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Hypoxia, angiogenesis and liver fibrogenesis in the progression of chronic liver diseases

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Abstract

Angiogenesis is a dynamic, hypoxia-stimulated and growth factor-dependent process, and is currently referred to as the formation of new vessels from pre-existing blood vessels. Experimental and clinical studies have unequivocally reported that hepatic angiogenesis, irrespective of aetiology, occurs in conditions of chronic liver diseases (CLDs) characterized by perpetuation of cell injury and death, inflammatory response and progressive fibrogenesis. Angiogenesis and related changes in liver vascular architecture, that in turn concur to increase vascular resistance and portal hypertension and to decrease parenchymal perfusion, have been proposed to favour fibrogenic progression of the disease towards the end-point of cirrhosis. Moreover, hepatic angiogenesis has also been proposed to modulate the genesis of portal-systemic shunts and increase splanchnic blood flow, thus potentially affecting complications of cirrhosis. Hepatic angiogenesis is also crucial for the growth and progression of hepatocellular carcinoma. Recent literature has identified a number of cellular and molecular mechanisms governing the cross-talk between angiogenesis and fibrogenesis,

with a specific emphasis on the crucial role of hypoxic conditions and hepatic stellate cells, particularly when activated to the myofibroblast-like pro-fibrogenic phenotype. Experimental anti-angiogenic therapy has been proven to be effective in limiting the progression of CLDs in animal models. From a clinical point of view, anti-angiogenic therapy is currently emerging as a new pharmacologic intervention in patients with advanced fibrosis and cirrhosis.

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Key words: Chronic liver diseases; Hepatic myofibroblasts; Hypoxia; Liver angiogenesis; Liver fibrogenesis

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INTRODUCTION

Angiogenesis can be envisaged as a dynamic, hypoxia-stimulated and growth factor-dependent ubiquitous process leading to the formation of new vessels from pre-existing blood vessels^[1-5], and should be distinguished from other terms that, although related to vessel formation and growth, define significantly different processes such as vasculogenesis, arteriogenesis and collateral vessel growth. Angiogenesis occurs virtually in almost all organs and tissues and is considered a critical step in either physiological conditions or in tissue repair and growth in several pathophysiological conditions^[1-5], including chronic liver diseases (CLDs)^[6-8].

Where the liver is concerned, physiological and pathological angiogenesis can occur during liver regeneration (after acute liver injury or after partial hepatectomy), in ischemic conditions, during chronic inflammatory and fibrogenic liver diseases as well as in hepatocellular carcinoma and in metastatic liver cancers^[6-8]. The steps and mechanisms of hepatic angiogenesis mostly overlap with those described in other organs or tissues but a number of liver parenchyma peculiarities are likely to make the overall scenario more complex^[6]. These include the existence of two different kinds of microvascular structures (portal vessels and liver sinusoids, lined by continuous or fenestrated and discontinuous endothelium, respectively), the expression of a putative liver specific angiopoietin-like peptide defined as ANGPTL3^[9] and, most relevant, the unique and heterogeneous phenotypic profile and functional role of hepatic stellate cells (HSCs) that, although regarded as liver specific pericytes in normal liver, also represent the most relevant pro-fibrogenic cell lineage^[10-13] in CLDs. HSCs, particularly in their activated and myofibroblast-like phenotype (HSC/MFs), are indeed emerging as cells that may have an active role in modulating angiogenesis that differs from the one attributed to microcapillary pericytes^[14]. The overall scenario is even more complex if one considers that in CLDs hepatic myofibroblast-like cells (MFs) constitute a heterogeneous population of pro-fibrogenic cells. These highly proliferative and contractile cells may also originate from portal (myo) fibroblasts, bone marrow-derived stem cells and, as recently suggested, also from hepatocytes or cholangiocytes through a process of epithelial to mesenchymal transition^[10-13]. In the following section of this editorial we will try to focus on those relevant features that link angiogenesis to liver fibrogenesis and then the progression of CLDs. The interested reader can refer to more comprehensive reviews - such as articles with more details on the basic principles and mechanisms involved in angiogenesis as well as the analysis of the role of angiogenesis in liver regeneration or hepatocellular carcinoma^[10-14].

HEPATIC ANGIOGENESIS AND ITS RELATIONSHIPS BETWEEN CHRONIC INFLAMMATION AND FIBROGENIC PROGRESSION OF CLDs

CLDs are characterized by reiteration of liver injury due to a number of aetiological conditions, including chronic infection by viral agents [mainly hepatitis B virus (HBV) and hepatitis C virus (HCV)] as well as metabolic, toxic/drug-induced (with alcohol consumption being predominant) and autoimmune causes, resulting in persistent inflammation and progressive fibrogenesis. Chronic activation of the wound healing response is the major driving force for progressive accumulation of

extracellular matrix (ECM) components, eventually leading to liver cirrhosis and hepatic failure. Oxidative stress and redox signalling, derangement of epithelial-mesenchymal interactions or, as recently proposed, the process of epithelial to mesenchymal transition represent additional mechanisms able to sustain fibrogenesis progression towards the final end-point of cirrhosis^[11,12,15-17].

Along these lines, cirrhosis should be regarded as an advanced stage of fibrosis characterized by the formation of regenerative nodules of parenchyma, surrounded and separated by fibrotic septa, and associated with significant changes in angio-architecture. The suggestion that angiogenesis may significantly contribute to fibrogenesis and disease progression relies first on the fact that vascular remodelling, irrespective of aetiology, is a common finding in human cirrhotic livers^[11,12,14,18,19]. Moreover, the formation of fibrotic septa, as well as capillarization of sinusoids, the latter due to early deposition of fibrillar ECM in the space of Disse, can result in an increased resistance to blood flow and oxygen delivery. These are the premises for hypoxia and the transcription of hypoxia-sensitive pro-angiogenic genes, usually modulated by the so-called hypoxia inducible factors (HIFs)^[20-23]. In addition, it is well known that in CLDs the inflammatory response gains the role of a dynamic state relevant for the progression of fibrogenesis towards the end-point of cirrhosis^[11-13,16]. Several mediators of the inflammatory response may stimulate other cells in the surrounding microenvironment to express vascular endothelial growth factor (VEGF) and other pro-angiogenic factors as well as to sustain angiogenesis^[24]. Moreover, cytokines or mediators produced during CLDs such as hepatocyte growth factor (HGF), platelet-derived growth factor (PDGF) and nitric oxide (NO), can play a role in the development of angiogenesis^[6,13,14]. In particular, one should consider that: (1) neo-vessels themselves can significantly contribute to perpetuation of the inflammatory response by expressing chemokines and adhesion molecules promoting the recruitment of inflammatory cells; (2) angiogenesis, early in the course of a CLD, may contribute to the transition from acute to chronic inflammation^[25].

A relevant additional point, as recently proposed^[20], is that depending on the specific pattern of fibrosis (post-necrotic or bridging fibrosis, pericellular or perisinusoidal fibrosis, biliary fibrosis or centrilobular fibrosis)^[12,26], the extent of neo-angiogenesis, in addition to favouring disease progression, may also represent a key limiting factor for fibrosis reversibility. This is potentially relevant for post-necrotic or bridging fibrosis, a pattern which is mainly seen in patients with advanced fibrosis or cirrhosis by chronic HBV or HCV infection. In this pattern of fibrosis, which is characterized by the formation of bridging septa between portal and central vein areas, angiogenesis, vascular remodelling and altered angio-architecture are particularly impressive.

PATHOLOGICAL ANGIOGENESIS AND PRO-ANGIOGENIC CYTOKINES HAVE BEEN DETECTED IN HUMAN CLDs

Current evidence suggests that angiogenesis and fibrogenesis are detectable and develop in parallel in any clinical condition of CLDs that can progress towards the end-point of cirrhosis, irrespective of aetiology, as well as in the most widely used experimental animal models of CLDs^[6,13,19,20].

Where clinical data are concerned, best relationships between angiogenesis and the pattern of fibrosis (i.e. bridging fibrosis)^[12,26] are usually found during chronic viral infection by either HBV or HCV. This is documented by either the abundant presence of endothelial cells (ECs) and neovessels/capillary structures found in inflamed portal tracts^[6] or by the over-expression of major pro-angiogenic molecules, including VEGF and Angiopoietin 1 (Ang-1) as well as their related receptors (VEGFR type II, Tie2) and HGF^[27-31]. In these clinical settings, PDGF, which is released by periportal inflammatory cells as well as by sinusoidal and perisinusoidal cells, may also play a pro-angiogenic role^[32]. In addition, selected viral proteins may have a multiple pro-angiogenic role like HBV-related X protein^[33]. This protein has been involved in disruption of inter-endothelial junctions by operating through a src-kinase-dependent signalling pathway, as well as in the up-regulation of inducible nitric oxide synthase (iNOS) through involvement of nuclear factor- κ B (NF- κ B) transcription factor^[34] or even by up-regulating membrane-type matrix metalloprotease (MT-MMP) expression, and then MMP-2 activation in hepatocytes^[35].

Angiogenesis has also been detected in biopsies from patients affected by either primary biliary cirrhosis (PBC) or autoimmune hepatitis as formation of neovessels by ECs positive for CD-31 and vascular endothelial-cadherin^[6]. These neo-vessels were located, particularly in PBC, mainly in portal areas in association with inflammatory infiltrate^[6,36]. Once again, enhanced expression of angiogenic molecules such as VEGF, Ang-1, Ang-2, Tie-2 and endoglin has also been characterized in these PBC patients.

Similar data have been reported for the most widely used experimental animal models of CLDs that have been instrumental in unequivocally documenting that angiogenesis and fibrogenesis develop in parallel during progression towards cirrhosis^[31,37-43].

Both human and experimental studies have also outlined that several peptide mediators other than VEGF, Ang-1 and HGF are likely to be involved in hepatic angiogenesis associated with the fibrogenic progression process in CLDs. Unequivocal data have been provided for the pro-angiogenic action of PDGF^[32] as well as for leptin, an adipocytokine that has been suggested to exert a pro-fibrogenic effect in promoting the development from non-alcoholic fatty liver disease (NAFLD) to non-alcoholic steatohepatitis (NASH)^[40,44]. Where leptin is concerned, it has been shown that leptin can up-regulate

the expression of VEGF and Ang-1, as well as the pro-inflammatory chemokine monocyte chemoattractant protein 1 or MCP-1^[44]. The role of angiogenesis in NASH development and fibrosis has been confirmed by a study performed on Zucker rats, animals that naturally develop leptin receptor mutations^[40], receiving the steatogenous choline-deficient and amino acid defined (CDAA) diet.

THE EMERGING ROLE OF HYPOXIA IN FIBROGENIC CLDs

Another major message coming mainly from experimental models of CLDs is that VEGF overexpression is strictly associated with hypoxic areas and is mostly limited to hepatocytes as well as to HSC/MFs^[31,37-43]. This concept fits well with the rational hypothesis that hypoxia is likely to represent the major stimulus for hepatic angiogenesis. The colocalization of hypoxic areas with VEGF overexpression and/or the association between VEGF expression and progression of fibrogenesis was first described in the model of bile duct ligation (BDL)^[37] and then confirmed in the diethyl-nitrosamine (DEN) model of fibrosis^[38], in the model of chronic treatment with CCl₄^[31,39,41] and in the choline-deficient and amino acid-defined diet rat model of NAFLD evolving into NASH and significant fibrosis^[40]. Of relevance, a recent study, has outlined in a mechanistic way, the strict relationships between hypoxia, angiogenesis, inflammation and fibrogenesis by using liver conditional HIF-1 α -deficient mice that were subjected to the BDL model of fibrosis^[43]. In this study, it was clearly shown that the appearance of HIF-1 α -positive hypoxic areas in the liver of BDL mice can take place as early as 3 d after surgery, before the development of detectable signs of fibrosis. In particular, within hypoxic areas HIF-1 α was found to be activated in hepatocytes and Kupffer cells. HIF-1 α -/- conditional mice subjected to BDL were characterized by a very significant decrease in collagen type I and α -SMA transcripts and protein levels, as well as of transcripts for PDGF-A, PDGF-B, plasminogen activator inhibitor-1 (PAI-1) and fibroblast growth factor 2 (FGF-2) as compared to wild type mice in which the typical scenario of biliary-type fibrosis and cirrhosis was associated with early and sustained up-regulation of HIF-1 α ^[43]. The authors of this study proposed that hypoxic hepatocytes, following activation of HIF-1 α and through the HIF-1 α -dependent release of these growth factors and mediators, may significantly contribute to either initial repair and revascularization of injured parenchyma as well as to fibrosis progression by targeting profibrogenic MFs. In a more recent study from the same group, this hypothesis was further investigated by exposing to hypoxia cultured hepatocytes obtained from normal mice as well as from HIF-1 α or HIF-1 β deficient mice^[23]. The overall scenario which emerged from the latter study indicated that hypoxic hepatocytes, through the involvement of HIF-1 α and HIF-2 α , can express and release PAI-1, VEGF and the vasoactive peptides adrenomedullin-1 (ADM-1)

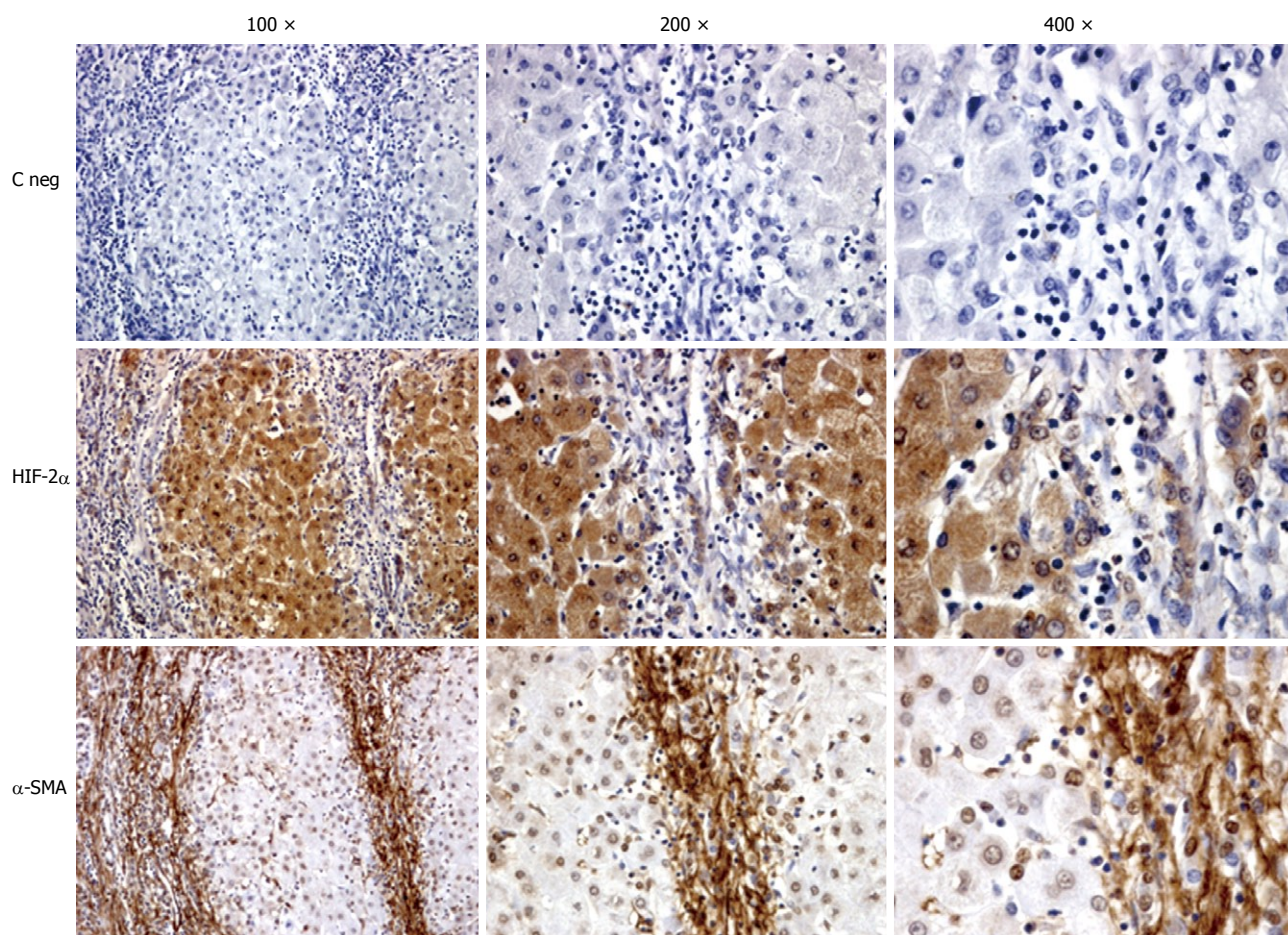


Figure 1 Immunohistochemical analysis performed on paraffin liver sections from patients with hepatitis C virus (HCV)-related liver cirrhosis (METAVIR F4). Sections (2 μ m thick) were incubated with specific antibodies raised against HIF-2 α or α -SMA that positively stain cells exposed to hypoxia (HIF-2 α) or myofibroblast-like cells (α -SMA). Primary antibodies were labelled by using EnVision, HRP-labelled System (DAKO) antibodies and visualized by 3'-diaminobenzidine substrate. Negative controls (C neg) were obtained by replacing the respective primary antibodies by isotype and concentrations matched irrelevant antibody. Original magnification is indicated.

and ADM-2^[23]. Hepatocytes exposed to hypoxia during CLD progression are then reasonably a relevant source of vasoactive mediators as well as of the master pro-angiogenic cytokine VEGF. This unavoidably underlines the relevance of the cross-talk between hypoxic hepatocytes and surrounding non-parenchymal cells like sinusoidal endothelial cells (SECs) and, as we will see later in this editorial, activated MFs. Indeed, VEGF has a well known major pro-angiogenic role by increasing vascular permeability as well as triggering endothelial cell proliferation and regulating neo-vessel lumen diameter^[1-8,14,19].

