < 0.05).

CONCLUSION

SIBO occurs more frequently in patients with MSAP or SAP than in those with MAP, usually ≤ 72 h after AP onset. Additionally, SIBO is associated with organ failure.

Key words: Severe acute pancreatitis; Small intestinal bacterial overgrowth; Hydrogen breath test; Complication

Core tip: The research on small intestinal bacterial overgrowth (SIBO) in acute pancreatitis is mostly confined to animal experiments. The traditional method of diagnosing SIBO is to take small intestinal fluid for bacterial culture, but it is difficult to achieve in clinical patients. In this study, a portable hydrogen expiratory detector was used to detect the patients' expired hydrogen concentration to diagnose SIBO. It was found that there were differences in the positive rates of SIBO in acute pancreatitis with different severity grades. Patients with more severe pancreatitis had a higher positive rate of SIBO. SIBO occurred mainly within 72 h of onset. Patients with SIBO were more prone to organ failure complications.

Zhang M, Zhu HM, He F, Li BY, Li XC. Association between acute pancreatitis and small intestinal bacterial overgrowth assessed by hydrogen breath test. *World J Gastroenterol* 2017; 23(48): 8591-8596 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i48/8591.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i48.8591

INTRODUCTION

Previous metagenomic studies have shown that the gut microbiome comprises up to 3.3 million microbial genes, 10 bacterial genetic logic gates, and more than 1000 bacterial species[1]. Maintenance of the dynamic equilibrium of the microbial ecosystem of the gut is crucial to human health, and abrupt and chaotic shifts in the gut microbiota can lead to many diseases. Small intestinal bacterial overgrowth (SIBO) is a type of dysbiosis and has no typical clinical presentation, and it is characterized by increasing numbers of intestinal bacteria and/or colonization of atypical microorganisms[2]. SIBO is related to various disorders, including irritable bowel syndrome, non-alcoholic fatty liver disease, inflammatory bowel disease, and pancreatitis[3-5]. Many experimental animal studies have shown that SIBO can affect the severity and progression of AP[6,7]. Paralytic ileus accompanied by bacterial overgrowth is an important mechanism of secondary pancreatic infection in patients with AP. Previous studies have shown that the pathogenic bacteria leading to pancreatic infection are similar to the opportunist species that overgrow in the small intestine, suggesting that SIBO plays a pivotal role in pancreatic infection[7,8]. A recent study suggested that prophylactic total colectomy in patients with AP induces SIBO involving both Gram-negative bacilli (*Escherichia coli*, *Proteus* spp.) and anaerobic bacteria[9]. We performed the present study to investigate the incidence of SIBO in AP with different severity grades and explore the correlation between SIBO and complications of AP.

MATERIALS AND METHODS

Patients

In total, 208 patients with AP admitted to the Department of Gastroenterology, Xuanwu Hospital, Capital Medical University from 2013 to 2016 were included in this study. The patients comprised 141 men and 67 women with an age range of 18 to 80 years and a mean age of 44.48 ± 0.26 years. There were no significant differences in sex or age among the three severity groups: severe AP (SAP), moderately severe AP (MSAP), and mild AP (MAP) (P > 0.05).

Diagnosis of AP

AP was diagnosed in accordance with the 2012 Atlanta Classification Criteria[10], which state that clinically confirmed AP should meet at least two of the following three characteristics: (1) signs of abdominal pain consistent with AP; (2) serum amylase and/or lipase level at least three times higher than the upper limit of normal; and (3) abdominal imaging findings consistent with the imaging features of AP.

Classification of disease severity

AP was divided into three categories according to severity: MAP, MSAP, and SAP. Patients with MAP had no local or systemic complications or organ failure and usually recovered within 1 to 2 wk. Patients with MSAP had local or systemic complications with transient organ failure that recovered within 48 hours. Patients with SAP had persistent organ failure (*i.e.*, respiratory, cardiovascular, or renal failure for ⩾ 48 h) that could not be restored without treatment and that may involve one or more organs.

Local complications of AP include acute fluid accumulation, acute necrosis, pancreatic pseudocyst formation, encapsulated necrosis, and pancreatic abscess formation. Systemic complications include organ failure (respiratory failure, circulatory failure, renal failure, *etc.*), systemic inflammatory response

syndrome, and systemic infection.

Inclusion criteria

The inclusion criteria for this study were as follows: age > 18 years; no colonoscopy or X-ray barium meal examination within the past 4 wk; and the presence of a clear consciousness, ability to communicate effectively with the physician, and ability to listen to instructions for completion of the breath-holding for 10 s.

Exclusion criteria

The exclusion criteria were postprandial hypoglycemia and a > 72-h duration from AP onset to hospitalization.

Study protocol

The hydrogen breath test was performed in all patients after admission using a lactulose hydrogen breath test instrument (Gastrolyzer 2; Bedfont Scientific Ltd., Maidstone, Kent, United Kingdom). The hydrogen concentration is expressed in ppm. The detection range was 0 to 500 ppm. The sensitivity was l ppm and the accuracy was ± 5%. The instrument was connected to the new D-type interface. The fasting expiratory hydrogen concentration was measured twice, and the highest value was used for analysis. The patients were instructed to quickly drink 10 g of lactulose in 100 mL of warm water, and the hydrogen concentration was then measured every 15 min for 3 h. The patients were asked to take a deep breath and hold it for 10 s, then breath. This was performed two consecutive times, and the maximum expiratory hydrogen concentration was recorded.

Result criteria

SIBO was defined as follows[11]: a fasting expiratory hydrogen concentration of < 10 ppm or an elevated oral expiratory lactate concentration of ⩾ 12 ppm after oral administration of lactulose. A fasting expiratory hydrogen concentration of > 20 ppm (with high defined as > 12 ppm) was more helpful for the diagnosis. The sum of the hydrogen breath values (lactulose hydrogen breath test set values) measured during the 90-min test was used as an index to evaluate intestinal bacterial growth.

Statistical analysis

Data are presented as mean ± SD. Student's *t*-test, Mann-Whitney *U* test, and Spearman's test were performed using SPSS v.19.0 software (IBM Corp., Armonk, NY, United States). A *P*-value < 0.05 was considered statistically significant.

RESULTS

Incidence of SIBO in patients with AP of different severity grades

Of the 27 patients with SAP, seven (25.92%) had SIBO. Of the 86 patients with MSAP, 22 (25.58%) had SIBO. Of the 95 patients with MAP, eight (8.42%) had SIBO. There were significant differences in these rates of SIBO positivity among patients with AP of different severities. Additionally, more severe AP was associated with higher rates of SIBO positivity ($P < 0.05$), as shown in Table 1.

Changes in small intestinal bacterial hydrogen production in early and late stages of AP

SIBO in patients with AP occurred mainly within 72 h of AP onset. The small intestinal bacterial hydrogen production (0-90 min) in patients with AP was 51.72 ± 1.63 ppm in the early stage (within 72 h) and 32.7 ± 0.69 ppm in the late stage (within 7 d). The intestinal bacterial hydrogen production was significantly lower in the late stage than in the early stage ($P < 0.05$), as shown in Table 2.

Correlation between SIBO and complications of AP

According to the results of the hydrogen breath test, the patients with AP were divided into an SIBO-positive group ($n = 37$) and an SIBO-negative group ($n = 171$), and the occurrence of complications was compared between the two groups. In the SIBO-positive group, 19 patients had respiratory failure, including two with acute renal failure and one with circulatory failure; the organ failure rate was 51.35%. In the SIBO-negative group, 44 patients had respiratory failure, including one with acute renal failure; the organ failure rate was 25.73%. The incidence of organ failure in the SIBO-positive group was significantly higher than that in the SIBO-negative group ($P < 0.05$). There was no significant difference in the other complications between the two groups, as shown in Table 3.

DISCUSSION

The effects of SIBO on the body mainly include the destruction of digestive enzymes and the decomposition of bile acids, causing indigestion of various nutrients[12]. The production of large numbers of harmful metabolites leads to acute and chronic toxicities within the body, including small intestinal motor disorders and reduced intestinal clearance. SIBO can be caused by an abnormal anatomy of the intestinal tract, a decreased ability of the ileocecal valve to block reflux of the colonic contents, a shift of bacteria into the small intestine, gastric acid, the decrease or lack of bile acid and proteolytic enzyme, or reduced secretion of immunoglobulins, which leads to their bactericidal or antibacterial action to weaken and induces SIBO. In addition, certain liver diseases can damage the small intestinal villi and the immune defense mechanism, leading to bacterial migration and SIBO[13-15]. The gold standard for the diagnosis of SIBO is culture of the small intestinal contents. Most

Table 1 Association between acute pancreatitis of different severity grades and small intestinal bacterial overgrowth

Disease classification	Total (*n*)	SIBO positive	SIBO negative	χ^2	*P* value
Mild	95	8	87	10.494	0.005
Moderate to severe	86	22	64		
Severe	27	7	20		

SIBO: Small intestinal bacterial overgrowth.

Table 2 Comparison of small intestinal bacterial hydrogen production in patients with acute pancreatitis in the early and late stages

Time	Hydrogen production (ppm)	T	*P* value
Early stage (within 72 h)	51.72 ± 1.63	4.734	0.000
Late stage (within 7 d)	32.7 ± 0.69		

Table 3 Correlation between small intestinal bacterial overgrowth and complications of acute pancreatitis

Complication	SIBO positive group (*n* = 37)	SIBO negative group (*n* = 171)	χ^2	*P* value
Organ failure				
Yes	19	44	9.456	0.002
No	18	127		
SIRS				
Yes	22	113	0.586	0.444
No	15	58		
Infection				
Yes	9	42	0.001	0.976
No	28	129		
Local complications				
Yes	12	73	1.324	0.250
No	25	98		

SIBO: Small intestinal bacterial overgrowth.

research has suggested that SIBO is present when the small intestinal bacterial count is $\geqslant 10^{10}$ CFU/mL or the proximal small intestinal fluid bacterial count is $> 10^5$ CFU/mL[16,17]. However, this diagnostic method is complex, repetitive, and painful for the patient; it is thus difficult to apply in the clinical setting. The hydrogen expiratory test is one of the most widely used diagnostic techniques for SIBO because it is simple, rapid, and noninvasive[18]. Animal studies have shown that SIBO is present in AP, even within 24 h of onset, and is related to AP disease progression[19,20]. Because of the attenuation of intestinal peristalsis, especially inhibition of the movement of compound waves, normal digestive fluid secretion is lacking, and intestinal immune dysfunction occurs due to a rapid increase in large and small intestinal bacteria. Anaerobic bacteria and lactobacilli significantly decrease, and this is accompanied by excessive growth of pathogenic bacteria. Intestinal bacterial overgrowth is related to the severity of the disease[21]. *Klebsiella* spp., which are resident bacteria of the colon, can reportedly be detected in the small intestine in cases of SIBO[22]. Our findings are consistent with this. We used the hydrogen breath test to detect SIBO in patients with different severities of AP. We found a significant difference in the rates of SIBO positivity among patients with different severities of AP, and the rate of SIBO positivity increased with more severe AP ($P < 0.05$). SIBO in patients with AP mainly occurred within 72 h of AP onset, and the amount of hydrogen production in the small intestine was significantly lower in the late stage of AP ($P < 0.05$).

We found that the incidence of organ failure was significantly higher in patients with SIBO than in those without ($P < 0.05$). SIBO may be an important risk factor for the progression of SAP. At present, the course of AP is divided into two different stages: the acute inflammatory response period (within the first week after onset) and the infectious complication period (2-6 wk after onset). It is helpful to evaluate the prognosis of AP and its prevention measures by thoroughly evaluating the characteristics of these two stages. Intestinal bacterial changes can reportedly occur in patients with varying degrees of pancreatitis and are closely related to the inflammatory response of AP[23]. Most of the bacteria in the intestinal tract are Gram-negative bacilli. SIBO results in the accumulation of large numbers of harmful metabolites, especially the release of large amounts of endotoxin[24]. This increases the systemic inflammatory response and promotes the occurrence of multiple organ dysfunction syndrome through the immune amplification effect. A correlation between SIBO and endotoxemia has been reported in patients with SAP. Endotoxemia is considered to be related to the occurrence and mortality of systemic complications of AP[25]. Endotoxin is a powerful immune system activator. It can act on neutrophils, macrophages, dendritic cells, and endothelial cells and result in the release of cytokines and inflammatory mediators, including interleukins (ILs) (*e.g.*, IL-6, IL-1, and IL-10) and tumor necrosis factor-α (TNF-α), which may in turn induce a cascade of inflammatory reactions. Zhang *et al*[26] found that serum endotoxin and intestinal mucosal permeability were higher in patients with SAP than in normal controls. In recent years, researchers have studied the relationship between the intestinal microecology and development of AP at the molecular level, such as cellular signal transduction. Wu *et al*[27] constructed a rhesus monkey model of AP and found that Toll-like receptor (TLR) 2 and TLR4 were expressed in the ileal mucosa of rhesus monkeys with AP. The lipopolysaccharide of *E. coli*, in combination with intestinal epithelial cell receptors such as TLR, activates NF-κB signal transduction and results in the release of excessive cytokines and inflammatory mediators, such as TNF-α, IL-6, and others. TNF-α level in the circulation is proportional to the degree of damage to the pancreas and lung tissue when AP occurs. IL-6

plays an important role in signal transduction during acute lung injury and is closely related to the severity of acute lung injury and organ failure.

SIBO may be an important cause of secondary infection in patients with AP. The mortality rate of patients with concurrent SAP and pancreatic infection ranges from 23% to 85%, which is higher than that in patients with SAP who do not have pancreatic infection[28]. Patients with AP have intestinal motility disorders, slow intestinal peristalsis, and intestinal stagnation. The colonic bacteria proliferate and migrate to the small intestine to multiply, causing SIBO. Because of these two factors, the proximal small intestine plays an important role in pancreatic necrosis and infection. Many researchers believe that bacterial translocation occurs in the small intestine in patients with AP and that bacterial translocation is associated with the subsequent infectious complications of AP. However, no direct correlation between SIBO and secondary infection was found in the patients with AP in the present study, which may be related to the fact that the number of SIBO-positive patients was lower than that of SIBO-negative patients or that SIBO was not the main risk factor for infection in patients with SAP.

In the present study, patients with MSAP and SAP were more susceptible to SIBO than patients with MAP. SIBO occurred mainly in the early stage of AP and was related to organ failure. How to intervene in the presence of SIBO in patients with SAP in the early stage will be of guiding significance to reduce early organ failure and late infectious complications.

ARTICLE HIGHLIGHTS

Research background

Current clinical and animal studies have shown that gut bacterial overgrowth and translocation is the main reason of pancreatic secondary infection. Small intestinal bacterial overgrowth (SIBO) is related to various disorders, including irritable bowel syndrome, non-alcoholic fatty liver disease, inflammatory bowel disease, and pancreatitis. Many experimental animal studies have shown that SIBO can affect the severity and progression of AP. Previous studies have shown that the pathogenic bacteria leading to pancreatic infection are similar to the opportunist species that overgrow in the small intestine, suggesting that SIBO plays a pivotal role in pancreatic infection. A recent study suggested that prophylactic total colectomy in patients with AP induces SIBO involving both Gram-negative bacilli (*Escherichia coli*, *Proteus* spp.) and anaerobic bacteria. We performed the present study to investigate the incidence of SIBO in AP with different severity grades and explore the correlation between SIBO and complications of AP.

Research motivation

Clinical studies have revealed that in SAP patients with intestinal flora disorders, gut barrier dysfunction has a significant impact on the disease occurrence, development, and prognosis. The research on SIBO in AP is mostly confined to animal experiments. The traditional method of diagnosing SIBO is to take small intestinal fluid for bacterial culture, but it is difficult to achieve in clinical patients. In this study, a portable hydrogen expiratory detector was used to detect the patients' expired hydrogen concentration to diagnose SIBO. In the early stages of AP disease, monitoring gut microbiota and timely treatment can improve the prognosis.

Research objectives

How to protect the integrity of the intestinal mucosal barrier, maintain its function, adjust the intestinal flora disorders, and reduce and prevent bacterial translocation of the intestine has become the key to control the development of SAP and reduce complications. We performed the present study to investigate the incidence of SIBO in AP with different severity grades and explore the correlation between SIBO and complications of AP.

Research methods

Hydrogen breath test principle: After taking lactulose, it reaches the colon and is fermented and decomposed by bacteria to produce hydrogen, causing a peak of hydrogen content in the colon. If there is SIBO, lactulose is fermented by overgrowing bacteria to produce hydrogen before entering the colon. Thus, a peak of hydrogen concentration in the small intestine peak occurs. Expiratory changes in hydrogen concentration can reflect the growth of bacteria in the small intestine. Different from the traditional monitoring methods, in this study, a portable hydrogen expiratory detector was used to detect the patients' expired hydrogen concentration to diagnose SIBO.

Research results

It was found that there were differences in the positive rates of SIBO in AP with different severity grades. Of the 27 patients with SAP, seven (25.92%) had SIBO. Of the 86 patients with MSAP, 22 (25.58%) had SIBO. Of the 95 patients with MAP, eight (8.42%) had SIBO. There were significant differences in these rates of SIBO positivity among patients with AP of different severities. Patients with more severe pancreatitis had higher positive rate of SIBO. SIBO occurred mainly within 72 h of onset. Patients with SIBO are more prone to organ failure complications. How to take timely and effective measures to deal with SIBO is a problem to be solved.

Research conclusions

In the present study, patients with MSAP and SAP were more susceptible to SIBO than patients with MAP. SIBO occurred mainly in the early stage of AP and was related to organ failure. How to intervene in the presence of SIBO in patients with SAP in the early stage will be of guiding significance to reduce early organ failure and late infectious complications.

Research perspectives

Which method can be used to effectively prevent or treat small intestinal bacterial overgrowth in patients with AP, while monitoring the intestinal mucosal barrier, is the future research direction.

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ORIGINAL ARTICLE

Observational Study

Endoscopic papillary large balloon dilatation with sphincterotomy is safe and effective for biliary stone removal independent of timing and size of sphincterotomy

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Abstract

AIM

To describe the efficacy and safety of endoscopic papillary large balloon dilatation (EPLBD) in the management of bile duct stones in a Western population.

METHODS

Data was collected from the endoscopic retrograde cholangiopancreatography (ERCP) and Radiology electronic database along with a review of case notes over a period of six years from 1st August 2009 to 31st July 2015 and incorporated into Microsoft excel. Statistical analyses were performed using MedCalc for Windows, version 12.5 (MedCalc Software, Ostend, Belgium). Simple statistical applications were applied in order to determine whether significant differences exist in comparison groups. We initially used simple proportions to describe the study populations. Furthermore, we used chi-square test to compare

proportions and categorical variables. Non-parametric Mann-Whitney *U*-test was applied in order to compare continuous variables. All comparisons were deemed to be statistically significant if *P* values were less than 0.05.

RESULTS

EPLBD was performed in 229 patients (46 females) with mean age of 68 ± 14.3 years. 115/229 (50%) patients had failed duct clearance at previous ERCP referred from elsewhere with standard techniques. Duct clearance at the Index* ERCP (1st ERCP at our centre) was 72.5%. Final duct clearance rate was 98%. EPLBD after fresh sphincterotomy was performed in 81 (35.4%). Median balloon size was 13.5 mm (10 - 18). In addition to EPLBD, per-oral cholangioscopy (POC) and electrohydraulic lithotripsy (EHL) was performed in 35 (15%) patients at index* ERCP. 63 (27.5%) required repeat ERCP for stone clearance. 28 (44.5%) required POC and EHL and 11 (17.4%) had repeat EPLBD for complete duct clearance. Larger stone size (12.4 mm *vs* 17.4 mm, $P < 0.000001$), multiple stones (2, range (1-13) *vs* 3, range (1-12), $P < 0.006$) and dilated common bile duct (CBD) (12.4 mm *vs* 18.3 mm, $P < 0.001$) were significant predictors of failed duct clearance at index ERCP. 47 patients (20%) had ampullary or peri-ampullary diverticula. Procedure related adverse events included 2 cases of bleeding and pancreatitis (0.87%) each.

CONCLUSION

EPLBD is a safe and effective technique for CBDS removal. There is no difference in outcomes whether it is performed at the time of sphincterotomy or at a later procedure or whether there is a full or limited sphincterotomy.

Key words: Endoscopic sphincterotomy; endoscopic papillary large balloon dilatation; Endoscopic retrograde cholangiopancreatography; Adverse events; Common bile duct stones

Core tip: This is one of the largest published series describing the management of difficult biliary stone disease utilizing large balloon sphincteroplasty in a Western population. Our findings suggest that large balloon sphincteroplasty can be performed and repeated safely and effectively in those with a sphincterotomy irrespective of its timing and size to establish complete duct clearance.

Aujla UI, Ladep N, Dwyer L, Hood S, Stern N, Sturgess R. Endoscopic papillary large balloon dilatation with sphincterotomy is safe and effective for biliary stone removal independent of timing and size of sphincterotomy. *World J Gastroenterol* 2017; 23(48): 8597-8604 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i48/8597.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i48.8597

INTRODUCTION

Common bile duct stones (CBDS) are diagnosed frequently throughout the world. The prevalence of CBDS in symptomatic gallstone disease is estimated between 10% and 20%[1-3]. Extraction of confirmed CBDS in symptomatic patients is recommended[4]. Endoscopic retrograde cholangiopancreatography (ERCP) is recognized as an effective treatment modality for CBDS extraction. Endoscopic sphincterotomy (EST) followed by stone extraction with balloon or basket has been used traditionally as a standard technique. Large or multiple stones however, may be technically challenging with standard techniques and may require additional treatment modalities including mechanical lithotripsy for successful stone extraction[5-7].

Endoscopic papillary balloon dilatation (EPBD) for biliary stones extraction was first reported in 1982[8]. EPBD without an EST has been reported but is associated with an increased risk of pancreatitis and reduced stone clearance rates[9]. In a Cochrane review, EPBD compared to EST has been associated with less risk of bleeding but increased risk of post ERCP pancreatitis[10].

EST followed by endoscopic papillary large balloon dilatation (EST+EPLBD) using a balloon sized 10-20mm was reported in 2003 as an effective and safe treatment of CBDS[11]. EPLBD with limited or full EST has subsequently emerged as an established endoscopic technique in the management of CBDS mainly in Asia based on published data[12]. Interestingly, more recent data has suggested that endoscopic papillary large balloon dilatation (EPLBD) alone without a preceding EST is a useful technique in CBDS removal with comparable outcomes to EST[13,14].

The safety profile and effectiveness of EPLBD with limited or full EST has been reported in most studies[15-27]. However, concerns remain regarding serious adverse events[28-32] including procedure related morbidity and mortality limiting wider application of EPLBD in the management of CBDS.

There remains a significant variation in practice when performing large balloon papillary dilatation with particular concerns to the timing and size of sphincterotomy prior to balloon dilatation. This study describes the safety profile and effectiveness of EPLBD in the management of CBDS and examines the impact of timing and size of sphincteroplasty in relation to EST in a Western population.

MATERIALS AND METHODS

Patients

This study from a United Kingdom academic referral centre involved 229 patients who had EPLBD for retrieval of CBDS from 1st August 2009 to 31st July 2015. Of these, 115 (50%) patients had previous unsuccessful stone extraction elsewhere. Inclusion

criteria were: adult patients with confirmed CBDS and who gave consent for the procedure. Excluded from the study were patients with coagulopathy (INR > 1.5), thrombocytopenia (platelet count < 50000/mL), and those who had distal biliary stricture.

Procedure

ERCP was undertaken predominantly with benzodiazepine and opioid sedation. General anesthesia was used when per-oral cholangioscopy (POC) (SpyGlass™DS, Boston Scientific, MA, United States) was performed.

ERCP was performed using side-viewing endoscopes (TJF-240; Olympus Optical Corporation, Tokyo, Japan). Standard wire guided EST was performed for native papilla. Stone extraction was attempted with extractor balloon catheter (Extractor™ Pro RX, 2 lumen extraction balloon, Boston Scientific, Cork, Ireland and/or Multi-3V Plus™, three lumen extraction balloon, Olympus Medical systems, Tokyo, Japan) and/or wire guided retrieval basket (Trapezoid™, Boston Scientific Limited, Ireland).

Where stone retrieval was unsuccessful with standard techniques, EPLBD (CRE™ Wire guided, Boston Scientific, Cork, Ireland) was performed for stone extraction. The balloon was inflated until disappearance of the waist (Figure 1). For complex and large stones, POC supplemented with electrohydraulic lithotripsy (EHL) (Nortech Autolith, Intracorporeal Electrohydraulic Lithotripter, Northgate Technologies INC, IL, United States) was used for stone extraction. Duct clearance was confirmed with an occlusion cholangiogram. Stone number, size and bile duct diameter were assessed with calibrated hospital radiology software tools on captured fluoroscopic images for accurate precision.

Study endpoints

The primary endpoint was common bile duct clearance. Secondary endpoints were procedure related adverse events (pancreatitis, bleeding, cholangitis, perforation and mortality).

Study design and statistical analysis

This was a cross-sectional study in which consecutive patients who underwent EPLBD during the defined period were evaluated based on study endpoints. Statistical analyses were performed using MedCalc for Windows, version 12.5 (MedCalc Software, Ostend, Belgium). Simple statistical applications were applied in order to determine whether significant differences exist in comparison groups. We initially used simple proportions to describe the study populations. Furthermore, we used chi-square test to compare proportions and categorical variables. Non-parametric Mann-Whitney *U*-test was applied in order to compare continuous variables. All comparisons were deemed to be statistically significant if *P* values were less than 0.05.

Table 1 Clinical characteristics by common bile duct clearance at the index endoscopic retrograde cholangiopancreatography *n* (%)

Variables	Group A (*n* = 166)	Group B (*n* = 63)	*P* value
Mean age	68.4 ± 14.3	68.4 ± 14.1	0.93
Sex (M: F)	70:96	17:46	0.046
HTN	65 (39.2)	26 (41.3)	0.88
IHD	19 (11.4)	7 (11.1)	0.87
DM	14 (8.4)	4 (6.3)	0.80
CLD	1 (0.6)	None	0.60
COPD	16 (9.6)	5 (7.9)	0.89
CKD	7 (4.2)	2 (3.2)	0.98

RESULTS

Two hundred and twenty-nine subjects (146, females) fulfilled the inclusion criteria (Table 1). Of these, 166 (72.5%) achieved duct clearance at index ERCP. In order to determine factors associated with duct clearance at index ERCP, subjects were divided into 2 groups according to duct clearance (A) and those who failed clearance (B). Only female gender was associated with failed duct clearance at index ERCP (*P* = 0.046).

Large stone size, multiple stones and dilated common bile duct were significant predictors of failed duct clearance at index ERCP (Table 2). Overall median balloon diameter used was 13.5 (10-18) mm (Table 3).

EST was performed at index ERCP in 81 (35.4%) and had previously been performed in 148 (64.6%) patients. Procedure time was significantly prolonged in those who failed to achieve duct clearance at index ERCP (30.5 min *vs* 44 min, *P* < 0.001) (Table 4).

POC supplemented with EHL was the most commonly used additional treatment modality for stone extraction at index and subsequent ERCPs (Figures 2 and 3).

Initial duct clearance rate with EPLBD at index ERCP was 72.5%. However, all cases of failed duct clearance (Group B) at index ERCP underwent further ERCP for duct clearance. Final duct clearance rate was 97.8%.

The procedure related adverse events are listed in Table 5. Post ERCP pancreatitis and haemorrhage were not significantly different between groups. There was no case of procedure related mortality.

DISCUSSION

We report an experience of EPLBD based on intention to treat analysis from an academic United Kingdom endoscopy centre. The study shows that EST followed by immediate or delayed EPLBD (EST + EPLBD) is a safe and an effective approach. Duct clearance was achieved in 73% of the patients at index ERCP and further procedures produced an overall duct clearance rate of 98%. Procedure related complications were reported in only four patients (1.7%). EPLBD was

Table 2 Outcome of index endoscopic retrograde cholangiopancreatography by stone characteristics and anatomical features of the biliary tree *n* (%)

Variables	Group A (*n* = 166)	Group B (*n* = 63)	*P* value
Number of stones	2 (1-13)	3 (1-12)	0.006
Median diameter of stones (mm)	12.4 (4.5-30)	17.4 (10.1-32)	< 0.000001
Median diameter of duct (mm)	12.4 (5-30)	18.3 (9.2-30)	0.001
Ampullary or peri-ampullary diverticulum	37 (22.3)	10 (15.9)	0.370
Intact GB	117 (70.5)	50 (79.4)	0.240
Primary stone disease	118 (71.1)	44 (69.8)	0.980
Recurrent stone disease[1]	48 (28.9)	19 (30.2)	0.900

[1]Recurrent stones were after previous EST.

Table 3 Diameter of the balloon used

Balloon size	*n*	Percent
10-11 mm	62	27%
12-15 mm	139	61%
16-18 mm	28	12%

performed immediately after sphincterotomy in 35% of the patients. The study results reveal comparable and even lower rates of complications including pancreatitis, perforation and bleeding than previously reported[10,28-30,32].

This is probably the largest single centre study in a Western population reporting the use of endoscopic large volume balloon dilatation in managing CBDS, in which 229 patients were included. A recent literature review reported data from 32 studies across the globe[12]. The largest study of this review, which was from China, included 169 patients.

In contrast to other published series reporting higher initial duct clearance rates, we observed relatively lower duct clearance rates at index ERCP. The reason for this is related to the more complex nature of stone disease in a referral population. Complexity of the stone disease in our cohort may be evident with the fact that all patients required large balloon sphincteroplasty in addition to the standard endoscopic techniques for stone extraction at the index ERCP. In addition to EPLBD using POC together with EHL could not achieve duct clearance in (3.5%) of patients at the index ERCP (Figure 2). Furthermore, 45% of the patients undergoing repeat ERCP for duct clearance required POC and EHL (Figure 3).

50% of the patients with first EPLBD performed at our centre had previously failed stone extraction with standard endoscopic techniques including EST followed by stone extraction with balloon and basket performed by the referring unit. Among those 50% with previous unsuccessful stone extraction, complete duct clearance was achieved with EPLBD in approximately 70% of the cases at index ERCP. Patients with failed duct clearance at index ERCP had significantly large stone size 17.4 (10.1-32) mm *vs* 12.4 (4.5-30) mm, $P < 0.000001$, multiple stones [2, range (1-13) *vs* 3, range (1-12), $P < 0.006$] and dilated bile duct 18.3 mm (9.2-30) mm *vs* 12.4 mm (5-30), $P < 0.001$.

In a systemic review and meta-analysis[33] initial duct clearance rates for stones larger than 15mm was 77% in the EPLBD group. Initial duct clearance rates with EST + EPLBD vary from 72.7% to 100% in published series[12] which are mainly from Asia. In a recent study from Australia[34] initial duct clearance rates were 70% in immediate EPLBD group and 74% in delayed EPLBD group.

Major adverse events (AEs) related to EST and EPLBD are pancreatitis, bleeding, and perforation. A recent Australian retrospective study[34] reviewed outcomes of EPLBD and EST in 136 patients. Only one case of non-fatal pancreatitis and a perforation was observed in the delayed EPLBD group. Three cases of non-fatal haemorrhage were observed in immediate and delayed EPLBD group ($P = 0.303$). The safety profile of EPLBD has been corroborated by a recent meta-analysis[33] in which four randomized controlled trials (RCT) comparing EST plus EPLBD *vs* EST alone and EST plus mechanical lithotripsy were assessed. In the three RCTs comparing EPLBD with EST alone there were no statistical differences in terms of each form of adverse events: PEP (3% *vs* 3.4%); haemorrhage (0.5% *vs* 1%); cholangitis (1.5% *vs* 1.9); perforation (0% *vs* 1%). In the RCT comparing EPLBD with EST plus ML higher rates of cholangitis were observed 0% *vs* 13.3 ($P = 0.026$). In one of these RCTs[23] less overall adverse events were observed with EST + EPLBD than after EST alone (4.4% *vs* 20%, $P = 0.049$). This emphasises the safety profile of EPLBD.

A review of 32 studies involving 1761 patients, demonstrated overall rates of AEs related to EST combined with EPLBD including perforation, pancreatitis, bleeding and cholangitis were 0.4%, 2.7%, 2.2% and 0.9% respectively[12]. A retrospective multicenter study[28] investigating 946 patients reported 9 perforations (0.95%), resulting in 3 deaths. Distal CBD stricture (OR = 17.08, $P < 0.001$) was an independent predictor for perforation. In a systemic review of 30 studies[35] the overall AEs were significantly lower in EST combined with EPLBD group than in EST alone (8.3% *vs* 12.7%, OR = 1.60, $P < 0.001$).

Our AEs rates were not only comparable but

Table 4 Procedure characteristics by outcomes of common bile duct stone extraction *n* (%)

Variables	Group A (*n* = 166)	Group B (*n* = 63)	*P* value
Procedure time (min)	30.5 (9-160)	44 (12-90)	0.0010
Number of ERCP (median)	1 (1-3)	2 (1-10)	0.0001
Pre-cut sphincterotomy	6 (3.6)	2 (3.2)	0.8000
Previous Sphincterotomy	106 (63.9)	42 (66.7)	0.8000
Fresh Sphincterotomy	58 (35.5)	23 (36.5)	0.9800
Median Balloon size diameter	13 (10-18)	13.5 (10-18)	0.2200

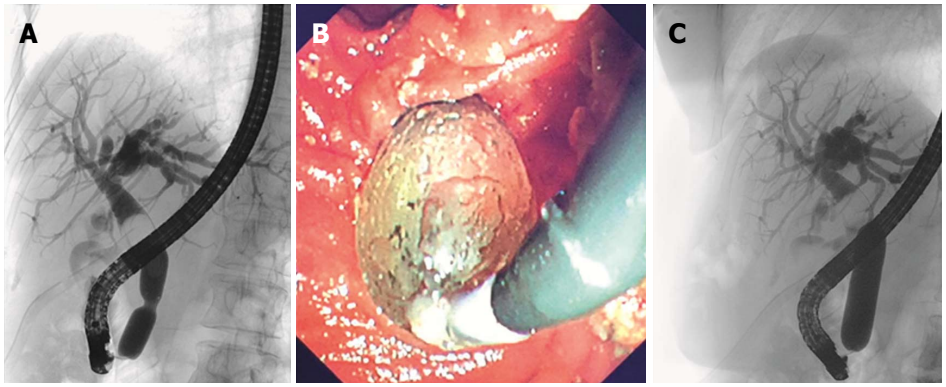

Figure 1 Demonstration of endoscopic papillary large balloon dilatation. A: Fluoroscopic view showing balloon waist with a proximal stone; B: Endoscopic view of ampullary orifice while performing EPLBD; C: Demonstration of disappearance of balloon waist with gradual inflation of balloon.

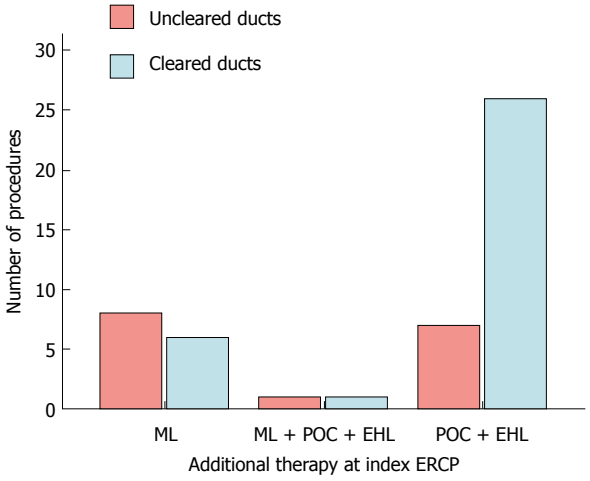

Figure 2 Types of additional therapy to endoscopic papillary large balloon dilatation at index endoscopic retrograde cholangiopancreatography for stone extraction. ML: Mechanical lithotripsy; POC: Per-oral cholangioscopy; EHL: Electrohydraulic lithotripsy.