Along these lines, it is worth mentioning that in the rat model of chronic administration of CCl₄, hypoxic hepatocytes have also been shown to contribute to the expression of the master profibrogenic cytokine transforming growth factor- β 1 (TGF- β 1)^[45]. This contribution has been reported to be relevant mainly in an advanced stage of fibrosis or cirrhosis, whereas TGF- β 1 is mainly produced by MFs and activated macrophages during early fibrosis and middle stage fibrogenic progression^[45].

A strict association between hypoxia and liver fibrogenesis can also be easily appreciated in the liver of human HCV cirrhotic patients by detecting HIF-2 α and α -SMA

in serial sections (Figure 1). HIF-2 α -positive staining in these human specimens is usually detectable in hepatocytes of pseudolobules as well as in some MFs at the interface between fibrotic septa and liver parenchyma or even within pseudolobules. Moreover, it is also evident that α -SMA-positive MFs are in close contact with HIF-2 α positive hepatocytes. Along these lines, it has been suggested that hypoxia may result in VEGF- and Angiopoietin 1-dependent increased migration and chemotaxis of HSC/MFs, contributing to their recruitment towards the site of injury and alignment with both nascent and established fibrotic septa, a relevant pro-fibrogenic feature^[31].

Hypoxia has also been suggested to result in the down-regulation of HGF expression by HSCs and in the inhibition of c-met expression by hepatocytes^[46], both events being able to significantly contribute to depressed liver regeneration during chronic liver injury.

THE ROLE OF PROFIBROGENIC CELLS IN LIVER ANGIOGENESIS

Hepatic stellate cells, due to their strategic location in the space of Disse and intimate contact with sinusoidal ECs,

have been described to behave as liver specific pericytes and then to play a role in physiological angiogenesis^[10]. Recent literature data suggest that HSC/MFs in CLDs are likely to represent a hypoxia-sensitive and cyto- and chemokine-modulated cellular crossroad between necro-inflammation, pathological angiogenesis and fibrogenesis. The latter statement is strongly suggested, in addition to exhaustive literature that has characterized the pro-fibrogenic and pro-inflammatory role of these cells, by a series of studies that outlined the following major concepts: (1) HSC and HSC/MFs can behave as pro-angiogenic cells able to react to conditions of hypoxia by up-regulating transcription and synthesis of VEGF, Ang-1 and their related receptors VEGFR-2 and Tie2^[31,44,47,48], the same behaviour has also been described for human HSC/MFs in which leptin was able to trigger a ERK1/2 and PI3-K-dependent nuclear translocation of HIF-1 α ^[44]; (2) HSC/MFs also represent a cellular “target” for the action of VEGF and Angiopoietin 1; VEGF has been reported to be able to trigger HSC/MFs proliferation^[39,49,50], increase deposition of ECM components^[38,39,50], as well as increase migration and chemotaxis^[31].

This is a scenario that is likely to be relevant in the progression of a CLD, as shown recently by *in vivo* morphological data obtained in human and rat fibrotic/cirrhotic livers^[31]. α -SMA-positive MFs, able to express concomitantly VEGF, Ang-1 or the related receptors VEGFR-2 and Tie-2, are found at the leading edge of tiny and incomplete developing septa, but not in larger bridging septa. This distribution may reflect the existence of two different phases of the angiogenic process during CLDs: an early phase, occurring in developing septa, in which fibrogenesis and angiogenesis may be driven/modulated by HSC/MFs, and a later phase occurring in larger and more mature fibrotic septa where the chronic wound healing is less active and fibrogenic transformation more established. In such a late setting, pro-angiogenic factors are expressed only by endothelial cells, a scenario that is likely to favour stabilization of the newly formed vessels.

A very recent and elegant experimental study^[51] has outlined another putative pro-angiogenic mechanism, that may have a role in vascular remodelling in cirrhosis. This mechanism is related to the action of so-called microparticles Hedgehog (Hh) ligands, which are known to be released during embryogenesis and to activate Hh signalling in endothelial cells. In this study, the authors showed that cholangiocytes and HSC/MFs can produce and then release, mainly in response to PDGF, Hh ligands in microparticles and that this event is relevant under conditions leading to experimental biliary cirrhosis (BDL model). The authors propose the following scenario: (1) in normal conditions the action of the low amount of Hh ligand released by rare immature ductular-type progenitors is counteracted by expression of Hh interacting protein (HIP) expressed by either quiescent HSC or fenestrated SEC; (2) under conditions of chronic injury, HIP expression is repressed and activation of

ductular-type progenitor cells may result in PDGF-BB up-regulation and release; this, in turn, is likely to lead HSC/MFs and ductular cells to produce Hh ligands. Hh ligands, apart from promoting proliferation and survival of both cholangiocytes and HSC/MFs, may also promote changes in SEC gene expression resulting in capillarization of sinusoids and the release of vasoactive factors such as nitric oxide, then contributing to vascular remodelling in cirrhosis^[51].

PATHOLOGICAL ANGIOGENESIS AS A POTENTIAL THERAPEUTIC TARGET IN CLDs

The analysis of the data and concepts presented in the previous sections, concerning the proposed relationships between angiogenesis, chronic wound healing and fibrogenesis and then disease progression, unavoidably leads to the following theoretical clinical goals: (1) detection of selected pro-angiogenic molecules (i.e. in serum or plasma) may serve as a non-invasive way to monitor both disease progression as well as the response to therapy; (2) anti-angiogenic therapy may be an effective tool for blocking or slowing down fibrogenic progression of CLDs.

We are indeed far from the first goal, and at present just a single study performed on 36 chronic HCV patients (*vs* 15 healthy controls) has tried to correlate circulating levels of molecules related to angiogenesis, disease progression and efficacy of standard pegylated interferon α -2b (IFN- α 2b) plus ribavirin therapy. VEGF, Ang-2 and soluble Tie-2 (sTie-2) were determined in the serum before and after therapy and authors reported increased levels of VEGF and Ang-2 that were significantly decreased after therapy in these patients^[52].

Where the efficacy of the angiogenic therapy is concerned, experimental data unequivocally indicate that anti-angiogenic therapy is indeed effective in preventing progressive fibrogenesis. Pioneering studies employed anti-angiogenic molecules or drugs like the semi-synthetic analogue of fumagillin TNP-470^[53] or antibodies able to neutralize either VEGFR-1 (Flt-1) and/or VEGFR-2 (Flk-1)^[39], both conditions being able to significantly inhibit angiogenesis, the number of α -SMA-positive cells and the development of fibrosis. The latter study also showed the *in vivo* predominance of VEGF interaction with VEGFR-2 to mediate angiogenesis during chronic liver injury. Neutralizing antibodies against VEGFR-2 were employed in other relevant studies, performed on a model of portal hypertensive rats, where VEGF expression and related angiogenesis were correlated to the development of porto-systemic collateral vessels and hyperdynamic splanchnic circulation^[54,55]. These data suggest that the increase in portal blood flow, which is an important contributor to portal hypertension, depends not only on vasodilation, but also on the enlargement of the splanchnic vascular tree caused by angiogenesis.

More recently, positive results have been obtained

in the chronic CCl₄ rat model of CLD by employing Sunitinib, a tyrosine kinase receptor inhibitor able to target VEGF and PDGF receptors^[41]. The treatment of cirrhotic animals with Sunitinib resulted in a significant decrease in hepatic vascular density, inflammatory infiltrate, abundance of α -SMA-positive mesenchymal cells, ECM deposition and even portal pressure.

Positive results have been also reported in another recent study in which mice undergoing BDL or chronic CCl₄ treatment received an adenovirus expressing soluble Tie-2 (AdsTie-2), the receptor for Ang-1, resulting in the blocking of Ang-1 signalling and in a significant prevention of both angiogenesis and fibrosis^[42].

A final and very recent experimental study reported a beneficial effect of Sorafenib^[56], a receptor tyrosine kinase inhibitor already approved for the treatment of hepatocellular carcinoma^[57,58], one of the most common complications of liver cirrhosis. In this study, oral administration of Sorafenib in rats with portal hypertension and cirrhosis (once a day for 2 wk) resulted in the inhibition of VEGF, PDGF and Raf kinase signalling; this, in turn, resulted in an approximately 80% decrease in splanchnic neovascularization and a very significant attenuation of hyperdynamic splanchnic and systemic circulations, as well as a significant decrease in the extent of portosystemic collaterals. Of relevance, Sorafenib treatment in cirrhotic rats also led to a 25% reduction in portal pressure and to a relevant improvement in liver injury, inflammation, fibrosis and angiogenesis.

The latter study has raised, in a more compelling way, the obvious question of whether anti-angiogenic therapy has an adequate rationale to be seriously considered for therapy in patients with cirrhosis and portal hypertension. As nicely pointed out by Shah and Bruix^[59], any future clinical trial employing Sorafenib in cirrhotic patients should assess a number of critical issues, the first being the optimal dosage to be used which may be theoretically lower than the dosage used in patients with hepatocellular carcinoma (HCC). The adverse effects of Sorafenib may represent a relevant concern: it is already known that more than 40% of HCC patients are forced to interrupt treatment^[57,58], and it has been shown that a major complication of angiogenic treatments employing Bevacizumab and Sunitinib in HCC patients is variceal bleeding^[60,61]. Although available data indicate that Sorafenib may be relatively safe in this complication, caution is in any case necessary because it has been authoritatively suggested that an intense anti-angiogenic effect may lead to significant damage of the vasa vasorum needed to maintain the structure of varices^[59].

Another note of caution has recently been provided by an experimental study performed in order to assess the anti-fibrotic potential of the inhibition of the vitronectin receptor integrin α v β 3, which has been shown to both promote angiogenesis by mediating migration and proliferation of SECs as well as to drive fibrogenic activation of HSCs^[62]. These authors employed the specific inhibitor of integrin α v β 3, Cilengitide which was administered orally in two different animal models of liver fibrosis, BDL and chronic administration of

thioacetamide (TAA). The relevant point was that this treatment was very effective in decreasing the overall formation of neo-vessels in both portal areas of BDL and septal areas of TAA fibrotic rats. Unfortunately, despite the anti-fibrogenic *in vitro* effect exerted by Cilengitide on cultured HSC/MFs, *in vivo* treatment with this inhibitor was associated in both models with a significant increase in liver collagen deposition and up-regulation of other profibrogenic genes and of matrix metalloproteinase-13, that is an overall worsening of liver fibrosis, with no relevant effect on inflammatory response^[62].

The use of anti-angiogenic drugs and, in particular, those drugs which have already been approved for the treatment of HCC, may then represent an attractive alternative therapeutic tool to prevent or significantly slow down fibrosis progression towards cirrhosis, which also represents the main risk factor for liver cancer development, as well as the development of portal hypertension and its complications. However, a tempered final message is that angiogenesis inhibitors should be used with caution and carefully balanced in these patients, bearing in mind that angiogenesis unavoidably is a relevant event for wound healing and excessive blocking of angiogenesis may not represent the desired therapeutic objective. Clinical trials with an appropriate design and primary end-points are needed.

CONCLUSION

Hepatic angiogenesis has been unequivocally described in CLDs, irrespective of aetiology, and in the most reliable experimental models of liver fibrosis and cirrhosis. Angiogenesis and related changes in angio-architecture have been proposed to favour fibrogenic progression of the disease towards the end-point of cirrhosis. Moreover, in CLDs these changes are believed to be involved in the increase of vascular resistance and portal hypertension as well as in the decrease of parenchymal perfusion. At the same time, hepatic angiogenesis has been proposed to modulate the genesis of portal-systemic shunts and increase splanchnic blood flow, potentially affecting complications of cirrhosis. Hypoxia and HIFs-related cellular responses are emerging as crucial in the overall scenario of CLD progression. Several cellular and molecular mechanisms have been identified which regulate the cross-talk between angiogenesis and fibrogenesis as well as between the different hepatic cell populations. In this scenario a major role is played by hypoxic hepatocytes, sinusoidal endothelial cells as well as hepatic MFs. Where MFs are concerned, whatever their origin, they are currently believed to represent a crucial cellular cross-road at the intersection between inflammation, angiogenesis and fibrogenesis. Indeed, these profibrogenic and pro-inflammatory cells also represent a cellular target for the action of pro-angiogenic cytokines as well as an effective source of VEGF and Ang-1.

Finally, experimental anti-angiogenic therapy has proven to be very effective in limiting the fibrogenic progression of animal models of CLDs. The use of

anti-angiogenic drugs, particularly of those that have already been approved for HCC therapy like Sorafenib, may then represent a rationale therapeutic option to limit the progression of human CLDs towards cirrhosis and its complications, including the development of HCC.

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Hepatic tight junctions: From viral entry to cancer metastasis

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Abstract

The tight junction (TJ) is a critical cellular component for maintenance of tissue integrity, cellular interactions and cell-cell communications, and physiologically functions as the "great wall" against external agents and the surrounding hostile environment. During the host-pathogen evolution, viruses somehow found the key to unlock the gate for their entry into cells and to exploit and exhaust the host cells. In the liver, an array of TJ molecules is localized along the bile canaliculi forming the blood-biliary barrier, where they play pivotal roles in paracellular permeability, bile secretion, and cell polarity. In pathology, certain hepatic TJ molecules mediate virus entry causing hepatitis infection; deregulation and functional abnormality of the TJ have also been implicated in triggering liver cancer development and metastasis. All these findings shed new insights on the understanding of hepatic TJs in the development of liver disease and provide new clues for potential intervention.

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Key words: Tight junctions; Hepatocytes; Blood-biliary

INTRODUCTION

The liver is the core metabolic center in the mammalian body and is responsible for major physiological functions such as carbohydrate/amino acid/fatty acid metabolism, bile secretion, and detoxification. The cellular composition of the liver comprises specialized cell types - hepatocytes and nonparenchymal cells. In a normal adult liver, hepatocytes are epithelial cells and alone account for 60%-70% of the total cell mass. Hepatocytes adopt a polarized architecture, resulting in the formation of numerous cell plates in the liver. Because of the highly vascularized nature of the liver, this hepatic parenchyma is infiltrated by an extensive microcirculatory network^[1,2]. In order to maintain this anatomical organization, hepatocytes are equipped basically with a vast variety of junctions, such as anchoring junctions, tight junctions (TJs), and gap junctions (GJs)^[3-5]. These junctions are situated at the surface of the hepatocytes so as to mediate cell-cell contact and communication. This editorial focuses on the hepatocyte TJs - as the "cements" of the building block and the "door" for entry of hepatitis viruses. Deregulation of TJ expression and function dismantles

the architecture of the hepatic parenchyma and causes liver diseases and cancer.