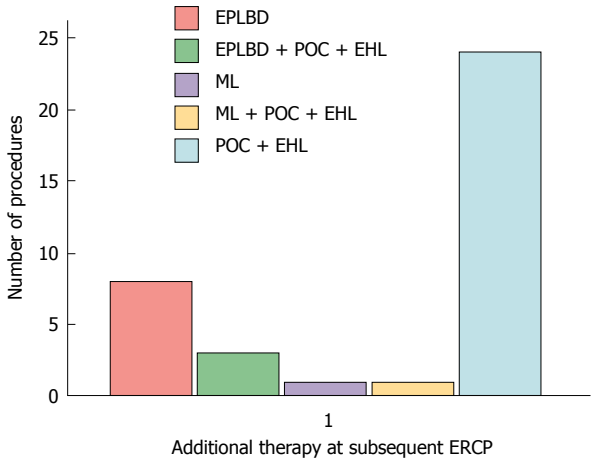

Figure 3 Types of endoscopic interventions at subsequent endoscopic retrograde cholangiopancreatography in those with failed duct clearance at index endoscopic retrograde cholangiopancreatography. EPLBD: Endoscopic papillary large balloon dilatation; ML: Mechanical lithotripsy; POC: Per-oral cholangioscopy; EHL: Electrohydraulic lithotripsy.

even much lower than most of the published data. Pancreatitis was defined according to the consensus criteria described elsewhere. The overall pancreatitis rate in our cohort was 2/229 (0.87%). Both cases had non-fatal pancreatitis and were managed conservatively. In both these cases duct clearance was not achieved at index ERCP with EPLBD. This is one of the principal emphases of the study that in our experience EPLBD was not associated with significantly increased risk of post ERCP pancreatitis. Peri-procedural optimization of patients is our standard practice to prevent post ERCP pancreatitis. This includes providing adequate intravenous hydration, prescribing rectal diclofenac and keeping a low threshold for placement of pancreatic stents for contrast injection and wire cannulation of pancreatic duct. These preventive measures along with rich high volume experience are probably the principal factors for a very low rate of post ERCP pancreatitis. Similarly, 2 cases (0.87%) of gastrointestinal haemorrhage were observed. One

Table 5 Adverse events after endoscopic papillary large balloon dilatation *n* (%)

Variables	Group A (*n* = 166)	Group B (*n* = 63)	*P* value
Pancreatitis	0	2 (3.2)	0.13
Bleeding	2 (1.2)	0	0.93
Cholangitis	None	None	NA
Perforation	None	None	NA
Mortality	None	None	NA

patient required blood transfusion and endoscopic haemostatic therapy. The other patient required two units of blood transfusion. There was no procedure related perforation, cholangitis or mortality.

Twenty percent of the patients in our study had periampullary diverticula (PAD), which did not impact on the incidence of the adverse events or duct clearance. In patients with and without PAD, overall duct clearance and adverse events rates were similar in retrospective studies[24,36] comparing EPLBD with limited EST or EPLBD alone.

In our study, the balloon was inflated until the disappearance of the waist rather than specific time duration. Hence, the balloon inflation time would have been relatively short. A meta-analysis[37] has reported an inverse relation of pancreatitis with balloon timing but this clearly was not the case in our scenario.

POC and EHL were planned in addition to EPLBD in 35 (15.3%) of the patients at index ERCP in view of previously demonstrated complex stone disease on cholangiographic appearances. In 8 (3.5%) patients, EPLBD even supplemented with POC and EHL could not clear the bile ducts due to complex nature of stone disease at index ERCP.

Prior to the introduction of EPLBD, ML was generally used after EST. In these circumstances, ML is traditionally used after EST. However, it may be time consuming and technically challenging with an increase risk of procedure related adverse events[23]. In our practice, EPLBD was the preferred treatment modality over ML and limited its use to 7% of the procedures. Recently published international consensus guidelines for EPLBD reaffirm our findings and recommend its use as preferred and alternative modality to ML in removing large bile duct stones[38].

Our experience of EST with immediate or delayed large balloon dilatation confirms its safety and effectiveness. We conclude that the safety of large balloon sphincteroplasty is not influenced by the presence of a periampullary diverticulum or a fresh sphincterotomy. Limited or full sphincterotomy followed by EPLBD does not influence the outcomes. Our results suggest that a significant proportion of patients with multiple stones and large stones may require additional treatment modalities including cholangioscopy and EHL where large balloon sphincteroplasty remains unsuccessful in achieving duct clearance.

ARTICLE HIGHLIGHTS

Research background

Endoscopic papillary large balloon dilatation (EPLBD) is an established technique for biliary stone extraction mainly in Asia. Serious adverse events related to EPLBD including pancreatitis, bleeding and perforation have limited its wider utility particularly in the Western world. Timing and size of preceding endoscopic sphincterotomy (EST) impose significant concerns regarding serious procedure related adverse events. This study describes the safety and efficacy of EPLBD in the management of CBDS and examines the impact of timing and size of sphincteroplasty in relation to EST in a Western population.

Research motivation

EPLBD is being performed very frequently in day to day practice. There is insufficient data on its efficacy and safety without any standardized techniques. There is little data on timing and hence an appreciation that there is variation in practice in some centres performing a sphincteroplasty on a subsequent and further endoscopic retrograde cholangiopancreatography. The principal motive of the study was to ascertain whether EPLBD could be performed safely as an effective and preferred endoscopic modality for complex biliary stone extraction following EST.

Research objectives

The main objectives of the study were to describe the efficacy and safety of EPLBD in the management of bile duct stones in a Western population from the experience in a busy tertiary referral unit.

Research methods

This was a retrospective observational study in which consecutive patients who underwent EPLBD during the defined period were evaluated based on study endpoints.

Research results

This study confers high safety profile of EPLBD for biliary stone extraction in relation to an EST. The study also ascertains EPLBD as an effective and preferred endoscopic modality than standard endoscopic techniques for complex bile duct stones management. The problems that remain to be solved are the predictive factors indicating the need for EPLBD and factors predicting failure.

Research conclusions

EPLBD is a safe and effective technique for biliary stone extraction. Safety of large balloon sphincteroplasty is not influenced by the presence of a periampullary diverticulum or a fresh sphincterotomy. Limited or full sphincterotomy followed by EPLBD does not influence the outcomes. Large and multiple stones may predict need for additional treatment modalities including cholangioscopy and electrohydraulic lithotripsy where large balloon sphincteroplasty remains unsuccessful in achieving duct clearance.

Research perspectives

This study suggests that large balloon sphincteroplasty can be performed and repeated safely and effectively in those with a sphincterotomy irrespective of its timing and size to establish complete duct clearance.

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ORIGINAL ARTICLE

Observational Study

Person-centered endoscopy safety checklist: Development, implementation, and evaluation

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Institutional review board statement: The Ethical Review Board of Stockholm regarded this study as a quality improvement project not requiring ethical approval (DNR: 2015/318-31/4).

Informed consent statement: All data collected from study participants were anonymous; therefore, written consent was not obtained. By completing questionnaires, the study participants gave their informed consent. Data from observations were completely anonymized, and due to the nature of the observations, informed consent was not possible to obtain. However, an ethical analysis based on the Declaration of Helsinki was undertaken by the authors.

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Abstract

AIM

To describe the development and implementation of a person-centered endoscopy safety checklist and to evaluate the effects of a "checklist intervention".

METHODS
The checklist, based on previously published safety checklists, was developed and locally adapted, taking patient safety aspects into consideration and using a person-centered approach. This novel checklist was introduced to the staff of an endoscopy unit at a Stockholm University Hospital during half-day seminars and team training sessions. Structured observations of the endoscopy team's performance were conducted before and after the introduction of the checklist. In addition, questionnaires focusing on patient participation, collaboration climate, and patient safety issues were collected from patients and staff.

RESULTS
A person-centered safety checklist was developed and introduced by a multi-professional group in the endoscopy unit. A statistically significant increase in accurate patient identity verification by the physicians was noted (from 0% at baseline to 87% after 10 mo, $P < 0.001$), and remained high among nurses (93% at baseline *vs* 96% after 10 mo, P = nonsignificant). Observations indicated that the professional staff made frequent attempts to use the checklist, but compliance was suboptimal: All items in the observed nurse-led "summaries" were included in 56% of these interactions, and physicians participated by directly facing the patient in 50% of the interactions. On the questionnaires administered to the staff, items regarding collaboration and the importance of patient participation were rated more highly after the introduction of the checklist, but this did not result in statistical significance ($P = 0.07$/$P = 0.08$). The patients rated almost all items as very high both before and after the introduction of the checklist; hence, no statistical difference was noted.

CONCLUSION
The intervention led to increased patient identity verification by physicians - a patient safety improvement. Clear evidence of enhanced person-centeredness or team work was not found.

Key words: Checklist; Communication; Endoscopy; Observation; Patient-centered care; Person-centered care; Patient safety; Teamwork

Core tip: With increasing indications and more technically advanced gastrointestinal endoscopy, finding strategies to prevent adverse events is important. Standardized methods, such as checklists and promoting patient involvement, are strategies for augmented patient safety. This paper describes the development of a novel endoscopy checklist that combined patient safety and a person-centeredness approach. After the introduction of the checklist, physicians' verifications of patients' identities before their examinations increased significantly. However, compliance to the checklist was suboptimal, possibly due to insufficient training. With more team training for all staff members, the checklist could be a tool for increased person-centeredness and safety.

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INTRODUCTION

In 1999, the United States Institute of Medicine (IOM) published the report "To Err is Human"[1], which raised awareness of major flaws in the American healthcare system and called for increased patient safety. In 2015, almost two decades later, the Swedish National Board of Health and Welfare[2] estimated that one in ten hospital patients suffered harm due to adverse events during their stay. Therefore, it is clear that this is an ongoing issue and that healthcare professionals worldwide are still seeking strategies to prevent mistakes being made in patient care and to improve their facilities' cultures of safety.

Indications for gastrointestinal endoscopy are increasing, and endoscopic examinations are becoming more technically advanced. However, possible complications during endoscopy procedures still include cardiopulmonary complications, sedation-related complications, allergic reactions, perforation, and bleeding[3,4]. Knowledge about a patient's health condition and proper monitoring of his or her vital functions are crucial to assess risks and prevent adverse events[3,4]. In addition, patient misidentification is a known safety risk[5,6] to which endoscopy teams should give special consideration as endoscopy is often a high-volume service.

One of the most well-known improvements of patient safety using standardized methods was the release of the World Health Organization's Surgical Safety Checklist in 2009 (WHO SSC)[7], which has been shown to contribute to improved surgical outcomes[8]. Improved communication in surgical teams, a factor known to be associated with improved patient outcomes[9], was another positive effect of the WHO SSC[10,11]. However, compliance with the WHO SSC varies widely among hospitals[12-14]. In a study by Conley *et al*[15] the quality of implementation was an important factor in the effectiveness of the checklist. In addition, "explaining why" and "showing how" were of great importance in motivating checklist use. Matharoo et al. came to a similar conclusion when assessing compliance to a checklist used in an endoscopy unit in the United Kingdom; they recommended that

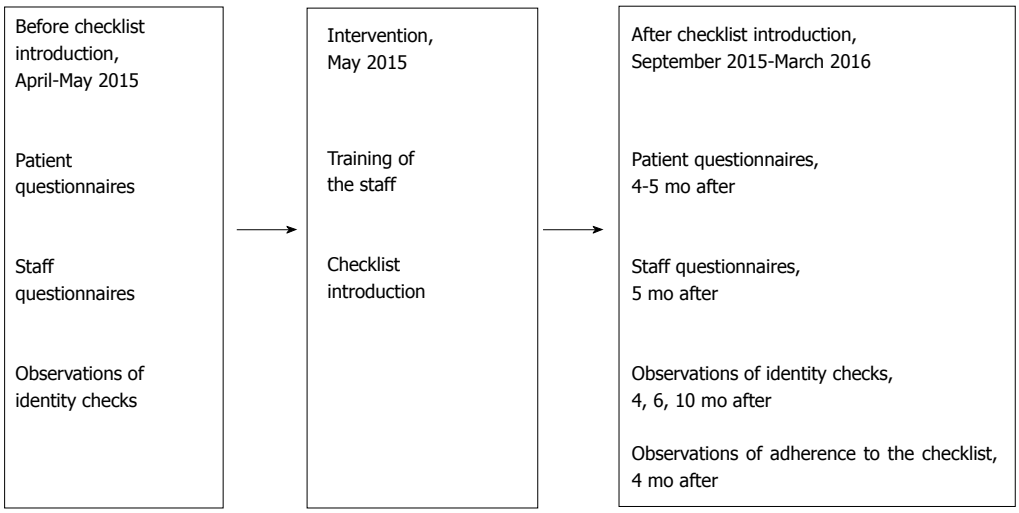

Figure 1 Timeline chart for checklist implementation, training, and evaluation.

adherence to the checklist could be better maintained by continued re-assessment and feedback[16].

Another method for improving healthcare quality is promoting patient participation. In a systematic review, McMillan *et al*[17] found that a patient-centered model seemed to increase patient satisfaction and their perceived quality of health. In addition, Greene *et al*[18] found a relationship between patient activation and positive health-related outcomes. Indeed, patient participation has been recognized as one of the key factors to patient safety[19,20]; therefore, healthcare professionals working directly with patients need training in facilitating patient participation and creating an open and safety-focused culture[21].

Person-centered care (PCC) views the patient as an accountable and capable individual who is an expert on his/her own condition. This approach to care emphasizes patient participation and empowerment[22]. One view of PCC is that the patient and the healthcare professionals should establish a partnership where the patient narrative - the personal account of the patient' s life situation and illness - is given key importance for shared decision making[23]. Despite the lack of a universal definition of PCC in the literature, it has been described as a collaborative and respectful partnership between healthcare professionals and the patient, including information exchange with both parts actively involved in the planning and the delivery of care with shared specified goals and strategies[24].

The aim of this study was twofold: Firstly, to describe the development and implementation of an endoscopy checklist at an inpatient and outpatient endoscopy unit that combined safety and person-centeredness, and secondly, to evaluate this "checklist intervention" in terms of patient safety, person-centeredness, and teamwork.

MATERIALS AND METHODS

The study was conducted in one of two endoscopy units at the Karolinska University Hospital in Stockholm, Sweden, as a part of a quality improvement project. At the unit, approximately 5000 endoscopic examinations are conducted each year. At the time of the study, ten nurses were employed at the unit (including the first author). Approximately 30 different physicians worked at the unit in varying degrees of frequency. The study design is presented in Figure 1.

Development and implementation of the checklist

The checklist was developed by a multi-professional group consisting of endoscopy nurses, senior physicians, and experts in person-centered care and teamwork. The WHO SSC[7] and the only endoscopy safety checklist found in the current literature and available at the time[25] were reviewed. Items considered relevant for endoscopy and of high impact for patient safety (*i.e.*, those that were in line with the hospital's incident reporting system) were incorporated into the endoscopy checklist. Local safety issues and routines were also taken into consideration. However, none of the reviewed checklists involved the patient; thus, a person-centered focus was introduced.

The implementation of the checklist was thoroughly planned and included factors identified as "enablers" to checklist uptake (*i.e.*, to explain why and show how)[15]. The project was supported by local management, and attendance at the checklist introduction seminars and team training sessions was mandatory for all endoscopy staff.

The checklist was introduced to the staff during three half-day sessions in multi-professional groups of five to seven members in May 2015. The sessions included oral presentations on safety culture, person-centered care, and the new checklist, which was illustrated with a short motivational instruction film. Scenario-based team training was then overseen by instructors from the hospital's Center for Advanced Medical Simulation and Training. The training focused on compliance to the checklist, non-verbal communication such as body language, and addressing the patient during the "summary" to enhance person-

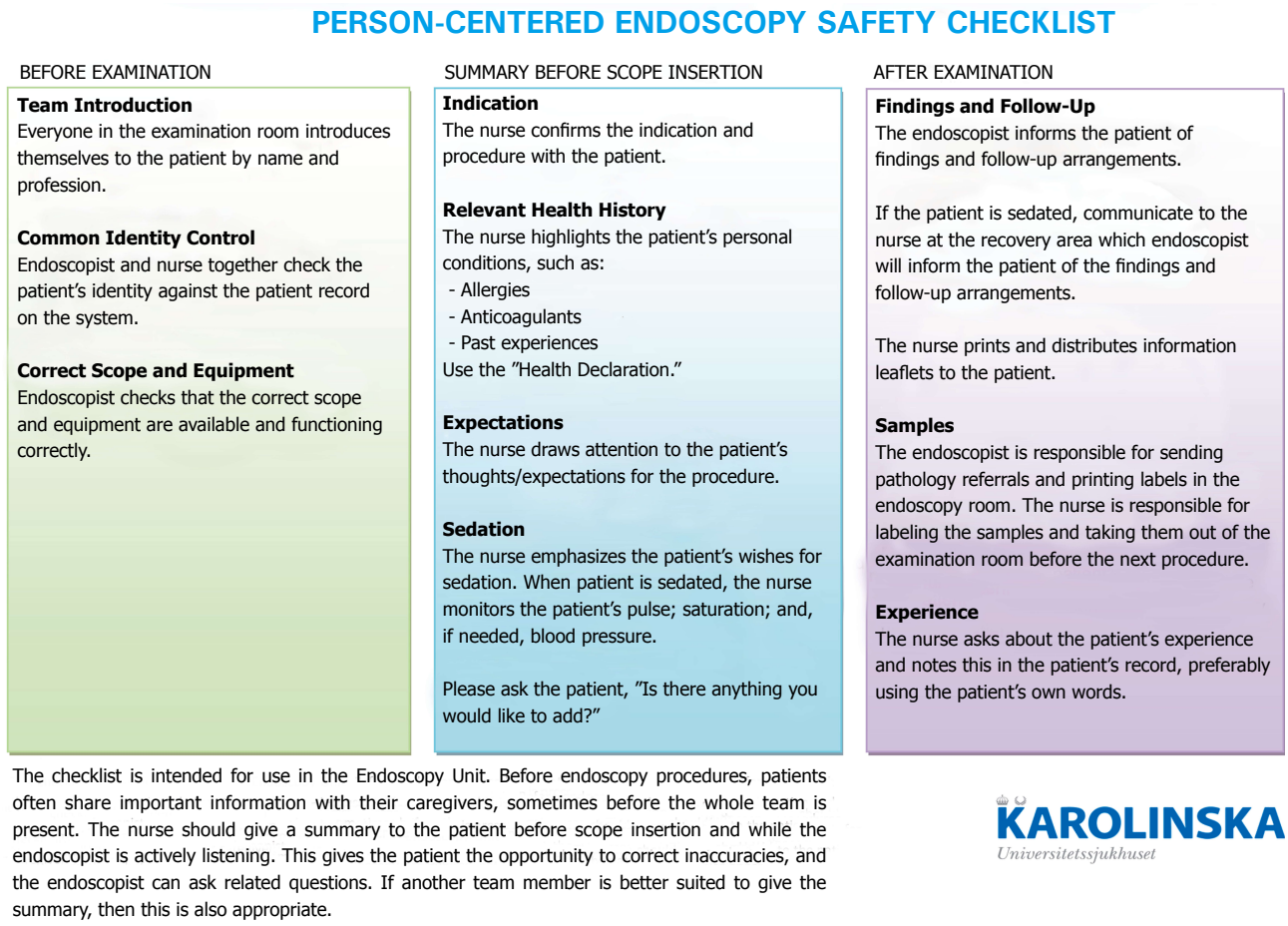

Figure 2 A person-centered endoscopy safety checklist (translated into English by the authors).

centeredness. Group discussions followed each training session.

Observations

The identity verifications of patients (*i.e.*, checking a patient's identity number against his or her medical record) performed by the nurses and physicians before an endoscopy were observed at baseline and at 4, 6, and 10 mo after the checklist was introduced. Staff were regularly informed that quality improvement observations were going to be performed but not told explicitly what was being observed or when the observations would take place. The observations were performed by the first author or by a research nurse who was in the examination room for other purposes. At 4 mo after the checklist intervention, adherence to the checklist was also observed using a standardized protocol, with a focus on the "summary" since it incorporated both safety issues and a person-centered approach. One of the purposes of the "summary" observations was to monitor if the staff addressed the patient and directly looked at him or her (Figure 2). The teams observed consisted of at least one physician and one nurse. The observations were performed on random days, with the ambition of observing as many different physician-nurse combinations as possible.

Evaluation: patient and staff questionnaires

An 18-item patient questionnaire was developed; its face validity was tested with patients (n = 10), revisions were made, and then a final version was implemented. It included questions regarding (1) information (about the procedure and findings); (2) patient participation; (3) identity check; (4) staff's behavior toward the patient; (5) perception of safety; (6) repeated questions from staff members; and (7) the perception of teamwork and safety measures within the medical team.

For 12 of the items, the response format was a 7-point Likert-type scale (1 = strongly disagree, 7 = strongly agree). Six questions had the response options of "yes," "no," or "partly/don't remember."

After their endoscopic examination, patients were asked if they would be part of the study and informed, both verbally and in writing, that the participation was voluntary and anonymous. The inclusion criteria for patients receiving a questionnaire were that they were older than 18 years and fluent in Swedish. Exclusion criteria were the following: (1) patients unable to fill out the questionnaire due to cognitive failure, poor

general condition, or heavy sedation; (2) patients who had already filled out the questionnaire in the past month; and (3) patients who had already been examined during the observations of the first author were excluded from completing the questionnaire to rule out the risk of influence on patient experiences. Nurses working in the unit collected the questionnaires at the very end of the patients' visits.

Prior to the introduction of the checklist, a baseline questionnaire was distributed to both inpatients and outpatients at the endoscopy unit over a 7-d period. In September to October 2015, patient questionnaires were randomly collected again.

In addition, a 14-item staff questionnaire contained questions/statements regarding (1) team collaboration; (2) working climate; (3) patient participation; and (4) patient safety. Items were partly adopted and incorporated from the validated Safety Attitudes Questionnaire[26]. The staff questionnaire was developed and tested in two steps: first by its face validity on the physicians (n = 3) and nurses (n = 2) at the hospital' s other endoscopy unit and then revising and testing through a test-retest process using physicians (n = 5) and nurses (n = 5) at the "twin unit." No need to further revise the questionnaire was found.

Staff was informed that their contributions *via* the questionnaire were voluntary and anonymous. Inclusion criteria for the staff questionnaire were that they worked at least 4 shifts per year in the unit and were fluent in Swedish. One nurse endoscopist at the unit was excluded from completing the questionnaire since she would not be able to participate anonymously. The baseline questionnaire was collected a few weeks prior to the intervention start, and the follow-up questionnaire was collected 5 months after the start of the intervention.

Statistical analysis

Observations: The frequency of identity checks performed by the nurses and the physicians was compared before and 10 months after the "checklist intervention" using the Fischer's exact test.

Patient questionnaires: Data are presented as medians and 25-75 percentiles. The Mann-Whitney U-test was used to test for statistically significant differences before and after the intervention. For the dichotomous responses, Fischer's exact test and χ^2, when appropriate, were used. The frequencies and proportions of responses to the questionnaires were calculated including only the response alternatives "Yes" or "No".

Staff questionnaires: Data are presented as medians and 25-75 percentiles. The Wilcoxon's matched-pairs signed-ranks test was used to test for statistically significant differences before and after the intervention. P-values less than 0.05 were considered statistically significant.

RESULTS

The endoscopy checklist

In the final checklist, the patients' individual conditions, experiences, expectations, and/or fears were highlighted in addition to safety-related items. Most importantly, the "summary," which corresponded to the "timeout" in the WHO SSC, directly addressed the patient before scope insertion was performed, giving him or her an opportunity to correct any inaccuracies. This "summary" always preceded any sedation of the patient and was performed by the nurse/endoscopy assistant who had followed the patient from the onset of the visit. The physician participated by first actively listening to the "summary" and then, if necessary, posing additional questions. The summary was ended by asking the patient if he or she had questions or wanted to add anything.

The checklist was displayed on large posters on the walls in all examination rooms; they were therefore visible to both the medical teams and the patients. In Figure 2, an English translation of the checklist is presented.

All nurses in the unit (n = 9), including the nurse endoscopist, attended the checklist introduction training. Out of the 20 physicians scheduled in the endoscopy department during the study period, seven participated in the training and two at attended an open lunch seminar. The remaining 11 physicians were briefed on the checklist by the first author and/or by viewing the instruction film.

Observations

During the baseline observations (n = 27), none of the physicians performed identity checks, while nurses did so for 96% of the observations. Follow-up observations (n = 45) were performed at 4, 6, and 10 mo after the intervention. The rate of identity checks for both the physicians and nurses remained high during the study period, reaching 87% at 10 mon (P < 0.001) for the physicians and 93% for the nurses (P = n.s.). Observations regarding the identity verifications are illustrated in Figure 3.

At 4 mo after the introduction of the checklist, a "summary" was initiated for 18 of 20 of the observations (90%) but with a varying degree of completeness (Figure 4). All parts of the "summary" box were included in 56% of the observations. The nurses fully addressed the patients during the "summaries" 89% of the time and partly addressed the patient 11% of the time. The physicians faced the patients 50% of the observed "summaries."

Patient questionnaire

Out of 168 patients examined during the baseline data collection period, 104 patients (62 %) completed the questionnaires. Reasons for not completing the

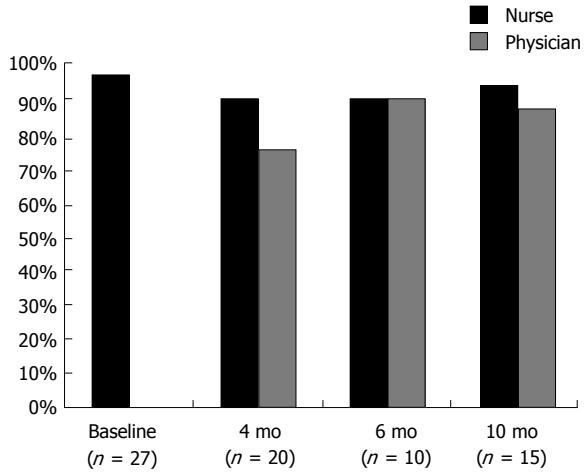

Figure 3 Observations of identity checks before/after introduction of the checklist.

Table 1 Patient background factors retrieved from the patient questionnaire *n* (%)

	Baseline questionnaire, *n* = 104	Follow-up questionnaire, *n* = 100
Men	49 (47)	38 (38)
Women	50 (48)	57 (57)
Age 18-29 yr	7 (7)	7 (7)
Age 30-64 yr	62 (60)	52 (52)
Age > 64 yr	34 (33)	37 (37)
Inpatients	10 (10)	17 (17)

questionnaires were the following: (1) The patient did not meet the criteria; (2) the patient declined to participate; or (3) the patient did not receive the questionnaire due to a heavier-than-normal workload for the nurses. At baseline data are missing regarding the number of patients per reason not to participate. During the follow-up, 183 patients were examined at the unit. Questionnaires were distributed to 141 patients; 42 patients did not receive the questionnaires due to a heavier-than-normal workload for the nurses collecting questionnaires. Out of the 141 patients, 30 did not meet the study criteria, leaving a sample of 111. Eleven patients declined participation. Completed questionnaires were received from 100 patients. Thus, the response rate was 55% if calculated according to the baseline and 90% if calculated based on eligible patients who received a questionnaire. Patient background factors are shown in Table 1. Missing values are not presented.

On the patient questionnaire, both at baseline and at follow-up, all Likert-scale ratings were 7 in median. The 25-75 percentiles were 7-7 for all responses but one, where it was 6-7. Data from these statements in the questionnaire are not presented further. The proportions (%) of patients' responses to the questions with the response options of "yes" or "no" are presented in Table 2.

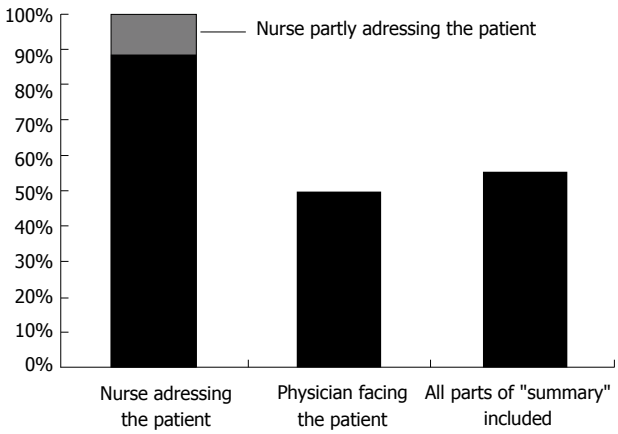

Figure 4 Observations of the checklist "summary" (*n* = 18) 4 mo after the intervention.

Staff questionnaire

The baseline staff questionnaire was distributed to nurses (*n* = 8) and physicians (*n* = 20) a few weeks prior to the intervention. The follow-up staff questionnaire was distributed 5 mo after the checklist introduction to the nurses (*n* = 8) and physicians (*n* = 18) who were eligible for inclusion at that time. The response rate for the nurses was 100% both before and after the implementation. All nurses were women. The response rate for the physicians was 65% (*n* = 13) at baseline and 72% (*n* = 13) in the follow-up period. Among physicians at the baseline, 3 respondents were women and 10 were male; at the follow-up, 2 physicians were women, 10 were men, and 1 did not report his or her gender. The staff questionnaire is presented in Table 3. No significant differences were found between the nurses' and physicians' answers; thus, they are presented together. In 2 of the 14 questions the changes were close to reaching statistical significance; *i.e.*, there were signs of increased quality in collaboration with nurses ($P = 0.07$) and an increased perception of the importance of patient participation ($P = 0.08$) after checklist implementation.

DISCUSSION

The aim of this study was to develop and implement a person-centered safety checklist at an endoscopy unit and to evaluate the "checklist intervention" in terms of patient safety, person-centeredness, and teamwork. To the best of our knowledge, this is the first checklist in an endoscopy setting that has combined patient safety with a person-centered approach. It was designed as a quality improvement project. During the team training, body language was emphasized, although non-verbal communication was not clearly incorporated into the checklist. The team training was mandatory for those working in the unit during the scheduled training days. Physicians not scheduled to work in the unit on those days were invited to attend either the team training

Table 2 Part of patient questionnaire (Translated from Swedish)

	Baseline questionnaire, *n* = 104 Yes/No (% Yes)	Follow-up questionnaire, *n* = 100 Yes/No (% Yes)	*P* value
I was offered sedatives before my examination	97/2 (98%)	95/2 (98%)	NS
I was given sedatives and/or analgesic drugs during my examination	77/26 (75%)	75/23 (76%)	NS
I was asked by the staff in the examination room to state my social security number/ show identification	96/6 (94%)	97/1 (99%)	NS
I had to answer the same questions several times while I was in the examination room	29/49 (37%)	47/33 (59%)	0.011
I received information concerning the result of the examination/treatment	74/5 (94%)	81/6 (93%)	NS
The nurse asked me how I experienced the examination	79/7 (92%)	79/7 (92%)	NS

Table 3 Staff questionnaire (Translated from Swedish)

	All Staff (Physicians and Nurses)		*P* value
	Baseline, *n* = 21 (Nurses *n* = 8, Physicians *n* = 13)	Follow-Up, *n* = 21 (Nurses *n* = 8, Physicians *n* = 13)	
Response options on a 7-point Likert-type scale: 1 = strongly disagree, 7 = strongly agree	Median (25-75 percentiles)		
The doctors and nurses work together as a well-coordinated team at the endoscopy unit	5 (4-6)	6 (6-7)	NS
I know the staff I worked with during my most recent shift at the endoscopy unit by first and last name	6 (3.5-7)	7 (3.25-7)	NS
Patient participation is considered important at the endoscopy unit	5 (5-6.5)	7 (6-7)	*P* = NS (0.08)
Patient safety is considered important at the endoscopy unit	7 (6-7)	7 (6-7)	NS
It is easy for patients to ask the staff questions at the endoscopy unit if there is something they do not understand	6 (5-6)	6 (5-7)	NS
It is easy for the staff to ask each other questions if there is something they do not understand	5.5 (5-7)	6 (5-7)	NS
We have clear routines for working in a patient-safe manner at the endoscopy unit	5 (4-6.5)	6 (6-7)	NS
I feel comfortable expressing a dissenting opinion to a colleague with another profession	5 (4.5-6)	6 (5-6.75)	NS
I feel comfortable expressing a dissenting opinion to a colleague with the same profession	6 (5-6)	6 (5-7)	NS
I would feel safe here as a patient	6 (5-7)	6 (5.25-7)	NS
Response options of very low, low, adequate, high, and very high are translated into numbers 1 to 5 below, with very low = 1 and very high = 5			
Describe the quality of your cooperation with the doctors at the endoscopy unit (answer also if you are a doctor)	4 (4-5)	4 (3.25-5)	NS
Describe the quality of the communication with the doctors at the endoscopy unit (answer also if you are a doctor)	4 (3-4)	4 (3-5)	NS
Describe the quality of your collaboration with the nurses at the endoscopy unit (answer also if you are a nurse)	4 (4-5)	5 (5-5)	P = NS (0.07)
Describe the quality of the communication with the nurses at the endoscopy unit (answer also if you are a nurse)	4 (4-4.5)	5 (4-5)	NS

or the open lunch seminar. Despite this invitation, however, only 7 physicians attended the training and 2 attended the lunch seminar. Therefore, one possible explanation for the physicians' deficient participation during the "summaries" could have been their lack of training. Previous research has shown that team training in non-technical skills is effective for increasing patient safety attitudes and situation awareness[27]. We therefore regard the team training and group discussions as crucial parts of the implementation process.

The most prominent finding was the increase in identity checks performed by the physicians, an effect that remained throughout the first year after the checklist introduction. In endoscopy, as well as any other type of healthcare, patient misidentification can lead to serious harm. Photo documentation and pathology referrals are two of the tasks that physicians are exclusively responsible for at the unit, and by minimizing the risk of patient misidentification by physicians, important safety improvements can be achieved.

Previous studies have shown improvements in collaborative climate and communication when using the WHO SSC[10,11], and a good working climate can have an indirect positive effect on patient safety. In addition, the perceived quality of communication among staff has previously been linked to improved patient outcomes[9]. Although one question on the staff questionnaire indicated improved collaboration in this study, statistical significance could not be shown, most likely due to the small number of participants; hence,

this result should be interpreted cautiously.

Although the checklist developed for this study is new, it contains similarities to the WHO SSC. Haynes *et al*[8] found that the WHO SSC improved patient outcomes, although other researchers have not noted this association[14]. In the current study, besides the increase in identity checks by the physicians, another positive effect on patient safety was the addition of the checklist "summaries," which most likely contributed to increased team situation awareness and possibly reduced the threshold for speaking up[28]. Other similar projects[16,29] have found that compliance to checklists is suboptimal. This can be seen in the physicians' lack of participation in half of the observed "summaries" in the current study. We concur with Catchpole and Russ[30], who consider safety checklists to be "complex socio-technical interventions" that should not be seen as quick solutions to safety issues. The implementation of new routines is a time-consuming process, and the implementation of this endoscopy checklist is not an exception. However, if properly used, this new endoscopy checklist could serve as a tool for improved patient safety, with a team training and group discussions serving as a basis for behavioral change.

Can a checklist enhance a person-centered approach? Since there are no clear definitions of person-centered care, we selected aspects of this approach that have been noted as important in the literature[23,24] and included them on the checklist and in the team training sessions. Our ambition was to evaluate if the checklist intervention could enhance person-centered care, but neither the staff questionnaire nor the patient questionnaire shed light on this matter. Although the staff reported an increased importance of patient participation at the unit, which might indicate a greater awareness of this important aspect of person-centered care, the *P*-value was just slightly over the cut-off value ($P = 0.05$).