TJ IN THE LIVER: FROM STRUCTURAL ARCHITECTURE TO SIGNALING NETWORK

In the liver, TJs can be found in 2 places, associating with either hepatocytes or bile duct epithelial cells (cholangiocytes). Those associated with the former cell type are alternatively called the blood-biliary barrier (BBB). Here we review the hepatocyte-associated TJ that concentrates at the specialized location surrounding the bile canaliculi. In addition to modulating paracellular passage of small molecules and ions, this BBB functions to keep bile in the bile canaliculi and apart from the blood circulation. TJ in the liver also segregates the apical surface from the basolateral surface of the hepatocytes, thereby maintaining cell polarity^[4]. Having the same TJ components as in other epithelia and endothelia, the TJ in the liver is also composed of claudins, occludin, junctional adhesion molecules (JAMs), and others such as coxsackievirus and adenovirus receptor (CAR)^[6-11].

Claudins constitute the largest TJ family; 24 claudins have been found in mammals and at least 7 of these, namely claudin-1, -2, -3, -4, -5, -7, and -10, have been studied in the liver. Most claudins are small molecules having molecular weights of approximate 22-27 kDa. They are tetra-span molecules with the amino-terminus and carboxyl-terminus in the cell cytoplasm, and they possess 2 extracellular loops and one intracellular loop. For sealing the intercellular gap, claudin needs to interact with other claudins in the adjacent cell through its extracellular loops^[12,13]. Occludin is the first studied TJ integral molecule. It has similar structural features to that of claudin, being a molecule with 4 transmembrane domains and utilizes a similar binding mechanism to that of claudin. However, it differs from claudin in its large molecular weight of 65 kDa^[11,14]. Another variant of occludin, occludin 1B, has been identified and it differs from occludin in having an extended amino-terminus. Both of them are found in mouse livers^[15].

JAM is a TJ molecule which has gained much attention recently. It is a single-pass membrane protein with its amino-terminus in the extracellular region and carboxyl-terminus in the cytoplasm. As the component of a barrier, a single JAM molecule needs to couple with another JAM in an adjacent cell. At least 4 members of JAM have been identified and JAM-1, JAM-2, and JAM-3 have been found in mouse livers^[10,11,16].

CAR was known originally as a new class of viral receptor, belonging to the immunoglobulin-like family and further studies demonstrated that CAR also had TJ functions^[17]. Of the 3 isoforms of CAR (i.e. CAR-1, CAR-2, and CAR-3), only CAR-2 has been positively identified in hepatocytes and both CAR-1 and CAR-2 are associated with cholangiocytes in mouse livers^[7]. As in other organs, these integral TJ proteins interact with

various scaffolding proteins to ensure structural integrity.

A plethora of adaptors and peripheral proteins are known to be present and associated with claudins, occludin, JAMs, and CARs. Adaptors such as zonula occludens-1 (ZO-1) act as a bridge linking the integral proteins to the underlying actin filament^[18-20]. Cingulin, symplekin, and MAGI-1 (membrane-associated guanylate kinase inverted-1) are other peripheral proteins at TJs, and some of them have also been demonstrated in the liver^[3,21-24]. By this way, a high degree of structural architecture is established at the TJ strand guarding the selective permeability barrier in the liver^[25] (Figure 1).

Apart from its barrier functions, recent studies have also elucidated the other roles of the TJ as a core component in the signaling network, in particular for those junctional complexes concentrating at the BBB. Accumulating evidence suggests that the junction does not function alone on the plasma membrane, but different junctions can interact with each other either directly or indirectly. Studies performed in different systems demonstrated a disruption of one junction type could lead to loss or gain of function of another junction type, emphasizing the significance of inter-junctional crosstalk^[26-28]. Epithelial cells, including hepatic cells, adopt this kind of junction-junction regulation. It is noted that enforced expression of connexin 32 into mouse hepatocytes derived from connexin 32-deficient mice results in TJ formation, accompanied by induced expression of occludin, claudin-1 and ZO-1, thereby leading to establishment of cell polarity^[29]. In addition, using the same experiment setup induced the expressions of another junction protein MAGI-1 at the TJ in connexin 32 transfectants^[30]. These findings unequivocally demonstrate the presence of a macrocomplex in the liver composed of at least a TJ and GJ^[4]. Solid evidence from other studies also suggested the possible involvement of the TJ in manipulating other junctions such as the adherens junction (AJ). For instance, an abnormality of JAM-1 in hepatoma HepG2 cells induced the production of an AJ protein E-cadherin^[31]. Our current understanding is that ZO-1 acts as the moderator in coordinating the cellular dynamics of various associated junctions and maintains the structural functionality of this multi-junctional network^[32-34].

Several cellular proteins, such as protein kinases and phosphatases, are some of the major regulators of junctions. Since most of them have numerous substrates, events of phosphorylation or dephosphorylation can modulate the status of components related to certain junctions. The p38 mitogen-activated protein kinase (MAPK) is a serine/threonine kinase that phosphorylates a handful of substrates including those associated with TJs^[35]. Treatment with SB203580, a p38 MAPK inhibitor, led to strengthening of the TJ with a concomitant increase in claudin-1 in rat livers after partial hepatectomy^[36]. Other events triggered by cytokines and growth factors are also involved in the regulation of TJ dynamics^[37,38]. For instance, incubation of rodent hepatocytes with a multifunctional cytokine oncostatin M triggered the

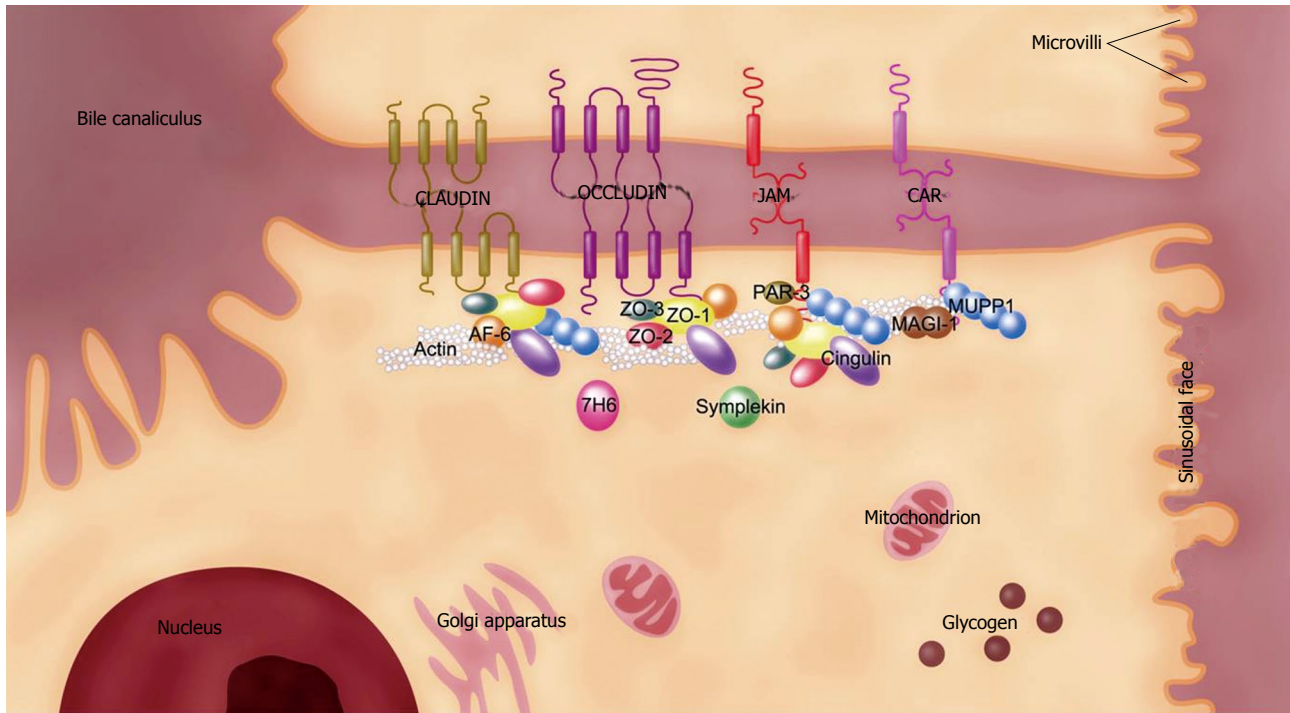


Figure 1 Molecular structure of the TJ in the mammalian liver. The TJ in the liver is associated with hepatocytes and bile duct cells. TJ location around the bile canalicular between 2 adjacent hepatocytes is shown. For simplicity, the molecular structure depicted in this figure represents the TJ molecules found in the mammalian liver. Claudin, occludin, JAM, and CAR are 4 core units for constituting TJ by uniting a panel of peripheral proteins like ZO-1 to form multiprotein complexes. TJ molecules display differential localizations in the mammalian liver, such that some of them like human symplekin and mouse CAR-2 are associated with both hepatocytes and bile duct cells while others, such as mouse CAR-1, are only found in the latter cell type. CAR: Coxsackievirus and adenovirus receptor; JAM: Junctional adhesion molecule; MAGI-1: Membrane-associated guanylate kinase inverted-1; MUPP1: Multiple PDZ domain protein-1; PAR-3: Partitioning defective 3 homolog; TJ: Tight junction; ZO: Zonula occludens.

production of claudin-2 and subsequently strengthened the TJ barrier^[39]. Further, the transforming growth factor- β (TGF- β) could reduce the production of claudin-1 and weaken the barrier function in rat hepatocytes^[40].

TJ AND LIVER-RELATED DISEASES

Hepatitis

Hepatitis is an infection of the liver caused commonly by hepatitis viruses such as hepatitis B and C. At least 7 hepatitis viruses are known today and new species are being identified^[41]. Hepatitis C virus (HCV) is the best studied for its ability to bind to TJ molecules on hepatocytes, and these molecules act as co-receptors for HCV entry^[42]. The concept of junction proteins mediating viral entry is not restricted to the hepatitis virus, but is apparent for several other viruses including the adenovirus and coxsackievirus^[43]. For the scenario of HCV infection, occludin and claudin-1 have been determined to be 2 key molecules for HCV entry^[44,45]. Some reports also demonstrated other molecules including CD81 and human scavenger receptor class B member 1 (SR-BI) as co-receptors for HCV entry^[46,47] and the expression levels of some of these receptors define the viral entry rate^[48]. Cooperation between these receptors and TJ molecules is essential for viral entry into hepatocytes. Several studies provided further evidence indicating that the events occurred in the hepatocytes

after HCV infection. Hepatoma HuH-7 cells, having genomic replicons of HCV, could alter TJ dynamics, such that a disarrangement of TJ components was found and retention of occludin in the endoplasmic reticulum was noted^[49]. Internalization of HCV is accompanied by an induced synthesis of fatty acid synthase, which is an enzyme responsible for fatty acid synthesis, and this event is associated with the production of claudin-1, but not CD81, in hepatoma HuH-7 cells^[50]. This can partly explain why HCV infection frequently leads to steatosis, a fatty liver-related disease. In addition to fatty acid synthase, protein kinase A has an important role in HCV infection, since an aberration in protein kinase A function in hepatoma HuH-7 cells led to a disorganization of claudin-1 and reduced the infection susceptibility^[51]. Together, these findings suggest an explicit role of TJ molecules, especially claudin-1 and occludin, in mediating HCV infection (Figure 2).

Liver cancer

Liver cancer or hepatocellular carcinoma (HCC) is one of the most aggressive liver malignancies worldwide. The development of HCC is a complex process that is not totally established even after several decades of research. In most cases, HCC results from a pre-neoplastic inflammation of the diseased liver and is the end stage of a progressive worsening of liver conditions originating from hepatitis or cirrhotic livers, encompassing 3 phases

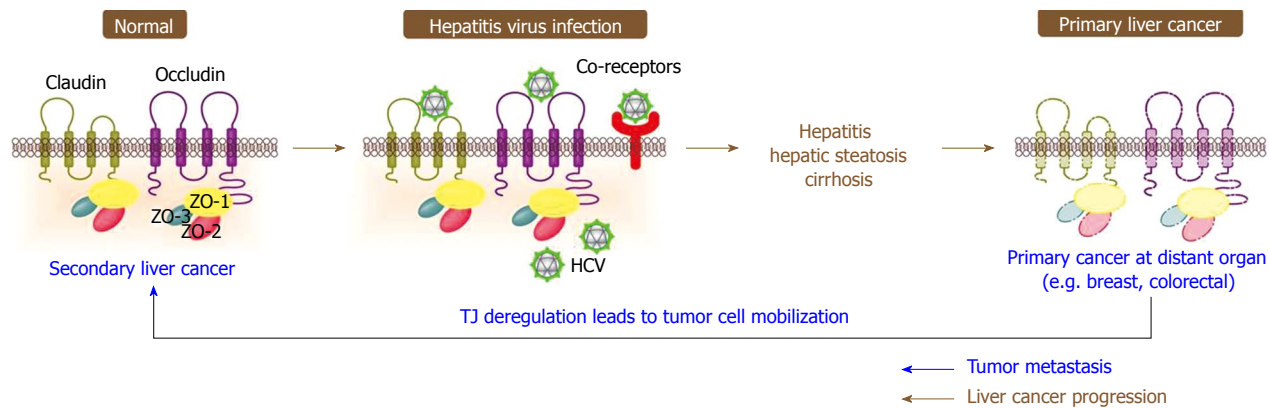


Figure 2 The TJ at different stages of liver cancer progression and distant liver metastasis. During viral infection, HCV binds onto several TJ molecules (claudin-1 and occludin) and co-receptors (CD81 and SR-BI) on hepatocytes before its internalization. This event leads to hepatic steatosis and/or cirrhosis before the subsequent development of primary liver cancer that is associated with TJ deregulation. For metastatic liver cancer originating from the intrahepatic site or distant organs such as breast and colorectum, there is an initial loss of TJ molecules at the primary tumor site and a subsequent gain of these molecules in the liver with tumor cell colonization. HCV: Hepatitis C virus; SR-BI: Scavenger receptor class B member-I.

Table 1 Clinical significance of TJ molecules in human HCC			
Molecules	Expression in HCC ¹	Clinical correlations	Ref.
CAR	Reduced	Poor tumor differentiation	[64]
Claudin-1	Reduced	Poor tumor differentiation, tumor invasion, poor survival	[63]
Claudin-10	Higher	Tumor recurrence	[67]
Occludin	Reduced	-	[9]
Symplekin	Reduced	Poor tumor differentiation	[3]
ZO-1	Reduced	-	[9]

¹Relative expression of TJ molecules in HCC tissues compared to normal or adjacent non-tumor tissues. TJ: Tight junction; HCC: Hepatocellular carcinoma; CAR: Coxsackievirus and adenovirus receptor.

of development - molecular, preclinical, and clinical^[52]. At the molecular level, this malignant transformation of the liver is accompanied by a stepwise change in genetic and proteomic information, which can be readily revealed using laboratory technologies including gene microarray and gel- or non-gel-based proteomics profiling, coupled with mass spectrometry^[53-55]. As a result of rapid developments in molecular and profiling techniques, the gene, protein, and microRNA data related to HCC are gradually being decoded^[56]. A handful of molecules, such as heat shock proteins and cadherins, and different pathways, such as Wnt and TGF- β pathways, have been determined to be HCC-related^[56-60]. The newly derived information assists the construction of the molecular network of HCC and enhances our knowledge of this cancer.

Primary tumors in the liver

Tumor nodules originating in the liver are generally termed as primary tumors. During hepatocarcinogenesis, the liver usually undergoes several phases of transition from pre-neoplasia, dysplasia, to neoplasia^[61]. Several TJ molecules are regarded as HCC biomarkers^[55,62]. An endogenous expression of certain claudins is found in the normal adult liver and their expression is attenuated when HCC develops. It is noted that claudin-1 has

reduced expression in cancerous liver when compared to its healthy counterpart^[63]. A general reduction in the levels of occludin, ZO-1, and CAR has been found in HCC when compared to normal liver^[9,64]. Also, there is a gradual decrease in the level of 7H6 TJ-associated antigen in rats during hepatocarcinogenesis^[65]. Apart from these molecules, other TJ molecules, such as JAM and cingulin, are present in hepatic cells^[31,66], but not all of these are associated with HCC. Those claudins with a high expression in normal liver have a role in liver physiology by maintaining a functional TJ barrier, whereas those claudins with elevated expression in the cancerous liver are likely to be involved in tumor formation. Sometimes these biomarkers not only indicate the advent of tumors, but can also be prognostic in nature (Table 1). A loss of claudin-1 expression in resected HCC indicates poor differentiation and high invasiveness of the tumor, and is associated with poor outcomes of patients^[63]. Similarly for other TJ molecules, a reduced CAR expression in resected livers is correlated with poor differentiation of HCC^[64]. However, Cheung *et al*^[67] linked the high expression of claudin-10 in HCC with the high incidence of postoperative tumor recurrence in patients.

With regard to biological significance, these biomarkers usually demonstrate gain- or loss-of-function in HCC. For instance, the expression of claudin-1 is associated preferentially with the fetal cell type of human hepatoblastoma, but not the highly proliferating embryonal cell type, with expression of proliferating cell nuclear antigen (PCNA) and Ki-67, suggesting its expression is negatively correlated with rapid cell growth and division^[68]. This anti-proliferative behavior of hepatic claudin-1 is further supported by a study showing a loss of claudin-1 associated with tumor aggressiveness^[63]. An overexpression of claudin-10 is linked to poor outcome of HCC patients after hepatic resection. To prove the tumorigenic features of this molecule, an overexpression experiment was performed in claudin-10-deficient Hep3B hepatoma cells and an induction of tumor phenotypes was observed. In the reciprocal experiment

using RNA interference (RNAi) to silence claudin-10 in HLE hepatoma cells with a high level of claudin-10, an alleviation of tumorigenic potential accompanied by reduced cell invasion was found^[69]. By these approaches, molecules can be studied for their tumorigenic properties. Besides their intrinsic tumorigenic properties being the subject of intense interest in research, TJ molecules have also been studied with regard to their associated pathways, some of which have been unfolded successfully. Borlak *et al.*^[70] utilized an epidermal growth factor-induced HCC mouse model showing a positive effect of this growth factor in inducing claudin-7 in small HCCs. Also, vascular endothelial growth factor (VEGF)-treated HepG2 hepatoma cells had disruptive TJs accompanied by reduced occludin expression, suggesting VEGF as one factor triggering the spread of tumor cells into the normal liver parenchyma^[71]. All these findings implicate the direct involvement of TJ molecules in the presentation of tumor phenotypes and the tumor-related signaling pathways, suggesting their interference may counteract the process of HCC. Therefore, TJ molecules may be another class of therapeutic target for HCC.

Metastatic tumors in liver

Metastatic tumors initiated elsewhere in the body may spread to and colonize the liver. Angiogenesis is a prerequisite process for tumor metastasis, enabling the migration of tumor cells through the circulatory system of the body from one site to the other^[72]. A number of factors such as growth factors and chemokines are important in triggering this event^[73,74]. For tumor cells to metastasize, loss of TJ function is usually observed in cancer cells prior to this process^[75]. Of the TJ molecules, claudin-7, ZO-1, and other emerging ones are associated with tumor metastasis. Thus, a decrease of claudin-7 in tumor tissues is associated with tumor metastasis in patients with breast carcinoma^[76]. This finding is further validated by a separate gene microarray study, in which breast cancer metastasis to the liver is associated with a reduced expression of a panel of TJ molecules including claudin-4 and ZO-1, in addition to claudin-7^[77]. For lung cancer, overexpression of claudin-1 in human lung adenocarcinoma cells reduced the metastatic potentials of tumor cells^[78]. Apart from these 2 cancer types, elimination of claudin-7 is frequently observed in colorectal cancer, and is clinically related to the event of tumor cell invasion into the blood circulation and the eventual development of tumor masses in the liver^[79]. In addition, malfunction of ZO-1 is observed after its phosphorylation, which also induces the migration of colorectal tumor cells into the liver^[80]. Interestingly, there is a restoration of the expression of TJ molecules in tumor cells after metastasis to the liver from a distant organ. It is noted that a re-expression of ZO-1 is observed in colorectal cancer cells after metastasis to the liver when compared to those developing metastatic potential at the primary cancer site^[80]. Similar re-expression of claudins such as claudin-1 and claudin-4 in liver-residing colorectal cancer cells is reported in another study examining colorectal tumor

metastasis^[81]. Therefore, it is clear that loss of TJ function is a key factor triggering the induction of metastatic potential in tumor cells to other sites including the liver, while a restoration of its function is needed for tumor cells to colonize in the liver.

CONCLUSION

In the liver, TJ is found to be associated with bile duct cells and hepatocytes. As hepatocytes are the most predominant cell type in the liver and the most studied in liver diseases, we focused our discussion on the role of hepatocyte-associated TJ molecules in hepatitis infection and liver cancer. TJs found in hepatocytes are also known as the BBB, keeping the bile in the bile canaliculi away from the blood circulation. Emerging evidence has further demonstrated the direct involvement of TJ molecules as co-receptors for HCV. On the other hand, accumulating evidence support the notion that deregulation of TJ molecules is frequently associated with increased incidence of HCC and poor prognosis of patients, signifying their putative use as biomarkers for diagnosis and prognosis of this liver malignancy. Molecules with induced expression in HCC are predicted to be tumor-inducing, while those with reduced expression are likely to be anti-tumor molecules. Proof-of-principle studies by means of RNAi or overexpression should enhance our knowledge of the roles of specific TJ molecules in liver diseases. Based on previous findings of TJ molecules as viral receptors, it is highly possible that a blocking peptide or antibody can be developed to prevent the binding of the viral particles to the TJ receptors on hepatocytes. This can open a new study area for therapeutic targeting of the TJ. Those TJ molecules with tumor-inducing properties are potential therapeutic targets for HCC. More in-depth studies should be performed to find antagonists to inhibit the functions of these molecules or to block their specific signaling pathways. With regard to advancement of HCC diagnosis, novel TJ molecules may be useful as HCC biomarkers. Further studies should be performed to investigate whether these biomarkers are eligible as supplemental criteria to diagnose HCC in patients with low levels of serum alpha-fetoprotein who constitute up to one-third of HCC cases. Therefore, study of the TJ in the liver can increase the clinical usefulness of this molecule in liver diseases.

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Update on collagenous sprue

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Abstract

Collagenous sprue has traditionally been defined as a small intestinal mucosal disorder characterized by persistent diarrhea, severe malabsorption with multiple nutrient deficiencies and progressive weight loss. Pathologically, a severe to variably severe "flattened" mucosal biopsy lesion with distinctive sub-epithelial deposits in the lamina propria region is detected. Histochemical stains and ultrastructural studies have confirmed that these deposits contain collagens. Often, an initial diagnosis of celiac disease is considered but no continued response to treatment with a gluten-free diet occurs. Recent reports indicate an intimate relationship between collagenous sprue and celiac disease, sometimes with concomitant T-cell enteropathy. In addition, permanent disappearance of these deposits after resection of a localized colon cancer suggested that this disorder could actually represent a paraneoplastic morphologic marker of an occult malignancy. Studies showing either gastric or colonic involvement (or both) with this unusual collagenous inflammatory mucosal process may also reflect a far more extensive and heterogeneous process than previously appreciated.

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Key words: Collagenous sprue; Celiac disease; Lymphoma; Paraneoplastic disease

INTRODUCTION

In 1970, Weinstein *et al*^[1] reported on a small intestinal mucosal biopsy lesion from a 51-year-old female initially thought to have celiac disease as histopathological changes included severely flattened villi. However, a long-term response to a gluten-free diet failed to develop. Subsequently, routine hematoxylin-eosin stained biopsies showed a prominent band-like deposit of sub-epithelial hyaline material in the lamina propria region of the small bowel. The deposit had the histochemical features of collagen and ultrastructural studies confirmed an electron-dense material with the typical 640 Å axial periodicity of collagen fibers. Her symptoms transiently improved with corticosteroids, but she then developed worsening diarrhea, severe malabsorption and progressive weight loss. Post-mortem examination showed very extensive pathologic changes in the proximal small intestine with sub-epithelial eosinophilic hyaline deposits of varying thickness. Short segments of normal mucosa were present in the distal small intestine. Two earlier reports by Schein^[2] in 1947 and Hourihane^[3] in 1963 may have reflected the same biopsy lesions (although in the latter, ileal involvement was also present).

Collagenous sprue was thought to be a "new" form of malabsorptive disorder with the specific clinical and pathological features: (1) persistent diarrhea with pan-malabsorption causing nutrient deficiencies and progressive weight loss; (2) a biopsy lesion included a unique

morphologic marker, a sub-epithelial band-like deposit with histochemical and ultrastructural features of collagen; (3) other pathologic changes of untreated celiac disease were present, but not responsive to a gluten-free diet; and (4) diffuse and patchy mucosal changes of variable severity, localized mainly in the proximal small intestine.

OTHER CAUSES OF SEVERE “FLAT” BIOPSY LESION

Traditionally, the diagnosis of celiac disease (or gluten-sensitive enteropathy) has been established pathologically and depended on two sequential criteria: first, documentation of the typical histopathologic features of untreated disease in small bowel biopsies, and, second, a response to a gluten-free diet. Otherwise, celiac disease, even if present, cannot be diagnosed with certainty. In some cases, a “flattened” biopsy appearance may be present, but a gluten-free diet response has not been documented. This may require months to years^[4]. Some investigators have loosely labeled these cases as refractory celiac disease, but this label should be reserved for those who show an initial (and documented) response to a gluten-free diet followed by later development of recurrent symptoms and biopsy changes. The most commonly reported causes for recurrent symptoms and biopsy changes include poor dietary compliance or inadvertent ingestion of a ubiquitous gluten-containing food (e.g. pill capsules, communion wafers). In these cases, removal of the offending gluten should be sufficient to resolve symptoms and biopsy changes. A second or superimposed cause (e.g. infection, folate or zinc deficiency) could also develop. In addition, another entirely separate cause for a “flat” biopsy lesion could be present^[5], as the initial true diagnosis (e.g. Crohn’s disease in duodenum without mucosal granulomas) may have been missed^[6] or an associated or complicating disease (e.g. collagenous colitis, lymphoma) could have developed. In these patients, symptoms and biopsy changes may be improved with specific treatment, but not with a gluten-free diet. Finally, another “wastebasket” group with a “flat” biopsy appearance that has never been responsive to a gluten-free diet may be present, so-called sprue-like intestinal disease or unclassified sprue^[7].

RELATIONSHIP WITH CELIAC DISEASE

Collagenous sprue has a “flat” biopsy appearance, like untreated celiac disease, but fails to show a persistent response to a gluten-free diet. In addition, collagenous sprue is characterized by the appearance of distinctive subepithelial collagen deposits. Some believed that this histopathological change might simply represent a prognostic pathologic marker for a poor outcome in celiac disease^[8]. Others, however, viewed collagenous sprue as a new and previously unrecognized small bowel disorder^[9]. Later reports have also described

further elements between celiac disease and collagenous sprue. Common clinical features include hyposplenism and positive endomysial antibodies that have been documented in both entities^[10]. In collagenous sprue, similar complications recorded in celiac disease may also occur, including both T-cell and B-cell lymphomas^[11,12].

NATURAL HISTORY AND LOCALIZATION

Collagen deposits may also be present in the colon (i.e. collagenous colitis) or even stomach (i.e. collagenous gastritis)^[13]. An associated inflammatory process in either colonic or gastric mucosa, or both, is also present, usually with epithelial lymphocytosis. Interestingly, collagenous or lymphocytic colitis as well as collagenous or lymphocytic gastritis are all associated with biopsy-defined celiac disease^[13-15]. These pathological changes also suggest that a far more extensive pathologic process may occur elsewhere in the gastrointestinal tract with collagenous sprue.

Previously published reports noted that the natural history of collagenous sprue was characterized by worsening malabsorption with an inevitably fatal outcome. In most patients, diarrhea and progressive weight loss occurred, and rarely, severe abdominal pain, sometimes with an associated vasculitis, was recorded^[16]. However, more recently, independent reports with extensive biopsy studies have documented complete resolution of the lesion for prolonged periods after corticosteroid treatment^[17,18] suggesting that the lesion may be reversed, at least temporarily, for extended periods, even years. Immunosuppressants have also been used in some cases.

DISEASE HETEROGENEITY

The etiology and pathogenesis of these collagenous deposits are not known, however, different causes could be responsible. In addition to celiac disease, collagenous sprue has not only been complicated with T-cell lymphoma^[12], but associated with its co-occurrence^[19]. Finally, collagen deposits in both small and large intestines were detected with an apparently coincidental, but localized, colon cancer^[20]. Later, clinical and histopathological changes were resolved after the cancer was resected, suggesting that these collagen deposits could represent a paraneoplastic morphologic marker of occult malignant disease.

FUTURE DIRECTIONS

Recent reports suggest that collagenous sprue may be more heterogeneous than previously appreciated. This has been reflected in frequently associated, but variable collagenous mucosal inflammatory changes elsewhere in the gastrointestinal tract, differential responses to treatment, particularly with steroids, and its association with other conditions, including malignant disease as a possible paraneoplastic morphologic marker.

Treatment of this condition remains an empirical exercise. Steroids and/or immunosuppressant agents have been used, but, to date, this approach has resulted in only rare success.

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Pulmonary involvement and allergic disorders in inflammatory bowel disease

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INTRODUCTION

Ulcerative colitis (UC) and Crohn's disease (CD) are the two major forms of chronic relapsing inflammatory bowel disease (IBD). Apart from overlapping epidemiological, clinical, radiological, endoscopic and histological characteristics^[1,2] between UC and CD, there are clear differences in the extent of inflammation in the gastrointestinal tract and in several immunological parameters^[3-5] suggesting that they are distinct disease processes. The pathogenesis of IBD seems to be more complex than one single cause and probably involves an interaction between genetic predisposing factors^[6-8], exogenous and endogenous triggers^[3,9-14], and modifying factors^[3,15,16]. The outcome of these interactions is a spontaneously relapsing and remitting inflammatory process in intestinal mucosa associated with recruitment and activation of lymphocytes, macrophages and other inflammatory cells^[3,17-20].

Extraintestinal and systemic manifestations occur frequently in patients with IBD^[20-25]. These various disease states can be diagnosed before, concomitant with, or after the diagnosis of a specific type of IBD. Two large case studies have demonstrated that between 25% and 36% of patients with either type of idiopathic IBD will have at least one such associated disease^[22-23]. Yamamoto-Furusho *et al*^[8] found that extraintestinal manifestations were present in 41.5% of 848 cases with UC. More than 100 systemic complications involving almost every organ system in the body have been described^[26]. The

Abstract

Inflammatory bowel disease (IBD) has been associated with either clinical or subclinical airway and parenchymal lung involvement and interstitial lung complications. Several studies have reported that atopy has a high prevalence in IBD patients. Overlapping allergic disorders seem to be present in both the respiratory and gastrointestinal systems. The purpose of this review is to update clinicians on recent available literature and to discuss the need for a highly suspicious approach by clinicians.

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Key words: Atopy; Inflammatory bowel disease; Pulmonary involvement

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spectrum, the frequency and the temporal relation of the complications have led to the hypothesis that IBD is a systemic disorder^[22-23,26].

IBD is associated with a wide variety of extraintestinal lesions in many organs and over some decades the pattern has changed and the lung is regarded as one of these affected organs^[21,26-31]. In recent years a few hundred cases with pulmonary involvement and IBD have been reported and in that way pulmonary involvement has been proved to be common. A growing number of studies in the literature have reported either clinical or latent pulmonary involvement in patients with IBD^[29,31-34]. In this review we will focus on the extraintestinal manifestations that are associated with lungs and airways. In an attempt to classify the reported lung manifestations in patients with IBD in a more useful way, the manifestations were distinguished as follows: (1) involvement of airways; (2) pulmonary function testing abnormalities; and (3) diffuse or localized interstitial lung complications caused by either disease or treatment received. Finally, studies regarding the relationship between allergy and IBD will be discussed in detail.