Associations between multi-professional teamwork and patients' perceptions of quality of care have previously been identified[31]. However, a similar effect could not be shown in this study since the patient baseline questionnaire - with maximal median scores on all items - did not leave room for improvement. Statistical significance was found for one item on the patient questionnaire, namely regarding the repetition of questions by the staff. However, due to the low number of responders to this specific item, it is not clear if this finding is fully representative.

In general patients tend to report high levels of satisfaction[32], which is why other research methods, such as qualitative interviews, might be useful to better understand patients' views on person-centered care in endoscopy settings.

Strengths and limitations

Some of the strengths in our study design were the inclusion of both patients and staff and the use of observations in addition to questionnaires for data collection. This quality improvement project was implemented and evaluated in a clinical setting with immediate consequences for patients and staff.

In our study, the observations contributed to an understanding of how the checklist was used by the staff. People who are being observed tend to perform differently than normal, so to minimize this bias, a research nurse performed parts of the observations simultaneously with her ordinary tasks.

Suitable existing questionnaires for our study could not be found, and we therefore developed our own based partly on existing validated instruments. Our questionnaires were tested for their usability and relevance. However, the lack of solid validation of the questionnaires is a limitation to our study.

As a quality improvement project, it was important that the implementation leaders were endoscopy staff members as this would increase their buy-in and sense of ownership of the checklist. However, our personal engagement in the research study could have affected the internal validity and generalizability of the findings. In addition, a one-group pretest-posttest design is vulnerable to internal validity threats. In future studies, a standardized model of implementation at multiple sites, together with validated instruments for measuring patient outcomes and potentially qualitative methods, could result in a greater understanding of the complexity of an endoscopy checklist that combines patient safety and person-centeredness and its effects.

CONCLUSION

The most prominent finding of this study was a statistically significant improvement in patient identity verification by physicians, which is an important patient safety measure. Compliance with the checklist was suboptimal. The staff questionnaires highlight a possible increase in staff's awareness of patient participation and improved collaboration. The patient questionnaires did not shed light on the study aims; therefore, further investigations at multiple units using a standardized implementation model and more sensitive instruments are needed to further evaluate this concept.

ARTICLE HIGHLIGHTS

Research background

One of the most well-known tools for improving patient safety is the World Health Organization's Surgical Safety Checklist (WHO SSC), which has been extensively evaluated. Studies have shown that implementing the WHO SSC contributes to better patient outcomes. Improved communication in surgical teams, a factor known to be associated with better patient outcomes, is another positive effect of the WHO SSC.

Within the field of endoscopy, the number of examinations continues to increase; at the same time, this diagnostic process has become more technically advanced. Therefore, knowledge about a patient's health condition

and proper monitoring of the patient's vital functions are crucial to prevent complications. Safety checklists similar to the WHO SSC that are specific to endoscopy have been described in the literature.

Another approach to improve healthcare quality and patient safety is promoting patient participation. Person-centered care has been described as a collaborative and respectful partnership between healthcare professionals and the patient. This study describes an attempt to combine patient safety with a person-centered approach in the endoscopy field, which to our knowledge has not been done before.

Research motivation

Our motivation was to explore if patient safety aspects could be combined with a person-centered approach in an endoscopy checklist. We also wanted to evaluate the impact of such a checklist. Would this novel checklist contribute to improved team communication and enhanced patient safety as previous checklists have done? Would the addition of a person-centered approach contribute to increased patient participation? Would the staff use the checklist as intended? The study contributes to the current literature through an innovative approach that could be adopted by other high-volume service areas in the medical field.

Research objectives

The main objectives of the study were to describe the development and implementation of a novel person-centered safety checklist and to evaluate the "checklist intervention" in terms of patient safety, person-centeredness, and teamwork.

Research methods

The intervention in this study was a newly developed endoscopy checklist at a university hospital's endoscopy unit in Sweden. The checklist was developed by a multi-professional group, and the introduction consisted of half-day sessions including lectures, a team training, and group discussions.

The intervention was evaluated using two methods: structured observations and pre/post questionnaires. The questionnaires were developed by the authors and were tested for their usability and relevance. Questionnaires were collected from both patients and staff. The structured observations included endoscopy teams of physicians and nurses. Anonymized data were analyzed using, when appropriate, the Mann-Whitney *U*-test, Fischer's exact test, the χ^2, and the Wilcoxon's matched-pairs signed-ranks test.

Research results

Our observations showed frequent attempts by the physicians and nurses to use the checklist, but with suboptimal compliance.

The most salient result in the study was the increase of patient identity verifications performed by physicians. At baseline, none of the physicians performed identity checks before scope insertion. At 10 mo after the intervention, the identity verifications performed by physicians were observed at 87%.

Neither the staff nor patient questionnaires had statistically significant differences. However, the staff reported an increased awareness of the importance of patient participation, which might indicate a greater emphasis on this important aspect of person-centered care (the *P*-value was slightly over the cut off value of $P = 0.05$). These results should be interpreted carefully and need to be investigated further in future studies using validated instruments or other research methods.

Research conclusions

This new endoscopy checklist, if properly used, could be a tool for improved patient safety, with a team training and group discussions serving as a basis for behavioral changes. The combination of patient safety aspects and a person-centered approach has been carried out and implemented with immediate positive consequences for patients and staff. However, further research is needed to evaluate the effects of the checklist, especially regarding a teamwork culture and person-centeredness.

Research perspectives

Since no suitable questionnaires were found for this context, the authors developed their own. Although this was an educative process, this method was not sufficient to draw conclusions for some of our research objectives. In future work, solid validation is necessary for such questionnaires. Qualitative methods could bring a deeper understanding of patient and staff experiences regarding patient participation and a teamwork culture. To measure patient safety, the checklist should be implemented using standardized methods at multiple sites and by using other patient outcome measures, such as complication rates or near misses.

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ORIGINAL ARTICLE

Randomized Clinical Trial

Multicenter, randomized study to optimize bowel for colon capsule endoscopy

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Abstract

AIM

To assess the cleansing efficacy and safety of a new Colon capsule endoscopy (CCE) bowel preparation regimen.

METHODS

This was a multicenter, prospective, randomized, controlled study comparing two CCE regimens. Subjects were asymptomatic and average risk for colorectal cancer. The second generation CCE system (PillCam® COLON 2; Medtronic, Yoqneam, Israel) was utilized. Preparation regimens differed in the 1st and 2nd boosts with the Study regimen using oral sulfate solution (89 mL) with diatrizoate meglumine and diatrizoate sodium solution ("diatrizoate solution") (boost 1 = 60 mL, boost 2 = 30 mL) and the Control regimen oral sulfate solution (89 mL) alone. The primary outcome was overall and segmental colon cleansing. Secondary outcomes included safety, polyp detection, colonic transit, CCE completion and capsule excretion ⩽ 12 h.

RESULTS

Both regimens had similar cleansing efficacy for the whole colon (Adequate: Study = 75.9%, Control = 77.3%; P = 0.88) and individual segments. In the Study group, CCE completion was superior (Study = 90.9%, Control = 76.9%; P = 0.048) and colonic transit was more often < 40 min (Study = 21.8%, Control = 4%; P = 0.0073). More Study regimen subjects experienced adverse events (Study = 19.4%, Control = 3.4%; P = 0.0061), and this difference did not appear related to diatrizoate solution. Adverse events were primarily gastrointestinal in nature and no serious adverse events related either to the bowel preparation regimen or the capsule were observed. There was a trend toward higher polyp detection with the Study regimen, but this did not achieve statistical significance for any size category. Mean transit time through the entire gastrointestinal tract, from ingestion to excretion, was shorter with the Study regimen while mean colonic transit times were similar for both study groups.

CONCLUSION

A CCE bowel preparation regimen using oral sulfate solution and diatrizoate solution as a boost agent is effective, safe, and achieved superior CCE completion.

Key words: Bowel preparation; Purgative; Capsule endoscopy; Endoscopy; Capsule colonoscopy; Large intestine

Core tip: Current bowel preparation boost agents for colon capsule endoscopy have associated risks and contraindications. This paper describes a new boost agent comprised of two very low dose hyperosmotic agents, oral sodium sulfate and diatrizoate solution, which appears to be an acceptable alternative regimen for colon capsule endoscopy.

Kastenberg D, Burch WC, Romeo DP, Kashyap PK, Pound DC, Papageorgiou N, Fernández-Urien Sainz I, Sokach CE, Rex DK. Multicenter, randomized study to optimize bowel preparation for colon capsule endoscopy. *World J Gastroenterol* 2017; 23(48): 8615-8625 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i48/8615.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i48.8615

INTRODUCTION

Colon capsule endoscopy (CCE) is a non-invasive procedure which effectively visualizes the entire colon. While CCE technology has advanced, the preparation regimen remains a challenge. Not only is a clean and fluid filled colon requisite, but the capsule must reach the hemorrhoidal plexus within its 12-h battery life. Furthermore, the capsule must dwell for a sufficient period of time within the colon as rapid transit (< 40 min) in combination with sub-optimal cleansing may lower the sensitivity for detecting polyps[1].

The CCE preparation regimen before capsule ingestion focuses on cleansing the colon, whereas post-capsule ingestion measures are aimed at propelling the capsule through the gastrointestinal (GI) tract and providing additional cleansing. A full dose purgative, typically polyethylene glycol-electrolyte solution (PEG-ELS), is administered as a split dose prior to capsule ingestion. After capsule ingestion, a pro-kinetic medication - in the United States metoclopramide -

may be administered if the capsule does not exit the stomach within an hour. Upon entering the duodenum, and then typically 3 and 5 h later if the capsule has not exited the colon, an agent ("boost") is administered to accelerate capsule transit and augment cleansing.

Generally the first and second boost consists of a hyperosmotic agent and, to date, sodium phosphate liquid (NaP) has most commonly been used[2-10]. However, numerous contraindications to the use of NaP makes it a non-viable option in the United States[11,12]. As an alternative to NaP, a large prospective CCE study used oral sulfate solution (OSS) for the first 177 mL (6 oz.) and second 89 mL (3 oz.) boost[1]. While overall bowel preparation adequacy was acceptable, an unexpected limitation of this regimen was a higher than expected rate of technically inadequate studies due to the combination of rapid colonic transit and inadequate colon cleansing.

Diatrizoate meglumine and diatrizoate sodium solution ("diatrizoate solution") is a hyperosmotic agent used in radiologic imaging, and when orally administered causes an influx of fluid into the GI tract resulting in diarrhea[13]. A small CCE study used diatrizoate solution alone as a boost agent and found high rates of adequate colon cleansing and CCE completion, while rapid colonic transit (< 40 min) was rare[14]. A larger study combined diatrizoate solution with NaP as a boost agent and observed adequate colon cleansing in 83%, and CCE completion in 98%, of subjects[15].

This study evaluated the efficacy and safety of a new CCE preparation regimen that combines low dose OSS with diatrizoate solution as a boost agent. The study was conducted at six centers in the United States.

MATERIALS AND METHODS

This was a multicenter, prospective, randomized, controlled study designed to assess the superiority of a new colon cleansing regimen for CCE. Six centers (2 academic) participated, and subjects were enrolled between 5/18/15 and 9/23/15. Each center obtained IRB approval prior to study initiation, and this protocol was registered with clinicaltrials.gov (ID# NCT02481219).

The second generation CCE system (PillCam® COLON 2; Medtronic, Yoqneam, Israel), was used in this study. This consists of an ingestible capsule, sensors attached to the abdominal wall that receive capsule signals, a data recorder, and software (RAPID, version 8.3) enabling image display and creation of reports.

The primary outcome was overall and segmental colon cleansing. Secondary outcomes included polyp detection (≥ 6 mm, ≥ 10 mm, and total), colonic transit time, CCE completion (defined as visualization of the hemorrhoidal plexus), excretion of capsule within 12 h of ingestion, and safety. Colon cleansing was assessed using a validated 4-point grading scale of excellent, good, fair, and poor for individual colon segments and for the overall colon[16]. Adequate cleansing was defined as a combination of good and excellent.

Subjects

Subjects were asymptomatic and average risk for colorectal cancer (CRC), and ranged in age from 50-75 years. Average risk was defined using the American Gastroenterology Association Guidelines on Colorectal Cancer Screening[17]. Subjects did not have a personal history of CRC or adenoma or inflammatory bowel disease, a first degree family member with CRC under age 60 or two or more first degree relatives with CRC at any age, or a personal or family history of a genetic syndrome high risk for CRC.

Furthermore, subjects were excluded if they had a negative colon evaluation within 5 years (colonoscopy, CTC, flexible sigmoidoscopy, barium enema, or stool testing for blood and/or DNA); a history of GI bleeding, heme positive stool, or iron deficiency; a contraindication to capsule technology including dysphagia or any swallowing disorder, a cardiac pacemaker or implanted electromedical device, anticipation of magnetic resonance imaging within 7 d of capsule ingestion, or increased risk for capsule retention including a suspected or known GI motility disorder, bowel obstruction, stricture or fistula; a history of renal disease**;** an allergy or contraindication to any component of the study regimens; pregnancy or active breast feeding; participation in another investigational study within 30 d that may interfere with the subject's safety or ability to participate in this study; a member of a vulnerable population (prisoner, intellectually challenged, *etc.*); or a severe medical condition such that participation is not appropriate due to increased risk, lack of benefit of screening, or survival anticipated to be less than 6 mo.

Study procedure

Subjects underwent a screening visit to assess eligibility. Demographic information as well as medical and surgical history was obtained, and an assessment of pregnancy potential was performed. Subjects of childbearing potential underwent a urine pregnancy test at the time of screening. During the screening visit, if eligibility criteria were satisfied, informed consent was obtained and subsequently subjects were randomized in a 1:1 ratio to receive the study bowel preparation regimen (Study) or the comparator control bowel preparation regimen (Control) (see bowel preparation regimen section below and Table 1). This study utilized randomization blocks using a standard envelope procedure. The CCE was performed within 45 days of the screening visit.

Table 1 Bowel preparation regimens

Time	Study regimen	Control regimen
2 d prior	⩾ 2400 mL (10 glasses) of liquid during the day; 4 senna tablets at bedtime	
1 d prior	Clear liquid diet all day; 2 L sulfate-free PEG-ELS[1] at about 7-9 pm (one 237 mL-296 mL cup (8-10 oz. cup) every 10-15 min)	
Day of capsule procedure		
45-75 min prior to capsule ingestion	2 L sulfate-free PEG-ELS	
1 h after capsule ingestion	Optional prokinetics (only if capsule in stomach > 1 h): 10 mg metoclopramide or 250 mg erythromycin	
1st boost: After capsule entry into small bowel[2]	89 mL (3 oz) OSS plus 60 mL diatrizoate solution[1]	89 mL (3 oz.) OSS
2nd boost: 3 h after 1st boost, only if capsule not excreted	89 mL (3 oz) OSS plus 30 mL diatrizoate solution[1]	89 mL (3 oz.) OSS
3rd boost: 2 h after 2nd boost, only if capsule not excreted	10 mg bisacodyl suppository	
2 h after 3rd boost, or after capsule passes (whichever occurs first)	Standard full meal	

[1]Diatrizoate solution = diatrizoate meglumine and diatrizoate sodium solution; [2]Clear liquids permitted after ingestion of 1st boost, PEG-ELS: Polyethylene glycol-electrolyte solution; OSS: Oral sulfate solution.

The CCE videos were read remotely by two readers who had extensive experience reading CCE studies and were unassociated with any study site. Video assignment to centralized readers utilized a 1:1 randomization stratified by bowel preparation group to minimize bias using MDT data manager (Medtronic, Mansfield, MA, United States). Videos were read within 3 wk of CCE completion, and no more than 5 videos per week were read by a single reader.

Readers used the aforementioned four-point scale to grade cleansing for 5 colon segments - cecum, right colon, transverse colon, left colon, and rectum. Additionally, readers provided an overall cleansing grade for the entire colon.

For each polyp, the location and size, as determined by the longest dimension using a software measuring tool, was recorded. The time for the capsule to reach the cecum, hepatic and splenic flexures, and exit the rectum was also measured using software. The cecum and last rectum image was identified by the reader, and then the other colon landmarks were identified by either the software or the reader.

The completed report was provided to the primary investigator at each enrollment site within 3 months of the capsule procedure**.** Follow-up of capsule findings was left to the discretion of the primary investigator at each site.

Bowel preparation regimens

The Study and Control bowel preparation regimens are summarized in Table 1. For both regimens, all subjects took 4 senna tablets the evening of Day -2, and 2 L of sulfate-free PEG-ELS (NuLYTELY®, Braintree Laboratories Inc., Braintree, MA, United States) the night before the capsule procedure and again the next morning with completion 45-75 min before capsule ingestion. If the capsule remained in the stomach more than 1 h, subjects in both study arms took metoclopramide 10 mg or erythromycin 250 mg orally.

All subjects (Study and Control) received a 1st boost once the capsule entered the duodenum, and a 2nd boost 3 h later if the capsule had not been excreted. Study and Control regimens differed in the composition of these boosts. For the 1st boost, the Study regimen used 89 mL (3 oz.) of OSS (SUPREP ® Braintree Laboratories Inc., Braintree, MA) diluted to 237 mL (8 oz.) with water and 59 mL (2 oz.) of diatrizoate solution (Gastrografin®, Bracco Diagnostics Inc., Monroe Township, NJ, United States) diluted to 207 mL (7 oz.) with water, and for the 2nd boost 89 mL (3 oz.) of OSS diluted to 237 mL (8 oz.) with water and 30 mL (1 oz.) of diatrizoate solution diluted to 89 mL (3 oz.) with water. For both the 1st and 2nd boosts, the Control regimen used 89 mL (3 oz.) of OSS diluted to 237 mL (8 oz.) with water. Subjects in both study arms drank at least 946 mL (32 oz.) of water with the first and second boosts.

Two hours after the 2nd boost, if the capsule had not been excreted, subjects in both study arms received a 3rd boost consisting of a 10 mg bisacodyl suppository. Diet was identical for subjects in both study arms.

Safety

Adverse events were recorded throughout the CCE preparation regimen. Subjects were called 5-9 d after completion of the capsule procedure to confirm capsule excretion and record any additional adverse events. Adverse events were classified as serious or non-serious. Non-serious adverse events were characterized and then classified as mild, moderate, or severe.

Comparison of study and control regimens with historic comparators

Preparation adequacy, CCE completion, capsule transit times, and polyp detection were compared between the study and control regimens and similar regimens used in two published CCE studies[1,15].The Study regimen was compared to Spada *et al*[15] as both used the combination of diatrizoate solution and a hyperosmotic

Table 2 Subject characteristics

	Study regimen, *n* = 62	Control regimen, *n* = 59	*P* value
Age (yr)	55.20	55.10	0.888
Gender (male:female)	45:55	49:51	0.660
BMI (mean)	28.50	28.50	0.978

purgative for the 1st and 2nd boosts. However, instead of OSS and diatrizoate solution, Spada *et al*[15]. used NaP in combination with diatrizoate solution [1st boost = 40 mL (1.4 oz.) NaP and 50 mL (1.7 oz.) diatrizoate solution in 1 L (34 oz.) of water; 2nd boost = 25 mL (0.8 oz.) NaP and 25 ml (0.8 oz.) diatrizoate solution in 0.5 L (17 oz.) of water].

The Control regimen was compared to a CCE preparation regimen used by Rex *et al*[1]. Both regimens were similar except for the OSS dose used for the 1st boost [Control = 89 mL (3 oz.) diluted to 237 mL (8 oz.) plus an additional 946 mL (32 oz.) of water, Rex *et al*[1] = 177 mL (6 oz.) diluted to 473 mL (16 oz.) plus an additional 946 ml (32 oz.) of water].

Similar to the Study and Control regimens, both Spada *et al*[15] and Rex *et al*[1] administered 4 senna tablets two days prior to CCE, split dose 4 L PEG-ELS beginning the night prior to CCE, and a prokinetic agent if the capsule remained in the stomach for more than 1 h.

Statistical analysis

The statistical methods of this study were reviewed by Mathilde Lourd, a biostatistician from Medtronic Inc.

Primary outcome: A sample size of 500 patients would provide > 80% power to detect a difference between the study groups for overall colon preparation adequacy with a two-sided test at a significance level of 0.05 (adequacy assumptions: 83% Study, 71% Control). Chi-square or Fisher's exact test, as appropriate, was performed to compare proportions of good and excellent cleansing between the two preparation regimens. Non-visualized colon segments were not graded for cleansing and not included in the colon segment preparation grading analysis.

Secondary outcomes: Chi-square or Fisher's exact tests were used for analyzing polyp detection (⩾ 6 mm, ⩾ 10 mm, and any polyp) in total for the whole colon and by colon segment, CCE completion rate, adverse events in relation to administration of diatrizoate solution, and colon capsule excretion within 12 h of ingestion. Colon capsule completion was defined as excretion of the capsule within 12 h of ingestion and complete visualization of the colon. *t*-tests for continuous variables were performed to evaluate the difference between the two preparation regimens for capsule transit time by colon segment and for the entire colon.

Plan for interim analyses: Interim analyses were planned after each group of 50 subjects until a final enrollment of 500. Pre-specified criteria were established for measures of performance and safety that would allow discontinuation of the trial. These included the following: (1) an increased prevalence (> 10%) of polyps (⩾ 6 mm and ⩾ 10 mm) in the Study group; (2) an increased incidence of adequate (good/excellent) cleansing (> 12%) for the whole colon in the Study group; and (3) adverse events in fewer than 10% of subjects in the Control group.

RESULTS

Subject flow is summarized in Figure 1. There were 126 subjects screened and consented, 122 subjects met eligibility criteria, and 121 ingested any of the preparation and were included in the analyses for subject characteristics and safety. After excluding 14 subjects for protocol deviations, 107 were included in the analyses for colon cleansing and polyp detection by colonic segment, CCE completion and transit times, and comparisons between the Control regimen and Rex *et al*[1]. for colon cleansing by segment. Both study groups were similar with regard to age, gender, and body mass index (BMI) (Table 2).

After excluding 5 additional subjects with ⩾ 1 unseen colon segment (cecum, ascending, or transverse), 102 subjects were analyzed for overall colon cleansing, polyp detection for the whole colon, and comparisons between the Study regimen and Spada *et al*[15] and the Control regimen and Rex *et al*[1] for overall cleansing of the colon. Five additional subjects were excluded who had both inadequate (fair or poor) overall colon cleansing and colonic transit < 40 min. The exclusions for this final group matched Spada *et al*[15] and Rex *et al*[1] to allow comparisons for polyp detection for the whole colon.

The a priori criteria for early termination of this study were met after the first group of 50 subjects. Because enrollment was rapid, by the time the analyses had been completed and the decision to halt the study made, more than 100 subjects were enrolled and the results are presented herein.

Cleansing efficacy

For overall colon cleansing, there was no significant

Table 3 Overall colon cleansing assessment

Overall cleansing assessment [95%CI]	Study regimen, *n* = 55	Control regimen, *n* = 52	*P* value
Adequate[1]	75.9 [62.4; 86.5]	77.3 [62.2; 88.5]	0.876
Excellent	16.7 [7.9; 29.3]	6.8 [1.4; 18.7]	0.216
Good	59.3 [45.0; 72.4]	70.5 [54.8; 83.2]	0.243
Fair	24.1 [13.5; 37.6]	22.7 [11.5; 37.8]	0.875
Poor	0.0 [0.0; 5.9]	0.0 [0.0; 6.6]	--

[1]Includes both excellent and good cleansing.

Table 4 Polyp detection by size for the whole colon

Polyp size [95%CI]	Study regimen, *n* = 55	Control regimen, *n* = 52	*P* value
⩾ 6 mm	36.4 [23.8; 50.4]	21.3 [10.7; 35.7]	0.096
⩾ 10 mm	14.6 [6.5; 26.7]	8.5 [2.4; 20.4]	0.346
Any polyp	58.2 [44.1; 71.3]	46.8 [32.1; 61.9]	0.251

Table 5 Colon capsule endoscopy completion and transit times

	Study regimen, *n* = 55	Control regimen, *n* = 52	*P* value
CCE completion [95%CI]	90.9 [80.0; 97.0]	76.9 [63.2; 87.5]	0.048[2]
CCE excretion ⩽ 12 h [95%CI]	90.9 [80.0; 97.0]	80.4 [66.9; 90.2]	0.121
GI tract transit - Ingestion to excretion[1], mean (SD) [95%CI]	5:54 (6:00) [4:18; 7:30]	9:00 (11:48) [5:36; 12:24]	0.107
Colonic transit time[1], mean (SD) [95%CI]	2:12 (1:36) [1:48; 2:42]	2:36 (1:30) [2:06; 3:06]	0.262
Colonic Transit < 40 min [95%CI]	21.8 [11.8; 35.0]	4.0 [0.5; 13.7]	0.007[2]

[1]All transit times measured as (h:min); [2]Statistically significant. CCE: Colon capsule endoscopy.

difference between the Study and Control regimens using the 4-point scale of excellent, good, fair or poor (Table 3). Overall adequate cleansing (good and excellent combined) of the whole colon was similar for both study groups (Adequate: Study = 75.9%, Control = 77.3%, *P* = 0.88). When the 4-point scale was used to grade individual colon segments, in no segment was there a significant difference between the Study and Control regimens for any grade (Supplementary Tables 1-4).

Polyp detection

Table 4 summarizes overall polyp detection for both study groups. There was a trend toward higher polyp detection for the whole colon with the Study regimen, although this was not statistically significant for any size category. When evaluated by colon segment, detection of polyps of all sizes (⩾ 6 mm, ⩾ 10 mm, and any polyp) was not significantly different between the Study and Control regimens (Supplementary Tables 5-7).

Capsule colon endoscopy completion and transit

Colon capsule completion and transit times are summarized in Table 5. Superior completion of the CCE procedure was achieved with the Study regimen (Study = 90.9%, Control = 76.9%; *P* = 0.048). Mean transit time through the entire GI tract, from ingestion to excretion, was shorter with the Study regimen while mean colonic transit times were similar for both study groups. Significantly more Study regimen subjects experienced capsule transit through the colon in less than 40 min (Study = 21.8%; Control = 4%, *P* = 0.007). Five subjects (9%) in the Study regimen arm had both inadequate colon cleansing and colonic transit < 40 min.

Safety

Adverse events occurred more often in subjects receiving the Study regimen [Study = 12 (19.4%), Control = 2 (3.4%); *P* = 0.0061], and these were primarily related to bowel preparation [Study = 8 (12.9%), Control = 1 (1.7%); *P* = 0.0327] and were gastrointestinal in nature (Table 6). The incidence of adverse events in Study regimen subjects was similar before and after administration of diatrizoate solution (before diatrizoate solution = 9.7%, after diatrizoate solution = 6.5%; *P* = 0.4142.) One serious adverse event occurred with the Study regimen (sinusitis), and this was judged unrelated to the preparation regimen or the capsule procedure. All adverse events in the Control regimen arm were graded as mild, and there was a non-significant trend toward a higher level of adverse event severity experienced by Study regimen

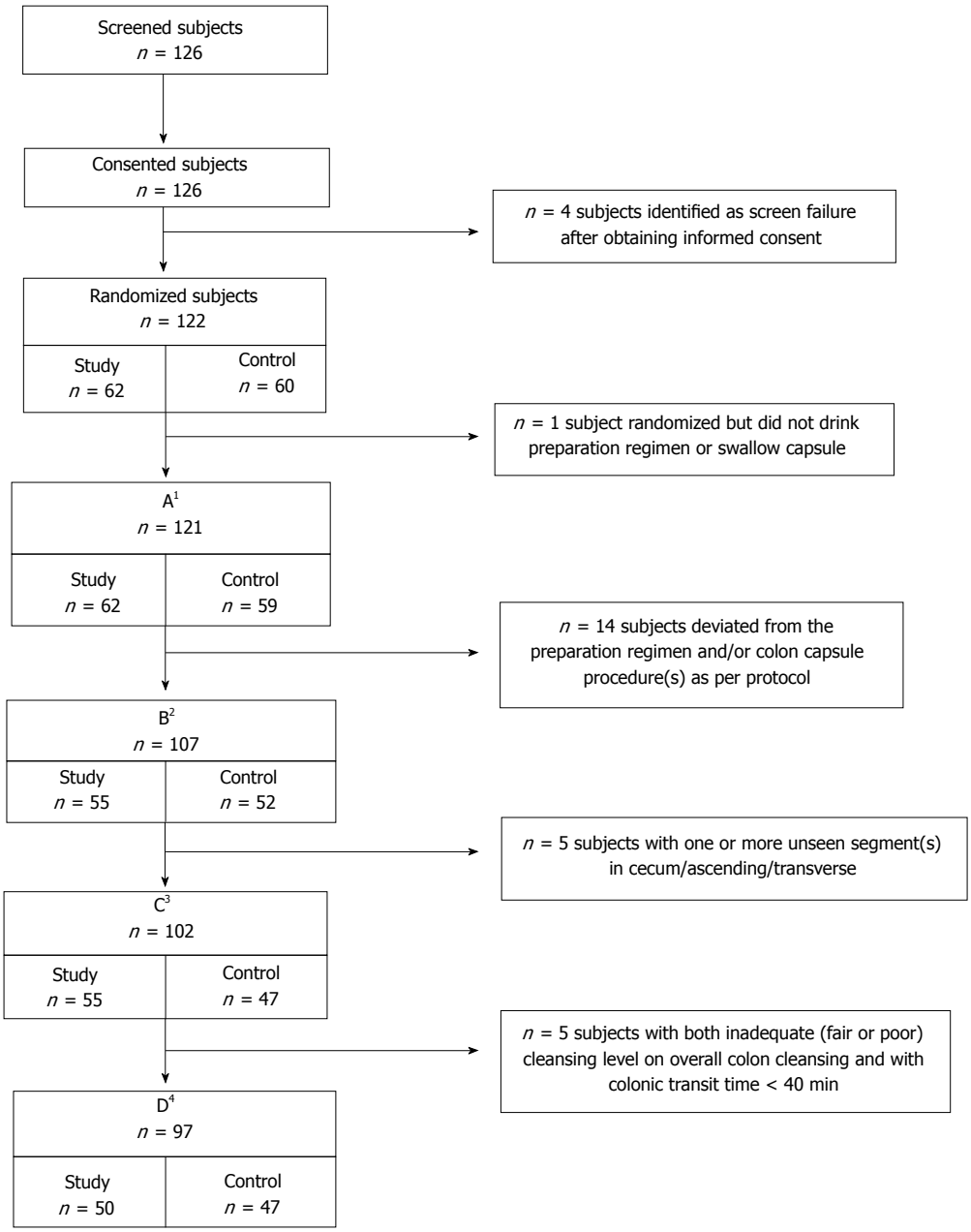

Figure 1 Patient flow diagram. [1]Group A received any of the CCE preparation regimen = Baseline subject characteristics, safety analysis, comparisons between Control regimen and Rex *et al*[1] and between Study regimen and Spada *et al*[15] for colon capsule completion and transit times. [2]Group B excluded subjects who withdrew before capsule ingestion, had a protocol violation, or experienced technical failure of the capsule = Colon cleansing by segment, CCE completion, CCE transit times, polyp detection by colon segment, comparisons between Control regimen and Rex *et al*[1] for colon cleansing by segment. [3]Group C excluded subjects listed above (B), and those with non-visualization of the cecum, right colon, or transverse colon = Colon cleansing for whole colon, polyp detection for the whole colon, comparisons between the Control regimen and Rex *et al*[1] and between the Study regimen and Spada *et al*[15] for overall cleansing of the colon. [4]Group D excluded subjects listed above (B and C) and those with both inadequate overall colon cleansing and colonic transit time < 40 min = Comparisons between Control regimen and Rex *et al*[1] and Study regimen and Spada *et al*[15] for whole colon polyp detection.

subjects (Table 6).

Comparisons to historical groups

Study regimen: The Study regimen demonstrated no significant difference as compared to Spada *et al*[15] for overall cleansing of whole colon, CCE completion, capsule transit through the entire GI tract, and overall polyp detection (Table 7).

Control regimen: The Control and Rex *et al*[1] regimens had similar overall cleansing efficacy for the whole colon and for individual colon segments. However, the Control regimen had a significantly lower rate of completion (Control = 77.6%, Rex *et al*[1] = 89%; $P = 0.041$) and a longer mean colonic transit time (Control = 2 h 48 min, Rex *et al*[1] = 1 h 52 min; $P < 0.001$). Polyp detection was not significantly

Table 6 Adverse events: Frequency and severity by subject

Adverse event	Study regimen, *n* = 62	Control regimen, *n* = 59	*P* value
Subjects with ⩾ 1, *n* (%) [95%CI]	12 (19.4) [10.4; 31.4]	2 (3.4) [0.4; 11.7]	0.006[2]
Occurring in > 2% of subjects, *n* (%) [95%CI]			
Headache	2 (3.2) [0.4; 11.2]	1 (1.7) [0.0; 9.1]	
Nausea	4 (6.5) [1.8; 15.7]	2 (3.4) [0.4; 11.7]	
Vomiting	2 (3.2) [0.2; 38.5]	0 (0) [0.0; 77.6]	
Maximum AE severity[1], *n* (%) [95%CI]			
Mild	6 (50) [21.1; 78.9]	2 (100) [22.4; 100.0]	
Moderate	5 (41.7) [15.2; 72.3]	0 (0) [0.0; 77.6]	0.308
Severe	1 (8.3) [0.2; 38.5]	0 (0) [0.0; 77.6]	

[1]Subjects were counted once according to maximum severity; 2nd Statistically significant.

Table 7 Comparisons between Study regimen and Spada *et al*[15]: Bowel cleansing, capsule transit, and polyp detection

Parameter	Study regimen	Spada *et al* (4)[1]	*P* value
Adequate preparation-whole colon (% [95%CI])	75.9 [62.4; 86.5]	78.0[64.0; 88.5]	0.820
CCE completion (% [95%CI])	90.2 [79.8; 96.3]	96.0 [86.3; 99.5]	0.291
GI transit time[2], mean (SD) [95%CI]	6:00 (6:12) [4:24; 7:36]	6:06 (4:48) [4:46; 7:26]	0.925
Polyp detection (% [95%CI])			
⩾ 6 mm	36.0 [22.9; 50.8]	25.0 [13.6; 39.6]	0.233
⩾ 10 mm	16.0 [7.2; 29.1]	12.5 [4.7; 25.2]	0.619

[1]1st boost = 40 mL sodium phosphate liquid (NaP) and 50 mL diatrizoate solution; [2]boost = 25 mL NaP and 25 mL diatrizoate solution; [2]All transit times measured as (hour: minutes). CCE: Colon capsule endoscopy.

Table 8 Comparisons between Control regimen and Rex *et al*[1]: Bowel cleansing, capsule completion and transit, and polyp detection

Parameter	Control regimen	Rex *et al* (7)[1]	*P* value
Adequate preparation–whole colon (% [95%CI])	77.3 [62.2; 88.5]	71.4 [68.1; 74.5]	0.369
CCE completion (% [95%CI])	77.6 [64.7; 87.5]	89.0 [86.8; 91.0]	0.041[3]
GI transit time[2], mean (SD) [95%CI]	2:48(1:36) [2:18; 3:18]	1:52 (1:40) [1:45; 1:59]	< 0.001[3]
Polyp detection (% [95%CI])			
⩾ 6 mm	21.3 [10.7; 35.7]	31.5 [28.1; 35.1]	0.100
⩾ 10 mm	8.5 [2.4; 20.4]	11.4 [9.1; 14.0]	0.810

[1]Used 6 ounces sulfate solution for 1st and 2nd boosts; [2]All transit times measured as (hour: minutes); [3]Statistically significant. CCE: Colon capsule endoscopy.

different between the two regimens, but there was a trend toward higher detection for polyps ⩾ 6 mm with the Rex *et al*[1] bowel preparation regimen (Table 8).

DISCUSSION

A new CCE preparation regimen using OSS + diatrizoate as a boost agent did not improve colon cleansing as compared to low dose OSS alone. The OSS + diatrizoate regimen resulted in more rapid transit of the capsule with a trend toward faster transit through the entire GI tract, superior CCE completion, and more frequent colonic transit less than 40 min. There was also a trend toward higher polyp detection with OSS + diatrizoate. More subjects receiving OSS + diatrizoate experienced adverse events which were primarily related to the bowel regimen and GI in nature, but did not appear to be related to diatrizoate solution. Patient related factors, not identified in this study, may have accounted for this difference in adverse events between the Study and Control arms.