IBD AND AIRWAYS

The manifestations of IBD in airways include, chronic bronchial suppuration particularly in patients with UC^[31,34-36], bronchiectasis^[32,37-40], localized obstruction of upper airways^[41], bronchiolitis obliterans organizing pneumonia^[32,42,43], granulomatous bronchiolitis^[44], tracheobronchitis^[45,46], bronchiolitis obliterans^[47], tracheobronchial stenosis^[48] and diffuse obstructive disease^[34,49]. Obstructive disease was not confirmed in some studies^[50]. An increased risk of both UC and CD in chronic obstructive pulmonary disease (COPD) patients has been reported in some studies, focusing attention on the association between airway diseases (AD) and IBD^[51]. We have reported a small airway dysfunction, detected by density dependence methods, in patients with IBD^[52]. In the study by Louis *et al*^[53], patients with IBD, free of pulmonary symptoms, independently of the presence of atopy, showed bronchial hyperresponsiveness. This interesting finding could lead to the hypothesis that local mucosal inflammation in the intestine is responsible for the mild airway inflammation and not atopy. This hypothesis is not new. Basal cell hyperplasia, membrane thickening and submucosal inflammation have been reported in patients with UC and bronchial suppuration^[36]. CD may affect the oral cavity and the colon^[26,54,55] while both UC^[34,41] and CD^[56] have been reported to involve the larynx. There are also some morphological and developmental similarities between colonic and bronchial epithelium. Both are derived from the primitive gut, whereas the lungs arise from the laryngo-tracheal bud. Both are composed of columnar epithelium with goblet cells and submucosal mucous glands. Furthermore, there is increasing evidence that an immune system specific to the gastrointestinal tract common to all mucosal surfaces exists^[57], in which lymphocytes are sensitized to antigens at one mucosal site and by circulation are localized and produce inflammation in other mucosal surfaces^[53,58-61].

PULMONARY FUNCTION TESTING ABNORMALITIES

Previous reports concerning pulmonary function abnormalities in patients with IBD are conflicting. In some studies no differences in pulmonary function tests (PFTs) between patients with IBD and the control group were found^[49,62]. In the study by Neilly *et al*^[49] airway obstruction was the most common finding affecting patients with CD (45%). However, the indices of airway obstruction were not significantly different from those obtained in age-, sex- and smoking-matched controls. As discussed above, Louis *et al*^[53] reported an increased bronchial responsiveness in IBD patients, while the baseline lung function tests were within the normal range. In the study by Mohamed-Hussein *et al*^[63], fifteen out of 26 patients with UC had an important impairment in PFTs. In the study by Herrlinger *et al*^[64], the impairment in PFTs was more pronounced in IBD patients with active disease than in those with inactive disease.

Pulmonary diffusion capacity (TLCO) is often impaired in IBD patients. Heatley *et al*^[65] found an increased prevalence of TLCO impairment in 25% of patients with CD. Reduction of TLCO in patients with IBD has been reported in various studies^[61,64,66-69]. Eade *et al*^[66] and Bonniere *et al*^[59] found that the reduced TLCO or other PFTs parameters were not correlated with the location and severity of IBD or with the concurrent medication mode^[59,66]. We examined 132 patients, 47 (17 female, 30 male) with CD and 85 (35 female, 50 male) mean age 40 years with UC. The main finding of our study was a high prevalence of impaired TLCO in patients with CD and UC suggesting involvement of the lung parenchyma^[70]. All other PFTs parameters were abnormal in a high percentage of patients, however, they did not show statistically significant differences from those in the control group. Our data suggest that the impairment of TLCO was statistically significantly higher in patients with exacerbation of disease than in remission^[70]. This finding is in accordance with other studies^[61,65,68-70] which reported a higher prevalence of impaired TLCO among patients with active IBD disease as compared to patients in remission. In contrast, Douglas *et al*^[71] reported a reduced gas transfer factor in 16% of 44 patients with IBD but these abnormalities were not related to disease activity. The reduction in gas transfer factor indicates damage to lung parenchyma. The nature of this lung involvement remains debatable. However, some explanations will be discussed in the following section concerning the relationship between IBD and interstitial lung complications.

INTERSTITIAL LUNG COMPLICATIONS

Interstitial lung involvement has been reported to accompany both clinical IBD entities, UC^[72-78] and CD^[79-83]. The interstitial lung infiltrates have been proven histologically to be either pulmonary vasculitis^[76,77,84] or more often granulomatous disease^[74,79,80,82,83,85,86].

Table 1 Studies on the relationship between IBD and atopic features

Group	Protocol	Atopy history	Skin prick tests	IgE	Ref.
CD: 11 UC: 19 Normals: 16	Skin prick tests SIgE	Allergic symptoms were more prevalent in IBD <i>vs</i> controls $P < 0.007$ (in UC $P < 0.004$)	IBD <i>vs</i> control $P < 0.02$	No statistically significant differences	[109]
UC: 14 CD: 20 Controls: 72	Atopy history IgA, IgG, IgM IgE	-	-	Increased IgG, IgM and IgE in patients <i>vs</i> controls ($P < 0.01$)	[114]
UC: 300 CD: 200 Controls: 254	Questionnaire	Asthma, hay fever, allergic rhinitis; UC <i>vs</i> Controls: $P < 0.02$; CD <i>vs</i> Controls: NS; Eczema-Any atopy-Family history; Both UC & CD <i>vs</i> controls: $P < 0.001$	-	-	[117]
UC: 39 CD: 35 Healthy: 37	Skin prick tests to various common allergens	23.1% in UC; 22.9% in CD; 21.4% in disease controls; 20% among healthy subjects	14/39 in UC and 12/35 in CD in food allergens ($P < 0.001$)	No differences	[119]
UC: 39 CD: 19 Normals: 20	Skin prick tests to milk proteins	Positive: 15.7% of UC and 13.3% of CD; Significant difference between patients and healthy subjects	No differences	No differences	[120]
UC: 63 CD: 59 Controls: 103	Skin prick tests to various common allergens	No difference between patients and healthy subjects	No difference between patients and healthy subjects	No difference between patients and healthy subjects	[121]
CD: 308 Normals: 930	Questionnaire	Atopic disease was more common in CD <i>vs</i> normal ($P = 0.001$); Atopic eczema was twice as common in CD <i>vs</i> normal ($P = 0.001$)	-	-	[124]
UC: 50 Healthy: 50	Skin prick/patch tests to airborne, food, contact allergens SIgE Atopy history and family history	Allergic symptoms were more prevalent in UC and first degree relatives than in controls ($P < 0.0001$, $P = 0.008$)	UC <i>vs</i> controls; Immediate type hypersensitivity $P = 0.01$; Delayed type hypersensitivity $P = 0.03$	IgE levels were higher in UC than in controls $P = 0.02$	[125]

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

Treatment with corticosteroids^[75,78] or with appropriate medication such as sulfasalazine or mesalamine for the basic gastrointestinal disease^[74] appeared to be satisfactory for both diseases. Pneumonitis, in contrast, due either to sulphasalazine or mesalamine is a well-recognized adverse drug reaction in these patients^[50,87-95].

The above observations and the histological similarities between CD and sarcoidosis in particular, have led several groups to investigate the number and types of cells recovered by bronchoalveolar lavage (BAL)^[58,59,96]. An increased percentage of alveolar lymphocytes was reported in the study by Wallaert *et al*^[58] in patients with CD. In the same study, a correlation between BAL differential cell count and PFTs abnormalities, drug treatment or CD site and activity was not reported^[58]. The same group reported an increased level of IgG and IgM in BAL recovered from patients with alveolitis but not in those with normal BAL^[97]. This observation of subclinical alveolitis was confirmed in the study by Bonniere and associates in 22 patients with CD^[59]. In the same study, a significant increase in superoxide anion production by alveolar macrophages related to spontaneous activation and alteration of pulmonary function was observed^[59,98]. Bartholo *et al*^[99] found lymphocytosis in induced sputum of patients with CD even without pulmonary symptoms. Raj *et al*^[100] reported a trend for higher lymphocyte counts in the sputum of patients with CD compared with UC. Smićan *et al*^[96] reported lymphocytosis alveolitis in patients with CD but not in patients with other inflammatory bowel

disorders including UC. In the same study, an increase in the CD4 lymphocyte subset (increased ratio of CD4/CD8) was found in patients with an active stage of CD similar to patients with sarcoidosis^[96]. Yamaguchi *et al*^[101] reported increased BAL lymphocytes with an elevated CD4/CD8 ratio and enhanced expression of CD2 antigen in lung T cells in 8 patients with CD. Ussov *et al*^[102] found a significant increase in the pulmonary vascular granulocyte pool in patients with CD. The meaning of this subclinical alveolitis and alterations in lung parenchyma is unclear. A subclinical inflammatory alveolitis as assessed by BAL cell analysis may be present in asymptomatic patients with immunological systemic disorders and with normal chest X-ray^[103]. The fact that pulmonary involvement is not as common during extrathoracic granulomatosis as CD, whereas subclinical alveolitis is frequent, suggests that the lung possibly downregulates, in some way, alveolar inflammation due to the systemic immune disorder. The alveolitis observed in IBD patients does not necessarily precede the development of pulmonary granulomatosis and fibrosis^[97,103]. Increased pulmonary permeability to diethylenetriaminepenta-acetate radiolabelled with 99m-technetium (^{99m}Tc-DTPA) related to abnormal BAL findings has also been reported in patients with CD^[104]. The reduction in diffusing capacity of the lungs (DLCO) is common and early manifestations of interstitial lung diseases^[64,68,97] and latent lymphocytosis alveolitis could explain, in part, the reduction in DLCO observed in patients with CD^[60].

ATOPY AND IBD

The gastrointestinal tract comes into direct contact with a great variety of foreign substances and under certain conditions these may act as antigens causing allergic reactions^[105]. On the other hand, atopic subjects are possibly susceptible to several inhalants or food allergens^[106], while clinical features of atopic disorders include many organs among them both the pulmonary and gastrointestinal systems. Hippocrates reported that milk could cause gastric upset and urticaria and was probably the first to relate general atopy with gastrointestinal allergy. Hammer *et al*^[107] found an increased prevalence of all atopic features. Asthma was also documented as being highly prevalent in a large study by Bernstein *et al*^[108]. Studies on the relation between IBD and atopy are listed in Table 1. Ceyhan *et al*^[109] reported that allergic symptoms and skin prick test positivity were more common in IBD patients (Table 1). Fireman *et al*^[110] reported a higher percentage of eosinophils in induced sputum in patients with UC. Several studies have tried to investigate the attractive hypothesis that IBD, in particular UC, may be an allergic response to food^[111,112] especially in individuals susceptible to various allergens. This hypothesis is supported by certain evidence that eosinophils and eosinophil-derived mediators contribute to the histopathology and pathophysiology of IBD^[19,113-116]. Most studies confirmed the observation that atopic features are more frequent in patients with IBD than in the general population^[114,117-120] (Table 1). This may be an explanation for the overlapping allergic disorders in both the respiratory and gastrointestinal systems. However, the frequency of bronchial hyperresponsiveness was significantly higher in IBD patients than in normal subjects (41% *vs* 5%), even when non-atopic subjects were considered^[53]. This finding is consistent with the hypothesis that another immune system common to both exists and may be responsible for the inflammation in both systems^[36]. Only one study by Troncone *et al*^[121] showed that there was no correlation between atopy and IBD.

Engkilde *et al*^[122] found an inverse association between a contact allergy and IBD. In this study although there was a chronic contact allergic dermatitis which was considered by the authors to have a Th2 profile, contact allergy has a Th1 profile. Engkilde *et al*^[122] suggested that this may be due to shared genetic factors, common environmental determinants or skewness of the immune system. Medoff *et al*^[123] suggested that T cell trafficking takes place in peripheral tissue in allergic asthma. It is suggested that this trafficking may involve several interactions between innate immune cells and T cells^[123]. Several explanations for this phenomenon have been given over the years, however, no definite conclusions have been reached. Hammer *et al*^[107] suggested a genetic predisposition, Myrelid *et al*^[124] implicated TNF mast cells and D'Arienzo *et al*^[125] suggested a Th2 or Th1 helper response. The mechanisms of atopy in IBD merit further investigation.

CONCLUSION

Three patterns of pulmonary involvement have been reported to accompany IBD: (1) airway disease including large airway stenosis, chronic bronchitis, small airway dysfunction, severe bronchial suppuration and bronchiectasis; (2) parenchymal lung involvement either as subclinical lymphocytic alveolitis or several types of pulmonary infiltrate such as granulomatous bronchiolitis and bronchiolitis obliterans; and (3) a reduction in the diffusing capacity of the lung is a well established abnormality of pulmonary function testing in some patients with IBD.

We propose that patients suffering from IBD should undergo pulmonary evaluation which should include physical examination, chest X-ray and pulmonary function testing with DLCO measurement. This pulmonary evaluation may be useful in detecting subclinical or clinical pulmonary involvement in IBD patients or as a baseline evaluation. In clinical cases with pulmonary manifestations, inhaled or systemically administered steroids appear to be an effective treatment. With regard to atopy, routine investigations should be considered, at least in patients with IBD who also present with airway dysfunction.

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Heparanase and hepatocellular carcinoma: Promoter or inhibitor?

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Abstract

Heparan sulphate proteoglycans (HSPGs) consist of a core protein and several heparan sulphate (HS) side chains covalently linked. HS also binds a great deal of growth factors, chemokines, cytokines and enzymes to the extracellular matrix and cell surface. Heparanase can specially cleave HS side chains from HSPGs. There are a lot of conflicting reports about the role of heparanase in hepatocellular carcinoma (HCC). Heparanase is involved in hepatitis B virus infection and hepatitis C virus infection, the activation of signal pathways, metastasis and apoptosis of HCC. Heparanase is synthesized as an inactive precursor within late endosomes and lysosomes. Then heparanase undergoes proteolytic cleavage to form an active enzyme in lysosomes. Active heparanase translocates to the nucleus, cell surface or extracellular matrix. Different locations of heparanase may exert different activities on tumor progression. Furthermore, enzymatic activities and non-enzymatic activities of heparanase may play different roles during HCC development. The expression level of heparanase may also contribute to the discrepant effects of heparanase. Growth promoting as well as

growth inhibiting sequences are contained within the tumor cell surface heparan sulfate. Degrading different HSPGs by heparanase may play different roles in HCC. Systemic studies examining the processing, expression, localization and function of heparanase should shed a light on the role of heparanase in HCC.

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Key words: Apoptosis; Heparanase; Heparan sulphate; Hepatocellular carcinoma; Infection; Metastasis

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INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common malignant tumors worldwide. Extracellular matrix (ECM) remodeling plays an important role in the development of HCC^[1].

Heparan sulphate proteoglycans (HSPGs), one of the main components of ECM, are abundant macromolecules associated with the cell surface and ECM of a wide range of cells of vertebrate and invertebrate tissues. The basic HSPG structure consists of a core protein and several heparan sulphate (HS) side chains covalently linked. Extracellular HSPGs can maintain the ECM self assembly and integrity with other macromolecules, while cell surface HSPGs may act as co-receptor for several signal pathway molecules. In fact, HS chains also bind a great deal of growth factors, chemokines, cytokines and enzymes to the ECM and cell surface. HSPGs can thus influence a

number of normal and pathological processes, among which are tissue repair, inflammation, tumor growth and metastasis, and angiogenesis^[2,3].

Recent discoveries indicated that HSPGs localized within the tumor microenvironment can be attacked by enzymes that alter proteoglycan structure resulting in dramatic effects on tumor growth and metastasis^[4,5]. Heparanase, an endoglycosidase, can specially cleave HS side chains from HSPGs and release a multitude of bioactive molecules. Then, the generated HS fragments and released bioactive mediators could facilitate tumor metastasis cooperatively. In addition, heparanase also exhibits non-enzymatic activities, including cell adhesion and survival, upregulation of vascular endothelial growth factor (VEGF) and tissue factor, induction of signal transduction, and enhancement of certain HSPG shedding from the tumor cell surface^[6-16].

A large body of evidence suggest that the expression of heparanase in the tumor closely relates with the potential for tumor invasion, angiogenesis and metastasis in most tumors examined^[7-10]. However, there are a lot of conflicting reports about the relationship between heparanase and HCC. It is timely to review the literature to evaluate the arguments for and against the possible roles of heparanase in HCC.

HEPARANASE AND HEPATITIS B VIRUS (HBV) AND HEPATITIS C VIRUS (HCV) INFECTION

Cell surface heparan sulfate mediates entry and initiation of infection of HBV and HCV, the most important pathogenic factors for HCC. Proper structure and sulfation levels of heparan sulfate are prerequisite for this mediation^[17-23]. Heparanase might inhibit HS-mediated HCV and HBV entry and the initiation of infection^[18,21]. Degradation of cell surface heparan sulfate by pretreatment with heparanases resulted in a marked reduction of HCV envelope glycoprotein E2 binding to HepG2 cells^[18]. Treatment of Namalwa B cells and human erythroleukemia K562 cells with heparinase I also reduced the cellular binding of HBV nucleocapsids^[21]. However, HCV E2 bound to target cells *via* putative receptors in a noncompetitive manner. Incomplete inhibition of heparan sulfate might lead to a partial E2 blockade and evasion of the host immune response^[23]. El-Assal *et al*^[24] reported that heparanase expression was significantly higher in HCV-related HCC compared with that in HCV-negative patients. It is possible to assume that HCV enhances heparanase expression that may be involved in the HCV-related pathological and malignant changes.

HEPARANASE EXPRESSION IN LIVER DISEASES

A biphasic pattern of heparanase expression is also significantly observed in rat liver following partial hepatectomy, peaking at 12 h and 96-168 h and decreasing

at 360 h post-surgery^[25]. Elevated heparanase levels are noted in the early stages of thioacetamide induced rat liver fibrosis, with no further increase evident in rats exhibiting higher fibrotic grades^[25]. Reduction or no significant difference in heparanase expression levels are found in liver fibrosis or cirrhosis samples resected from human patients^[24,26-31].

There are conflicting reports about the expression level of heparanase in HCC. Examining HCC patients' specimens by reverse transcriptase-polymerase chain reaction (RT-PCR) or Real-Time Quantitative RT-PCR, *in situ* hybridization, Western blotting, immunohistochemistry and tissue microarrays (TMAs), five out of the seven studies reported that heparanase was over-expressed in HCC^[24,28-31]. However, two studies indicated that the expression level of heparanase was lower than that in adjacent noncancerous tissue^[26,27] (Table 1).

HEPARANASE AND HCC

Heparanase and metastasis of HCC

Metastasis is a sequential process including breaking off from the primary tumor, traveling through the bloodstream and stopping at a distant site. Heparanase enhances HCC metastasis by degrading ECM and releasing ECM-resident growth factors and angiogenic factors. Furthermore, non-enzymatic activities of heparanase, such as promoting cell adhesion, might also play a role in HCC metastasis^[6-16].

Hepatoma heparanase was first purified from a human hepatoma cell line Sk-hep-1 in 1998^[32]. El-Assal *et al*^[24] reported that expression of heparanase mRNA was significantly correlated with larger tumor size, potential for tumor invasion and tumor microvessel density. Many research studies also support the concept that heparanase expression closely relates with metastasis and recurrence of HCC, tumor differentiation and tumor stage^[28-31]. More recently, some researchers reported that down-regulating heparanase expression either by antisense oligodeoxynucleotide or by RNA interference could significantly inhibit the invasiveness, metastasis, and angiogenesis of human HCC SMMC7721 cells both *in vitro* and *in vivo*^[33]. Yang *et al*^[34] reported that two polypeptide antibodies, anti-MAP1 (multiple antigenic peptides)- and anti-MAP2-antibody, can effectively inhibit the heparanase activity of HCCLM6 human hepatocellular carcinoma cells *in vitro* and influence their invasive ability. Recently, PI-88, an heparanase inhibitor, showed preliminary efficacy as an adjunct therapy for post-operative HCC^[35]. Glycosaminoglycan mimetics may also compete with cellular heparan sulfate chains for the binding to CXC-chemokine Stromal cell-Derived Factor-1 (SDF-1)/CXCL12 and may affect heparanase expression, leading to inhibition of SDF-1/CXCL12-mediated migration and invasion of the Huh7 human hepatoma cells^[36].

However, Ikeguchi *et al*^[26] reported that heparanase mRNA in HCC was significantly lower than that of noncancerous liver tissue and heparanase expression did not correlate with tumor differentiation, tumor stage, or patient prognosis. In another study conducted by Ikeguchi's group, the expression level of heparanase was low in HCC

Table 1 Studies examining the pro-metastatic role of heparanase in HCC

Studies	No.	Methods	Positive rate of HCC tissue	Correlation between heparanase expression and HCC progression
El-Assal <i>et al.</i> ^[24] , 2001	55 ¹	RT-PCR	47%	Significant positive correlation
Ikeguchi <i>et al.</i> ^[26] , 2002	50 ²	QRT-PCR	< adjacent noncancerous tissue	No significant correlation
Ikeguchi <i>et al.</i> ^[27] , 2003	48 ²	QRT-PCR	< adjacent noncancerous tissue	Significant negative correlation
Xiao <i>et al.</i> ^[28] , 2003	11 ³	QRT-PCR, WB, ISH, IHC	> normal and cirrhosis tissue	Significant positive correlation
Chen <i>et al.</i> ^[30] , 2004	33 ²	RT-PCR	48.5%, > adjacent tissue	Significant positive correlation
Liu <i>et al.</i> ^[31] , 2005	33 ⁴	RT-PCR	48.5%, > paracancerous and normal tissue	Significant positive correlation
Chen <i>et al.</i> ^[29] , 2008	120 ⁵	IHC in TMAs	45.83%, > adjacent tumor tissue, cirrhosis, and normal liver tissue	Significant positive correlation

¹55 HCC tissue samples; ²Both HCC tissue samples and non-cancerous liver samples were obtained from the same patients; ³16 normal liver tissue samples, 14 liver cirrhosis tissue samples and 11 HCC tissue samples; ⁴HCC tissue samples and paracancerous tissue samples were obtained from 33 HCC patients; paracancerous tissues of 9 cases of benign liver tumor were used as normal controls; ⁵48 cases of adjacent HCC liver, 62 cases of cirrhosis, and 23 cases of normal liver tissues. HCC: Hepatocellular carcinoma; RT-PCR: Reverse transcriptase-polymerase chain reaction; QRT-PCR: Real-time quantitative RT-PCR; WB: Western blotting; ISH: *In situ* hybridization; IHC: Immunohistochemistry; TMAs: Tissue microarrays.

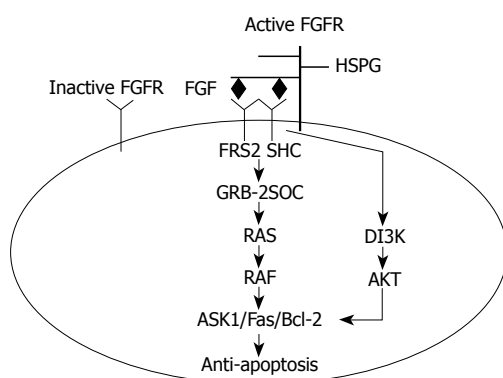


Figure 1 Heparan sulphate proteoglycan (HSPG) and fibroblast growth factor (FGF)-induced signal transduction. Basic FGF (bFGF) enhances tumor progression by protecting tumor cells from apoptosis. Cell surface HSPGs could act as a co-receptor for formation of a bFGF high-affinity receptor complex. The alteration of cell surface HSPGs resulting from heparanase might down-regulate HSPG-mediated bFGF-induced signal pathway, resulting in apoptosis of tumor cells.

and a high expression level of heparanase was associated with better disease-free 5-year survival rate^[27]. Ogawa *et al.*^[37] established rat HCC cell lines with a high metastatic potential and found that one cell line, showing high levels of lung metastasis when injected subcutaneously in nude mice, exhibited decreased heparanase mRNA expression compared with other cell lines.

In a study of fibroblasts transfected with various oncogenes, one cell line exhibiting a metastatic phenotype was not found to have a significant increase in heparanase activities, though another one having the highest metastatic potential was shown to contain the greatest heparanase activity^[38]. The hypothesis is that high heterogeneity of HCC might contribute to such discrepancy. Growth promoting as well as growth inhibiting sequences are contained within the tumor cell surface heparan sulfate^[39]. Degrading different HSPGs by heparanase may play different roles in the complex process of metastasis.

Heparanase and apoptosis of HCC

The HS side chains of HSPGs could bind a multitude

of growth factors, chemokines, cytokines and enzymes in ECM and cell surface, such as basic fibroblast growth factor (bFGF), vascular endothelial growth factor and hepatocyte growth factor. The cleaving of HSPGs by heparanase could release of HS-bound growth factors and exhibit complicated effects^[7-10]. bFGF might enhance endothelial cell and tumor cell proliferation, contributing to HCC progression^[40,41]. El-Assal *et al.*^[24] reported that bFGF and heparanase co-expressed in HCC patients' specimen and this co-expression was associated with higher tumor microvessel density than that in specimens with expression of either factor alone.

Heparanase is involved in the activation of several signal pathways including bFGF-induced signal transduction^[42-47] (Figure 1). In an *in vitro* study of melanoma cells, heparanase seemed necessary for phosphorylation of extracellular signal-related kinase (ERK) or focal adhesion kinase (FAK) in response to bFGF^[46]. Kato *et al.*^[47] reported that in postmastectomy wound fluids, syndecan-1 was converted from an inhibitor to an activator of bFGF by the degrading activities of heparanase. The release of bFGF and HS degrading fragments by heparanase might promote bFGF-receptor binding and activation^[42-47].

Heparanase expression closely related with apoptosis of several tumor cells, including HCC cells^[27,48,49]. Ikeguchi *et al.*^[27] found a significant positive correlation between heparanase mRNA expression levels and the percentages of apoptotic hepatocytes in liver tissues. In addition to mitogenic effects, bFGF also could enhance some tumor progression by protecting tumor cells from apoptosis^[50-56]. Targeting bFGF by neutralizing antibody or antisense oligonucleotides could result in apoptosis of some tumor cells^[57-59]. Cell surface HSPGs could not only act as co-receptors for formation of bFGF high-affinity receptor complexes, but could also function directly as receptors for bFGF-induced signal transduction, depending on core protein or HS specific manner^[60-62]. One possibility is that the alteration of cell surface HSPGs resulting from heparanase might down-regulate HSPG-mediated bFGF-induced signal pathways, resulting in apoptosis of tumor cells.

Location of heparanase in HCC

Human heparanase is synthesized as a 65 kDa inactive precursor within late endosomes and lysosomes. Then heparanase undergoes proteolytic cleavage, yielding 8 and 50 kDa protein subunits that heterodimerize to form an active enzyme in lysosome. Active heparanase translocates to the nucleus, cell surface or ECM^[6,63]. Different locations of heparanase may exert different activities. Cell surface expression and secretion of heparanase in EB mouse lymphoma cells markedly promotes tumor angiogenesis and metastasis compared with intracellular enzyme^[64]. However, nucleus heparanase induces differentiation of some tumor cells, such as esophageal cancer cells, mammary cancer cells and leukemic cells. Furthermore, a nuclear location of heparanase represents a better prognosis in tumor patients than its cytoplasmic location^[65-70].

During liver regeneration, the location of heparanase exhibits a dramatic alteration from cytoplasm to cell surface in a time-dependant manner^[25]. Xiao *et al.*^[28] and Chen *et al.*^[29] reported that high heparanase expression in HCC was localized within the cytoplasm of tumor cells and there was a significant correlation between the expression level of heparanase mRNA and tumor stage. Does the role of heparanase in HCC depend on its location?

CONCLUSION

There are a lot of conflicting reports about the role of heparanase in HCC. Several questions are intriguing and shouldn't be ignored. (1) In a human glioma cell xenograft tumor model, moderate heparanase expression levels significantly enhanced tumor development, whereas high heparanase expression levels inhibited tumor growth^[71]. Another study also showed that extensive heparanase inhibited bFGF binding in human metastatic melanoma 70W cells, while treatment of 70W cells with low heparanase concentrations enhanced bFGF binding^[46]. Does the effect of heparanase depend on its expression level in HCC? (2) During the course of colon adenoma-carcinoma progression, active heparanase increases in the early stage, while latent heparanase predominantly increases in the late stage. The possibility was that enzymatic activities and non-enzymatic activities of heparanase have different roles in the early and late stages of colon cancer development^[72]. Do enzymatic activities and non-enzymatic activities of heparanase play different roles during HCC development? (3) HSPGs may have promoting or inhibiting activities depending on the core protein and localization^[73]. For example, Glypican-3 and syndecan-1 might act as promoter and inhibitor during the development of HCC, respectively^[74,75]. Degrading different HSPGs by heparanase may play different roles in HCC; and (4) Researchers have already observed that heparan sulfates could occur in hepatic nucleus and hypothesized that alteration of heparan sulfates detected in HCC might be involved in HS-related gene expression^[76-81]. For example, DNA topoisomerase I activity is modulated by heparan sulfates present in normal liver cells but is markedly reduced or absent in their transformed counterparts^[80]. In-

terestingly, active heparanase also could translocate to the nucleus and degrade nuclear HS^[65-70,81]. Is heparanase the criminal for the lack of biologically active HS in HCC? Do the effects of heparanase depend on its location in HCC? Systemic studies examining the processing, expression, localization and function of heparanase should shed a light on the role of heparanase in HCC.

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***Helicobacter pylori* and EBV in gastric carcinomas: Methylation status and microsatellite instability**

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cagA⁺ and Epstein Barr virus (EBV) infections in gastric adenocarcinomas.

METHODS: Methylation-specific PCR (MSP) assay was performed in 89 primary gastric carcinomas (intestinal and diffuse types). Microsatellite instability (MSI) analysis was performed using the BAT26 primer set and PCR products were analyzed with the ABI PRISM 3100 Genetic Analyzer using Genescan 3.7 software (Applied Biosystems). Detection of *H. pylori* and genotyping were performed by PCR, using specific primers for *ureaseC* and *cagA* genes. The presence of EBV was assessed by *in situ* hybridization. Statistical analyses were performed using the χ^2 or Fisher's exact test.

RESULTS: The most frequent hypermethylated gene was *COX-2* (63.5%) followed by *DAPK* (55.7%), *CDH1* (51%), *CDKN2A* (36%) and *hMLH1* (30.3%). Intestinal and diffuse adenocarcinomas showed different methylation profiles and there was an association between methylation of *E-CDH1* and *H. pylori-cagA*⁺ in the intestinal adenocarcinoma type. MSI was correlated with *hMLH1* methylation. There was an inverse correlation between *DAPK* hypermethylation and MSI.

CONCLUSION: We found a strong association between *CDH1* methylation and *H. pylori-cagA*⁺ in intestinal-type gastric cancer, association of MSI and better prognosis and an heterogeneous *COX-2* overexpression.

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Key words: Gastric cancer; Methylation; Microsatellite instability; *Helicobacter pylori*; Epstein Barr virus

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Abstract

AIM: To verify the methylation status of *CDH1*, *DAPK*, *COX2*, *hMLH1* and *CDKN2A* genes and to evaluate their association with *Helicobacter pylori* (*H. pylori*)-

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INTRODUCTION

Gastric cancer (GC), one of the most common cancer types, is associated with high mortality rates^[1,2]. The prognosis of GC remains poor, especially when the diagnosis is undertaken at advanced stages^[3]. Thus, studies to elucidate the mechanisms acting in gastric carcinogenesis and which search for possible markers to assist in both earlier diagnosis and therapeutic approaches are relevant.

Gastric adenocarcinomas can be divided into intestinal or diffuse histological types. Environmental factors appear to be related to the intestinal type, which may play a role in carcinogenesis characterized by precursor lesions of gastric mucosa, followed by intestinal metaplasia that can lead to dysplasia and GC. In contrast, in the diffuse carcinoma no precursor lesions have been identified to date^[4].

Many studies have identified the transcriptional silencing by DNA methylation as a mechanism responsible for tumor suppressor inactivation. Methylation of promoter CpG islands leads to DNA structural changes and, consequently, gene inactivation^[5]. Several cancers, including gastric tumors, show methylation of multiple genes including *CDH1*, *DAPK*, *COX2*, *hMLH1* and *CDKN2A*^[6,7].

Microsatellite instability (MSI) reflects an erroneous form of DNA replication in repetitive microsatellite sequences and has been considered a hallmark of mismatch repair gene inactivation. MSI has been associated with less aggressive tumor behavior and favorable prognosis in sporadic colorectal cancer^[8-10]. MSI status has been determined by means of BAT26 mononucleotide repeats because this marker is quasi-monomorphic in normal DNA and has shown high sensitivity and specificity in the identification of MSI phenotype^[11].

Helicobacter pylori (*H. pylori*), carcinogen class I^[12], colonizes the gastric epithelium and causes a severe inflammatory reaction that depends on factors including host genetic susceptibility, immune response, age at the time of initial infection, and environmental and virulence factors such as cytotoxin-associated gene A (*cagA*)^[13-15]. The complex interactions among the different types of *H. pylori*, inflammation and genetic features of the host could promote a cascade of morphological events leading to GC^[16].