The combination of a hyperosmotic colon purgative (NaP) and hyperosmotic diatrizoate solution for use as a boost agent has previously been shown to be effective[15]. The study reported herein evaluated the use of OSS as an alternative hyperosmotic colon purgative. As a boost agent, OSS + diatrizoate performed similarly to the combination of NaP and diatrizoate solution with respect to colon cleansing adequacy, CCE completion, overall GI transit time, and polyp detection. While these results support OSS as a boost agent in place of NaP, use of a historical comparator is a limitation.

A large prospective CCE study by Rex *et al*[1] found an unexpectedly high number of capsule studies (approximately 10%) could not be evaluated due to a combination of inadequate preparation and rapid (< 40 min) colonic transit. A similar frequency (approximately 9%) of CCE studies with both inadequate cleansing and colon transit < 40 min was observed with the

Study regimen. It is unknown whether this trend would have continued had enrollment not been terminated early. Our Control regimen differed from Rex *et al*[1] only in the OSS dose for the 1st boost [Control = 89 mL (3 oz.), Rex *et al*[1] = 177 mL [6 oz.)]. As compared to Rex *et al*[1] boosts comprised of low dose OSS alone did slow transit but at a cost of inferior CCE completion. These data suggest that while CCE transit is too often fast with the Rex *et al*[1]. regimen, transit may be too slow when the 1st and 2nd boost utilize low dose OSS alone[1]. Again, these conclusions are limited by the use of a historical comparator.

Like colonoscopy, CCE's effectiveness depends on a complete colon exam and adequate preparation. Achieving these endpoints with colon capsule is a complicated endeavor in that the preparation regimen must both cleanse the colon and propel the capsule. The lumen must be fluid filled and clear of debris, and the capsule needs to traverse the entire colon within the limits of its battery life - but not too quickly such that findings could be missed. And, just like colonoscopy, complete passage through the colon and adequate preparation are basic requirements and no guarantee that lesions will not be missed. Using colonoscopy as the gold standard, a meta-analysis showed that the second-generation colon capsule utilized in our study had high sensitivity and specificity for polyps ⩾ 6 mm[18]. The acceptable standards for CCE completion and adequate bowel preparation remain to be determined, but it is reasonable to believe that higher thresholds will translate into better outcomes.

Our study has some additional limitations. While enrollment was terminated early using pre-specified criteria, outcomes may have varied if full enrollment was achieved. While the difference between the Study and Control regimens for polyp detection seen after the first interim analysis at 50 subjects persisted after enrollment closed, this was not the case for preparation adequacy or adverse event incidence. Colon cleansing was graded per segment and overall (primary endpoint), but a preparation receiving an overall grade of adequate could have one or more individual segments graded as graded as fair or poor. Looking to colonoscopy and the Boston Bowel Preparation Scale for guidance, a total colon score of > 5 is associated with both superior polyp detection and high adherence to guidelines for screening and surveillance[19]. Yet, individual colon segment scores are important, and segments scored < 2 are at greater risk for missing polyps[20]. For colon capsule, whether an overall cleansing grade of adequate is sufficient for quality measures such as polyp detection and compliance with interval performance of this procedure remains to be determined. Finally, in the study we have presented, polyp detection by CCE was not confirmed with colonoscopy. However, a meta-analysis demonstrating high sensitivity and specificity for polyps ⩾ 6 mm and ⩾ 10 mm with the colon capsule utilized for this study provides reassurance regarding its accuracy for polyp detection[18].

In summary, a CCE preparation regimen combining two hyperosmotic agents, OSS and diatrizoate solution, as a boost agent was not superior to low dose OSS alone for achieving adequate overall colon cleansing. The OSS + diatrizoate regimen did achieve a high rate of colon capsule completion, was associated with trend toward higher polyp detection, and performed similarly to a historic comparator boost regimen comprised NaP + diatrizoate. The combination of inadequate preparation and rapid colonic transit seen in some subjects receiving OSS + diatrizoate, which may lower the accuracy of CCE, is a potential limitation of this regimen and requires additional investigation. Future research efforts should continue to focus on improving cleansing efficacy and patient tolerance while maintaining high rates of colon capsule completion. While permitting sufficient capsule dwell time in the colon is important to minimize the risk for missing lesions, the effect of rapid colonic transit may be mitigated with adequate cleansing.

ARTICLE HIGHLIGHTS

Research background

The technical performance of colon capsule endoscopy (CCE) has made great strides, but this technology remains highly dependent on the purgative procedure. The ideal capsule preparation would (1) adequately cleanse the colon; (2) propel the capsule through the GI tract within its battery life; (3) enable sufficient dwell time within the colon for accurate visualization; (4) be tolerable and safe; (5) be easily generalizable for the vast majority of patients. Each of these areas require improvement, and this study's significance is that it moves this field forward by evaluating a new boost agent.

Research motivation

Tailoring the preparation procedure based on patient characteristics and real-time capsule feedback ("personalized" medicine) will likely improve CCE performance, tolerability, safety, and subsequently acceptance. Technological advancements that reliably visualize mucosa obscured by debris, and assist in the detection of polyps, would be immensely helpful as well.

Research objectives

The main objective was to evaluate the efficacy of new boost agent consisting of low dose sulfate solution combined with diatrizoate solution ("Study" regimen), and comparing this to low dose sulfate solution alone ("Control" regimen). We found that colon cleansing was similar between the two regimens, but CCE completion and the proportion of subjects in which the capsule passed through the colon in less than 40 min were significantly greater with the Study regimen. Also observed was numerically greater, not statistically superior, polyp detection with the Study regimen. This suggests that it is reasonable to incorporate the boost regimen of low dose sulfate solution and diatrizoate solution into the preparation procedure for CCE.

Research methods

This was a multicenter, prospective, randomized, controlled study comparing two preparation regimens for CCE at six United States sites, 2 of which were academic centers. CCE studies were read centrally by experienced readers who were blinded to subject randomization, and a validated cleansing scale for CCE was utilized.

Research results

The study regimen did not result in superior colon cleansing, but did result in a superior rate of CCE completion and higher proportion of studies with colonic transit less than 40 min. Increased polyp detection, though not significant, was observed in the Study arm suggesting that polyp detection was not compromised in this group. However, this study was not powered for polyp detection. We also observed a greater incidence of adverse events, primarily GI, in the Study group. This observation in the Study group did not appear to be related to the boost agent. Progress needs to be made in further improving the efficacy of colon cleansing, with the goal for CCE being more closely aligned with that established for traditional colonoscopy. Further, simplifying the regimen, shortening overall GI transit time, and lessening the incidence of adverse events related to the preparation regimen are all worthy of further study.

Research conclusions

Diatrizoate solution augments the performance of sulfate solution to create an effective and very low dose hyperosmotic boost agent for CCE. The combination of low dose sulfate solution and diatrizoate solution was superior to low dose sulfate solution alone with respect to CCE completion and the frequency that the capsule traversed the colon in less than 40 minutes. These findings, along with a trend toward higher polyp detection with the combination Study regimen, support the use of low dose sulfate solution combined with diatrizoate solution as a boost agent in place of low dose sulfate solution alone.

Research perspectives

While our findings represent an improvement in the preparation regimen for CCE, there is still much progress to be made in this arena. In particular, the rate of preparation adequacy needs to increase. Personalized medicine may play a role in optimizing the regimen for CCE. Using patient characteristics and real time capsule data, individual adjustments might include varying the volume of PEG-ELS, utilizing additional agents, adjusting the frequency and timing of medication administration, and more.

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SYSTEMATIC REVIEWS

Fusobacterium's link to colorectal neoplasia sequenced: A systematic review and future insights

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Abstract

AIM

To critically evaluate previous scientific evidence on Fusobacterium's role in colorectal neoplasia development.

METHODS

Two independent investigators systematically reviewed all original scientific articles published between January, 2000, and July, 2017, using PubMed, EMBASE, and MEDLINE. A total of 355 articles were screened at the abstract level. Of these, only original scientific human, animal, and in vitro studies investigating *Fusobacterium* and its relationship with colorectal cancer (CRC) were included in the analysis. Abstracts, review articles, studies investigating other colonic diseases, and studies written in other languages than English were excluded from our analysis. Ninety articles were included after removing duplicates, resolving disagreements between the two reviewers, and applying the above criteria.

RESULTS

Studies have consistently identified positive associations between *Fusobacterium*, especially *Fusobacterium nucleatum* (*F. nucleatum*), and CRC. Stronger associations were seen in CRCs proximal to the splenic flexure and CpG island methylator phenotype (CIMP)-high CRCs. There was evidence of temporality and a biological gradient, with increased *F. nucleatum* DNA detection and quantity along the traditional adenoma-carcinoma sequence and in CIMP-high CRC precursors. Diet may have a differential impact on colonic *F. nucleatum* enrichment; evidence suggests that high fiber diet may reduce the risk of a subset of CRCs that are *F. nucleatum* DNA-positive. Data also suggest shorter CRC and disease-specific survival with increased amount of *F. nucleatum* DNA in CRC tissue. The pathophysiology of enrichment of *F. nucleatum* and other *Fusobacterium* species in colonic tissue is unclear; however, the virulence factors and changes to the local colonic environment with disruption of the protective mucus layer may contribute. The presence of a host lectin (Gal-GalNAc) in the colonic epithelium may also mediate *F. nucleatum* attachment to CRC and precursors through interaction with an *F. nucleatum* protein, fibroblast activation protein 2 (FAP2). The clinical significance of detection or enrichment of *Fusobacterium* in colorectal neoplasia is ambiguous, but data suggest a procarcinogenic effect of *F. nucleatum*, likely due to activation of oncogenic and inflammatory pathways and modulation of the tumor immune environment. This is hypothesized to be mediated by certain *F. nucleatum* strains carrying invasive properties and virulence factors such as FadA and FAP.

CONCLUSION

Evidence suggests a potential active role of *Fusobacterium*, specifically *F. nucleatum*, in CRC. Future prospective and experimental human studies would fill an important gap in this literature.

Key words: Colon microbiota; *Fusobacterium*; Systematic; review; *Fusobacterium nucleatum*; Colorectal cancer; Colorectal polyps; Carcinogenesis

Core tip: This is, to our knowledge, the first review to systematically examine the heterogeneous literature linking *Fusobacterium* to colorectal neoplasia. Accumulating evidence suggests that *Fusobacterium*, specifically *Fusobacterium nucleatum* (*F. nucleatum*), is more frequently detected in colorectal neoplasia, especially the pathway involving microsatellite instability. Multiple observational and animal experimental studies also suggest a procarcinogenic effect of *F. nucleatum*, likely due to activation of oncogenic and inflammatory pathways and modulation of the tumor immune environment. Virulence factors of *F. nucleatum* may contribute to its procarcinogenic effect. This information may be used to create novel strategies targeting colorectal cancer detection and chemoprevention.

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INTRODUCTION

Colorectal cancer (CRC) is the most common gastrointestinal cancer, as well as the third leading cancer by incidence and mortality, in the United States[1]. The majority of CRC cases are sporadic, where a complex interaction among genetic and environmental factors impacts the carcinogenesis process. The underlying molecular changes follow at least two distinctive pathways of genomic dysfunction: the chromosomal instability (CIN) and CpG island methylator phenotype (CIMP)-high pathways. CIN is observed in 70%-85% of sporadic CRCs and describes aneuploidy due to gains or losses of whole or large portions of chromosomes[2,3]. CIN is likely due to defects in chromosome segregation pathways and is likely initiated by the adenomatous polyposis coli (APC) mutation with subsequent β-catenin/Wnt-signaling pathway activation[4]. APC mutation is followed by a cascade of molecular changes in a multistep fashion, as the flat mucosa evolves into a progressively larger adenoma that ultimately turns into cancer (adenoma-carcinoma sequence). CIMP-high CRCs include microsatellite instability (MSI)-high CRCs, with serrated polyps representing the main precursors[3]. These account for 15% of all CRCs and are characterized by inactivation of mismatch repair enzymes and other tumor suppressor genes via mutations or hypermethylation[5-8].

The human large intestine is a complex bacterial ecosystem that plays a significant role in health and disease. Increasing evidence suggests that a healthy symbiotic relationship between the host and microflora may be disrupted, leading to chronic metabolic and inflammatory changes promoting colorectal carcinogenesis[9,10]. Although the technology to define the microbiome continues to evolve, the prevalence of some bacteria appears to be elevated in CRC. These include *Fusobacteria, Alistipes*, *Porphyromonadaceae*, *Coriobacteriaceae*, *Staphylococcaceae*, *Akkermansia,* and *Methanobacteriales*. Conversely, other bacteria exhibit reduced prevalence in CRC, including *Bifidobacterium*, *Lactobacillus*, *Ruminococcus*, *Faecalibacterium* spp., *Roseburia*, and *Treponema*[11]. Although more research is warranted to establish firm causative links between CRC and flora diversity, patterns, specific microbial populations, and microbial functions, we are particularly intrigued by current data regarding *Fusobacterium*, a genus of the strictly anaerobic *Fusobacteria* phylum. Oral *Fusobacterium* consists mainly of the species *Fusobacterium nucleatum* (*F. nucleatum*), an

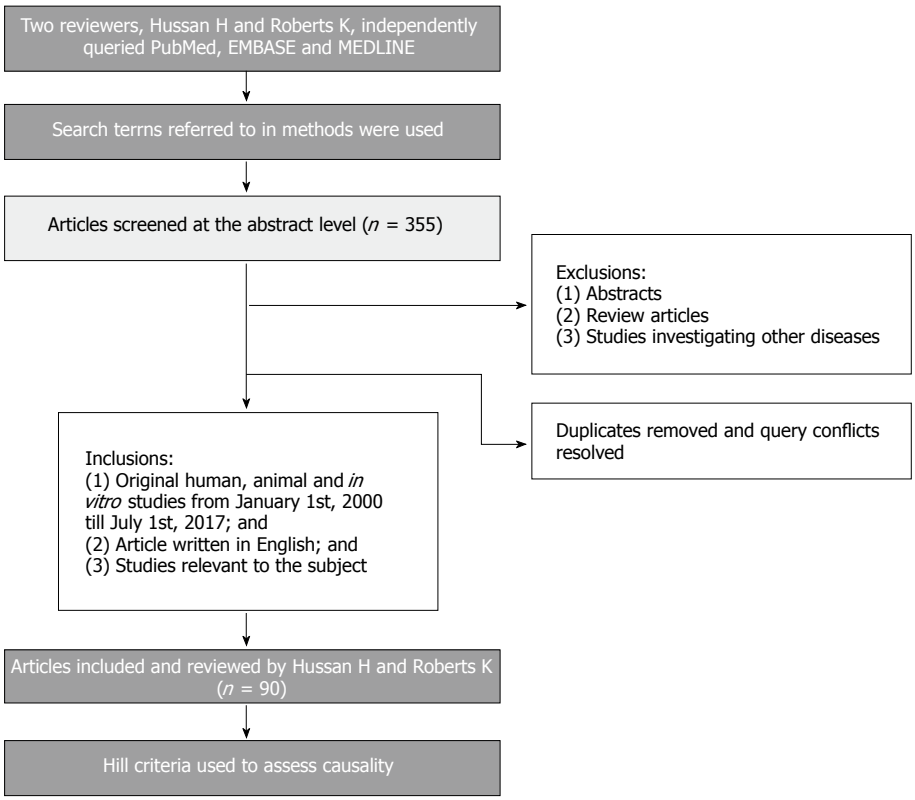

Figure 1 Diagram illustrating systematic review flow and methods.

adherent[12], invasive[13], and proinflammatory[14,15] bacterium that is linked to periodontal disease[16]. *F. nucleatum* is also the first anaerobic species to colonize the mouths of infants, indicating a potential prolonged exposure to *F. nucleatum* in adults who harbor it[17-19]. *F. nucleatum* is classified into subspecies *animalis*, *fusiforme*, *nucleatum*, *polymorphum*, and *vincentii*[20]. *F. varium* is another *Fusobacterium* species and has been associated with ulcerative colitis[21, 22]. Other *Fusobacterium* species, such as *F. naviforme*, are mainly oral commensals and are associated with periodontal health[19,23]. The presence of *Fusobacterium* in the colon, specifically *F. nucleatum*, is increasingly linked to CRC through a variety of recent studies, albeit with significant heterogeneity in study methods and findings. Thus, a critical evaluation of the scientific literature regarding the link between *Fusobacterium/F. nucleatum* and CRC may contribute to the development of more comprehensive and novel studies to better define this relationship.

MATERIALS AND METHODS

Two independent reviewers (HH and KR) systematically queried PubMed, Embase, and Medline using the following search terms: ("*Fusobacterium* " {All fields} OR "*Fusobacteria*" {All fields}) AND ("colon" {All fields}), "rectum" {All fields}, "colorectal" {All fields}, "colorectal cancer" {All fields}, "polyps" {All fields}, "adenomas" {All fields}), "serrated" {All fields}, "SSA" {All fields}, "SSP" {All fields}, "CIMP" {All fields}, "MSI" {All fields}, OR "microsatellite" {All fields}). On the basis of this search, 355 articles were screened at the abstract level. The following inclusion criteria were used: (1) Original human, animal, and in vitro studies investigating *Fusobacterium* and colorectal neoplasia that were published between January 1, 2000, and July 1st, 2017; (2) articles written in English; and (3) studies relevant to colorectal neoplasia. We excluded: (1) abstracts; (2) review articles; and (3) studies investigating other colonic diseases, such as ulcerative colitis. Ninety original articles were included after removing duplicates, resolving disagreements between the two reviewers, and applying the above criteria. The resulting 90 articles were then independently reviewed at the manuscript level by HH and KR. We used the Hill criteria to assess causality in the current evidence linking *F. nucleatum* and CRC[24]. A brief illustration of our methods is shown in Figure 1.

RESULTS

Associations with CRC

Associations between *Fusobacterium* and CRC: Consistent case-control studies using various samples including both stool and fresh and formalin-fixed paraffin-embedded (FFPE) colonic tissue demonstrated increased detection, quantity, and/or relative percentage of *Fusobacterium* rDNA copies in CRC tissue compared with matched adjacent noncancerous tissue and compared with healthy controls without colorectal neoplasia, as

summarized in Table 1[25-40]. The histopathology of these findings is ambiguous, but some data suggest that *Fusobacteria* have been observed within the colonic bacterial biofilms, in the colonic mucus layer, within colonic crypts, and invading the colonic epithelium[33,41-43]. *F. nucleatum* was the detected species of *Fusobacterium* in CRC tissue in 13 out of the 15 studies that presented species-level analysis[41,44-55]. In two out of three studies that presented subspecies-level analysis, *F. nucleatum* subspecies *animalis* was the most frequent subspecies of *F. nucleatum* in CRC tissue[40,54,56]. Other *Fusobacterium* species, such as *F. periodonticum, F. varium, F. ulcerans F. necrophorum,* and *F. gonidiaformans*, were also identified in CRC tissue in the five remaining studies[51,54,56-58]. *F. nucleatum*, *F. periodonticum, F. varium*, and *F. ulcerans* species can actively invade host cells, independently of mucosal compromise or presence of coinfection with other bacteria[59,60]. Conversely, *F. necrophorum* and *F. gonidiaformans* are termed passive invaders, and their presence in CRC could be due to the disruption of the mucus layer seen with CRC or to coinfection with other invasive bacteria. In the largest study comparing genes of *Fusobacterium species*, active invaders such as *F. nucleatum* were found to harbor larger genomes encode adhesions, and contain twice as many genes encoding membrane-related proteins compared with other *Fusobacteria*l species termed passive invaders[59]. Thus, the presence of multiple *Fusobacteria*l species could be due to their virulence and/or to early changes in the colonic environment that facilitate their presence in CRC tissue. Further studies are warranted to answer this question.

No major associations were found between *F. nucleatum* and characteristics of CRC patients such as age, gender, ethnicity, body mass index (BMI), smoking, or alcohol consumption, except in one South African study, where researchers found an association between *Fusobacterium* and both African race and age less than 60[29,31,42,49,50,54,61,62]. In all studies, the prevalence of *F. nucleatum* rDNA in CRC tissue varied between 8.6% and 87.1%. This wide variability could be explained by heterogeneity in study design, sampling, analysis methodology, population, geographic location, or diet; these variations are summarized in Table 1[38,46-48,52,63,64]. For instance, higher *F. nucleatum* detection is seen when using CRC tissue samples and whole-genome shotgun metagenomics sequencing methods, as compared with fecal samples or bacterial 16s sequencing[10,26,50]. Furthermore, caution should be taken when interpreting studies of FFPE samples, because FFPE samples provide a less accurate assessment of the microbiome when compared with fresh-frozen samples[65]. Finally, patients typically undergo bowel preparation when tissue samples are collected, which may affect *Fusobacterium* detection or abundance in tissue samples[66]. We conclude that consistent associations are seen between *Fusobacterium*, mainly *F. nucleatum*, and CRCs, with variable prevalence of *F. nucleatum* in CRC subjects, which is likely due to heterogeneous methodologies. It would be of value for future studies to utilize unprepared fresh-frozen colonic samples (for instance, unprepped flexible sigmoidoscopy with biopsies) when possible, combined with whole-genome shotgun metagenomic sequencing, in order to potentially yield more accurate detection and quantification of *Fusobacterium* and *F. nucleatum*.

Relation between *Fusobacterium* and dietary characteristics of CRC patients: Low-fiber, high-fat Western diet administration over 2 wk to 20 Native Africans was associated with altered colonic microbiome and increased number of *F. nucleatum* rDNA copies in colonic tissue, in association with increases in early colonic biomarkers of CRC[63]. It is interesting to note that colonic biopsies quantity of *F. nucleatum* rDNA copies did not decrease in 20 African Americans switched from a Western diet to a high-fiber, low-fat diet for 2 wk. This could be due to the small sample size, or it could take longer than 2 wk for dietary changes to reverse *F. nucleatum* rDNA abundance in colonic tissue[63]. Recently, vegetable consumption was also inversely associated with relative concentration of *Fusobacterium* rDNA in stool of patients with advanced adenomas[54]. However, the same study did not find associations between relative concentration of *Fusobacterium* rDNA in stools of 46 CRC patients and dietary habits such as consumption of red meat, processed meat, any meat, vegetables, or whole grains. This finding may be due to the cross-sectional nature of the study or to the researchers' superficial assessment of dietary habits and use of fecal samples as opposed to colonic tissue in their study[54]. Mehta *et al*[64] prospectively investigated long-term dietary patterns in a cohort of 137217 patients using validated food frequency questionnaires. There were 1019 incidences of CRCs, which were classified in into *F. nucleatum*-positive or *F. nucleatum*-negative CRCs based on presence or absence of *F. nucleatum* rDNA in CRC tissue respectively. They identified that, when compared with a Western diet, a diet rich in whole grains and dietary fiber (prudent diet) was associated with a lower risk of *F. nucleatum*-positive CRCs, with a hazard ratio (HR) of 0.43 (95%CI: 0.25-0.72; $P = 0.003$). No associations between prudent diet and *F. nucleatum*-negative CRC risk was identified, indicating a differential impact of prudent diet on CRC risk that are *F. nucleatum*-positive specifically[64]. These inverse associations between prudent diet and *F. nucleatum*-positive CRCs were more pronounced when comparing high fiber intake (> 26 g/d for men and > 19 g/d for women) with the lowest fiber intake quartile (< 18 g/d for men and < 13 g/d for women; $P = 0.04$). Cereal-derived fiber had the strongest inverse association with *F. nucleatum*-positive CRCs (HR = 0.58; 95%CI: 0.34-0.99; $P = 0.03$)[64]. Fruit consumption was also shown to reduce the risk of both *F. nucleatum*-positive and *F. nucleatum*-negative CRCs, with no specific relation to *F. nucleatum* status of the CRC[64]. The researchers observed no impact of prudent diet subgroups (vegetables, legumes, or whole grains), on *F. nucleatum*-positive CRC risk, as also previously demonstrated[29,54,64].

Table 1 Studies examining association between *Fusobacteria* and colorectal neoplasia

Authors	Year	Cohort information	Specimen type	Detection method	Relation to CRC location	CRC features	*Fusobacteria* sequencing depth and associations with colorectal neoplasia
Ahn *et al*[25]	2013	United States cohort: 47 CRCs and 94 healthy controls. Matched for age, sex, BMI and hospital	Stool from cases and controls	16S rRNA sequencing	-	-	Genus level: *Fusobacterium* rDNA was significantly more detected in of CRCs (31.9%) *vs* of controls (16%)
Vogtmann *et al*[26]	2016	United States cohort: 52 CRCs and 52 healthy controls, recruited between 1985-1987, matched by sex and BMI. Controls did not have a colonoscopy evaluation to rule out large polyps French validation cohort: 53 CRCs and 83 controls (61 healthy colons and 27 small adenomas) recruited from 2004-2006	Stool from cases and controls	Whole-genome shotgun sequencing Compared to16S rRNA sequencing from Ahn *et al*[25] study	-	-	Genus level: Whole genome analysis: *Fusobacterium* rDNA was significantly more detected in CRCs (76.9%) *vs* controls (48.1%). 16S rRNA sequencing: *Fusobacterium* rDNA was significantly more detected in CRCs (31.9%) *vs* controls (16%) Genus level: *Fusobacterium* rDNA was significantly more detected in CRCs (10.08%) *vs* controls (0.01%)
Gao *et al*[27]	2015	Chinese cohort: 31 CRCs and 30 healthy controls	Fresh-frozen tissue from cancer, adjacent non-cancerous tissue and normal mucosa samples at the time of surgery/ colonoscopy and after colonoscopy bowel preparation	16S rRNA sequencing	Genus level: *Fusobacterium* rDNA was more detected in distal CRC compared to proximal CRC	-	Genus level: Higher relative percentage of *Fusobacterium* rDNA copies (relative to other bacterial rDNA) in CRCs (9.58%) *vs* adjacent non-cancerous tissues (0.57%)
Park *et al*[28]	2016	Korean cohort: 8 TAs, 10 SSA/Ps and 8 CRCs	Fresh-frozen tissue after colonoscopy bowel preparation	16S rRNA sequencing	All CRCs were distal	-	Phylum level: *Fusobacterium* rDNA was detected in 37.5% of TAs, 50% of SSA/Ps and 100% of CRCs. Higher relative percentage of *Fusobacterium* rDNA copies in CRC tissue (33.8%) *vs* TA (4.3%) and SSA (1.9%). No difference in relative concentration of *Fusobacterium* rDNA copies between TAs and SSA/Ps
Feng *et al*[29]	2015	Austrian cohort: 46 CRCs, 44 advanced adenomas and 57 healthy controls. Ages between 45-86 yr, both genders and White race	Stool from cases and controls	Metagenomics Whole-genome shotgun sequencing	-	-	Genus level: Higher relative percentage of *Fusobacterium* rDNA copies in CRCs *vs* Carcinoma *in situ*. Higher relative percentage of *Fusobacterium* rDNA copies in CRCs *vs* advanced adenomas *vs* controls Genus level: No association between relative percentage of *Fusobacterium* rDNA copies and anthropometric measures or diet (meat, fiber, vegetables and fruit intake)

Burns *et al*[30]	2015	United States cohort: 44 CRCs	Fresh-frozen tissue from cancer and adjacent non-cancerous tissue at the time of surgery and after bowel preparation	16S rRNA sequencing	-	-	Species level: No specific species identification. Higher relative concentration of *Fusobacterium* rDNA copies in CRCs *vs* adjacent non-cancerous tissues. Correlated enrichment with Providencia Species level: No specific species identification. Tumor microbiome was enriched with genes encoding virulence and toxin proteins that were associated with and dependent on the presence of *Fusobacterium* and *Providencia*
Viljoen *et al*[31]	2015	South African cohort: 55 CRCs. 70.4% mixed ancestry, 14.8% White, 11.1% African, equal gender, 7 MSI-high (4 CRCs with Lynch syndrome), 3 MSI-low. 41.5% received chemo-radiation prior to sample collection. 18 FFPE CRCs that are MSI high (2 CRCs with Lynch syndrome)	Fresh-frozen tissue from cancer and adjacent non-cancerous tissue at the time of surgery and after bowel preparation. FFPE samples after bowel preparation	16S rRNA sequencing Metagenomics	No association between number of *Fusobacterium* rDNA copies in CRC tissue and colon *vs* rectum location	Association between higher number of *Fusobacterium* rDNA copies in CRC tissue and MSI-H status	Species level: No specific species identification. *Fusobacterium* rDNA was detected in 82% of CRCs and 81% adjacent non-cancerous tissues with concurrent presence in 80% of paired CRC and adjacent tissue. Higher number of *Fusobacterium* rDNA copies in CRCs *vs* adjacent non-cancerous tissues.Species level: No specific species identification. Higher number of *Fusobacterium* rDNA copies in CRC tissue was associated with African race and age < 60. Species level: No specific species identification. Higher number of *Fusobacterium* rDNA copies in CRC tissue correlated with pks-positve *E. coli* in normal tissue; but no correlation with Enteropathogenic *Escherichia coli*, *Streptococcus gallolyticus*, *Enterococcus faecalis*, Enterotoxigenic *Bacteroides* fragili or afaC-positive *E. coli*
Zhou *et al*[32]	2016	Chinese cohort: 97 CRCs and 48 healthy controls. Age and sex matched	Fresh-frozen tissue from cancer, adjacent non-cancerous tissue and normal mucosa samples at the time of surgery/ colonoscopy and after bowel preparation	16S rRNA sequencing	No association between relative percentage of *Fusobacterium* rDNA copies in CRC tissue and colon *vs* rectum location	No association between relative percentage of *Fusobacterium* rDNA copies in CRC tissue and CEA, P53, EGFR, Ki67, CA199 or CRP	Species level: No specific species identification. Higher number of Fusobacterium rDNA copies correlated positively with presence of chronic inflammation in CRC tissue Species level: No specific species identification. *Fusobacterium* rDNA was detected in 72.16% in CRCs *vs* 67.01% of adjacent non-cancerous tissues, both higher than controls. Species level: Higher number of *Fusobacterium* rDNA copies correlated positively with that of Enterotoxigenic *Bacteroides fragilis*, *E. faecalis* in CRC tissue when compared to adjacent non-cancerous tissue

Dejea *et al*[33]	2014	United States cohort: 30 CRCs, 6 adenomas and 22 healthy controls Malaysian cohort: 21 CRCs and 1 adenoma.	Fresh-frozen, formalin fixed tissue from tumors (adenomas and cancers), adjacent normal tissue and normal mucosa samples at the time of surgery/ colonoscopy after bowel preparation	16S rRNA sequencing	Similar relative percentage of *Fusobacterium* rDNA copies (≥ 25%) in CRC tissue proximal to hepatic flexure vs more distal CRC	-	Phylum level: *Fusobacterium* rDNA was detected in 32% of CRC, none of the matched normal tissue or healthy controls
Marchesi *et al*[34]	2011	Netherlands cohort: 6 CRCs	Fresh-frozen tissue from cancer and adjacent non-cancerous tissue at the time of surgery and after bowel preparation	16S rRNA sequencing	-	-	Genus level: *Fusobacterium* rDNA was more detected with also higher relative percentage of *Fusobacterium* rDNA copies in CRC compared to adjacent non-cancerous tissue
Thomas *et al*[35]	2016	Brazilian cohort: 18 rectal cancers (no prior neoadjuvant therapy) and 18 healthy controls	Fresh-frozen tissue from cancer and normal mucosa samples at the time of surgery/ colonoscopy and after bowel preparation	16S rRNA sequencing	Rectal cancers only	-	Species level: No specific species identification. Higher number of *Fusobacterium* rDNA copies in rectal cancers compared to normal controls
Wang *et al*[36]	2012	Chinese cohort: 46 CRCs and 56 healthy controls	Stool, prior to bowel preparation	16S rRNA sequencing	-	-	Genus level: Higher relative percentage of *Fusobacterium* rDNA copies in CRC tissue compared to controls
Gao *et al*[37]	2017	Chinese cohort: 65 CRC patients	Fresh-frozen tissue from cancer and matched adjacent non-cancerous tissue at the time of surgery after bowel preparation	16S rRNA sequencing	Genus level: *Fusobacterium* rDNA was more detected in distal CRC compared to proximal CRC	No association between relative percentage of *F. nucleatum* rDNA copies in CRC tissue and presence of K-ras mutation	Phylum level: *Fusobacterium* rDNA was significantly more detected in CRC (8.5%) compared to matched normal tissue (4.13%)
Allali *et al*[38]	2015	United States and Spanish cohorts: 90 CRC patients	Fresh-frozen tissue from cancer and adjacent non-cancerous tissue at the time of surgery after bowel preparation	16S rRNA	-	United States cohort: Higher relative percentage of *Fusobacterium* rDNA copies in both CRC and matched normal tissue in the right colon and splenic flexure *vs* left colon and sigmoid colon Spanish cohort: Higher relative percentage of *Fusobacterium* rDNA copies in CRC tissue in the left colon	Phylum level: Higher relative percentage of *Fusobacterium* rDNA copies in CRC *vs* matched normal tissue in Spanish cohort but not United States cohort. Higher relative percentage of *Fusobacterium* rDNA copies in matched adjacent non-cancerous tissue of the United States cohort compared to matched adjacent non-cancerous tissue of the Spanish cohort
Zackular *et al*[39]	2014	United States and Canadian cohort: 30 CRC, 30 TA and 30 healthy controls	Frozen fecal samples prior to colonoscopy and bowel preparation.	16S rRNA	No relation between relative percentage of *Fusobacterium* rDNA copies in CRC tissue to CRC location	-	Genus level: Higher relative percentage of *Fusobacterium* rDNA copies in CRC compared to both adenoma and healthy controls

Zeller *et al*[40]	2014	French cohort (population F): 53 CRC, 42 TAs, and 61 healthy control patients. German cohort (Population G): 38 CRC patients. German, Danish and Spanish cohorts (Population H): 297 IBD and healthy controls. German cohort (48 CRC patients at the time of CRC surgery)	Populations F and G: Stool prior to colonoscopy bowel preparation Population H: Stool German cohort: CRC and matched normal tissue	16S rRNA	-	-	Species level: Higher relative percentage of *Fusobacterium* rDNA copies in CRC compared to controls Subspecies level: *F. nucleatum* ssp. vincentii and *F. nucleatum* ssp. animalis are predominant in CRC tissue
Castellarin *et al*[41]	2012	Canadian cohort: 99 CRCs	Frozen tissue from cancer and adjacent non-cancerous tissue at the time of surgery after bowel preparation	Metagenomics *F. nucleatum* quantitative PCR	No association with proximal *vs* distal CRC location	Association between higher relative percentage of *F. nucleatum* rDNA copies in CRC and tumor involvement of more than 50% of the colon circumference	Subspecies level: Higher relative percentage of *F. nucleatum* subsp. *Nucleatum* rDNA copies in CRCs compared to matched normal tissues Species level: Confirmed *in vitro* invasion of *F. nucleatum* into human epithelial colonic cells Species level: No association between *F. nucleatum* and history of treatment or patient age
Chen *et al*[42]	2017	Chinese cohort: 14 CRCs 98 FFPE CRC	Frozen tissue at the time of surgery after bowel preparation FFPE CRC tissue after bowel preparation	16S rRNA FISH F. nucleatum-targeted probe	Higher frequency of *F. nucleatum* rDNA detection in proximal CRC than distal location	-	16s rRNA: Phylum level: *Fusobacterium* was a dominant phylum in CRC. FISH analysis. Species level: *F. nucleatum* rDNA was detected in 62.2% of FFPE CRC tissues No. difference in patients gender or age between CRCs that are *F. nucleatum* positive (detected) or absent
McCoy *et al*[43]	2013	United States Cohort: 10 CRCs, 48 adenomas and 67 healthy controls	Fresh-frozen normal rectal biopsies from adenoma and controls after bowel preparation Frozen tissue from CRC and adjacent non-cancerous tissue at the time of surgery after bowel preparation	*F. nucleatum* quantitative PCR 16S rRNA	-	Association between high number *F. nucleatum* rDNA copies in tissue and IL-6, IL-10, IL-17 and TNF-α in adenoma cases but no similar associations were seen in controls	Species level: Higher number of *F. nucleatum* rDNA copies seen in adenoma cases *vs* controls Species level: No association between *F. nucleatum* rDNA copy numbers and adenomas size/number Phylum level: Increased number of *Fusobacterium* rDNA copies in CRCs compared to matched normal tissues
Fukugaiti *et al*[44]	2015	Brazilian cohort: 7 CRCs and 10 healthy controls	Stool, prior to bowel preparation	16S rRNA sequencing	-	-	Species level: Both *F. nucleatum* and *Clostridium difficile* had higher number of rDNA copies in stool of CRC patients when compared to controls
Warren *et al*[45]	2013	Canadian cohort: 65 CRCs	Frozen tissue from cancer and adjacent non-cancerous tissue at the time of surgery after bowel preparation	Metatran-scriptomics	-	-	Species level: Higher relative percentage of *F. nucleatum* rDNA copies in CRC compared to matched normal tissue Genus level: Co-occurrence of *F. nucleatum* with *Campylobacter* (in vitro co-aggregation with *C. showae*) and *Leptotrichia* in CRC tissue Genus level: Higher relative percentage of *Fusobacterium* rDNA copies in tumor tissue was correlated with host immune response genes and oncogenes

Ito *et al*[46]	2015	Japanese cohort: 138 Microvesicular HPs, 129 SSAs, 102 TSAs, 131 adenomas and 544 CRCs with matched adjacent non-cancerous tissue as well as 20 healthy controls	FFPE CRC tissue after bowel preparation	*F. nucleatum* quantitative PCR	No relation between *F. nucleatum* detection or higher number of rDNA copies in CRC and CRC location (Rectum to splenic flexure *vs* Transverse colon to cecum) Gradual increase in percentage of SSAs that are *F. nucleatum* positive from sigmoid colon to cecum; no similar finding seen for TA, TSA and HP	High number of *F. nucleatum* rDNA copies in CRC was associated with MLH1 methylation, CMP-high status and MSI-high status. No association between detection or number of *F. nucleatum* rDNA copies in CRC to KRAS mutation, PIK3A mutation or miRNA 31 expression	Species level: *F. nucleatum* rDNA was detected in 56% of CRCs. Higher number of *F. nucleatum* rDNA copies in CRC tissue compared to matched normal tissue. *F. nucleatum* rDNA was not detected in 17/20 of healthy controls; no significant difference in number of *F. nucleatum* rDNA copies between matched normal tissue and healthy controls Species level: *F. nucleatum* rDNA detected in 24% of HPs, 35% of SSAs, 30% of TSAs and 33% of TAs. No difference in frequency of *F. nucleatum* rDNA detection between these groups Species level: *F. nucleatum* rDNA more frequently detected in CRCs compared to polyps after adjustment for confounders Species level: High number of *F. nucleatum* rDNA copies in CRC was positively associated with large tumor size
Nosho *et al*[47]	2016	Japanese cohort: 511 CRCs	FFPE CRC tissue after bowel preparation	*F. nucleatum* quantitative PCR	-	*F. nucleatum* rDNA presence in CRC was associated with MSI high status	Species level: *F. nucleatum* rDNA was present in 8.6% of CRCs
Mima *et al*[48]	2015	United States cohort: 598 CRCs from the Nurses' Health Study and Health Professionals Follow-up Study	FFPE CRC tissue after bowel preparation	*F. nucleatum* quantitative PCR	-	High number *F. nucleatum* rDNA copies in CRC tissue was associated with lower CD3+ T-cells density. No association with CD8+, CD45RO+, or FOXP3+ T-cells density in CRC. No significant association with Crohn's-like histology, or tumor infiltrating lymphocytes	Species level: *F. nucleatum* rDNA was more frequently detected in CRCs (13%) compared to matched normal tissue (3.4%)
Kostic *et al*[49]	2012	Spanish, United States and Vietnamese cohort: 95 CRCs	Frozen tissue from cancer and adjacent non-cancerous tissue at the time of surgery after bowel preparation	Whole genome sequencing 16S rRNA *F. nucleatum* quantitative, PCR	No association with CRC location	No association with CRC purity, inflammation, necrosis, and vascularization	Species level: Higher relative percentage of *Fusobacterium* rDNA copies in CRC compared to matched normal tissue Detected species: *F. nucleatum* (most dominant species), *F. necrophorum*, *F. mortiferum*, and *F. perfoeten*. Species level: Higher relative percentage of *Fusobacterium* rDNA copies in Spanish *vs* US/Vietnamese cohorts Species level: No association with age, gender, ethnicity
Flanagan *et al*[50]	2014	Czech cohort: 49 CRCs German cohort: 45 CRCs. Irish cohort:	Frozen tissue from cancer, adjacent non-cancerous tissue at the time of surgery after bowel preparation. Stool	*F. nucleatum* quantitative PCR	No association between relative percentage of *F. nucleatum* rDNA copies in CRC and colon *vs*	Association between higher relative percentage of *F. nucleatum* rDNA copies	Species level: Higher relative percentage of *F. nucleatum* rDNA copies in CRC and HGD compared to matched normal tissue. Similar relative

		28 CRCs and 52 TAs Stool from 7 CRCs, 24 TAs patients (10 adenoma with HGDs, 12 TVAs and 2 adenomas) and 25 healthy controls			rectum location	in CRC tissue and TP53 mutation in the Irish cohort (Small sample size) Association between higher relative percentage of *F. nucleatum* rDNA copies in CRC tissue with KRAS mutation No association between *F. nucleatum* and BRAF mutation. No association between *F. nucleatum* and CRC grade	percentage of *F. nucleatum* rDNA copies in TA, TVA compared to their respective matched normal tissue Species level: Increased relative percentage of *F. nucleatum* rDNA copies during the adenoma-carcinoma progression in cancerous and matched normal tissue (TA to TVA to HGD to CRC) Species level: Increased relative percentage of *F. nucleatum* rDNA copies in stool of CRC patients compared to adenomas. Stool relative percentage of *F. nucleatum* rDNA copies is similar between adenoma and healthy controls patients. No significant correlation in relative percentage of *F. nucleatum* rDNA copies between disease tissue (CRC and adenomas) and stool samples from same patient
Wu *et al*[51]	2013	Chinese cohort: 19 CRCs and 20 healthy controls. Matched for age, sex and body mass index	Stool	16S rRNA sequencing			Species level: Higher relative percentage of Fusobacterium rDNA copies compared to controls. Several species involved: *F. nucleatu*m, *F. periodonticum*, *F. necrophorum*, *F. ulcerans*, *F. varium*, and *F. gonidiaformans*
Li *et al*[52]	2016	Chinese cohort: 101 CRC patients	Fresh-frozen tissue from cancer and adjacent non-cancerous tissue at the time of surgery after bowel preparation	*F. nucleatum* quantitative PCR	-	-	Species level: Increased relative percentage of *F. nucleatum* rDNA copies in 87.13% of CRCs compared to controls Higher relative percentage of *F. nucleatum* rDNA copies in CRC compared to controls
Mira-Pascual *et al*[53]	2015	Spanish cohort: 7 CRC, 8 TA, 7 healthy controls	Frozen fecal samples prior to colonoscopy and bowel preparation Biopsies from normal rectal mucosa of controls and neoplasm of cases after bowel preparation	*F. nucleatum* quantitative PCR	-	-	Species level: *F. nucleatum* rDNA more frequently present in fecal and tissue samples of tumor group (CRCs and polyps) compared to controls
Amitay *et al*[54]	2017	German cohort of patients aged 50 years old and above: 46 CRC, 113 advanced adenomas (TA > 1 cm in size, TVA, or with HGD), 110 adenomas, and 231 healthy controls	Frozen fecal samples prior to colonoscopy and bowel preparation. Median time between collection and storage was 7 d	*F. nucleatum* quantitative PCR	-	-	Subspecies level: *Fusobacterium* rDNA was more frequently present in CRC (54.3%) than advanced adenoma (23.9%), TA (23.6%) and healthy controls (25.1%) ($P < 0.001$) rDNA sequence of *F. periodonticum* was more detected in CRC compared to controls ($P = 0.003$). No difference in detection of rDNA of *Fusobacterium simiae*, *F. nucleatum* ssp. nucleatum, *F. nucleatum* ssp. animalis, *F. nucleatum* ssp. vincentii and *F. nucleatum* ssp. polymorphum between CRC and controls No significant difference between relative concentration of *F. nucleatum* rDNA copies in advanced adenomas/TAs *vs* Controls

Yu *et al*[55]	2015	Chinese cohort: 42 CRCs, 47 TAs and 52 healthy controls	Stool Left colonic biopsies	*F. nucleatum* quantitative PCR 16S rRNA	-	-	Species level: Relative percentage of *F. nucleatum* rDNA copies per sample gradually increased from healthy control to TAs and to CRC. Results seen in both stool and tissue samples
Ye *et al*[56]	2017	United States cohort: 25 CRC patients	Fresh-frozen tissue from CRC and adjacent non-cancerous tissue at the time of surgery after bowel preparation	*F. nucleatum* quantitative PCR 16S rRNA	-	Increased Chemokine (C-C motif) ligand 20 (CCL20) chemokine expression in all stages of CRC suggesting it is an early event in carcinogenesis. *F. nucleatum* ssp. Animalis induced CCL20 cytokine expression in CRC cell lines and monocytes. Monocytes are activated and migrate in the presence of *F. nucleatum* ssp. Animalis, this effect is inhibited by blocking CCL20 No control bacteria used in this experiment	Genus level: Increased relative percentage of *Fusobacterium* rDNA copies in CRC tissue *vs* normal matched tissue Species level: CRC samples contained *F. periodonticum*, *F. canifelinum*, *F. varium*, *F. simiae*, and *F. nucleatum*. *F. nucleatum* was the most frequently detected among Fusobacterium species Subspecies level: *F. nucleatum* ssp. Animalis most dominant among *F. nucleatum* subspecies in CRC samples
Chen *et al*[57]	2012	Chinese cohort: 46 CRCs and 56 healthy controls. BMI range 20-24, matched by sex	Stool and fecal swabs from cases and controls prior to bowel preparation Fresh-frozen tissue from cancer and adjacent non-cancerous tissue from cases at the time of surgery after bowel preparation	16S rRNA sequencing	-	-	Species level: Increased relative concentration of *F. varium* rDNA copies in fecal swabs of CRCs compared to controls Genus level: Increased relative concentration of Fusobacterium rDNA copies in CRC tissues *vs* stool specimens (4.97% *vs* 0.47%, $P < 0.001$) Genus level: Unifrac PCA analysis found no difference in microbial composition of cancers and adjacent non-cancerous tissues
Kasai *et al*[58]	2016	Japanese cohort: 9 CRCs (3 invasive and 6 carcinoma in adenoma), 50 TAs and 49 healthy controls	Stool prior to colonoscopy bowel preparation	16S rRNA sequencing	-	-	Species level: Increased relative percentage of *F. varium* rDNA copies in carcinoma in adenomas *vs* not detected in controls
Tahara *et al*[61]	2014	United States cohort: 149 CRCs and 89 adjacent tissues	Fresh-frozen CRC and adjacent non-cancerous tissue	Pan *Fusobacterium* and *F. nucleatum* quantitative PCR	-	Association with CIMP-high CRC, wild type TP53, MLH1 methylation, MSI-high and CHD7/8 mutation positivity	Genus and species level: Fusobacterium and *F. nucleatum* rDNA were more frequently detected in CRCs (74.3% and 52.3% respectively) compared to adjacent normal appearing mucosa (52.8% and 30.3% respectively). Higher relative percentage of *Fusobacterium* and *F. nucleatum* rDNA in CRC compared to normal appearing mucosa

Yazici *et al*[62]	2017	United States cohort: CRC (97 African Americans and 56 Whites). Healthy controls (100 African Americans and 76 Whites)	Fresh-frozen CRC, adjacent non-cancerous tissue and normal mucosa samples at the time of surgery/ colonoscopy and after bowel preparation	*F. nucleatum* quantitative PCR 16S rRNA	-	-	Genera level: *Fusobacterium* was most abundant sulfidogenic bacteria identified in the study. No difference in relative concentrations of *Fusobacterium* rDNA copies between normal mucosa of cases and controls. Increased sulfidogenic bacteria in African Americans compared to non-Hispanic whites
Park *et al*[69]	2017	South Korean cohort: 160 MSI-high CRC. Excluded rectal carcinoma post neoadjuvant chemotherapy	FFPE CRC tissue after bowel preparation	*F. nucleatum* quantitative PCR	No association between relative concentrations of *F. nucleatum* rDNA copies in CRC and proximal versus distal/rectal CRC location	Association between high relative concentrations of *F. nucleatum* rDNA in CRC and BRAF mutation, CDKN2A (P16) promoter hypermethylation, tumor-infiltrating pan-macrophage density and CD68+ macrophages in tissue when compared with *F. nucleatum*-low/ negative CRCs No association between high relative concentration of *F. nucleatum* rDNA in CRC tissue and CRC infiltrating CD3+ lymphocyte; PD-L1 expression status; Kras mutation; expression of MLH1, MSH2, MSH6 or PMS; CIMP status; or MLH 1 methylation when compared to CRCs that were *F. nucleatum*-low/ negative	Species level: Of the MSI-high CRCs, 9% had high *F. nucleatum* rDNA relative concentrations, 58% had low *F. nucleatum* rDNA relative concentrations, and 33% of CRCs had no detected *F. nucleatum* in the tissue
Wei *et al*[70]	2016	Chinese cohort: 180 CRCs, all stages, median follow up 47 months	Fresh-frozen tissue from cancer and adjacent non-cancerous tissue at the time of surgery after bowel preparation	*F. nucleatum* quantitative PCR	No association between relative concentration of *F. nucleatum* rDNA copies in CRC and CRC location in colon *vs* rectum	High relative concentration of *F. nucleatum* rDNA copies in CRC was associated with high TNF-α, MMP9, NF-κB, β-catenin, CTNNB, KRAS and BRAF expression as well as lower MLH1 expression No association between relative concentration of *F. nucleatum* rDNA copies in CRC and COX1 or COX2 protein levels	Species level
Mima *et al*[72]	2016	United States cohort: 1,102 CRCs	FFPE CRC tissue after bowel preparation	*F. nucleatum*	Percentage of CRCs	-	Species level

		from the Nurses' Health Study and Health Professionals Follow-up Study. Median follow up of 10.7 years		quantitative PCR	with high number of *F. nucleatum* rDNA copies increased gradually from rectum (2.5%) to cecum (11%) with a linear trend (P < 0.0001)		
Mima *et al*[73]	2015	United States cohort: 1069 CRCs from the Nurses' Health Study and Health Professionals Follow-up Study. Median follow up of 10.7 years	FFPE CRC tissue after bowel preparation	*F. nucleatum* quantitative PCR	Association with proximal CRCs location	Association between high number of *F. nucleatum* rDNA copies in CRCs and poor tumor differentiation Association between high number of *F. nucleatum* rDNA copies in CRCs and MSI-high CRC independent of CIMP and BRAF status Association between high number of *F. nucleatum* rDNA copies in CRC and MLH1methylation	Species level:
Yoon *et al*[78]	2017	North Korean cohort: 6 CRCs, 6 TAs, 6 SSAs and 6 healthy controls. Equal male female distribution	Normal rectal mucosa after bowel preparation	16S rRNA	-	-	Species level: *F. nucleatum* found in only one SSA patient rectal biopsy
Kostic *et al*[79]	2013	United States and United Kingdom cohorts: 27 CRCs, 28 TAs and 31 healthy controls	Fresh-frozen tissue from adenomas and adjacent normal tissue at the time of colonoscopy after bowel preparation Stool	*Fusobacterium* quantitative PCR	-	-	Species level: No specific species identified *Fusobacterium* detected in 48% of adenomas. Increased number of *Fusobacterium* rDNA copies in adenomas compared to matched normal tissue Species level: No specific species identified Higher *Fusobacterium* detection and number of copies in stool from CRC and adenoma compared to controls

CRC: Colorectal cancer; MSI: Microsatellite instability; FFPE: Formalin-fixed paraffin- embedded; BMI: Body mass index; PCR: Polymerase chain reaction; TAs: Tubular adenomas; SSA/Ps: Sessile serrated adenomas/polyps.

Limitations to the Mehta *et al*[64] study include the use of FFPE as opposed to fresh colonic samples and the study's observational design.

One explanation for the relationship between diet and colonic *F. nucleatum* is the potential impact of diet on oral *Fusobacterium* abundance. However, in a population-based case-control study, no associations were found between fiber intake and presence of oral *Fusobacteria*, and only modest positive correlations were found between consumption of saturated fatty acids, vitamin C, B vitamins, and vitamin E, on the one hand, and oral *Fusobacteria* abundance, on the other (P < 0.01)[67]. Furthermore, no associations were observed between oral *Fusobacterium* presence or abundance and CRC[68]. However, that could be due to the study design, whereby patients' oral microbiomes were sampled after CRC resection and treatment; the study also lacks oral hygiene data[68].

In summary, diet may have a differential impact on colonic *F. nucleatum* enrichment, with increased abundance of *F. nucleatum* in the colons of patients consuming a Western diet. Long-term consumption of a high fiber diet may reduce the risk of a subset of CRCs that are *F. nucleatum*-positive. Future studies investigating the mechanisms of the impact of diet on colonic *Fusobacterium* and, subsequently, CRC risk are

needed in order to determine dietary patterns targeting CRC chemoprevention and treatment. Furthermore, these data underline the importance of considering the impact of diet when investigating the links among *Fusobacterium*, other bacteria, and CRC.

***Fusobacterium* associations with CRC anatomic location:** Many previous publications have reported no difference in presence or relative percentage of *Fusobacterium*/*F. nucleatum* rDNA copies in tissue or stool with respect to CRC location, as illustrated in Table 1[31-33,39,43,46-48,69,70]. This could be due to varying definitions of high versus low *F. nucleatum* enrichment, unmeasured dietary confounders, and comparisons of colon versus rectum cancers, as opposed to proximal versus distal location. However, a few research teams have observed differences by CRC location. Yu *et al*[71] identified an increased *F. nucleatum* prevalence and relative concentrations in CRCs proximal to the splenic flexure as opposed to more distal CRCs[42]. A recent report by Mima *et al*[72,73] looked primarily at *F. nucleatum* enrichment in relation to CRC location and found significant relationships between *F. nucleatum*-high CRC and location. The study used FFPE samples from a large United States CRC cohort and found a gradual linear increment in CRCs that had high number of *F. nucleatum* rDNA copies from rectum to cecum (2.5% *vs* 11%, respectively; $P < 0.0001$)[72,73]. Contradictory findings were reported in two studies involving Chinese and Spanish cohorts with an increased detection and relative concentrations of *Fusobacterium* in CRCs distal to splenic flexure. These results could be due to small sample sizes, sampling bias, different geographic location and associated dietary patterns, or looking at *Fusobacterium* as opposed to *F. nucleatum* specifically[27,37,38]. The increased prevalence of *F. nucleatum* in proximal CRC coincides with the presence of invasive bacterial biofilms in 89% of right-sided colonic cancers and their surrounding normal mucosa, which may suggest a more active bacterial role in right CRC carcinogenesis[33]. Thus, current evidence is conflicting, but *F. nucleatum* may be more prevalent in CRC proximal to the splenic flexure, with a gradual increase in *F. nucleatum*-high CRCs from rectum to cecum. The increased *F. nucleatum* in proximal CRCs maybe due to *F. nucleatum* favoring anaerobic conditions, the presence of bacterial biofilms that facilitate its presence or to the differential impact of colonic lumen content on *F. nucleatum* abundance[63,64]. These associations are summarized in Figure 2A.

***Fusobacterium* associations with CRC molecular features:** Associations have been observed between *Fusobacterium*/*F. nucleatum* and certain subsets of CRC, such as MSI-high and CIMP-high phenotypes (Table 1)[31,46,47,61,73]. *F. nucleatum* has also been associated with higher expression of *BRAF* and decreased *MLH1* expression, both of which are seen in MSI-high sporadic CRCs[46,61,69,70,73]. *KRAS* mutations are usually associated with lower CRC methylation (CIMP negative), and conflicting results were seen when the relation of *F. nucleatum* to *KRAS* mutation was evaluated[37,46,50,69,70,74]. However, a large study by Ito *et al*[46] found no association between KRAS mutations and detection or number of *F. nucleatum* rDNA copies in CRC, which is consistent with an *F. nucleatum* predilection to CIMP-high CRCs. A recent, more in-depth investigation showed that presence of high number of *F. nucleatum* rDNA copies in CRC was associated with a 5-fold increased risk of having MSI-high CRCs, irrespective of CIMP-high status or *BRAF* mutation status[73]. This suggests that CRCs with MSI-high status are linked to *F. nucleatum*, whether owing to inherited, somatic, or epigenetic inactivation of MLH-1[5,6]. Further testing that was restricted to MSI-high CRCs showed that high relative concentrations of *F. nucleatum* rDNA was associated with *CDKN2A* (P16) promoter hypermethylation, a tumor suppressor gene associated with CIMP-high CRC[69].Thus, there is increasing evidence linking MSI-high CRC to *Fusobacterium*, but ambiguity exists regarding whether the increased detection of *Fusobacterium* is a cause or consequence of MSI-high status and associated molecular findings in colorectal neoplasia.

***Fusobacterium* associations with CRC stage and prognosis:** Previous investigation has assessed *Fusobacterium* in relation to CRC staging and patient survival with variable results, as summarized in Table 2. High percentage of *Fusobacterium* rDNA copies in CRC tissue was associated with worse depth of invasion in two large studies that looked specifically at CRC prognosis[70,73]. Heterogeneity was seen when *F. nucleatum* abundance was evaluated in relation to lymph node metastasis[32,41,42,70,71,73]. None of the aforementioned studies found any associations between *F. nucleatum* and distal metastatic disease. Lastly, higher *Fusobacterium* rDNA was associated with more advanced CRC stage in 2 out of 11 studies, suggesting a lack of correlation between *Fusobacterium* abundance and the Duke's or tumor, node, metastasis (TNM) staging classifications[31,32,41,46,50,72,73]. Conflicting observations were made when *F. nucleatum* was investigated as a predictor of CRC-specific survival. However, in two large studies by Wei *et al*[70] and Mima *et al*[73] with 10-year follow up, high quantity of *F. nucleatum* rDNA copies in CRC samples was associated with shorter CRC-specific survival after adjustment for multiple confounders. CRC-specific survival was assessed only secondarily in the other studies, with negative results[47,48,70,73]. Heterogeneous observations were made when *Fusobacterium* enrichment was assessed in relation to overall survival in CRC patients[41,46,50,70,73]. A comprehensive evaluation by Mima *et al*[73] adjusted for many confounders and found no association between high, low, or negative *F. nucleatum* rDNA copies presence in CRC tissue and CRC patients' overall survival. The other two studies showing worsened overall survival had a shorter follow-up period and adjusted for fewer

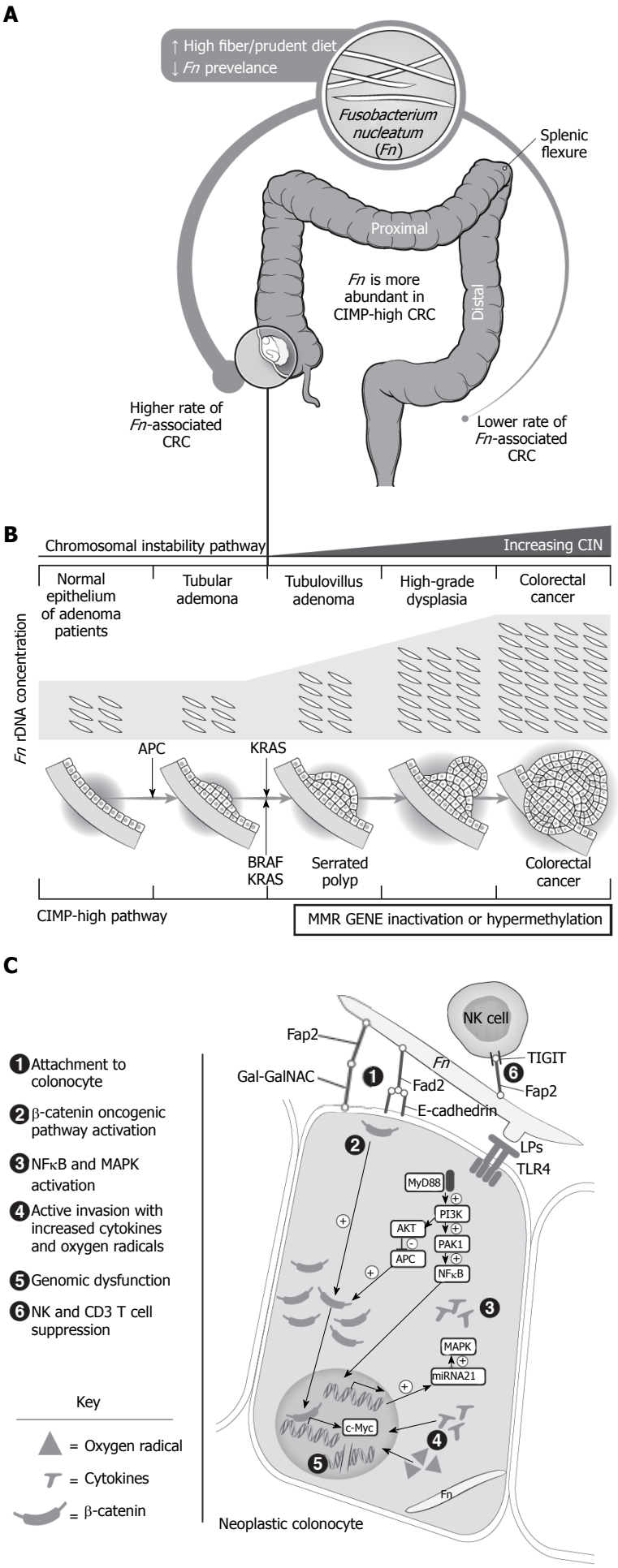

Figure 2 A simplified figure illustrating the link between *Fusobacterium nucleatum* and colorectal cancer. A: Association between *Fusobacterium nucleatum* (*F. nucleatum*) and colorectal cancer (CRC) pathways and location; B: *F. nucleatum* prevalence along chromosomal instability (CIN) and the CpG island methylator phenotype (CIMP)-high CRC pathways and their precursors; C: Postulated mechanisms of *F. nucleatum* induced colorectal carcinogenesis.

Table 2 Studies looking at *Fusobacterium* associations with colorectal cancer stage and prognosis

Authors	Sample size	CRC depth of invasion	CRC lymph nodes metastasis	CRC metastatic disease	CRC stage	CRC prognosis
Viljoen *et al*[31]	South African cohort: 55 CRCs.	-	-	-	Association between higher number of *F. nucleatum* rDNA copies and late stage CRC (stage III and IV compared to stage I and II)	-
Zhou *et al*[32]	Chinese cohort: 97 CRCs	No association with relative percentage of *Fusobacterium* rDNA copies	No association with relative percentage of *Fusobacterium* rDNA copies	No association with relative percentage of *Fusobacterium* rDNA copies	No association with relative percentage of Fusobacterium rDNA copies	-
Zackular *et al*[39]	United States and Canadian cohort: 30 CRC, 30 TA, 30 healthy controls	-	-	-	No association with relative percentage of *F. nucleatum* rDNA copies in CRC	-
Castellarin *et al*[41]	Canadian cohort: 99 CRCs	-	Association between relative percentage of *F. nucleatum* rDNA copies in CRC and regional lymph nodes metastasis	-	No association with relative percentage of *F. nucleatum* rDNA copies in CRC	No association to between relative percentage of *F. nucleatum* rDNA copies in CRC and CRC overall survival
Chen *et al*[42]	Chinese cohort: 98 CRCs	-	No association with presence of *F. nucleatum* rDNA in CRC	-	-	-
Ito *et al*[46]	Japanese cohort: 544 CRCs	-	-	-	No association with detection or number of *F. nucleatum* rDNA copies	No association between detection or number of *F. nucleatum* rDNA copies and CRC overall survival-unknown follow up period
Nosho *et al*[47]	Japanese cohort: 511 CRCs	-	-	-	No association with detection of *F. nucleatum* rDNA in CRC	No association between *F. nucleatum* rDNA presence in CRC and CRC-specific survival-unknown follow up period
Mima *et al*[48]	United States cohort: 598 CRCs.	-	-	-	-	No relation between *F. nucleatum* rDNA copies in CRC and CRC-specific survival or CRC overall survival- unknown follow up period
Flanagan *et al*[50]	Czech, German and Irish cohorts: 122 CRCs	-	-	-	No association with relative percentage of *F. nucleatum* rDNA copies	Higher relative percentage of *F. nucleatum* rDNA copies was associated with shorter CRC overall survival within 3-5 years follow up (HR = 19.96, 95%CI: 1.42-281.42) (no adjustment for other confounders)
Li *et al*[52]	Chinese cohort: 101 CRC	No association	Association between relative percentage of *F. nucleatum* rDNA copies in CRC and lymph nodes metastasis	-	No association with relative percentage of *F. nucleatum* rDNA copies in CRC	-
Amitay *et al*[54]	German cohort: 46 CRC	-	-	-	Relative percentage of *F. nucleatum* rDNA copies in CRC was associated with advanced stage [stage I *vs* II (P = 0.012) and stage I *vs* III (P = 0.042)]	-

Park *et al*[69]	South Korean cohort: 160 MSI-high CRC.	-	No association with *F. nucleatum* rDNA detection or number of copies	-	No association with *F. nucleatum* rDNA detection or number of copies	No association between *F. nucleatum* rDNA detection or number of copies and disease-free survival
Wei *et al*[70]	Chinese cohort: 180 CRCs	Association between high relative percentage of *F. nucleatum* rDNA copies in CRC and depth of invasion	Association between high relative percentage of *F. nucleatum* rDNA copies in CRC and lymph nodes metastasis	-	-	High relative percentage of *F. nucleatum* rDNA copies in CRC was associated shorter CRC overall survival within 3 years follow up [HR = 1.993 (1.024 to 3.879)] High relative percentage of *F. nucleatum* rDNA copies in CRC was associated with shorter CRC disease-free survival within 3 years follow up [HR = 1.829 (1.000 to 3.345)]
Yu *et al*[71]	Chinese cohort: 88 CRCs	-	*F. nucleatum* rDNA was more frequently detected in metastatic lymph nodes of proximal *vs* distal CRC *F. nucleatum* detected in 100% of metastatic lymph nodes compared to 40% of lymph nodes without metastasis ($P < 0.001$)	-	-	-
Mima *et al*[73]	United States cohort: 1069 CRCs	Association between number of *F. nucleatum* rDNA copies in CRC and higher pT of the TNM staging	No association with number of F. *nucleatum* rDNA copies in CRC	No association with number of *F. nucleatum* rDNA copies in CRC	No association with number of *F. nucleatum* rDNA copies in CRC	High number of *F. nucleatum* rDNA copies in CRC was associated with shorter CRC-specific survival within 10.7 years follow up [HR = 1.58 (1.04 to 2.39)] for *F. nucleatum*-high *vs F. nucleatum*-negative CRCs]. (Multivariable models included CRC stage, age, sex, year of diagnosis, family history of CRC, CRC location, MSI status, CIMP status, KRAS status, BRAF, PIK3CA and CRC LINE-1 methylation.) No association between *F. nucleatum* rDNA copies in CRC and CRC overall mortality

CRC: Colorectal cancer; CIMP: CpG island methylator phenotype.

covariates than did Mima *et al*[50,70]. In one study, *Fusobacterium* subspecies were predominantly present in the goblet-like transcriptional CRC subtype[74-76]. This CRC subtype confers good prognosis in chemotherapy-untreated patients, but it has a detrimental effect on prognosis when adjuvant chemotherapy or chemoradiation are used[77]. Thus, CRC treatments, as well as CRC molecular subtypes, need to be investigated when looking at the impact of *F. nucleatum* presence on CRC survival. The above evidence is conflicting but suggests a more aggressive CRC biology with shorter CRC disease-specific survival periods in the presence of *F. nucleatum*; however, there are no relations between *F. nucleatum* and CRC staging. Thus, clarification is warranted of whether *F. nucleatum* modulation in CRC tissue is associated with better disease-free and CRC-specific survival rates after accounting for CRC molecular features and treatments.

Temporality and biological gradient

F. nucleatum prevalence and enrichment was evaluated in CRC precursors in order to assess temporality and an earlier role in CRC carcinogenesis (Figure 2B and Table 1). The number of rDNA copies of *F. nucleatum* was higher in normal rectal/left colonic biopsies of patients with tubular adenomas (TAs) compared with controls[43,55]. The exception is a study that found no evidence of *F. nucleatum* in rectal biopsies of controls, TA patients, or CRC patients; this result is likely due to a small sample size[78]. Higher presence

and relative percentage of *Fusobacterium*[29] and *F. nucleatum*[55,79] rDNA copies was seen in stools of patients with TAs compared with those of controls. On the contrary, two studies found no significant difference in relative percentage of *Fusobacterium*[39] and *F. nucleatum*[54] rDNA copies in fecal samples of patients with TAs, advanced TAs, and controls. Reasons for this discrepancy could include the use of fecal samples, which may represent transit from oral microbiome and may not necessarily correlate with true *Fusobacterium* abundance in colonic tissue, as well as absence of information on prior antibiotic use[10,26,50]. Detection and relative percentage of *Fusobacterium*[28] and specifically *F. nucleatum*[46,50,55] rDNA copies were found to increase in colonic tissue as it progressed through the CIN pathway (healthy control *vs* TA *vs* tubulovillous adenoma [TVA] *vs* high grade dysplasia *vs* CRC). Interestingly, no difference in relative percentage of *F. nucleatum* DNA copies was observed between TA and TVA tissue when compared with surrounding normal tissues[28,46,50]. This finding maybe be due to presence of bacterial biofilms or precancerous molecular changes in the surrounding normal mucosa, despite normal histological appearance, which may favor the attachment or invasion of *Fusobacterium*[33,80]. Data are limited but suggest no relation between adenoma size or burden and *F. nucleatum* rDNA copy numbers in rectal tissue[43]. Finally, *F. nucleatum* was associated with CIMP-high and right-sided sessile serrated adenomas/polyps (SSA/Ps) when SSA/Ps were compared with TAs[28,46,71]. Limitations to the above studies include an absence of information on concomitant preneoplastic tissue in patients who had hyperplastic polyps and the simultaneous use of FFPE and colonic preparation in specimens collected, which could reduce *F. nucleatum* detection[66,81]. All these data suggest that there may be a stepwise increase in *F. nucleatum* rDNA quantity and detection as colorectal neoplasms progress through the CIN pathway. Furthermore, *F. nucleatum* may play an earlier role in the CIMP-high CRC pathway. These results suggest temporality and a biological gradient of *F. nucleatum* in CRC development.

Plausible mechanisms and experimental evidence

Despite the accumulating associations between *Fusobacterium*/*F. nucleatum* and colorectal neoplasia, establishing direct causality is challenging with the absence of prospective human studies supported by correlative laboratory science. In brief, multiple observational and animal experimental studies suggest plausible mechanisms by which *F. nucleatum* may contribute to CRC development, and these warrant additional investigation (Figure 2C).