Apart from the accepted role of *H. pylori* in the pathogenesis of GC, the Epstein Barr virus (EBV) has been associated with gastric carcinoma in at least 10% of cases^[17]. Countries with the highest incidences are Japan (19.3%) and Germany (18%)^[17,18]. In Brazil, frequencies of EBV infec-

tion ranging between 8% and 11% have been described^[19,20].

Some studies have linked DNA hypermethylation with *H. pylori-cagA*⁺ and EBV infection but these data are not conclusive and the studies did not examine both agents at the same time. By examining 89 primary gastric carcinomas, the present study verifies MSI frequency and the methylation status of the *CDH1*, *DAPK*, *COX2*, *hMLH1* and *CDKN2A* genes and evaluates their association with *H. pylori* (*cagA*⁺ and *cagA*) and EBV infections and also with clinicopathological features of gastric carcinomas.

MATERIALS AND METHODS

Samples

Eighty-nine gastric adenocarcinomas and their corresponding adjacent normal tissue were obtained surgically from Brazilian patients at the Federal University of Ceara State, the Clinical Hospital at the UNESP Medical School in Botucatu, Sao Paulo State and the Amaral Carvalho Hospital, and immediately frozen in liquid nitrogen until micro-dissection and DNA extraction. The Research Ethics Committees of the respective institutions approved this study and each subject signed an informed consent form before tissue was obtained. Histopathological analyses determined that the tumor specimens consisted mainly (> 80%) of tumor tissues and that the adjacent tissue was free of tumor cells. The histological classification was made according to the Laurén classification system^[4] and the tumors were staged according to the TNM criteria^[21]. DNA was extracted using standard methods^[22].

Bisulfite modification and methylation-specific PCR (MSP)

DNA from both tumoral and normal tissues was subjected to treatment with sodium bisulfite as described by Herman *et al.*^[23]. The modified DNA was amplified with primers specific for either the methylated or unmethylated sequences of *hMLH1*, *COX2*, *DAPK*, *CDKN2A* and *CDH1* (Table 1). PCR was individually performed in 25 µL reaction volumes, containing 1 × Platinum Taq buffer, 1.5 mmol/L MgCl₂, 0.2 mmol/L of each dNTP, 0.4 µmol/L of each primer set, 1 U of Platinum Taq DNA Polymerase (Invitrogen) and 1 µL of treated DNA. DNA methylated *in vitro* by *Sss-I* methylase (New England Biolabs) was used as a positive control, and water and DNA from peripheral lymphocytes of healthy donors were used as negative controls. PCR products were separated on silver-staining 6% non-denaturing polyacrylamide gels^[22].

Bisulfite sequencing analysis

To confirm reaction specificity, MSP-PCR products from each gene analyzed were cloned with TOPO TA Cloning Kit (Invitrogen) and sequenced using the ABI PRISM® BigDye Terminator v3.0 Cycle Sequencing Kit (Applied Biosystems) and ABI Prism 3100 DNA Sequencer (Applied Biosystems).

Table 1 Primer Sequences and PCR conditions for methylation-specific PCR (MSP) analysis

Gene	Primer (5'-3') forward	Primer (5'-3') reverse	Size (bp)	T (°C)	Ref.
COX2	M TTAGATACGGCGGCGCGGC	TCITTTACCCGAACGCTTCCG	161	68	[24]
	U ATAGATTAGATATGGTGGTGGTGGT	CACAATCTTTACCCAAACACTTCCA	171	67	
DAPK	M GGATAGTCGGATCGAGTTAACGTC	CCCTCCCAAACGCCGA	98	65	[25]
	U GGAGGATAGTTGGATTGAGTTAATGTT	CAAAATCCCTCCCAAACACCAA	116	65	
CDH1	M TTAGGTTAGAGGGTTATCGCGT	TAACATAAAATTCACCTACCGAC	115	61	[26]
	U TAAATTTAGGTTAGAGGGTTATTGT	CACAACCAATCAACAACACA	97	59	
<i>hMLH1</i>	M TATATCGTTCGTAGTATTCTGTT	TCCGACCCGAATAAACCCAA	153	65	[26]
	U TTTTGATGTAGATGTTTATTAGGGTGT	ACCACCTCATCATAACTACCCACA	124	63	
CDKN2A	M TTATTAGAGGGTGGGGCGGATCGC	GACCCCGAACCGCGACCGTAA	150	70	[21]
	U TTATTAGAGGGTGGGGTGGATTGT	CAACCCCAAACCAACCATAA	151	70	

Microsatellite instability analysis

MSI analysis was performed using the BAT26 primer set (5'-TGACTACTTTTGACTTCAGCC-3', sense and 5'-AACCATTCAACATTTTAACCC-3', antisense). Sense primer was labeled with 6-FAM. PCR was performed in a final volume of 25 µL containing 1 × PCR buffer, 3.0 mmol/L MgCl₂, 0.2 µmol/L dNTPs, 0.4 µmol/L of each primer, 2 U of Platinum Taq DNA Polymerase (Invitrogen) and 50 ng of DNA. The thermal conditions were 94°C/5 min followed by 40 cycles (94°C/1 min, 50°C/1 min and 72°C/1 min) and a final extension at 72°C/7 min. The dye-labeled PCR products were analyzed with ABI PRISM 3100 Genetic Analyzer using Genescan 3.7 software (Applied Biosystems). Both tumoral and normal samples were analyzed. Negative (SW480 cells) and positive (HCT116 cells) controls for MSI had been included in all the analyses. Deletions or insertions of at least 4 bp were required to satisfy the definition of instability^[27]. All cases were repeated twice.

H. pylori and *CagA* detection

Detection of *H. pylori* in gastric samples was performed for PCR amplification with primers specific to *H. pylori ureaseC* gene. The primer sequence used (5'-AAGCTTT-TAGGGGTGTTAGGGGT'TT-3', sense and 5'-CT-TACTTTCTAACACTAACGC-3', antisense)^[28] amplifies a 294 bp fragment. To detect *cagA*, the primer set 5'-ATAATGCTAAATTAGACAACCTTGAGCGA-3' (sense) and 5'-TTAGAATAATCAACAAACATAACG CCAAT-3' (antisense)^[29] was utilized to amplify a 297 bp fragment. Each primer set (*ureaseC* and *cagA*) was used in an independent PCR reaction in a final volume of 25 µL containing 1 × PCR buffer [20 mmol/L Tris-HCl (pH 8.4) and 50 mmol/L KCl], 1.5 mmol/L MgCl₂, 0.2 mmol/L of each dNTP, 0.4 µmol/L of each primer set, 1 U of Platinum Taq DNA Polymerase (Invitrogen) and 100 ng of DNA, under the following conditions: for *ureaseC* PCR, initial denaturation at 94°C/3 min followed by 35 cycles of denaturation at 94°C/30 s, annealing at 58°C/30 s and extension at 72°C/2 min and final extension at 72°C/5 min. The *cagA* PCR included an initial denaturation at 94°C/3 min followed by 40 cycles of denaturation at 94°C/30 s, annealing at 58°C/45 s and extension at 72°C/2 min and final extension at 72°C/5 min. Both tumoral and normal samples were analyzed. Negative and

positive controls were assayed in each run. PCR products were separated by silver-stained 6% non-denaturing polyacrylamide gel electrophoresis^[22].

EBER1 *in situ* hybridization

The presence of EBV was assessed by RNA *in situ* hybridization reaction with a 30 bp biotinylated probe (5'-AGACACCGTCCTCACCACCCGGGACTTG TA-3') complementary to the RNA *EBER1*. EBV transcript was shown in high amounts in the nuclei of latently infected cells. Signal amplification was employed with anti-biotin antibody (clone BK, mouse, dilution 1:20; DakoCytomation®) and biotinylated anti-immunoglobulin antibody (polyclonal, rabbit, dilution 1:100; DakoCytomation®). The reaction was detected with the streptavidin-biotinperoxidase complex (DakoCytomation®) and diaminobenzidine chromogen (DakoCytomation®). The slides were counterstained with Harris's hematoxylin. A case of nasopharyngeal carcinoma was used as positive control.

Statistical analysis

For statistical analysis the χ^2 test or Fisher's exact test was used. *P* values ≤ 0.05 were considered statistically significant.

RESULTS

Patients and tumor characteristics

The clinicopathological and epidemiological features of patients are shown in Table 2.

Methylation status

Of the five genes analyzed, the *COX2* gene was the one most frequently hypermethylated (63.5%) followed by *DAPK* (55.7%), *CDH1* (51%), *CDKN2A* (36%) and *hMLH1* (30.3%). Figure 1 displays representative examples of the MSP products.

In the diffuse adenocarcinoma cases, methylation of *CDH1*, *COX2* and *CDKN2A* presented higher frequencies in early stage tumors (0- I) with a tendency to decrease along with the tumor grades (mainly *CDH1* and *COX2*). On the other hand, in the intestinal type, the methylation status of *CDH1*, *COX2*, *hMLH1* and *CDKN2A* tended to increase from the earliest stages

Table 2 Clinicopathological and epidemiological features of patients in the four tumor stages *n* (%)

Variables	Patients	Tumor stage			
		0- I	II	III	IV
Age (yr)					
≤ 50	19	3 (15.8)	2 (10.5)	9 (47.4)	5 (26.3)
> 50	70	8 (11.4)	11 (15.7)	30 (42.9)	21 (30)
Gender					
Male	60	8 (13.3)	8 (13.3)	27 (45)	17 (28.3)
Female	29	3 (10.3)	5 (17.2)	12 (41.4)	9 (31)
Anatomic subsite					
Proximal (Cardia)	13	1 (7.7)	1 (7.7)	5 (38.5)	6 (46.1)
Distal (Antrum/body)	46	2 (4.3)	9 (19.6)	21 (45.7)	14 (30.4)
Mixed	3	0 (0)	1 (33.3)	2 (66.7)	0 (0)
Laurén type					
Diffuse	31	4 (12.9)	4 (12.9)	12 (38.7)	11 (35.5)
Intestinal	57	7 (12.3)	9 (15.8)	27 (47.4)	14 (24.6)
Mixed	1	0 (0)	0 (0)	0 (0)	1 (100)

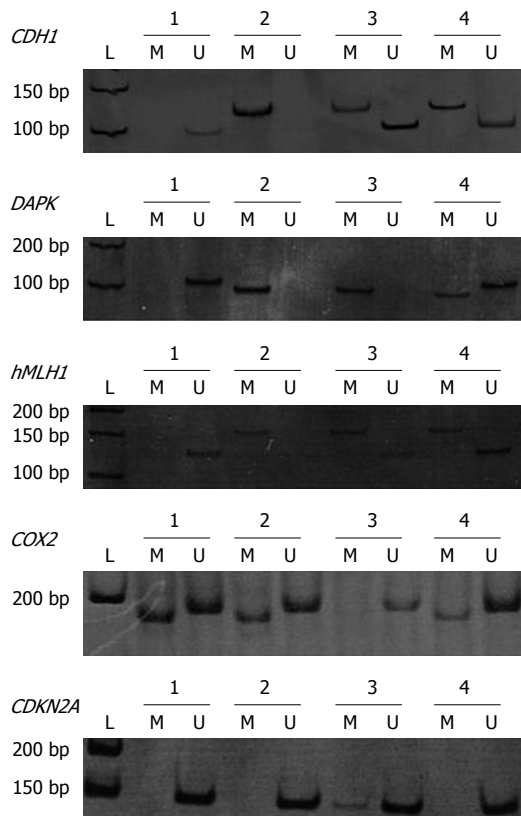


Figure 1 Example of results from methylation-specific PCR analysis performed in tumoral samples from patients with gastric cancer. The studied gene is given on the right of the each panel. Lane U: Amplified product with primers recognizing unmethylated sequences; Lane M: Amplified product with primers recognizing methylated sequences; L: Ladder 50 bp.

(0- I) to advanced stages (II-IV) ($P < 0.001$, Figure 2A and B). The methylation status tended to increase with age; the highest frequency of cases with promoter methylation was found in patients between 45 and 64 years old. Patients aged more than 50 years had a higher frequency of methylation in *CDKN2A* ($P = 0.035$).

Table 3 summarizes overall results of the correlation

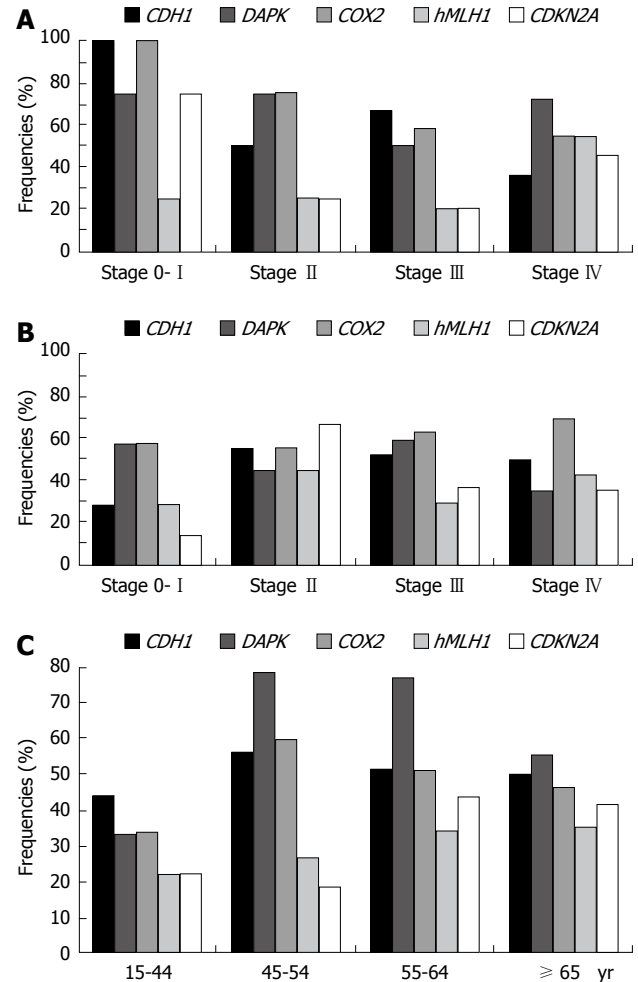


Figure 2 Frequencies of methylation at *CDH1*, *DAPK*, *hMLH1*, *COX2* and *CDKN2A* genes in gastric carcinomas. A: Frequencies of methylation at *CDH1*, *DAPK*, *hMLH1*, *COX2* and *CDKN2A* distributed according to tumor stages in diffuse adenocarcinoma; B: Frequencies of methylation at *CDH1*, *DAPK*, *hMLH1*, *COX2* and *CDKN2A* distributed according to tumor stages in intestinal adenocarcinoma; C: Frequencies of methylation at *CDH1*, *DAPK*, *hMLH1*, *COX2* and *CDKN2A* distributed with regard to age groups.

between the promoter methylation frequency of each gene and relevant clinicopathological parameters.

Microsatellite instability analysis

MSI was observed in 17.2% of GCs. Most of the patients with MSI tumors (86.7%) had an advanced form of the disease and 66.7% of them shown lymph node metastasis. Intestinal and diffuse adenocarcinomas had similar MSI frequencies. There was a significant correlation between MSI and *hMLH1* methylation ($P = 0.001$). Conversely, a significant inverse correlation was demonstrated between *DAPK* methylation and MSI ($P = 0.012$).