***F. nucleatum* transmission to colorectal neoplastic tissue:** Rats treated with 1,2-dimethylhydrazine (DMH) had increased *Fusobacterium* detection in tumors, whereas it was absent in nontreated controls, indicating a predilection of *Fusobacterium* to tumor tissue[82]. Oral administration of *F. nucleatum* into *APC*$^{min/+}$ and DMH-treated mice led to colorectal colonization and promoted colorectal neoplasia development, suggesting an active role of *F. nucleatum* in CRC neoplasia[55,79]. Similar findings were not seen in wild-type mice, suggesting that *F. nucleatum* can be contracted through oral ingestion if individuals are already predisposed to CRC. The mechanism by which *F. nucleatum* reaches the colonic epithelium are unclear. However, some *F. nucleatum* strains display the potential to disrupt the colonic mucosal barrier, suggesting that it can be transmitted from the colonic lumen to the epithelium, potentially causing colorectal disease[60]. Other *Fusobacteria* may take advantage of coinfection with other invasive bacteria or of disruption of the mucosal layer, seen with CRC. Another mechanism by which *Fusobacteria* home and localize to dysplastic colorectal epithelium is the blood-borne route[83]. In a novel study, a host lectin (Gal-GalNAc) was shown to mediate *F. nucleatum* attachment to CRC and precursor cells through interaction with an *F. nucleatum* protein, fibroblast activation protein 2 (FAP2)[83]. The expression of Gal-GalNAc is increased in a stepwise fashion in colorectal adenoma and matched surrounding normal tissue to villous adenomas with highest levels seen in CRC[83,84]. In a prior study, Gal-GalNAc was also more abundant in visually normal colonic epithelium of patients with CRC and its precursors, when compared to healthy controls[85]. Gal-GalNAc is mainly expressed in embryonic colonic goblet cells, and, in parallel, *Fusobacterium* was predominantly present in the goblet-like transcriptional CRC subtype[76,85-87]. The above data suggest that *F. nucleatum* can be localized through the lumen, or it can be blood borne. *F. nucleatum*'s preferential adherence to colorectal neoplasia maybe due to increased colonic epithelial Gal-GalNAc expression potentially due to goblet-like transformation of colorectal dysplastic epithelium. This increased Gal-GalNAc expression may explain *F. nucleatum's* prevalence in visually normal colonic tissue of predisposed individuals, as well as *F. nucleatum's* stepwise abundance through the adenoma-carcinoma sequence. Further evaluations confirming the goblet-like transformation of visually normal appearing colonic tissue of CRC patients in relation to *Fusobacterium* and bacterial biofilm formation are warranted.

***F. nucleatum* leads to increased expression of oncogenic and inflammatory factors early in CRC development:** Stool metabolomics and CRC tissue inflammosome analysis supported associations between *Fusobacterium*[31,88], specifically *F. nucleatum*[45,89], and inflammatory metabolites as well as pathways implicated in colon carcinogenesis: Interleukin (IL) 6, IL8, IL10, IL17F, IL21, IL22, the Regenerating gene family, tumor necrosis factor (TNF), Matrix metallopeptidase 9 (MMP9) and Nuclear Factor kappa B (NF-κB)[70,76,90-92]. Quantity of *F. nucleatum* rDNA copies and inflammatory markers were both higher

in visually normal rectal mucosa of adenoma patients compared with healthy controls[43,89]. Fluorescence in situ Hybridization (FISH) further confirmed the presence of *F. nucleatum* in the mucus layer and within colonic crypts of normal appearing colonic mucosa[43]. Bacterial biofilms were also found to cover normal appearing colorectal mucosa adjacent to CRC; and this was associated with an increase in colonic epithelial proliferation, *IL6* and *STAT3* activity as well as decreased E-cadherin in the normal appearing colonic epithelium[33]. All this suggests that *F. nucleatum* is associated with increased colorectal inflammation in CRC tissue. There is also an association between of presence of *F. nucleatum* rDNA and inflammation in visually normal appearing colorectal epithelium. The presence of inflammation in normal appearing colonic epithelium could potentially be due to presence of bacterial biofilms. These findings are interesting since inflammation is considered to be a marker of carcinogenesis which suggest a potential early role for *F. nucleatum* in carcinogenesis even prior to adenoma formation[93].

Indeed, data showed that incubation of *F. nucleatum* with CRC cell lines promotes proliferation and invasion of CRC cell *in vitro* and mice xenograft modules[91]. Experimental mouse data using *APC* $^{Min/+}$ and DMH models are supportive showing that *F. nucleatum* administration increases the number and size of aberrant crypt foci and colorectal tumors, with activation of JAK/STAT and MAPK/ERK pathways critical for CRC development[55,79,91]. The mechanism for *MAPK* activation is thought to be due to recognition of *F. nucleatum* lipopolysaccharide by toll-like receptor 4 (TLR4) surface protein present on CRC cells leading to initiation of the TLR4/MYD88/NF-κB pathway, with subsequent binding of NF-κB to the micro RNA (miRNA)-21 promoter site[42,91]. This leads to increased expression of miRNA 21 which regulate RASA1 gene with subsequent activation of the MAPK pathway[91]. Similarly, *F. nucleatum* lipopolysaccharide possibly activates the TLR4/p21-activated kinase 1 (PAK1) cascade with subsequent increased β-catenin expression[42]. In parallel, it is proposed that *F. nucleatum*'s adhesion molecule, FadA, mediates induction of E-cadherin/β-catenin with subsequent abundance of target genes, such as *C-myc* and *CCND1*[89,91]. These proposed mechanisms are described in Figure 2C. In a recent study, *F. nucleatum 2* equipped with FadA and FAP2 proteins did not increase inflammation or promote CRC in *APC*$^{Min/+}$ nor in *IL10*-knockout mice, suggesting that FadA and FAP2 are necessary but not sufficient to promote CRC[94]. This could be due to some *F. nucleatum* strains having distinct virulence factors and/ or distinct lipopolysaccharides that are associated with more invasive and inflammatory behavior[60]. Functional pathway analysis supports this hypothesis, with increased bacterial virulence and motility protein pathways in *F. nucleatum*-invading CRC tumors[30]. Finally, *F. nucleatum* invasion and survival inside colorectal cells may cause increased production of reactive oxygen species[92,95]. The resultant activation of inflammatory cascades is hypothesized to induce DNA damage and epigenetic silencing of key targets, such as the mismatch repair gene MLH1, potentially leading to MSI seen frequently with *F. nucleatum*[46,61,89,90-92]. Additional investigation is warranted focusing upon the relationship between virulent *Fusobacterium* strains, specifically *F. nucleatum*, and induction of an inflammatory microenvironment that facilitates epigenetic and genetic alterations involved in early colorectal carcinogenesis.

***F. nucleatum* modulates the tumor immune microenvironment favorably towards carcinogenesis:** Mounting evidence suggests that *F. nucleatum* modulates the microenvironment at the interface between the developing cancer and the host immune response. For instance, *F. nucleatum rDNA* abundance in tumor tissue was correlated with host immune response genes and oncogenes[45]. *F. nucleatum* can impact tumor T-cell abundance by inducing T-cell apoptosis, as well as by reducing T-cell proliferation, activation and response to certain mitogens and antigens[79,96-102]. This effect could be due to the FAP2 protein of *F. nucleatum* directly interacting with T-cell immunoreceptor with immunoglobulin (Ig) and ITIM domains (TIGIT), leading to the inhibition of natural killer (NK) cell-induced tumor cytotoxicity. Other tumor-infiltrating CD3+ T cells (CD4+ and CD8+) also have TIGIT and are possibly inhibited by FAP2[103]. This is consistent with the observation that *Fusobacterium*-high CRC cases are inversely associated with the density of CD3+ T cells, a type of T cell that is usually associated with better patient survival[48]. In parallel, Forkhead box P3 (FOXP3)-low T cells do not possess tumor suppressive activity and can secrete proinflammatory cytokines. FOXP3-low T-cell-infiltrated CRCs show increased expression of inflammation and immune-mediated genes such as IL12A, IL12B, transforming growth factor (TGF)-beta 1, and TNF, and they are associated with *F. nucleatum* abundance, paradoxically conferring better CRC-free survival[104]. *F. nucleatum* also recruits CD11b myeloid-derived immune cells, which are precursors to macrophages, consistent with the finding of increased tumor macrophages in the presence of *F. nucleatum*[69,79,105]. Furthermore, *F. nucleatum* induces activation of the *CCL20*/*CCR6* axis in monocytes and CRC cells, potentially promoting monocyte migration and CRC development[56]. Thus, *F. nucleatum* abundance is associated with increased CD68 tumor-infiltrating macrophages, monocytes, and FOXP3-low T cells, but lower infiltration of CD3 lymphocytes. These findings support the hypothesis that *F. nucleatum* may exert an immunosuppressive effect in the cancer microenvironment that promotes the sustained survival of CRC cells. It may also explain the mystery of why the high load of MSI-induced antigens does not lead to immune eradication of MSI-high CRCs; this could be due to infiltration by *F. nucleatum* and associated immunosuppression. The relation between the immune

microenvironment and prognosis is still controversial, and future studies linking bacteria such as *Fusobacterium* to survival through peripheral immune modulation are warranted.

Practical applications of fn in CRC prevention

The accumulating literature linking *F. nucleatum* to CRC led to efforts investigating the utility of *F. nucleatum* in CRC detection. Fecal-based *F. nucleatum* polymerase chain reaction (PCR) can serve as a noninvasive tool for CRC detection, with even better results when using digital PCR based on water-oil emulsion droplet technology[39,54,106-109]. Compared with PCR, loop-mediated isothermal amplification (LAMP) is a simple, noncostly and accurate method for bacterial testing that was shown to be more sensitive than PCR for *F. nucleatum* detection[110]. Two drawbacks of LAMP are the potential for false positivity and the complex design primer used. Metagenomic analysis of fecal microbiome across European and Chinese cohorts also showed that butyryl-CoA dehydrogenasegene *F. nucleatum* gene markers accurately distinguished CRC cases from controls, with area under the curve (AUC) = 0.84 and an odds ratio of 23[111]. Finally, Wang *et al*[112] demonstrated that *F. nucleatum* can also induce a serological anti-*F. nucleatum*-IgA immune response that is higher in CRC patients compared with patients with benign colonic polyps, those with inflammatory bowel disease, and healthy controls. In that study, the combination of anti-*F. nucleatum*-IgA and carcinoembryonic antigen (CEA) was found to better for diagnosing CRC compared with either one alone (sensitivity: 53.10%; specificity: 96.41%; AUC = 0.848).

The finding that diet can alter the microbiome and associated colonic carcinogenesis led to efforts investigating *F. nucleatum* modulation in CRC chemoprevention and therapeutics through the use of probiotics and herbals[63]. Probiotics including *Bifidobacterium longum*, *Lactobacillus acidophilus* and *Enterococcus faecalis* significantly reduced *Fusobacterium* levels by nearly 5-fold in CRC surgery patients when compared with placebo probiotics (10.08% *vs* 1.91%, respectively; $P = 0.03$)[113]. Limitations of that study include the variable length of probiotic treatment and the presurgery use of antibiotics and bowel preparation, which can alter the microbiome[113]. Berberine (BBR) is an isoquinoline alkaloid and a component of the Chinese herb *Coptis chinensis*. BBR was shown to prevent insulin resistance and obesity in mice fed a high-fat diet, in association with an impact on the intestinal microbiome[114]. Administration of BBR to *APC* $^{Min/+}$ and DMH mice inoculated with *F. nucleatum* led to reduced tumorigenesis and *Fusobacterium*-induced activation of the JAK/STAT and MAPK/ERK pathways[55]. Both probiotics and herbals may provide tactics for modulating *F. nucleatum*, but the implications are still under investigation.

Conclusions

Fusobacteria are significantly more abundant in colorectal tissues and stools of patients with CRC than in healthy controls. The histopathology of these findings is ambiguous, but the few available data suggest that *Fusobacteria* have been observed within the colonic biofilms, the colonic mucus layer, colonic crypts, and inside the colonic epithelium. *F. nucleatum* has been associated with proximal CRCs and CRCs with MSI-high features, a finding warranting additional investigation. Findings also suggest temporality and a biological gradient with presence of fusobacteria in CRC precursors. Further, researchers have observed increased detection and quantity of *F. nucleatum* rDNA in the visually normal mucosa of colorectal neoplasia patients when compared with healthy controls. The pathophysiology and significance of this finding is unclear, as is its relation to cancer progression. *Fusobacteria* are usually indigenous to healthy mouth microbiota, highly adherent to teeth and oropharyngeal epithelium in the presence of a low viscous saliva environment, and unspecialized for viscous environment. Therefore, they are normally only transient in the colon, which is protected by a mucus layer. Disruption of the colonic mucus layer or coinfection with other invasive bacteria may facilitate the presence of *Fusobacteria*l species in CRC tissue. Furthermore, some *Fusobacteria*l strains, specifically *F. nucleatum*, are considered active invaders, giving them the potential to disrupt an intact colonic mucosal barrier and potentiate colorectal disease. The presence of a host lectin (Gal-GalNAc) in the colon may also mediate *F. nucleatum* blood-borne transmission and attachment to CRC and precursors through interaction with an *F. nucleatum* protein, FAP2. *F. nucleatum* was demonstrated to have cancer-promoting properties in several rodent models, supporting its role in the human colon cancer cascade. This is thought to be due to its activation of inflammatory and oncogenic pathways associated with colon carcinogenesis, as well as its modulation of the immune microenvironment in a manner that favors cancer progression. The lack of prospective human studies is a large limitation of current literature regarding the temporality of *Fusobacterium* and cancer; most human studies to date were cross-sectional case-control studies. Thus, more evidence is needed to confirm causality and inform future detection and therapeutic efforts targeting *F. nucleatum* and other microbiota involved in CRC.

ARTICLE HIGHLIGHTS

Research background

The presence of *Fusobacterium*, specifically *Fusobacterium nucleatum* (*F. nucleatum*), in the colon is increasingly linked to colorectal cancer (CRC). However, significant heterogeneity in study methods and findings poses challenges to interpretation. An evaluation of this rapidly expanding literature will help direct future studies to answer unresolved questions and to avoid previous design pitfalls in order to further our knowledge in this exciting field.

Research motivation

A critical evaluation of the scientific literature regarding the link between *Fusobacterium/F. nucleatum* and CRC may contribute to the development of more comprehensive and novel studies to better define this relationship and its potential applications in CRC treatment and prevention.

Research objectives

This systematic review evaluated the clinical and experimental evidence linking *Fusobacterium* and CRC. The authors reviewed studies investigating the relationship between *Fusobacterium* and the following variables: CRC, CRC patients' characteristics and dietary patterns, CRC anatomic location, CRC molecular features, and CRC stage and prognosis. The authors also reviewed studies looking at presence of *Fusobacterium* in pre-neoplastic lesions, as well as experimental evidence testing the procarcinogenic potential of *Fusobacterium.* Finally, the authors looked at the implications of *Fusobacterium* for CRC detection and treatment. Elucidating these heterogeneous studies may impact our understanding of the relationship between *Fusobacterium* and CRC, as well as improve detection and chemoprevention tactics for CRC.

Research methods

This is, to our knowledge, the first systematic review of the scientific evidence surrounding the link between *Fusobacterium* and CRC. Using PubMed, Embase, and Medline, the authors systematically reviewed all original studies investigating *Fusobacterium/F. nucleatum* and CRC published between January 1st, 2000, and July 1st, 2017. All abstracts were screened to identify original human, animal, and in vitro research. Out of the 355 articles that were screened, 90 articles were included in this review. Articles were excluded if diseases other than CRC were included and if they were written in languages other than English. All review articles and citations including only an abstract were excluded from analysis.

Research results

An accumulating body of evidence supports the hypothesis that *Fusobacterium,* especially *F. nucleatum* is more frequently detected in colorectal neoplasia, especially the microsatellite instability neoplastic pathway and proximal CRC. Studies investigating *F. nucleatum* in colorectal precancerous tissue suggest temporality and a biological gradient; however, ambiguity still exists on whether this increased detection of *Fusobacterium* is a cause or consequence of colorectal neoplasia. Diet may have a differential impact on colonic *F. nucleatum* enrichment, high fiber diet potentially reducing the risk of a subset of CRCs that are *F. nucleatum*-positive. Evidence also suggests a shorter CRC disease-specific survival in the presence of *F. nucleatum*, albeit with no relations between *F. nucleatum* and CRC staging. The homing of *Fusobacteria* and *F. nucleatum* to the colonic epithelium maybe partly due to increased Gal-GalNAc expression on colonic cells, virulence factors of *F. nucleatum* and other *Fusobacteria*, and changes to the local colonic environment with disruption of the protective mucus layer. Experimental evidence suggests that *Fusobacterium nucleatum* has a procarcinogenic potential that is likely mediated by activation of oncogenic and inflammatory pathways, as well as modulation of the tumor immune environment. The lack of prospective human studies is a large limitation of current literature. Furthermore, it will be essential to further delineate mechanisms and timing of *Fusobacterium* homing to the colonic mucosa, as well as its relation to cancer progression. This review may be used to develop hypotheses for novel strategies targeting colorectal cancer detection and prevention. Future robust analysis would also benefit from adjusting for confounders, such as *Fusobacterial* strain virulence factors, colonic preparation, antibiotic use, and diet.

Research conclusions

Accumulating evidence supports the hypothesis that *F. nucleatum* may enhance colorectal carcinogenesis, especially the neoplastic pathway involving defects in microsatellite instability. Virulence factors of *F. nucleatum* may contribute to its procarcinogenic effect. The lack of prospective human studies is a large limitation of current literature regarding the link between *Fusobacterium* and CRC. This review may be used to guide novel strategies targeting colorectal cancer detection and prevention.

Research perspectives

This review gathers an ample evidence implicating *Fusobacterium* in CRC etiology and highlights the promising global efforts aimed at testing the role of *Fusobacterium* in CRC detection, chemoprevention and outcomes. There are multiple gaps in knowledge and the current evidence lacks prospective human studies. To advance our knowledge, future prospective studies need to clarify the timing and mechanisms of *Fusobacterium* transmission to the colon in relation to colorectal carcinogenesis and histopathology of these findings. In order to potentially design future CRC therapies, additional investigations are also warranted to delineate the relationships between virulent *Fusobacterium* strains, specifically *F. nucleatum*, and induction of inflammatory, pro carcinogenic and immune mechanisms involved in early colorectal carcinogenesis. Furthermore, future efforts need to prospectively test the impact of diet, probiotics and other chemopreventative agents on colonic *Fusobacterium* and whether modulation of colonic presence/concentrations of *Fusobacterium* will alter the risk or outcomes of CRC. Finally the role of *Fusobacterium* in CRC screening is intriguing and studies combing *Fusobacterium* testing with other CRC screening methods such as stool DNA testing or even colonoscopy may potentially improve CRC detection and preventative efforts. This study outlines the significant heterogeneity in the methods used and the need for more consistent design. In order to attain more robust results, the authors suggest future studies to: (1) use a blinded prospective randomized controlled design and/or large sample size when possible; (2) perform better sampling by collecting unprepared colonic tissue stored as fresh frozen samples; (3) have more detailed microbiome sequencing using whole-genome shotgun metagenomic sequencing, FISH and other methods in order to assess *Fusobacterium* sub-species concentrations, the presence of virulence factors and location of *Fusobacterium* in relation to the colonic epithelium; and (4) adjust for potential dietary, geographic and racial variables that may have an impact on *Fusobacterium/F. nucleatum* presence or concentration within specimens.

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SYSTEMATIC REVIEWS

Psychiatric morbidity after surgery for inflammatory bowel disease: A systematic review

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Abstract

AIM

To examine the evidence about psychiatric morbidity after inflammatory bowel disease (IBD)-related surgery.

METHODS

PRISMA guidelines were followed and a protocol was published at PROSPERO (CRD42016037600). Inclusion criteria were studies describing patients with inflammatory bowel disease undergoing surgery and their risk of developing psychiatric disorder.

RESULTS

Twelve studies (including 4340 patients) were eligible. All studies were non-randomized and most had high risk of bias. Patients operated for inflammatory bowel disease had an increased risk of developing depression, compared with surgical patients with diverticulitis or inguinal hernia, but not cancer. In addition, patients with Crohn's disease had higher risk of depression after surgery compared with non-surgical patients. Patients with ulcerative colitis had higher risk of anxiety after surgery compared with surgical colorectal cancer patients. Charlson comorbidity score more than three and female gender were independent predictors for depression and anxiety following surgery.

CONCLUSION

The review cannot give any clear answer to the risks of psychiatric morbidity after surgery for IBD studies with the lowest risk of bias indicated an increased risk of depression among surgical patients with Crohn's disease and increased risk of anxiety among patients with ulcerative colitis.

Key words: Inflammatory bowel disease; General surgery; Psychiatry; Depression; Anxiety; Postoperative complications

Core tip: Patients with inflammatory bowel disease (IBD) have higher risk of depression after surgery compared with patients operated for diverticulitis or inguinal hernia but not cancer. Patients with ulcerative colitis (UC) might have higher risk of anxiety after surgery compared with patients with colorectal cancer. Compared with nonsurgical patients, patients operated for UC have higher risk of anxiety and patients operated for Crohn's disease have higher risk of depression. Among patients with IBD, female gender and Charlson comorbidity score > 3 are risk factors for both anxiety and depression following surgery.

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INTRODUCTION

Compared with other chronic disease populations, patients with inflammatory bowel disease (IBD) are younger at the time of diagnosis but they have normal length of lifespan[1]. Hence, they live many years with a chronic disease. Despite the use of immunomodulators, which have reduced the need of surgery, 12% of patients with Crohn's disease (CD) and 6% with ulcerative colitis (UC) still undergo IBD-related surgery within one year of diagnosis[1]. Many of these patients end up with a permanent or temporary stoma.

Previous research has shown a high incidence of psychiatric disorders among patients with IBD, especially those with active disease[2-4]. The burden of psychiatric disorders is enormous[5]. This includes both the personal burden and the cost for the society. Comorbid mental disorders in other chronic diseases are the main reason for functional impairment[6]. This is also likely to be true in IBD. Therefore, preventing development of mental disorders in these patients is imperative.

Already in 1986, the medical society showed interest in the psychological effects of stoma creation among patients with IBD, cancer, and diverticulitis[7]. Recently, in 2014, a narrative review assessed the psychological impact of surgery on patients with IBD[8]. The study found improvement in quality of life, but also an increased risk of depression and anxiety compared with the general population. This seemed contradictory. There are still controversies regarding psychiatric comorbidity in IBD compared to other chronic medical illnesses[9,10].

The objective of this review was to examine the evidence about psychiatric morbidity after IBD-related surgery compared to non-IBD surgery. This systematic review assessed the following study questions, which has not been described in previous publications: (1) do patients with IBD have higher risk of psychiatric disorder after surgery compared with other surgical patients? (2) do patients with IBD have higher risk of psychiatric disorder after surgery compared with non-surgical patients with IBD? and (3) among surgical patients with IBD, how do we identify patients needing extra attention to prevent development of psychiatric disorder?

MATERIALS AND METHODS

This systematic review followed PRISMA guidelines[11]. To reduce risk of bias, a study protocol was made at an early stage and stated precise eligibility criteria. The protocol was registered in PROSPERO[12] (registration number CRD42016037600).

Eligibility criteria

Inclusion criteria: (1) studies about patients with IBD (CD and/or UC) undergoing IBD-related surgeries; (2) studies assessing outcomes of psychiatric comorbidity in terms of: ICD (International Classification of Diseases) or DSM (Diagnostic and Statistical Manual of Mental Disorders); change in psychiatric rating scales indicating psychiatric morbidity [Hospital Anxiety and Depression Scale (HADS)], Depression Anxiety and Stress Scale (DASS), State Trait Anxiety Inventory (STAI), Beck depression inventory, or Rorschach content interpretation for anxiety). Those psychiatric rating scales were chosen because they indicate presence of anxiety and/or depression. Yet, they are not alone diagnostic; and (3) clinical trials, retrospective- and prospective cohort studies, systematic reviews, meta-analysis, and case-control studies with no language restrictions.

Exclusion criteria: (1) studies describing psychiatric disorders prior to surgical intervention; (2) studies with delirium diagnosis as outcome; (3) studies exclusively reporting quality of life or single psychological symptoms as part of larger questionnaires not assessing psychiatric morbidity; and (4) case-series, case reports, commentaries, letters, conference abstracts, narrative reviews, and editorials.

The search was performed in the following databases: MEDLINE, PsycINFO, EMBASE, and the Cochrane Library. A search strategy was developed combining MeSH terms (Major Subject Headings) and free-text terms. The search from MEDLINE is reported in the supplementary figure. The search from the three other databases contained the same keywords. The last search in all databases was performed on May

1, 2016. Reference lists from all included articles were screened for relevant studies.

The selection of studies was performed using "Covidence" management tool[13]. Two independent reviewers, blinded to the other reviewer's decision, completed the selection of studies in two steps. First, title-abstract was screened and afterwards full-text screening of included abstracts was performed. Disagreements were resolved by consensus.

Zangenberg MS performed the data collection on: authors, publication year, study design, details of populations (CD, UC or mixed IBD), intervention details (type of surgery), risk factor for psychiatric disorder after surgery, outcome measures (ICD, DSM, rating scale), any comparisons used, and results. In articles with mixed participants, data of the IBD population was extracted and reported. One author was contacted to clarify results of study tables[3]. The author of two studies[14,15] was contacted to make sure the patients were not duplicates.

The methods for assessing risk of bias are described in "supplementary methods". The risk of bias assessment was carried out by Zangenberg MS.

No meta-analysis was conducted due to heterogeneity in methodology and outcome reporting in the included studies. We chose to report our results in three sections according to outcome.

RESULTS

Study selection

A total of 980 studies were identified using the above-mentioned search strategy. Seventy-one studies were chosen for the full-text screening, from which 12 studies were included in the synthesis of results (Figure 1 shows PRISMA flowchart). No additional studies were found screening the reference list of the included articles. All selected studies for inclusion were in English. Translation was therefore not needed. The 12 included studies covered a total of 4340 patients (n = 2047 patients with UC and n = 2293 patients with CD). All studies found were non-randomized. Characteristics of included studies can be found in Table 1.

Quality assessment was done using a modified Newcastle-Ottawa scale described in "supplementary methods". Most studies had high risk of bias. See Figure 2 for a total result of the quality assessment.

Psychiatric rating scales

The included studies used four different psychiatric rating scales to assess psychiatric morbidity. The scales are mentioned in the methods. The most commonly used scale was HADS which consists of seven items for depression and seven items for anxiety. Each item covers a score from 0-3, where 3 indicates greatest severity. The range of each subscale is 0-21 and the score can be divided in different categories. Most studies use the categories normal/non-cases (0-7), mild/doubtful cases (8-10), moderate (11-15), and severe (16-21). Some mix the last two categories and call is cases/probable mental disorders (11-21).

Outcomes regarding depression

Eleven studies described depression after IBD-related surgery (Table 2). Two studies found insignificant associations between past history of surgery and depression[3,4]. One study showed that patients operated for IBD had a greater five-year post-operative risk of depression compared with patients operated for diverticulitis or inguinal hernia[16]. The same study looked at CD treated surgically compared with non-surgical CD cases and found a significant increased risk of depression (Table 2). In patients with UC treated with surgery versus non-surgical cases there was no increased risk. These results indicate that patients with CD may be more prone to depression after surgery compared with patients with UC.

Patients with colorectal cancer and a colostomy scored higher on Beck depression inventory than patients with UC and an ileostomy[17]. In another study, Beck depression inventory was used preoperative, 3, 6, and 24 mo after elective bowel resection for CD. Depression scores at three and six month follow-up declined compared with the preoperative score[18]. After 24 mo, improvement was only seen in the group still in remission. The results suggest decreased risk of depression, but did not control for disease activity, which can be an important confounder.

Three studies measured depression after surgery (HADS-score $\geqslant$ 11) and found a prevalence of 4%-16%[14,15,19]. The highest prevalence (16%) was found in the cohort consisting of only patients with Crohn's disease, which could indicate that patients with CD are more prone to depression after stoma surgery compared with mixed cohorts including patients with UC. None of the studies measuring HADS compared the scores with non-surgical patients or other surgical patients.

Three studies investigated ileal pouch-anal anastomosis (IPAA) as an intervention[20-22]. A study of patients with UC having IPAA found no difference in HADS between groups with IPAA and patients without[20]. Two studies looking at subgroups of IPAA cohorts found increased depression scores in patients with irritable pouch syndrome[21,22]. This indicates that only patients with problems (*e.g.,* irritable pouch syndrome) after IPAA has increased risk of depression.

Collectively, most studies using statistical comparisons found no increased risk of depression following surgery for IBD, although some studies indicate that patients with CD undergoing surgery are more prone to depression compared with patients medically treated for CD.

Outcomes regarding anxiety

Ten studies described anxiety outcome following

Table 1 Characteristics of included studies

Ref.	Year	Country	Study design	Population	Surgery	Outcome measures	Comparisons
Makkar *et al*[22]	2015	Canada	Cross sectional study	137 patients with UC > 18 yr who were > 1 yr from the final stage of their total IPAA surgery.	IPAA	DASS-21 including subscales for stress, anxiety and depression	Subgroup analysis comparing normal pouch, irritable pouch syndrome and pouch inflammation. All groups had IPAA
Panara *et al*[4]	2014	United States	Retrospective cohort study	393 patients > 18 yr with UC (121) or CD (272)	History of surgical stoma or seton placement as risk factor (from surgical records)	ICD-9-CM (International Classification of Diseases, Clinical Modification) codes for depression	None
Ananthakrishnan *et al*[16]	2013	United States	Retrospective cohort study	707 with CD and 530 with UC	Bowel resection surgery (ICD records)	ICD-9 codes for depressive disorders or generalized anxiety given after 30days after surgery. Analyses of independent predictors of depression and anxiety following IBD-surgery	IBD patients not having surgery and patients undergoing surgery for other diseases
Knowles *et al*[14]	2013	Australia	Cross sectional study	83 mixed IBD. (62.7% UC) Age between 18-40 yr	Stoma surgery (self-reported)	HADS (normal = 0-7, mild severity = 8-10, moderate severity = 11-15, severe severity = 16-21)	none
Knowles *et al*[15]	2013	Australia	Cross sectional study	31 with CD	ostomy	HADS (normal = 0-7, mild severity = 8-10, moderate severity = 11-15, severe severity = 16-21)	none
Nahon *et al*[3]	2012	France	Cross sectional study	1663 with IBD (63.9% CD and 37.1% UC or indeterminate colitis	Past history of surgery as risk factor	HADS > 11 on either subscale was considered "significant" cases of psychological comorbidity	none
Schmidt *et al*[21]	2007	Germany	Cross sectional study	43 with UC	IPAA	HADS ⩾ 11 on either subscale (depression/anxiety) indicative of a probable mental disorder	IPAA patients in remission, with pouchitis and with irritable pouch syndrome
Häuser *et al*[20]	2005	Germany	Cross sectional study	101 with UC	IPAA	HADS ⩾ 11 on either subscale was considered "significant" cases of psychological comorbidity Use of psychopharmacological agents	UC patients with IPAA *vs* general german population and UC patients with IPAA *vs* UC patients without IPAA.
de Oca *et al*[23]	2003	Spain	Cross sectional study	100 with UC and 12 with CD (discovered postoperative)	IPAA	STAI for Anxiety	Only subgroup (CD *vs* UC) comparisons
Nordin *et al*[19]	2002	Sweden	Cross sectional study	331 with UC and 161 with CD (all in the range of 18-70 yr of age)	Ileostomy, ileoanal anastomosis and ileorectal anastomosis	HADS where ⩽ 7 = "non-case"; 8-10 = "doubtful case"; ⩾ 11 = "case"	none
Tillinger *et al*[18]	1999	Austria	Prospective cohort study	16 with CD	Elective ileum or colon resection	Beck depression inventory within one week before operation, three, six and 24 mo postoperative	none
Keltikangas-Järvinen *et al*[17]	1983	Finland	Cross sectional study	32 with UC operated with ileostomy	Operation with ileostomy (follow up = 7 ± 1.2 yr. after the operation)	Beck's depression scale and Rorschach content interpretation for anxiety	34 colorectal cancer patients having colostomy

HADS: Hospital Anxiety and Depression Scale; DASS: Depression, Anxiety and Stress Scale; STAI: State-Trait Anxiety Inventory; IPAA: Ileal Pouch-Anal Anastomosis; CD: Crohn's disease; UC: Ulcerative colitis.

Table 2 Results regarding depression and anxiety

Ref.	Depression results	Anxiety results
Nahon *et al*[3], 2012	Multivariate analysis of predictive factors found no association between past history of surgery and depression (OR = 0.93, 95%CI: 0.50-1.72)	Multivariate analysis of predictive factors found past history of surgery to be significantly associated with decreased risk of anxiety (OR = 0.47, 95%CI: 0.31-0.71)
Panara *et al*[4], 2014	Multivariate analysis: history of surgery had a non-significant HR = 1.3 (95%CI: 0.92-1.76; P = 0.13).	-
Ananthakrishnan *et al*[16], 2013	Chi-square test: Higher 5 yr postoperative risk in IBD group (16%) compared with diverticulitis (9%) and inguinal hernia group (7%) (P < 0.05). Higher risk in CD surgery group compared with non-surgical group (OR = 1.34, 95%CI: 1.01-1.77). No significant increased risk in UC surgery group compared with non-surgical group (OR = 1.21, 95%CI: 0.93-1.58).	no significant increased OR in CD-surgery group compared with non-surgical group (OR = 1.20, 95%CI: 0.93-1.55) or UC-surgery group compared with non-surgical group (OR = 1.26, 95%CI: 0.96-1.65).
Keltingas-Jarvinen *et al*[17], 1983	Comparisons of means in Beck depression inventory – type of analysis not stated: UC < colorectal cancer	Comparisons of means in Rorschach content interpretation for anxiety – type of analysis not stated: UC > colorectal cancer
Tillinger *et al*[18], 1999	Wilcoxon test: significantly improved score three and six months postoperatively (P = 0.0038 and 0.0013 respectively). 24 mo postoperatively only improved scores for patients still in remission.	-
Nordin *et al*[19], 2002	Percentage of population divided on HADS depression subscales: 87% "non-cases"; 9% "doubtful cases"; 4% cases Subgroup analysis of depression: unpaired *t*-test showed no difference between CD and UC patients with ileostomies and those without ileostomies.	Percentage of population divided on HADS anxiety subscale: 71% "non-cases"; 14% "doubtful cases"; 15% cases. Subgroup analysis of anxiety: unpaired t-test showed no difference between CD and UC patients with ileostomies and those without ileostomies.
Knowles *et al*[14], 2013	Percentage of population divided on HADS depression subscales: 84% normal; 6% mild; 10% moderate; 0% severe	Percentage of population divided on HADS anxiety subscale: 50% normal; 24% mild; 16% moderate; 10% severe.
Knowles *et al*[15], 2013	Percentage of population divided on HADS depression subscales: 58% normal; 26% mild; 16% moderate-severe	Percentage of population divided on HADS anxiety subscale: 51% normal; 39% mild; 10% moderate-severe
Häuser *et al*[20], 2005	Student's t-test: no increased probable (HADS ⩾ 11) mental disorder in UC with IPAA *vs* the general German population. Wilcoxon Mann-Whitney test: no difference in HADS depression subscales between UC patients with IPAA† compared to UC without IPAA.	Student's *t*-test: no increased probable (HADS ⩾ 11) mental disorder in UC with IPAA *vs* the general German population. Wilcoxon Mann-Whitney test: no difference on HADS anxiety subscale between UC patients with IPAA compared to UC without IPAA.
Schmidt *et al*[21], 2007	Kruskal-Wallis test showed no significant difference in HADS depression subscales between IPAA subgroups	Kruskal-Wallis test showed no significant difference on HADS anxiety subscale between IPAA† subgroups
Makkar *et al*[22], 2015	ANOVA: Significant difference between DASS among patients with irritable pouch syndrome (11.7 ± 9.7), pouch inflammation (8.1 ± 9.1) and normal pouch (4.4 ± 6.2), P =0.012.	ANOVA: no significant difference between DASS among patients with irritable pouch syndrome (8.1 ± 7.0), pouch inflammation (6.0 ± 6.8), and normal pouch (4.2 ± 4.9), P = 0.1
de Oca *et al*[23], 2003	-	Student's *t*-test: CD < UC on anxiety values of the STAI (P = 0.014)

OR: Odds ratio; ANOVA: Analyses of variance; HADS: Hospital Anxiety and Depression Scale; DASS: Depression, Anxiety and Stress Scale; STAI: State-Trait Anxiety Inventory; IPAA: Ileal Pouch-Anal Anastomosis; CD: Crohn's disease; UC: Ulcerative colitis.

IBD-related surgery (Table 2). In one study, it was shown that IBD-related surgery was not significantly associated with development of anxiety[16]. Another study found a quite strong association between history of surgery and a decrease of anxiety (OR = 0.47, 95%CI: 0.31-0.71, P < 0.0001)[3].

Three studies used HADS (defined by cases scoring ⩾ 11) to describe anxiety prevalence after IBD-related surgery and found 10%-26%[14,15,19]. The highest prevalence was found in a mixed IBD cohort having stoma surgery. The remarkably lower incident in the CD stoma cohort could indicate that anxiety problems are greater among patients with UC. Yet, the studies are heterogeneous and precautions need to be paid before giving any conclusions.

One study compared colorectal cancer patients with colostomies and patients with UC and ileostomy[17]. The patients with UC scored highest on Rorschach content interpretation for anxiety.

Four studies investigated development of anxiety following IPAA[20-23]. Three studies found no difference in anxiety scores between IPAA and non-IPAA treated patients or subgroups of IPAA treated patients[20-22]. A study with 100 patients with UC and 12 patients with CD showed significantly lower levels of anxiety in the group with CD[23]. Unfortunately, it was not stated if the STAI score for the UC group was high enough to indicate possible anxiety disorder.

Taken together, the only study comparing with other surgical patients found that patients with UC had higher risk of anxiety after surgery compared with colorectal patients. There was no difference in anxiety prevalence between patients with UC having IPAA and non-surgical patients with UC. Also, patients with UC seems to be more prone to anxiety than patients with CD.

Independent predictors of depression and anxiety following surgery

Independent predictors of depression in patients with

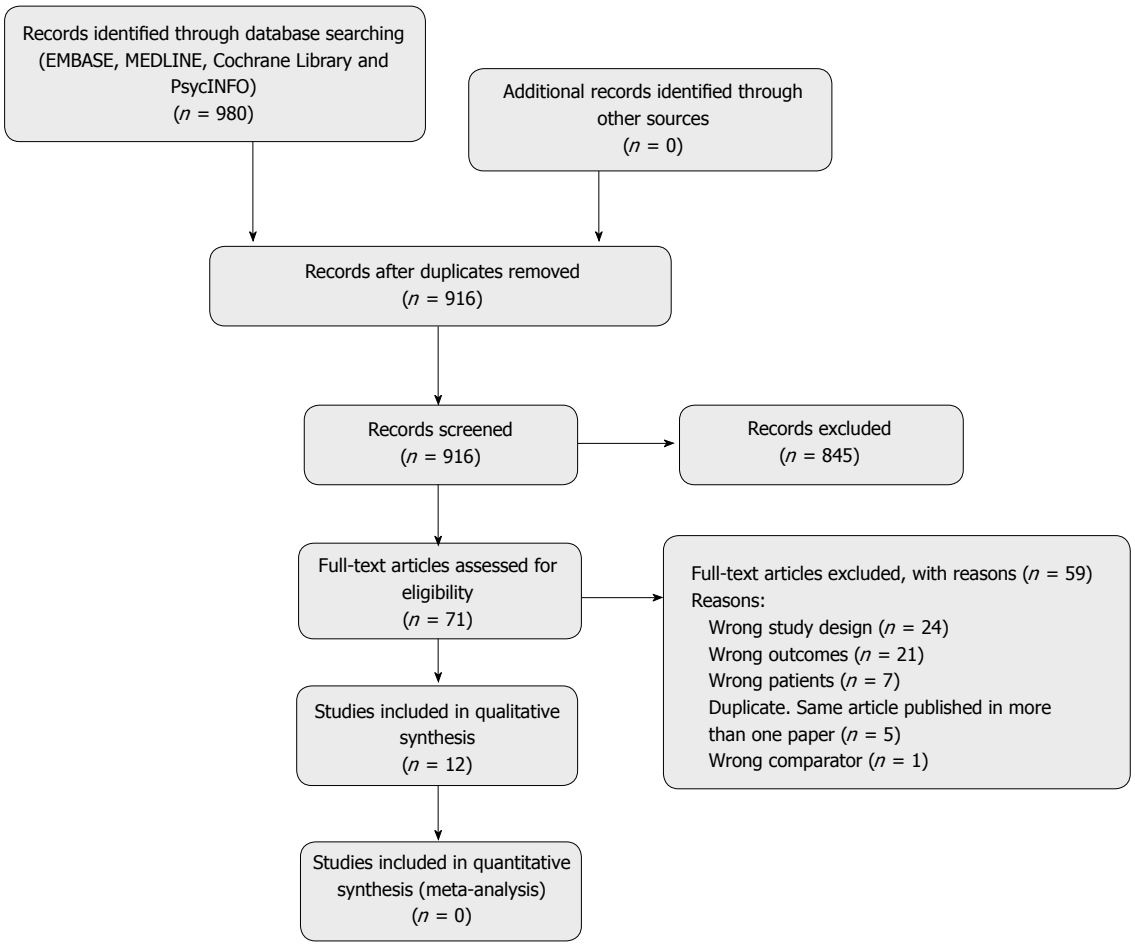

Figure 1 PRISMA flowchart.

CD were identified using multivariate analyses[16]. The independent predictors were: Charlson comorbidity score three or more (OR = 4.3, 95%CI: 2.82-6.57), stoma surgery (OR = 1.90, 95%CI: 1.15-3.13), female gender (OR = 1.77, 95%CI: 1.16-2.71), perianal disease (OR = 1.64, 95%CI: 1.01-2.69), immunomodulatory use (OR = 1.56, 95%CI: 1.03-2.38), and surgery within three years (OR = 1.54, 95%CI: 1.01-2.37). For patients with UC only Charlson score three or more (OR = 3.73, 95%CI = 2.33-5.97) and female gender (OR = 2.92, 95%CI: 1.80-4.76) were identified as risk factors for depression[16].

The same multivariate analysis was performed for development of anxiety in the same two populations. In patients with CD, the following factors were identified: surgery within three years (OR = 2.19, 95%CI: 1.44-3.33), female gender (OR = 2.07, 95%CI: 1.35-3.19), Charlson comorbidity score three or more (OR = 1.84, 95%CI: 1.19-2.84), two surgeries (OR = 1.79, 95%CI: 1.09-2.93), and stoma surgery (OR = 1.73, 95%CI: 1.05-2.85). Again, for patients with UC, only Charlson score (OR = 3.26, 95%CI: 1.98-5.38) and female gender (OR = 1.84, 95%CI: 1.18-2.87) was identified as independent risk factors[16]. The multivariate analysis showed no significant correlation between age at surgery and depression or anxiety after surgery in IBD patients.

It seems that patients with IPAA who develop irritable pouch syndrome have higher risk of depressive symptoms but not anxiety symptoms[22].

DISCUSSION

We found evidence that patients with IBD have higher post-operative risk of depression compared with patients operated for diverticulitis and inguinal hernia[16]. Yet, looking at patients with UC separately, they scored lower on Beck depression inventory after stoma surgery compared with colorectal cancer patients, which might be related to the fact that surgery is curative for UC[17]. In terms of anxiety, patients with UC and an ileostomy scored higher on Rorschach content interpretation compared with patients with colorectal cancer and a colostomy[17]. In the comparison of patients with IBD with other disease populations, there is a risk that the results simply reflect the difference between the disease groups and not the effect of surgery on the different diseases. This is likely sine earlier studies have shown a higher risk in patients with IBD in general compared with other diseases[2].

Comparing patients with IBD undergoing surgery

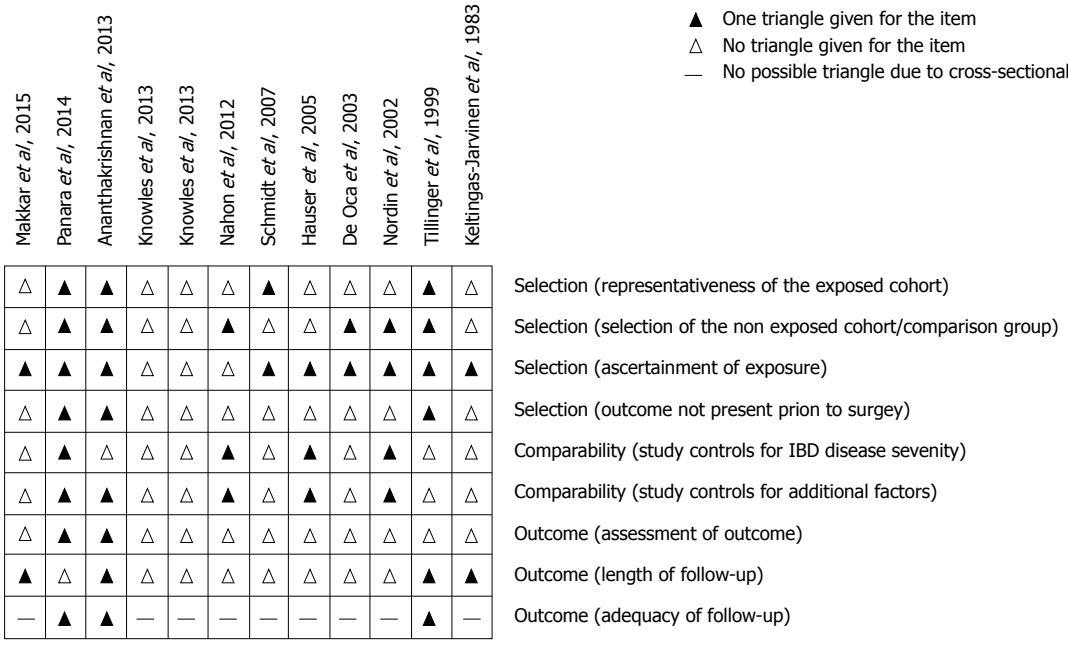

Figure 2 Quality assessment.

with non-surgical IBD cases we only found studies assessing specifically CD or UC. Some studies indicate that patients with CD have higher risk of depression while UC might have higher risk of anxiety. The multiple surgical interventions in CD *vs* curative nature of surgery in UC might be an explanation, while worries about permanent stoma may lead to anxiety in UC. Also, we know very little about identifying patients needing extra attention to reduce the incidence of psychiatric comorbidity after surgery for IBD. One study showed that predictors of both anxiety and depression in patients after IBD-related surgery are female gender and comorbidity. This is not surprising, since more women than men suffer from depression and anxiety in general[24,25]. Also, comorbidity and disability is associated with anxiety and depression in the general population[25]. Age at surgery was not an independent risk factor, emphasizing that the awareness on psychological impact of surgery is important in all age groups.

There is strong evidence that increased incidence of psychiatric disorders among patients with IBD is strongly correlated to disease activity[2]. Few of the included studies adjusted for this possible confounding factor.

The limitations of this review were mainly related to the non-randomized study designs and the heterogeneity of the included studies. Differences in methodology and outcome reporting makes the generalization to the broad surgical IBD population very difficult and interpretation of the results need to be precautious.

The bulk of the included studies were cross-sectional studies and different types of bias can be suspected. The risk that different patients have different likeability to answer questionnaires raised concerns regarding nonresponse bias. It can be hypothesized that patients with greater psychological difficulties will be less likely to return questionnaires due to lack of psychological capacity to do so. This could underestimate the incidence of psychiatric comorbidity. On the other hand, patients with psychiatric problems could be more motivated to return questionnaires assessing this matter. If this was the case, the incidence in the included observational studies could be overestimated. In observational studies with questionnaires there is a risk of recall bias. It could be, that patients who already have an outcome, in this case psychiatric disorders, would report differently about the risk factors they have had in the past. Also, there is a risk of detection bias due to different rating scales and outcome parameters. For risk of bias across studies, the proportion of information from studies of high risk of bias is sufficient to affect the interpretation of the results. A big problem with cross-sectional studies is the question of causality. Because the risk factors and the outcomes are assessed at the same point in time, it is difficult to know if the risk factors actually preceded the outcomes. In cross sectional studies using questionnaires, no outcome could be assessed prior to the intervention to make sure that any psychiatric morbidity wasn't present prior to surgery.

Many of the included studies lacked a control group, which made it difficult to answer our study questions. We need analyses of both CD and UC within the same population using the same scores and comparing with other diseases treated surgically, plus non-surgical IBD cases. Measuring *e.g*, HADS before and after surgical intervention in a prospective manner would create

representative results.

In conclusion, the review cannot give any clear answer to the risks of psychiatric morbidity after surgery for IBD. Studies with the lowest risk of bias show increased risk of depression among surgical patients with CD and increased risk of anxiety for patients with UC. Among patients planning to undergo IBD-related surgery, females and those with comorbidities need extra attention to prevent the development of psychiatric disorders.

ARTICLE HIGHLIGHTS

Research background

Previous research has shown a high incidence of psychiatric disorders among patients with inflammatory bowel disease (IBD), especially those with active disease this may lead to personal burden and prohibitive costs for the society.

Research motivation

In patients with IBD might have a higher risk for postoperative psychiatric disorders compared with other patients undergoing same type of surgery. This risk may simply reflect the difference between the disease groups and not the effect of surgery on the different diseases.

Research objectives

The aim of this review was to examine the evidence about psychiatric morbidity after IBD-related surgery.

Research methods

This is a systemic review which adheres to PRISMA guidelines. Research question and protocol were published at PROSPERO (CRD42016037600). Inclusion criteria were studies describing patients with inflammatory bowel disease undergoing surgery and their risk of developing psychiatric disorder. Studies describing psychiatric disorders prior to surgical intervention and studies exclusively reporting quality of life or single psychological symptoms as part of larger questionnaires not assessing psychiatric morbidity were excluded.

Research results

Patients with IBD have higher risk of depression after surgery compared with patients operated for diverticulitis or inguinal hernia but not cancer. Patients with ulcerative colitis (UC) might have higher risk of anxiety after surgery compared with patients with colorectal cancer. Compared with nonsurgical patients, patients operated for UC have higher risk of anxiety and patients operated for Crohn's disease have higher risk of depression. Among patients with IBD, female gender and Charlson comorbidity score > 3 are risk factors for both anxiety and depression following surgery.

Research conclusions

Patients with IBD have higher postoperative risk for anxiety and/or depression.

Research perspectives

Large multi-center prospective studies are warranted to show and quantify the risk of postoperative psychiatric disorders in patients with IBD.

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CASE REPORT

Stricturing Crohn's disease-like colitis in a patient treated with belatacept

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Abstract

Cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) modifying agents have been involved in the development of intestinal inflammation, especially therapeutic

monoclonal antibodies directed against CTLA-4. Here we report the appearance of a severe stricturing Crohn's disease-like colitis in a patient with a kidney allograft who was treated with belatacept, a recombinant CTLA-4-Ig fusion protein.

Key words: Crohn's disease; Colitis; Belatacept; HHV-6; Inflammatory bowel disease

Core tip: Belatacept is a cytotoxic T-lymphocyte-associated antigen 4 Ig fusion protein used for kidney transplant rejection prophylaxis. We report the appearance of a severe stricturing Crohn's disease-like colitis in a patient who was treated with belatacept. After belatacept withdrawal, complete mucosal healing was observed with the persistence of a non-ulcerated left-sided colonic stricture which did not allow passage of the colonoscope. So, in patients treated with belatacept who develop digestive symptoms such as diarrhea or intestinal bleeding, we recommend performing early colonoscopy and considering belatacept withdrawal in case of suggestive endoscopic and histologic findings in order to avoid colonic sequela.

Bozon A, Jeantet G, Rivière B, Funakoshi N, Dufour G, Combes R, Valats JC, Delmas S, Serre JE, Bismuth M, Ramos J, Le Quintrec M, Blanc P, Pineton de Chambrun G. Stricturing Crohn's disease-like colitis in a patient treated with belatacept. *World J Gastroenterol* 2017; 23(48): 8660-8665 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i48/8660.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i48.8660

INTRODUCTION

Belatacept is a cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) Ig fusion protein mostly used for kidney transplant rejection prophylaxis, in association with steroids and mycophenolate mofetil[1]. Belatacept selectively inhibits T cell activation and was recently demonstrated to be superior to cyclosporin in patients with renal transplants in terms of renal function with equivalent survival[2]. Frequent adverse events associated with belatacept are anemia, disturbance of bowel habits and infections, especially urinary tract infections, with no need to stop treatment in most of cases. Here, we report a case of severe stricturing Crohn's disease (CD)-like colitis due to belatacept administration in a renal transplant recipient.

CASE REPORT

We report the case of a 62-year-old man who received a first kidney allograft from a deceased donor in September 2013. His end-stage renal disease was attributed to chronic glomerulonephritis. After an induction treatment by thymoglobulin and methylprednisolone pulses, the immunosuppression regimen consisted of tacrolimus, mycophenolate mofetil and steroids. There was no immediate complication after transplantation and the nadir of serum creatinin was 2.24 mg/dL. Rapidly, after two months, tacrolimus was withdrawn due to nephrotoxicity (histologically proven) and replaced with everolimus which was also stopped due to development of lymphocele and proteinuria. In February 2014, belatacept was started at a dose of 5 mg/kg intravenously every month in association with mycophenolate mofetil in order to decrease corticosteroids to the level of 10 mg/d.

From the start of mycophenolate mofetil treatment, the patient had anorexia and diarrhea, with liquid stools without blood. He underwent in March 2014 an upper gastrointestinal endoscopy and a colonoscopy which showed no mucosal abnormalities. Duodenal biopsies demonstrated normal mucosal histology. In October 2015, because of worsening of the diarrhea, a stool culture was performed which was positive for *Campylobacter jejuni*. Antibiotics course was prescribed with some efficacy but the diarrhea persisted.

In February 2016, the patient was hospitalized for bloody stools with anemia and abdominal pain for which blood transfusion was necessary. A colonoscopy was rapidly performed showing large round shaped deep ulcers with normal surrounding mucosa. These ulcers were located in the caecum, transverse colon, left colon and sigmoid colon and were compatible with the diagnosis of CD (Figure 1). The terminal ileum and rectum were normal. Histologic examination of the colonic biopsies showed acute colitis with ulcerations, crypt abscesses, lymphocytes and neutrophil polymorphonuclear leukocyte infiltration. Neither crypt dystrophy nor granuloma was found (Figure 2). A small bowel wireless capsule endoscopy was also performed without mucosal abnormalities.

Due to suspicion of mycophenolate mofetil involvement in the acute colitis, this treatment was withdrawn in March 2016, but belatacept was pursued with an increase of steroid therapy to 20 mg/d. A follow-up colonoscopy was performed in June 2016 which showed persistence of the large ulcers previously described and the appearance of a passable ulcerated inflammatory stricture at the left colonic flexure (Figure 1). Histologic examination showed acute colitis without signs of chronic inflammation (Figure 2). No signs of cytomegalovirus colitis were found on histology, such as owl's eye inclusion bodies. Polymerase chain reaction (PCR) analysis of colonic biopsies was positive for human herpes virus 6 (HHV-6) and negative for cytomegalovirus and herpes simplex virus. Also, PCR analysis for cytomegalovirus in the blood was negative. Belatacept was therefore withdrawn in June 2016 and the patient was treated with low dose tacrolimus for

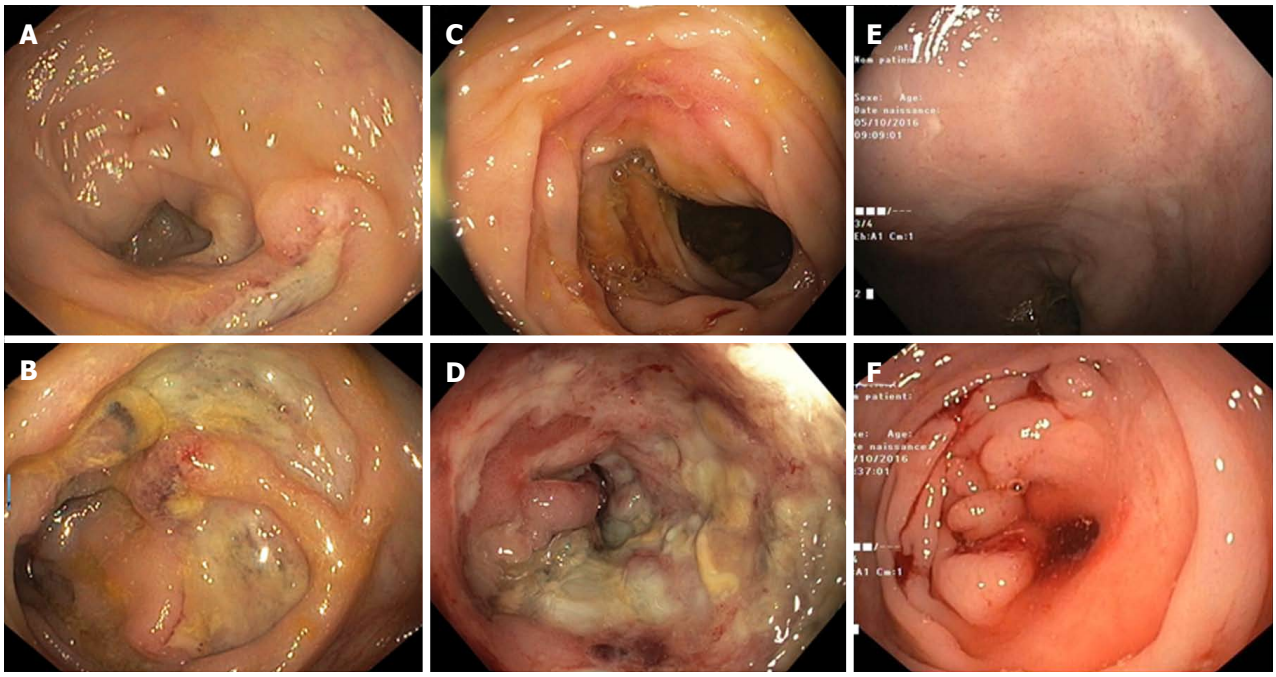

Figure 1 Endoscopic finding before and after withdrawal of belatacept. The first colonoscopy showed the presence of disseminated ulcers on the colonic mucosa (A) with more severe lesions at the left colonic flexure (B). The patient was still on mycophenolate mofetil and belatacept. After mycophenolate withdrawal and continuation of belatacept, the second colonoscopy showed the persistence of disseminated ulcerations (C) and a worsening of lesions at the left colonic flexure with appearance of an inflammatory ulcerated stricture (D). Five months after belatacept withdrawal, follow up colonoscopy showed complete healing of disseminated ulcerations in the left colon (transverse and right colon were not visualized due to the stricture, (E) and healing of the left colonic flexure with persistence of a non-inflammatory stenosis (F) which did not allow passage of the colonoscope.

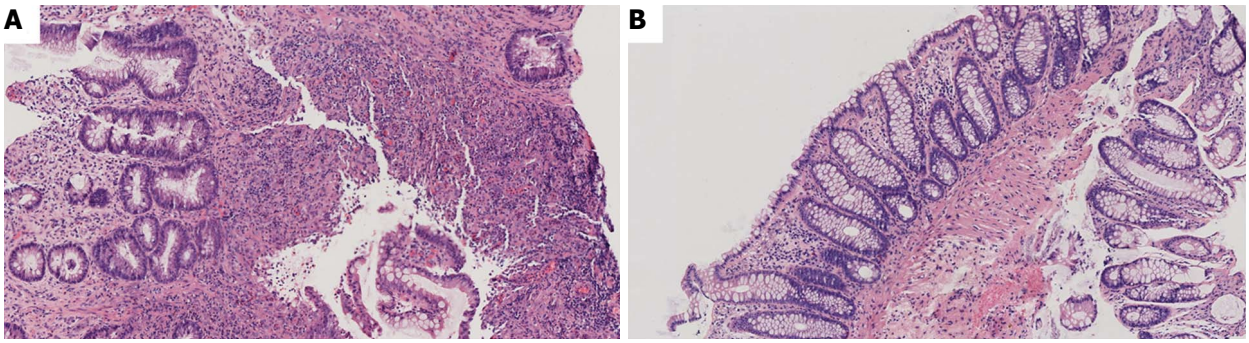

Figure 2 Histologic findings of belatacept-induced colitis. A: Histologic examination of colonic biopsies showed acute colitis with ulcerations, crypt abscesses, lymphocytes and neutrophil polymorphonuclear leukocyte infiltration. Neither crypt dystrophy nor granuloma was found; B: After belatacept withdrawal, colonic biopsies showed complete healing of the mucosa with no signs of chronic mucosal inflammation.

prevention of allograft rejection and an increased dose of steroids to treat colonic inflammation. In October 2016, the patient was free from diarrhea but described left-sided abdominal pain with partial obstructive symptoms probably due to the colonic stricture. The colonoscopy performed four months after belatacept withdrawal showed a complete healing of the ulcers in the left colon and a non-inflammatory stricture of the left colonic flexure which could not be passed (Figure 1). The stricture was not dilated as clinical symptoms were mild. PCR for HHV-6 was negative on colonic biopsies and only slightly positive on biopsies targeted to the colonic stricture. At the last clinical outpatient visit in January 2017 the patient was asymptomatic with no signs of acute renal rejection.

DISCUSSION

We have described here for the first time the case of a severe stricturing CD-like colitis occurring in a patient with a kidney allograft treated with belatacept. Gastrointestinal side effects are well known in renal transplant recipients receiving immunosuppressive therapy, especially mycophenolate mofetil (MMF). Indeed, MMF has multiple side effects and those affecting the gastrointestinal tract mostly occur during the first 6 mo after the onset of treatment[3]. These side effects include nausea, vomiting, abdominal pain,

Table 1 Case reports of colitis induced by cytotoxic T-lymphocyte-associated antigen 4-Ig fusion proteins

	Type of CTLA-4-Ig fusion protein	Delay between CTLA-4-Ig introduction and colitis (mo)	Endoscopic findings	Histological findings	CTLA-4-Ig withdrawal	Colitis treatment	Evolution of the colitis
Amezcua-Guerra *et al*[7] *Gut* 2005	Abatacept	15	UC-like colitis	Lymphoplasmocytic infiltration/cryptitis/ Intraluminal abscesses	Yes	Mesalazine	Clinical remission
Motohashi *et al*[8] Case 1 *Scand J Gastroenterol* 2014	Abatacept	25	UC-like colitis	Neutrophil infiltration/crypt abscesses	Yes	Infliximab + Mesalazine	NA
Motohashi *et al*[8] Case 2 *Scand J Gastroenterol* 2014	Abatacept	5	UC-like colitis	Neutrophil infiltration/crypt abscesses	Yes	Mesalazine + Prednisolone + Granulocytapheresis	NA
Present case	Belatacept	23	CD-like colitis	Ulcerations/crypt abscesses/ lymphocytes and neutrophil infiltration	Yes	Prednisolone	Clinical and endoscopic remission

CD: Crohn's disease; CTLA-4: Cytotoxic T-lymphocyte-associated antigen 4; NA: Not available; UC: Ulcerative colitis.

and diarrhea, whereas bleeding is less reported. A recent retrospective study investigated endoscopic findings in patients treated with MMF having diarrhea. In most of the cases the colonic mucosa was normal and the common lesion was simple erythema without deep ulcer or stricture[3]. Our patient had diarrhea after introduction of MMF with liquid stools but no bleeding, and this diarrhea improved after MMF withdrawal. Although acute colitis was first thought to be due to MMF, appearance of bleeding, large ulcers without erythema at colonoscopy and worsening of endoscopic lesions after MMF withdrawal led us to suspect the involvement of belatacept.

Ipilimumab and tremelimumab, two therapeutic monoclonal antibodies against CTLA-4 and prescribed in cancer patients, have previously been implicated in the development of severe and extensive forms of inflammatory bowel disease with colonic ulcerations[4]. CTLA-4 is a homologue of CD28 that binds CD80 and CD86 with higher affinity, and thereby down-regulates T cell activation. Anti CTLA-4 monoclonal antibodies block its interaction with CD80 and CD86 and favour CD28 engagement and consequently T cell activation and proliferation. The overactivation of the immune system in patients treated with anti CTLA-4 antibodies associated with a specific gut microflora may explain the development of treatment-mediated CD-like colitis[5]. Abatacept and belatacept are two recombinant fusion proteins comprising a fragment of the Fc domain of human IgG1 and the extracellular domain of human CTLAA-4. Similar to CTLA-4, abatacept and belatacept compete with CD28 for CD80 and CD86 binding to block co-stimulatory signaling, thus selectively modulating T-cell activation. In comparison to abatacept, belatacept confers higher affinity for CD 80/86 ligands and has a slower dissociation rates. It could also alter regulatory T cell development, which plays an important part in intestinal inflammation. Abatacept is effective for rheumatoid arthritis and juvenile idiopathic arthritis[6], and belatacept is currently used for kidney transplant rejection prophylaxis[1]. It has been showed that abatacept was not effective for the treatment of active ulcerative colitis and CD[6]. It may be surprising that belatacept induces CD-like colitis given it has the opposite effect from anti-CTLA-4 antibodies. However, cases of colitis have been also described in patients treated with abatacept (Table 1). A first case of ulcerative colitis was reported in 2006 in a 55 year old male patient treated with abatacept for rheumatoid arthritis[7]. The diagnosis of ulcerative colitis was made 15 mo after start of abatacept and digestive symptoms improved after abatacept withdrawal and mesalamine treatment. Two other cases of ulcerative colitis developing five and 23 mo after abatacept introduction in rheumatoid arthritis patients were reported. The first one was treated with mesalamine and infliximab and the second one with prednisolone and mesalamine[8]. Similarly to these case reports, the severe colitis occurred 23 mo after belatacept introduction in our patient. Macroscopically, the endoscopic lesions were more in favor of CD compared to abatacept-induced colitis, and the large deep ulcerations were similar to anti CTLA-4 enterocolitis. The histological findings in our patients described acute colitis with polymorph leucocyte infiltration and crypt abscesses without atrophy, distortion, branching or budding of crypts. These findings were also described in abatacept and anti CTLA-4 colitis. The most striking finding in our case is the development on belatacept treatment of an inflammatory stricture of the left colon. After belatacept withdrawal and prednisolone treatment, we observed complete healing of colonic lesions, but with persistence of a non-inflammatory colonic stricture which could be passed.

Although belatacept seems to be involved in the development of colitis in our patient, the exact mechanisms of this colitis are unclear. It may be a direct effect of belatacept, which could alter the development of regulatory T cells, and therefore lead to uncontrolled intestinal inflammation. Another hypothesis may be an

indirect effect of belatacept which confers a profound immunosuppression leading to the development of infectious colitis. Cytomegalovirus colitis was ruled out by careful histologic examination and negative PCR analyses in the blood and in colonic biopsies. PCR in biopsies was however, strongly positive for HHV-6. HHV-6 reactivation in patients with solid organ or hematopoietic stem cell transplantation has been reported to be associated with intestinal disease[9,10]. Moreover, HHV-6 was found in colonic mucosa of inflammatory bowel disease patients in 44% of the cases and associated with disease activity and use of immunosuppressive therapy[11]. HHV-6 intensity also correlated with endoscopic severity in ulcerative colitis. After belatacept withdrawal and mucosal healing, PCR for HHV-6 in colonic biopsies was found to be negative or slightly positive in our patient.

Thus, we report here a case of CD-like colitis in a patient treated with belatacept. Despite belatacept withdrawal, the patient developed a severe colonic stricture which may impact quality of life and necessitate subsequent colonic surveillance. Therefore in patients treated with belatacept who develop digestive symptoms such as diarrhea or intestinal bleeding, we recommend performing early colonoscopy and considering belatacept withdrawal in case of suggestive endoscopic and histologic findings.

COMMENTS

Case characteristics

A 62-year-old man with kidney allograft treated with belatacept and mycophenolate mofetil presented a diarrhea with rectal bleeding and abdominal pain.

Clinical diagnosis

Abdominal tenderness associated to liquid stools and rectal bleeding.

Differential diagnosis

Diarrhea associated to mycophenolate mofetil, viral enterocolitis, bacterial enterocolitis, Crohn's disease or ulcerative colitis.

Laboratory diagnosis

Normal stool cultures and blood tests ruled out opportunistic infections.

Imaging diagnosis

Colonoscopy showed large colonic ulcers with normal surrounding mucosa disseminated along the colonic tract and a passable ulcerated inflammatory stricture at the left colonic flexure.

Pathological diagnosis

Histologic examination of the colonic biopsies showed acute colitis with ulcerations, crypt abscesses, lymphocytes and neutrophil polymorphonuclear leukocyte infiltration. Neither crypt dystrophy nor granuloma was found. No signs of cytomegalovirus colitis were found on histology, such as owl's eye inclusion bodies.

Treatment

Withdrawal of belatacept and corticosteroid therapy.

Related reports

Previous cases of colitis in patients treated with abatacept, another Cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) Ig fusion protein, have been also described.

Term explanation

Drug-induced colitis is described with numerous agents, especially mycophenolate mofetil or antibodies against CTLA-4. Pathophysiological mechanisms are not fully understood. Endoscopic and histologic findings are not specific showing acute colitis and withdrawal of the drug which leads to complete resolution in most of the cases, confirms the diagnosis.

Experiences and lessons

In patients treated with belatacept who develop digestive symptoms such as diarrhea or intestinal bleeding, early colonoscopy should be performed and belatacept withdrawal should be considered in case of suggestive endoscopic and histologic findings in order to avoid colonic sequela.

Peer-review

The report has high novelty, clinically important information, which is relevant in therapeutic settings.

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CASE REPORT

Emphysematous pancreatitis associated with penetrating duodenal ulcer

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Abstract

In the "proton pump inhibitors era", a penetrating peptic ulcer (PPU) represents an exceptional cause of abdominal pain, and was more frequently observed in the past where there was not an effective antacid treatment. Ulcer-induced pancreatitis is very rare, too, and manifests with persistent, intense pain radiating to the back. A mild to severe pancreatitis with peripancreatic fluid collection can be observed at imaging. However, only a few cases of association between PPU and emphysematous pancreatitis (EP) have been published in the literature. EP is a rare but potentially fatal form of acute necrotizing pancreatitis in which gas grows in and outside the pancreas, and typically involves the whole parenchyma in diabetic individuals.

Here we report an extremely rare case of a duodenal ulcer penetrating the pancreas and complicated with EP.

Unlike the classic form of EP, which involves the whole parenchyma and has a poor prognosis, we found that the emphysematous involvement of the pancreas by PPU has a benign course if a conservative therapy is promptly established. Gas is confined to the site of penetration, usually the pancreatic head, and ulcers most often involve the duodenum.

Key words: Acute; Pancreatitis; Emergency department; Gas-forming bacteria

Core tip: Penetrating peptic ulcers (PPU) represent an extremely rare cause of abdominal pain, and can sometimes manifest with mild to severe acute pancreatitis. However, only a few cases of association between PPU and emphysematous pancreatitis (EP) have been published so far in the literature. Here we report an extremely rare case of a duodenal ulcer penetrating the pancreas and complicated with EP. Unlike the classic form of EP, which involves the whole parenchyma and has a poor prognosis, we found that the focal emphysematous involvement of the pancreas by PPU has a benign clinical course if an appropriate therapy is promptly established.

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INTRODUCTION

In the "proton pump inhibitors era", a penetrating peptic ulcer (PPU) represents an exceptional cause of abdominal pain, and was more frequently observed in the past where there was not an effective antacid treatment. Ulcer-induced pancreatitis is very rare too, and manifests with persistent, intense pain radiating to the back[1-3]. A mild to severe pancreatitis with peripancreatic fluid collection can be observed at imaging[4]. However, only a few cases of association between PPU and emphysematous pancreatitis (EP) have been published in the literature, and complete information regarding epidemiology are unknown. Here we report an extremely rare case of a duodenal ulcer penetrating the pancreas and complicated with EP.

CASE REPORT

A 49-year-old man presented with an acute complaint of epigastric pain radiating to the back 30 min after an excessive intake of fried, high fat food. He also reported a daily intake of nonsteroidal anti-inflammatory drugs for low back pain for over a year. There was no history of alcohol consumption. Physical examination revealed fever (38 ℃), abdominal rigidity, tenderness and torpid peristalsis. Laboratory exams showed an increase in erythrocyte sedimentation rate (46 mm/h), c-reactive protein (27.01 mg/dL), lipase (226 U/L), and leucocytosis with neutrophilia (20.1 and 18.4 × 10^3/μL, respectively). Hemoglobin, amylase and liver tests were normal. Procalcitonin was 1.45 ng/mL. Abdominal X-ray was negative. Ultrasound (US) did not reveal gallstones or free fluids; bile ducts were not dilated. Pancreas was not evaluable due to meteorism. Blood cultures were collected. Abdominal contrast-enhanced computed tomography (CECT) showed the presence of gas within the pancreatic head, suggestive of focal EP (Figure 1A), and oedema of the duodenum (Figure 1B). There was no free air suggestive of perforation. Clinical examination, laboratory tests and imaging did not reveal signs of obstructive jaundice; there was therefore no indication for an early endoscopic retrograde cholangiopancreatography. The mild oedema of the duodenum in close relation to the damaged pancreas suggested a duodenal origin of the pancreatic gas, we therefore suspected a PPU as the cause of symptoms. Esophagogastroduodenoscopy (EGD) revealed a small duodenal ulcer with the hole at the bottom, confirming the diagnosis of PPU. The surgeon indicated conservative therapy (IV rehydration, bowel rest, proton pump inhibitors, antibiotics), and clinical, laboratory and imaging findings progressively improved. After 4 wk, symptoms resolved and we observed a clear improvement of the pancreatic damage at CECT follow-up.

DISCUSSION

EP is a rare but potentially fatal form of acute necrotizing pancreatitis in which gas grows in and outside the pancreas, and typically involves the whole parenchyma in diabetic individuals[5]. Acute pancreatitis is classified as oedematous and necrotizing, and most common causes are gallstones and alcohol use. Less frequently, metabolic causes (hypertriglyceridemia, hypercalcemia), drugs, trauma, infections, autoimmune and genetic causes can be found[6-11]. Rarely, peptic ulcers can penetrate the pancreas and cause pancreatitis, which usually manifests with mild to severe pancreatitis with peripancreatic fluid collections. In the last few years, the incidence of complicated peptic ulcers has decreased by one-half and one-third in males and females, respectively. However, complete information regarding epidemiology of association between PPU and EP is still lacking[12]. The overall mortality of EP is high, estimated around 32.8%. The presence of advanced age, hypotension, gas outside the pancreas on CT, multiorgan failure and

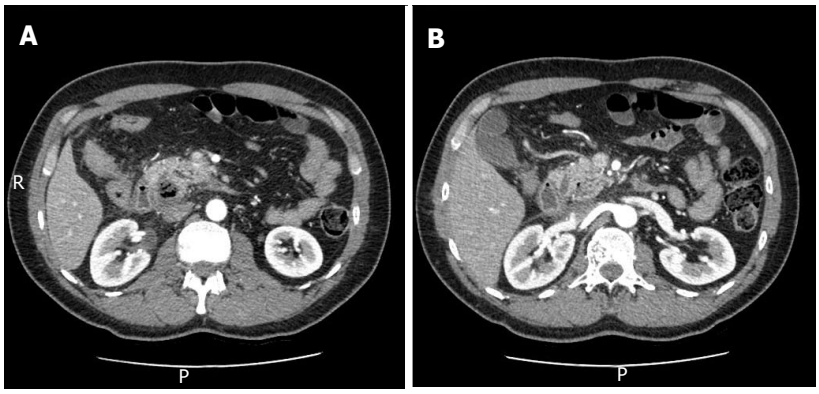

Figure 1 Abdominal contrast-enhanced computed tomography showing the presence of gas within the pancreatic head. A: Suggestive of focal emphysematous pancreatitis; B: Oedema of the duodenum. There was no free air suggestive of perforation.

initial surgical evacuation is associated with higher mortality. Therefore, less invasive approaches have been replacing surgery for their better outcome[13]. Percutaneous or endoscopic aspirations are indeed useful to drain collections and to isolate bacteria by aerobic/anaerobic cultures[5].

The clinical picture is characterized by epigastric pain, nausea, vomiting, and development of multi-organ failure in severe cases[14]. Laboratory tests are non-specific; only a slight elevation of amylase and lipase can be found, and the presence of pancreatic enzyme elevation does not correlate with the severity of the disease[14]. US evaluation can hardly detect a focal emphysematous involvement and, unlike other abdominal inflammatory processes[15-22], cannot be used alone to achieve the correct diagnosis. CECT is useful to reveal the presence and severity of gas in and outside the parenchyma[23], and gas-forming microorganisms may enter from the bowel by haematogenous and lymphatic routes, through the ampulla of Vater or by transmural passage from the adjacent colon. Endoscopy or sphincterotomy can be associated with EP, and a causal relation between EP and PPU has also been hypothesized[24]. Only a few cases have been documented so far, and gas is confined to the site of penetration, usually the pancreatic head, and ulcers most often involve the duodenum[25].

The diagnosis of PPU is made with EGD, which reveals an ulcer with the hole at the bottom[3]. Imaging, such as CECT, is useful to reveal the focal presence of gas within the pancreatic parenchyma but is less useful to reveal peptic ulcers, in particular if they are small and posterior[14,26,27]. In our case, CECT showed focal emphysema of the pancreatic head and also mild oedema of the duodenum, suggesting the intestinal origin of the gas.

Unlike the classic form of EP, which involves the whole parenchyma and has a poor prognosis, we found that the focal involvement of the pancreas by PPU has a benign clinical course if a conservative therapy is promptly established. In view of the clinical and laboratory stability of this patient and the absence of signs of perforation, we in fact preferred to treat him conservatively obtaining a clinical and imaging improvement over time, as documented by CECT follow-up.

In conclusion, the focal presence of gas within the pancreas is a rare condition which should be carefully investigated because it can be associated with an underlying condition not evident on imaging. PPU can be suspected as the cause of EP when the pancreas is involved focally without any other apparent cause of inflammation. A prompt conservative treatment is associated with a good outcome if established promptly, especially if the parenchymal involvement is mild and the patient is hemodynamically stable.

COMMENTS

Case characteristics

A 49-year-old man presented with an acute complaint of epigastric pain radiating to the back after an excessive intake of fried, high fat food and of nonsteroidal anti-inflammatory drugs for low back pain.

Clinical diagnosis

A focal emphysematous pancreatitis due to a penetrating duodenal ulcer was observed.

Differential diagnosis

Perforated ulcer.

Laboratory diagnosis

Laboratory tests are non-specific; only a slight elevation of amylase and lipase can be found, and the presence of pancreatic enzyme elevation does not correlate with the severity of the disease.

Imaging diagnosis

Contrast-enhanced computed tomography is useful to reveal the presence and severity of gas in and outside the pancreatic parenchyma.

Treatment

A prompt conservative treatment is associated with a good outcome if

established promptly, especially if the parenchymal involvement is mild and the patient is hemodynamically stable.

Related reports

Very few cases of association between penetrating ulcers and emphysematous pancreatitis have been published in the literature. This case shows that the focal presence of gas within the pancreas is a rare condition which should be carefully investigated because it can be associated with an underlying condition not evident on imaging.

Term explanation

A penetrating peptic ulcer represents an exceptional cause of abdominal pain and was more frequently observed in the past where there was not an effective antacid treatment.

Experiences and lessons

Focal emphysematous pancreatitis is a rare condition that could be associated with penetrating ulcers and has a benign clinical course if a conservative therapy is promptly established.

Peer-review

This is a nice case report on a rare case of a duodenal ulcer penetrating the pancreas and complicated with emphysematous pancreatitis. This manuscript is generally of interest. The authors provided the complete review of this issue.

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CASE REPORT

Infiltrative xanthogranulomatous cholecystitis mimicking aggressive gallbladder carcinoma: A diagnostic and therapeutic dilemma

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Abstract

Xanthogranulomatous cholecystitis (XGC) is an uncommon variant of chronic cholecystitis. The perioperative findings in aggressive cases may be indistinguishable from those of gallbladder or biliary tract carcinomas. Three patients presented mass lesions that infiltrated the hepatic hilum, provoked biliary dilatation and jaundice, and were indicative of malignancy. Surgical excision was performed following oncological principles and included extirpation of the gallbladder, extrahepatic bile duct, and hilar lymph nodes, as well as partial hepatectomy. Postoperative morbidity was minimal. Surgical pathology demonstrated XGC and absence of malignancy in all three cases. All three

patients are alive and well after years of follow-up. XGC may have such an aggressive presentation that carcinoma may only be ruled out on surgical pathology. In such cases, the best option may be radical resection following oncological principles performed by expert surgeons, in order that postoperative complications may be minimized if not avoided altogether.

Key words: Hepaticojejuostomy; Xanthogranulomatous cholecystitis; Gallbladder carcinoma; Hepatectomy; Hilar cholangiocarcinoma

Core tip: Though it is a benign disease process, xanthogranulomatous cholecystitis may have an aggressive presentation suggestive of a carcinoma of the gallbladder or biliary tract. In such cases, the best option may be surgical resection performed by expert surgeons following oncological principles, in order to cure affected patients without provoking postoperative morbidity.

Nacif LS, Hessheimer AJ, Rodríguez Gómez S, Montironi C, Fondevila C. Infiltrative xanthogranulomatous cholecystitis mimicking aggressive gallbladder carcinoma: A diagnostic and therapeutic dilemma. *World J Gastroenterol* 2017; 23(48): 8671-8678 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i48/8671.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i48.8671

INTRODUCTION

Xanthogranulomatous cholecystitis (XGC) is an uncommon variant of chronic cholecystitis characterized by focal or diffuse severe inflammatory destruction of the gallbladder. The incidence of XGC is variable and has been described among series of cholecystectomies to range between 0.6% and 10%[1]. Regarding pathogenesis, the prevailing theory holds that chronic outflow obstruction provokes mucosal ulceration and/or rupture of Rokitansky-Aschoff sinuses and extravasation of mucin and bile into subepithelial tissue. Extravasated bile provokes inflammation, and macrophages phagocytose bile lipids and cholesterol to form ceroid-laden and foamy histiocytes (xanthoma cells). The chronic phase is characterized by repair of the inflammatory reaction, resulting in fibrosis[2-6]. The inflammatory process may be severe and extend into adjacent organs, such as the liver, and fistulae may develop into surrounding hollow viscuses (namely the duodenum and transverse colon)[7].

Given the relative scarcity of the disease process and the fact that it may be difficult to differentiate from gallbladder carcinoma (GBC) based on clinical presentation and preoperative imaging, it is not uncommon that patients with XGC are taken to the operating room without a clear diagnosis. We describe three such cases in which preoperative studies and intraoperative findings were highly suggestive for malignancy, and radical resection following oncological principles was performed. In all three, surgical pathology was ultimately benign, and the postoperative courses were uneventful.

CASE REPORT

Case 1

The first patient is a 42-year-old woman with no significant past medical history who presents with loss of 6 kg over the course of two months and a two-week history of epigastric pain and jaundice. No abdominal mass is palpated on physical exam. Initial laboratory tests are significant for cholestasis, with serum bilirubin of 9.3 mg/dL. Computed tomography (CT) and magnetic resonance cholangiopancreatography (MRCP) imaging reveal a gallbladder with stones and asymmetrical malignant-appearing wall thickening and a contiguous hepatic hilar mass. The mass infiltrates hepatic segment Ⅳb as well as the common and bilateral hepatic ducts, with intrahepatic biliary dilatation (Figure 1). There is extensive contact between the mass and the right portal vein, without any apparent plane of separation. No hilar lymphadenopathy is observed. Given these imaging findings suggestive for resectable biliary tract cancer, the decision is made to perform radical surgery. Intraoperatively, a petrous lesion enveloping the gallbladder and the biliary confluence, with retrograde biliary dilatation, is observed. Right trisectionectomy with cholecystectomy and complete extirpation of the extrahepatic bile duct, hilar lymphadenectomy, and double Roux-en-Y hepaticojejunostomy is performed. The specimen is not opened, but the proximal and distal bile duct margins are sent for perioperative frozen-section analysis (negative for malignancy). The intraoperative and postoperative courses are uneventful, and the patient is discharged home on postoperative day thirteen. Pathological analysis of the surgical specimen reveals chronic cholecystitis with areas of xanthogranulomatous inflammation and absence of malignancy. With over ten years of follow-up, the patient remains well and asymptomatic.

Case 2

The second patient is a 66-year-old man with no significant past medical history that is referred to our center for suspected gallbladder versus hilar cholangiocarcinoma. The patient arrives at our emergency department with complaints of abdominal pain, fever, jaundice, acholic stools, and choluria. A left-sided external biliary drain has been placed at the referring center. The patient is cachectic and presents pain on palpation of the right upper quadrant, but

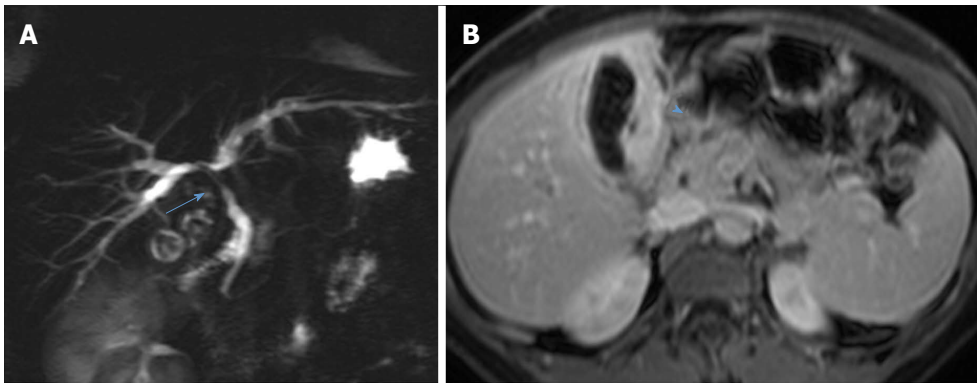

Figure 1 Preoperative magnetic resonance cholangiopancreatography from case 1 demonstrates stenosis of the common bile duct and biliary confluence (arrow, A) and retrograde biliary dilatation. The transverse section demonstrates diffuse asymmetrical gallbladder wall thickening (arrowhead, B) and contiguous hilar mass.

no mass is appreciated. Initial laboratory evaluation at our center is significant for a serum bilirubin of 3 mg/dL (status post biliary drainage) and white blood cell count of 18.5 × 10^9/L. The external biliary drain is exchanged for an internal-external biliary drain. Imaging studies, including CT and MRCP, are performed, revealing gallstones and a collapsed gallbladder, with focal malignant-appearing wall thickening. There is apparent contiguous infiltration of hepatic segment IVb, the biliary confluence, the right hepatic duct and second-order biliary radicals on the right, and the proximal and middle thirds of the common bile duct, with intrahepatic biliary dilatation. There is also focal contact with the right hepatic artery and a suspicious-appearing spiculated 1-cm hilar lymph node (Figure 2). Exploratory laparoscopy is performed to rule out peritoneal carcinomatosis and intraabdominal metastatic disease followed by laparotomy. Perioperative frozen-section analysis of the suspicious hilar lymph node is negative for malignancy. Radical surgery, including right trisectionectomy with cholecystectomy and complete extirpation of the extrahepatic bile duct, hilar lymphadenectomy, and double Roux-en-Y hepaticojejunostomy, is performed. The postoperative course is complicated by signs of mild hepatic insufficiency (grade 1-2 hepatic encephalopathy and peak serum bilirubin of 8.5 mg/dL on postoperative day 2) and self-limited bile-tinged output in the abdominal drain. The patient is ultimately discharged home on postoperative day thirteen. Pathological analysis of the surgical specimen reveals XGC, without any evidence of malignancy. With almost nine years of follow-up, the patient remains well and asymptomatic.

Case 3

The third case is a 65-year-old man with active cigarette use (one pack per day), and a personal history of arterial hypertension, glucose intolerance, and left nephrectomy over forty years prior. He presents to the emergency department with a five-day history of jaundice, choluria, and anorexia. Initial laboratory examination is remarkable for a serum bilirubin of 6.8 mg/dL. Abdominal imaging demonstrates gallstones and asymmetric gallbladder wall thickening, affecting primarily the infundibulum, with a contiguous hilar mass infiltrating bilateral hepatic ducts and contacting the right hepatic artery and portal vein, without any apparent plane of separation (Figure 3). Intrahepatic biliary dilatation was also present. Given a differential diagnosis including GBC centered at the infundibulum versus hilar cholangiocarcinoma, radical surgery is indicated. Perioperative frozen-section analysis of hilar lymphadenopathy is negative for malignancy. Ultimately, *en bloc* resection of the gallbladder, hepatic segments IVb and V, and the extrahepatic bile duct, as well as hilar lymphadenectomy and Roux-en-Y hepaticojejunostomy, is performed. The intraoperative and postoperative courses are uneventful, and the patient is discharged home on postoperative day nine. Pathological analysis of the surgical specimen reveals chronic cholecystitis with focal areas of xanthogranulomatous inflammation and absence of malignancy (Figure 4). The patient remains well after almost seven years of follow-up.

DISCUSSION

Herein, we present three cases of aggressive XGC where the preoperative studies and intraoperative findings demonstrated widely infiltrative disease processes that could only be removed by radical surgical excision. From a technical standpoint, when severe chronic inflammatory changes of XGC have extended into the hepatic hilum, resection of adjacent organs and the extrahepatic bile duct might be necessary, regardless of the ultimate diagnosis. Such radical interventions should always be performed by surgeons with appropriate expertise, in order that postoperative complications may be minimized, if not avoided altogether.

The difficulty in reaching a definitive diagnosis preoperatively in cases of aggressive XGC lies in the considerable overlap they may present with GBC. Both share peak incidences in the sixth and seventh

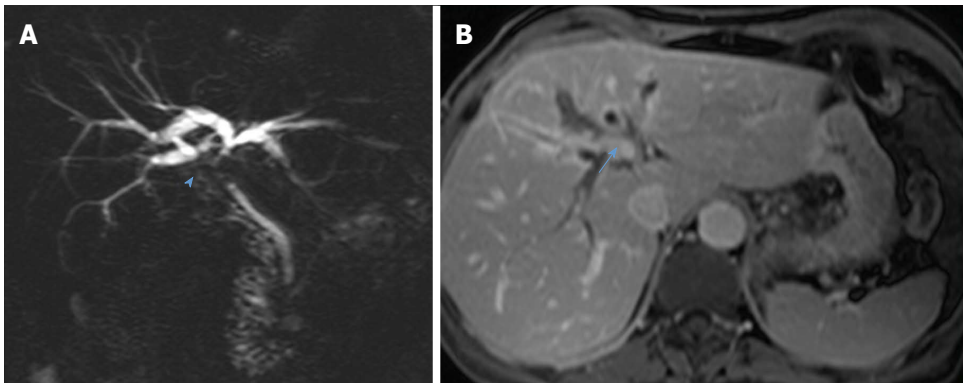

Figure 2 Preoperative magnetic resonance cholangiopancreatography from case 2 demonstrates stenosis of the proximal and middle thirds of the common bile duct, biliary confluence (arrowhead, A), and right hepatic duct and second-order biliary radicals, with retrograde biliary dilatation; a suspicious-appearing spiculated hilar lymph node is seen on transverse section (arrow, B).

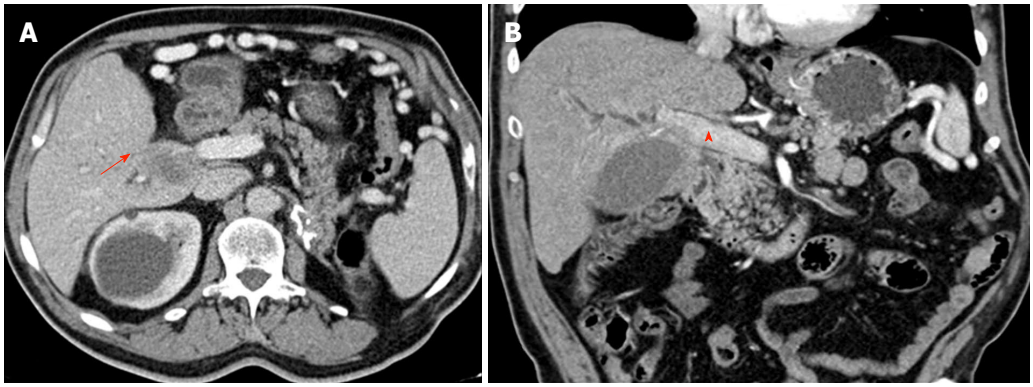

Figure 3 Preoperative CT images from case 3 demonstrating a dilated intrahepatic bile duct (arrow, A) that ends abruptly at the biliary confluence. An ill-defined hilar mass is seen infiltrating the right hepatic artery (arrow, B) and bilateral hepatic ducts and contacting focally with the portal vein (arrowhead, B).

decades of life, arise more commonly in women[8], have been associated with cholelithiasis and chronic inflammation, and present vague clinical signs and symptoms suggestive of biliary colic or acute or chronic cholecystitis[9]. Jaundice and cholestasis may be seen in both, though jaundice in the setting of GBC portends worse prognosis.

None of the three patients in our series had elevated serum tumor markers. However, in the diagnosis of patients with XGC, serum tumor markers (*e.g.*, CA-19.9) are of little utility, as they are not infrequently elevated (and in some cases extremely so)[3,9]. Also, patients who are Lewis antigen negative (10% of the Caucasian population) do not express CA-19.9.

Radiological findings in XGC may include the presence of gallstones and gallbladder wall thickening (diffuse 80%-90%, focal 10%-20%), intramural hypoattenuated nodules, and continuous mucosal line enhancement. Though typically considered characteristic of XGC, intramural nodules may also be seen in well-differentiated GBC with abundant mucin production[8]. Features more commonly associated with malignant pathology, including mass lesion, hepatic invasion, and enlarged lymph nodes, may also be seen in XGC[5,9]. While involvement of the biliary tree by the inflammatory process ("xanthogranulomatous choledochitis") may be present, intrahepatic biliary dilatation is often absent[7,8]. Findings in our cases that were indicative of potentially malignant processes include hilar mass lesions, intrahepatic biliary dilatation, and images suggestive of vascular infiltration in all three.

When the diagnosis is clear at the time of surgical intervention, simple cholecystectomy is sufficient therapy[1,5,6]. Contiguous organ involvement may necessitate performing more extensive resection, however, even when it is known preoperatively that the underlying disease process is entirely benign. The three cases presented in our series were rather complex, due to the presence of widely infiltrative hilar mass lesions with associated vascular affectation and retrograde biliary dilatation and jaundice, and the interventions that were performed were necessary to remove the masses and adequately relieve biliary obstruction. In general, the laparoscopic approach is not indicated for XGC (associated with conversion rates of up to 80%)[1], and open approaches are often used initially due to suspicion of cancer and/or the anticipation of technical difficulty.

It has been repeatedly suggested that intra-

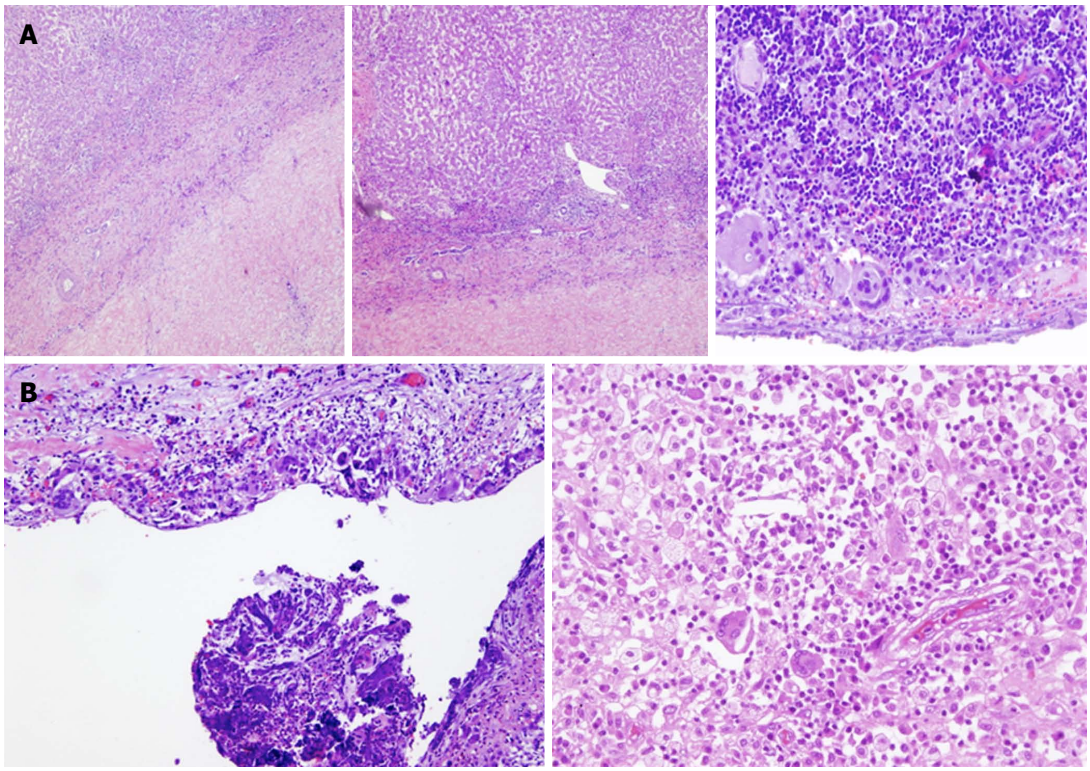

Figure 4 Histological examination of the surgical specimens from the three cases of xanthogranulomatous cholecystitis reveals findings of chronic cholecystitis and marked inflammatory infiltrate, including lymphocytes, plasma cells, foamy histiocytes, and spindle-shaped cells. A: Focal formations of pseudocysts, with multinucleated foreign-body giant cells and cholesterol clefts, are also observed; B: Hyalinization and fibrosis of the gallbladder wall reflects chronic inflammation. Typically, the xanthogranulomatous reaction occupies a limited portion of the gallbladder wall, while the remainder shows signs of conventional chronic cholecystitis. Polymorphonuclear lymphocytes, reflecting acute inflammation, are also occasionally seen. The mucosa presents focal ulceration and erosion and reactive changes that consist in papillary hyperplasia and mucinous and cardial-type glandular metaplasia. Dysplastic changes and malignant features are absent in all three cases.

operative frozen-section analysis may be useful when diagnosis is in doubt, in order to avoid an unnecessarily aggressive/"mutilating" intervention[3,4,9]. This approach is problematic, however, for a couple of reasons. GBC may co-exist with XGC in up to 31% of cases (and may actually provoke outflow obstruction or serve as an entry point for bile, lipids, etc., into subepithelial tissues)[2,4,9-13], and GBC may be missed due to sampling error when the two are present simultaneously[4,9,10,14]. Also, opening a potentially cancerous gallbladder to examine the mucosa risks cutting across tumor and disseminating malignant disease. Authors who describe doing so relate cases where surgical pathology was ultimately benign (XGC), but they typically do not describe cases operated in this manner where the ultimate diagnosis was GBC. In general, retrospective series of rare and highly selected patients that criticize the "overtreatment" of this benign disease with an oncological resection can be misleading and should be regarded with caution. In order to adequately analyze the risk for overtreatment, it is important to take into account the percentage of patients with aggressive radiological features that are ultimately diagnosis with GBC, which is the great majority[3].

Complete resection with negative margins remains the only curative treatment for patients with GBC. According to the National Comprehensive Cancer Network (NCCN) 2017 Guidelines for the management of GBC, if there is a mass on imaging suspicious for GBC, perioperative biopsy is not necessary. Also, suspicious mass lesions found during cholecystectomy should not be biopsied, as doing so might risk peritoneal dissemination. If expertise is available and there is convincing clinical evidence of cancer, definitive resection (radical cholecystectomy including segments IVb and Ⅴ, lymphadenectomy, and extended hepatectomy or biliary resection as needed to obtain negative margins) should be performed. If expertise is not available, the patient should be referred to a center/surgeon capable of performing radical/definitive resection[15].

Table 1 provides an overview of single-center series (including our own) and case reports published to date that include patients undergoing radical resection following oncological principles (associating, at a minimum, cholecystectomy with resection of hepatic segments Ⅳb and Ⅴ and hilar lymphadenectomy) for what ultimately turned out to be XGC. Among these 68 patients, the great majority (72%) presented mass lesions and almost half (47%) hepatic invasion. Postoperative outcomes were reported for 42 patients,

Table 1 Case series and reports on radical resection for xanthogranulomatous cholecystitis

Ref.	*n*	Age (yr)	M:F	Perioperative findings	Intervention	Outcome
Agarwal *et al*[12] *Gastrointest Surg*, 2013	31	50 ± 13	1:3.3	Cholelithiasis 55% Continuous mucosal line enhancement 48% GB wall thickening 19% Hepatic invasion 81% Intramural hypoattenuating nodules 42% Jaundice 7% Mass lesion 100%	Radical cholecystectomy	Postoperative mortality 3%
Rammohan *et al*[3] *Gastroenterol Res*, Pract 2014	16	56 ± 12	1:1.5	Cholelithiasis 69% Continuous mucosal line enhancement 50% GB wall thickening 37% Intramural hypoattenuating nodules 56% Jaundice 13%	Radical cholecystectomy	NR
Suzuki H, *World J Gastroenterol* 2015	6	64 ± 10	2:1	Cholelithiasis 83% Continuous mucosal line enhancement 50% GB wall thickening 50% Intramural hypoattenuating nodules 50% Jaundice 17% Retrograde biliary dilatation 17%	Radical cholecystectomy	NR
Nacif Souto L, 2017	3	65 (42-66)	2:1	Cholelithiasis 100% Continuous mucosal line enhancement 100% GB wall thickening 100% Hepatic invasion 67% Intramural hypoattenuating nodules 33% Jaundice 100% Mass lesion 67% Retrograde biliary dilatation 100%	Cholecystectomy + right trisectionectomy + CBD excision + hilar lymphadenectomy + double hepaticojejunostomy (n = 2), radical cholecystectomy + CBD excision + hilar lymphadenectomy + hepaticojejunostomy (n = 1)	Asymptomatic after ⩾ 6 yr f/u
Krishna R, *J Gastrointest Surg* 2008[7]	3	55 (48-56)	2:1	Cholelithiasis 100% GB wall thickening 100% Jaundice 100% Mass lesion 33%	Cholecystectomy + CBD excision + hepaticojejunostomy (n = 1), right hepatectomy + CBD excision	Asymptomatic after ⩾ 1 yr f/u
Enomoto T, *Hepato-gastroenterology* 2003	1	64	M	Hepatic invasion, jaundice, mass lesion, retrograde biliary dilatation	Cholecystectomy + right hepatectomy + Whipple's procedure	NR
Garg P, J *Gastrointest Canc* 2014	1	32	F	Hepatic invasion, jaundice, mass lesion, retrograde biliary dilatation	Radical cholecystectomy + CBD excision + hepaticojejunostomy	Asymptomatic
Goldar-Najafi A, *Semin Liver Dis* 2003	1	45	M	Cholelithiasis, GB wall thickening, jaundice, retrograde biliary dilatation	Whipple's procedure	NR
Kawate S, *World J Gastroenterol* 2006	1	34	F	Jaundice, mass lesion, retrograde biliary dilatation	Cholecystectomy + extended right hepatectomy + CBD excision + hepaticojejunostomy	NR
Makino I, *World J Gastroenterol* 2009	1	76	M	GB wall thickening, hepatic invasion	Radical cholecystectomy	Asymptomatic after 8 mo f/u
Martins P, *Hepatobiliary Pancreat Dis Int* 2012	1	35	M	GB wall thickening, hepatic invasion, jaundice	Cholecystectomy + left trisectionectomy + CBD excision + hilar lymphadenectomy + hepaticojejunostomy	Asymptomatic after 6 mo f/u
Pantanowitz L, *Pathol Int* 2004	1	75	F	Mass lesion, retrograde biliary dilatation	Cholecystectomy + extended left hepatectomy	NR
Sharma D, *ANZ J Surg* 2009	1	52	F	Cholelithiasis, hepatic invasion, mass lesion	Radical cholecystectomy	Uneventful postoperative course
Spinelli A, *World J Gastroenterol* 2006	1	46	F	Cholelithiasis, jaundice, mass lesion, retrograde biliary dilatation	Cholecystectomy + right hepatectomy + CBD excision + segmental duodenal resection + right hemicolectomy + partial omentectomy + hepaticojejunostomy + ileotransversostomy	Asymptomatic after 1 yr f/u
Total	68	53 ± 7	1:1.7	Cholelithiasis 62% Continuous mucosal line enhancement 43% GB wall thickening 35% hepatic invasion 47% Intramural hypoattenuating nodules 38% Jaundice 25% Mass lesion 72% Retrograde biliary dilatation 15%		Postoperative mortality 1%

Single-center series and case reports published to date in which radical resection following oncological principles was performed for what ultimately turned out to be xanthogranulomatous cholecystitis. CBD: Common bile duct; f/u: Follow-up; GB: Gallbladder; NR: Not reported.

and the majority experienced an uneventful postoperative course. There was only one postoperative death (1%).

In conclusion, thought it is ultimately a benign condition, XGC may have such an aggressive presentation that carcinoma may only be definitively ruled out on surgical pathology. Considering the implications of undertreatment when diagnosis is in doubt, the fact that both XGC and GBC may co-exist, and the fact that lesser surgery might not be technically feasible (especially when there is a mass lesion with extensive involvement of the biliary tree), the best option may be to err on the side of overtreatment. In such cases, surgical intervention should be undertaken by a skilled surgeon capable of performing radical resection and reconstruction and curing the patient of his or her disease process, with little-to-no short- or long-term secuelae.

ARTICLE HIGHLIGHTS

Case characteristics

Three patients presented with jaundice and variable other symptoms, including abdominal pain and weight loss.

Clinical diagnosis

Clinical findings were suggestive of neoplastic processes affecting directly or indirectly the biliary tree.

Differential diagnosis

Serum bilirubin was elevated in all three cases, while serum CA-19.9 levels were normal.

Laboratory diagnosis

Laboratory tests and imaging studies were performed to clarify the diagnosis.

Imaging diagnosis

Abdominal imaging studies, including CT and magnetic resonance cholangiopancreatography, demonstrated widely infiltrative hilar mass lesions with associated vascular affectation and retrograde biliary dilatation.

Pathological diagnosis

Since all three patients had aggressive yet apparently resectable lesions, surgery was undertaken without previous biopsy.

Treatment

All three interventions were performed according to oncological principles and included, at a minimum, radical cholecystectomy, common bile duct excision, hilar lymphadenectomy, and hepaticojejunostomy.

Related reports

There are a few previous reports that describe radical resection of very aggressive cases of what ultimately turned out to be xanthogranulomatous cholecystitis, and most describe little-to-no postoperative morbidity or mortality.

Term explanation

In xanthogranulomatous cholecystitis, mucin and bile are extravasated into subepithelial tissues and phagocytosed, resulting in inflammation, xanthoma formation, and processes of repair and fibrosis that, in some cases, produce pseudotumors that may be confused with malignancy.

Experiences and lessons

For clinicians confronting similar cases, we recommend direct surgical intervention performed by an experienced hepatobiliary surgeon capable of removing all diseased tissue, reconstructing the patient's anatomy, and effectively curing the patient of his or her disease process.

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