Detection of *H. pylori*

The presence of *H. pylori* was identified in 98% of GC patients, of whom 63.2% were *cagA*⁺. The frequency of *H. pylori-cagA*⁺ infection was similar between intestinal and diffuse adenocarcinomas (64.5% *vs* 59.6%, respectively). In the intestinal type, *CDH1* methylation was more frequent in *H. pylori-cagA*⁺ cases (55.8%) than in

Table 3 Associations between gene methylation status and clinicopathological features, age, *H. pylori*, MSI and EBV *n* (%)

	Total	Methylated genes									
		<i>CDH1</i>	<i>P</i> -value	DAP-kinase	<i>P</i> -value	<i>COX2</i>	<i>P</i> -value	<i>hMLH1</i>	<i>P</i> -value	<i>CDKN2A</i>	<i>P</i> -value
Mean age (yr)		60.2		59.9		60.9		63.1		62.5	
Age group (yr)											
≤ 50	19	10 (52.6)	0.883	8 (44.4)	0.282	9 (50)	0.179	6 (33.3)	0.903	3 (15.8)	0.035 ¹
> 50	69	35 (50.7)		41 (58.6)		45 (67.2)		21 (31.8)		29 (42)	
Gender											
Male	59	30 (50.8)	0.938	33 (55.9)	0.946	34 (59.6)	0.289	21 (35)	0.787	21 (35.6)	0.830
Female	29	15 (51.7)		16 (55.2)		20 (71.4)		11 (37.9)		11 (37.9)	
Nodal metastases											
Absent	17	8 (47.1)	0.708	9 (50)	0.586	11 (64.7)	0.910	6 (33.3)	0.903	6 (33.3)	0.764
Present	71	37 (52.1)		40 (57.1)		43 (63.2)		21 (31.8)		26 (37.1)	
Tumor site											
Cardia	13	8 (61.5)	0.855	9 (69.2)	0.427	10 (83.3)	0.228	4 (30.8)	0.304	8 (61.5)	0.266
Antrum	38	21 (55.3)		20 (52.6)		21 (56.8)		14 (37.8)		14 (35.9)	
Body	10	5 (50)		7 (70)		7 (70)		1 (11.1)		4 (40)	
Mixed	3	3 (100)		2 (66.7)		3 (100)		0		0	
Lauren											
Diffuse	30	17 (56.7)	0.503	10 (33.3)	0.628	19 (63.3)	0.973	8 (27.6)	0.481	10 (33.3)	0.628
Intestinal	57	28 (49.1)		22 (38.6)		34 (63)		19 (35.2)		22 (38.6)	
Mixed	1	0 (0.0)		0		1 (100)		0		0	
<i>H. pylori</i>											
<i>CagA</i> ⁺	55	31 (56.4)	0.261	30 (54.5)	0.782	35 (66)	0.536	15 (28.8)	0.41	22 (40)	0.468
<i>CagA</i> ⁻	34	15 (44.1)		19 (57.6)		19 (59.4)		12 (37.5)		11 (32.4)	
MS status											
MSI	14	5 (35.7)	0.174	4 (26.7)	0.012 ¹	8 (53.3)	0.349	10 (66.7)	0.001 ¹	4 (26.7)	0.405
MSS	72	40 (55.6)		44 (62)		45 (66.2)		16 (23.5)		27 (38)	
EBV											
Positive	5	1 (20)	0.101	4 (80)	0.346	4 (80)	0.694	0	0.179	3 (60)	0.462
Negative	48	28 (58.3)		28 (58.3)		33 (71.7)		15 (31.9)		21 (42.9)	

¹Statistically significant result.

H. pylori-cagA⁻ cases (39.1%), a phenomenon not observed in the diffuse type. Also, *cagA*⁺ among the intestinal cases displayed a higher frequency of *hMLH1* methylation than among diffuse *cagA*⁺ cases (31.8% *vs* 15.8%, respectively). On the other hand, methylation of *DAPK* and *COX2* did not vary when the samples were grouped by histotype and *cagA* status. With regard to MSI and the presence of *cagA*, MSI was inversely correlated with the *cagA* gene (*P* = 0.012), as shown in Table 3.

Detection of EBV

Fifty-four tumors were analyzed for EBV, of which 5 (9.3%) specimens were EBV-positive (EBV⁺). According to histological classification, 4 patients presented intestinal type adenocarcinoma and one was diffuse. All EBV⁺ cases were advanced grade (III and IV stages) and presented lymph node metastases. Two cases were EBV/*H. pylori*. The EBV⁺ cases were found to be associated with *H. pylori*, but only one was *H. pylori-cagA*⁺. Although the number of EBV⁺ cases was small, most of them displayed *DAPK*, *COX2* and *CDKN2A* methylation.

DISCUSSION

Our results show that methylation status tends to increase with age. This finding corroborates the fact that GC occurs at a higher frequency in older individuals^[1,2].

Moreover, previous studies showed that the age-related phenomenon of methylation of some tumor-suppressor and tumor-related genes can also be present in various non-neoplastic tissues, suggesting an association between age-related methylation and GC development^[30-32].

In this study, we demonstrate that 84.3% of GCs in our sample present methylation for about one in five of the genes analyzed. *COX2* was the gene found to be most commonly hypermethylated (63.5%) followed by *DAPK* (55.7%), *CDH1* (51%), *CDKN2A* (36%) and *hMLH1* (30.3%). An interesting observation in this study was related to the difference in methylation profiles between diffuse and intestinal adenocarcinoma types: in diffuse cases, the global methylation status, especially of *CDH1*, *COX2* and *CDKN2A*, has the highest frequency in early stage tumors (0- I) with a tendency to decrease along with tumor grades; while in the intestinal-type, the methylation status for *CDH1*, *COX2*, *hMLH1* and *CDKN2A* tended to increase from the earliest (0-I) to advanced stages (II-IV), as shown in Figure 2A and B. *CDH1* methylation was more frequent in the diffuse histotype. In fact, a vast difference was verified in stage I tumors where all diffuse-type tumors presented *CDH1* promoter methylation (100%) compared with only a small fraction (28.6%) in the intestinal-type, suggesting that *CDH1* methylation is an early event occurring in diffuse-type GCs. Since *CDH1* plays a fundamental role in maintaining cell differentiation,

polarity and normal tissue architecture^[33], the diffusely-growing and low cell cohesion characteristics of diffuse-type GC could be a function of *CDH1* down-regulation. Differences in the clinicopathological features between the intestinal and diffuse GC histological subtypes may be determined by different pathogenic processes^[16,34]. The data presented in this study show that methylation in the same crucial genes could be an important pathway for the development of diffuse types. Identification of epigenetic differences between these two pathways could be of great importance in understanding gastric carcinogenesis and useful in the delineation of new therapeutic strategies.

The mechanisms that are implicated in *CDH1* promoter methylation are yet to be identified. The role of *H. pylori* in the regulation of *CDH1* expression has been described in recent studies showing that after *H. pylori* eradication, *CDH1* methylation is decreased^[35,36]. In our study, we observed that in the intestinal adenocarcinoma cases, methylation in the *CDH1* promoter was more frequent in the group *H. pylori-cagA*⁺ (55.8%) than in those with *H. pylori-cagA*⁻ (39.1%), indicating that *H. pylori-cagA*⁺ may be involved in *CDH1* methylation in these tumors.

DAPK methylation has been shown to be associated with aggressive and metastatic phenotypes in some human cancers^[25,37]. In the present study, we found a substantial frequency of *DAPK* methylation and observed that positive lymph node cases showed a slightly higher frequency of *DAPK* methylation than unmethylated cases (57.1% *vs* 42.9%). An important finding in this study was the inverse correlation observed between *DAPK* methylation and MSI. Although the mechanisms linking MSI to *DAPK* methylation are not known, this finding may provide a clue towards a better understanding of the association between MSI and better prognosis, since *DAPK* participates in the positive control of apoptosis.

Similar to previously reported results^[38], 36.4% of the cases displayed hypermethylated *CDKN2A*. The EBV seems to play an important role in *CDKN2A* methylation^[38,39]. However, the fact that, in this study, 60% of the EBV⁺ cases showed methylated *CDKN2A* should be interpreted cautiously because of the low number of such cases. Methylation in *CDKN2A* was more frequent in patients over 50 years of age ($P = 0.035$), and was also present in some samples of non-neoplastic gastric epithelia (data not shown) suggesting a link between aging and cancer *via* an increase in methylation. However, it is noteworthy that younger patients (< 50 years) who did not present *CDKN2A* methylation still developed GC, which suggests that other factors may account for the gastric carcinogenesis in these patients. In order to better evaluate in our population the correlation between *CDKN2A* and EBV it will be necessary to increase the number of tumors analyzed for EBV.

Most of the gastric tumors in our sample (63.5%) exhibit aberrant methylation of *COX2*. The correlation between *COX2* methylation and gene downregulation has been well documented in the literature^[40] although overexpression of *COX2* has also been reported in GCs and some precancerous tissues^[41]. *COX2* overexpression

is associated with enhanced proliferation, angiogenesis, resistance to apoptosis and tumorigenesis^[41]. Despite the apparent selective advantage given by *COX2* overexpression, the results from our research group and others^[42] suggest that *COX2* overexpression may not be essential in all cases of gastric tumorigenesis. Recent studies have documented that *H. pylori*-induced inflammation is linked to *COX2* overexpression^[43] and these findings led us to investigate whether *cagA* presence was related to the methylation status of *COX2*. In the present study, no significant correlation between *cagA* and *COX2* methylation status was found. Thus, *cagA* presence appears not to be the only mechanism involved in the control of *COX2* expression in *H. pylori*⁺ gastric cells.

Methylation of promoter regions is reported to be the main cause of inactivation of *hMLH1*^[44]. The present study revealed a significant relationship between *hMLH1* methylation and MSI ($P = 0.001$), corroborating findings from other studies^[44,45]. In fact, methylation of this mismatch repair gene has been shown to play a major role in MSI in several cancers. The data from MSI analyses found in our study (17.2%) corroborated other studies in the literature which demonstrated MSI ranging from 13% to 39%^[46]. The association between MSI and clinicopathological characteristics of GC remains unknown. While some studies reported that MSI gastric tumors are associated with distal tumor location, intestinal histotype, fewer lymph node metastases and better prognosis^[46,47], others have found the absence of an association among these parameters^[48]. In our data, 33.3% of MSI patients had no positive lymph nodes (N0) *vs* 18.1% for MSS (stable) patients, suggesting a tendency for a better prognosis. Previous studies detected that MSI frequency in *H. pylori*-positive groups was significantly higher than in *H. pylori*-negative ones^[49-51] indicating that this agent may play an important role in genetic stability. Several studies have suggested that the virulence attributed to the *H. pylori-cagA*⁺ strain is associated with a severe inflammatory response. It is known that in the gastric mucosa, reactive oxygen species are released as a result of *H. pylori* infection^[51] and that the expression of DNA mismatch repair proteins in mismatch-competent cells might be transiently suppressed in the presence of oxidative stress^[52]. In the present study, when the cases were divided into two groups (*H. pylori-cagA*⁺ and *H. pylori-cagA*⁻) we observed that, in contrast to our expectations, MSI was inversely correlated with *cagA* ($P = 0.012$), suggesting that, apart from *cagA*, other factors may contribute to MSI occurrence. These results require further exploration with larger numbers of cases.

In conclusion, our results confirm that methylation is an early epigenetic event in the molecular pathogenesis of GC. The methylation pattern of the genes studied suggests that gastric tumorigenesis can occur through different pathways. It appears that in diffuse adenocarcinoma tumors, methylation can be an early and crucial event in enabling tumorigenesis, where methylation in *CDH1* assumes an important role and that MSI is associated with *hMLH1* methylation, although this event is infrequent. The inverse association discovered between *DAPK* methylation

and MSI provides new data for elucidating the mechanisms involved in the association of MSI and better prognosis. Analysis of a larger number of patients is necessary to confirm our findings and to ascertain the significance of the association between promoter methylation, MSI and the presence of infectious agents in gastric carcinogenesis. We observed that *H. pylori-cagA*⁺ may be involved in the methylation process of *CDH1* in intestinal adenocarcinoma. Microsatellite instability was inversely correlated with the *cagA* gene, suggesting that other factors may contribute to MSI occurrence. *COX-2* overexpression does not occur in all GC cases.

COMMENTS

Background

Gastric cancer (GC) is one of the most common cancer types and it is associated with high mortality rates. The prognosis of this disease remains poor, especially when the diagnosis is undertaken at advanced stages. The most common GC is adenocarcinoma divided into two types, intestinal and diffuse, and these differ substantially in epidemiology, pathogenesis, genetic profile and prognostic features. *Helicobacter pylori* (*H. pylori*) is one of the more important etiological factors in GC. The *cagA* gene codes for one of the major *H. pylori* virulence proteins. Bacterial strains that have the *cagA* gene are associated with an ability to cause a stronger inflammatory response in the stomach and patients are at a greater risk of developing peptic ulcers or stomach cancer. The second infectious agent found to be associated with a subset of GCs is the Epstein Barr virus (EBV). DNA methylation is an epigenetic modification found in physiological events, however, when it is aberrant it can be associated with inactivation of tumor suppressor genes. Microsatellite Instability (MSI) reflects an erroneous form of DNA replication in repetitive microsatellite sequences and has been considered a hallmark of DNA mismatch repair gene inactivation and therefore consequently leads to genetic instability.

Research frontiers

Some studies have linked DNA hypermethylation and MSI with *H. pylori-cagA*⁺ and EBV infection but these data are not conclusive and the studies did not look at both agents at the same time. Therefore it is very important to analyze the same cases for both etiological factors and correlate them with MSI genetic and epigenetic alterations as well as with clinical and epidemiological data.

Innovations and breakthroughs

The present study suggests that intestinal-type and diffuse-type GC show different methylation profiles in the genes analyzed and a strong association was found between methylation of *CDH1* and *H. pylori-cagA*⁺ in the intestinal-type GC. In addition, a very significant inverse correlation was found between methylation of *DAPK* gene (*DAPK* has a pivotal role in regulation of apoptosis and survival in cells) and MSI, providing new evidence for the association of MSI and better prognosis.

Applications

The data presented in this article represent important information about methylation profiles for intestinal-type and diffuse-type GC. Results show the association between *H. pylori-cagA*⁺ and methylation in an important gene involved in metastasis (*CDH1*) in addition to showing the inverse association between *DAPK* methylation and MSI, providing new data for elucidating the mechanisms involved in the association of MSI and better prognosis. This will add to the available body of knowledge about gastric carcinogenesis and aid in future research into this important disease.

Peer review

The manuscript by Ferrasi *et al* describes the methylation status and MSI frequency in the context of *H. pylori* and EBV infections in GC. This topic is of scientific and of clinical interest.

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Helicobacter pylori HopE and HopV porins present scarce expression among clinical isolates

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METHODS: *H. pylori* *hopE* and *hopV* genes derived from strain CHCTX-1 were cloned by polymerase chain reaction (PCR), sequenced and expressed in *Escherichia coli* AD494 (DE3). Gel-purified porins were used to prepare polyclonal antibodies. The presence of both genes was tested by PCR in a collection of *H. pylori* clinical isolates and their expression was detected in lysates by immunoblotting. Immune responses against HopE, HopV and other *H. pylori* antigens in sera from infected and non-infected patients were tested by Western blotting using these sera as first antibody on recombinant *H. pylori* antigens.

RESULTS: PCR and Western blotting assays revealed that 60 and 82 out of 130 Chilean isolates carried *hopE* and *hopV* genes, respectively, but only 16 and 9, respectively, expressed these porins. IgG serum immunoreactivity evaluation of 69 *H. pylori*-infected patients revealed that HopE and HopV were infrequently recognized (8.7% and 10.1% respectively) compared to *H. pylori* VacA (68.1%) and CagA (59.5%) antigens. Similar values were detected for IgA serum immunoreactivity against HopE (11.6%) and HopV (10.5%) although lower values for VacA (42%) and CagA (17.4%) were obtained when compared to the IgG response.

CONCLUSION: A scarce expression of HopE and HopV among Chilean isolates was found, in agreement with the infrequent seroconversion against these antigens when tested in infected Chilean patients.

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Key words: *Helicobacter pylori*; Gene expression; HopE and HopV porins; Antigens; Immune response

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Abstract

AIM: To evaluate how widely *Helicobacter pylori* (*H. pylori*) HopE and HopV porins are expressed among Chilean isolates and how seroprevalent they are among infected patients in Chile.

Lienlaf M, Morales JP, Díaz MI, Díaz R, Bruce E, Siegel F, León G, Harris PR, Venegas A. *Helicobacter pylori* HopE and HopV porins present scarce expression among clinical isolates. *World J Gastroenterol* 2010; 16(3): 320-329 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v16/i3/320.htm> DOI: <http://dx.doi.org/10.3748/wjg.v16.i3.320>

INTRODUCTION

Helicobacter pylori (*H. pylori*) are Gram-negative, micro-aerophilic, spiral-shaped bacteria isolated from human gastric biopsies in 1983^[1]. In order to survive in this aggressive environment, *H. pylori* are able to neutralize their close surrounding space by production of urease, which catalyzes the conversion of urea into ammonium and CO₂, raising pH close to neutral. In addition, to colonize the epithelium, this bacterium is able to bind to the epithelial cell surface, partially avoiding its removal by natural peristalsis or mucus renewal. These characteristics allow *H. pylori* to persist for decades.

H. pylori infection affects one half of the world population, roughly 73% in Chile^[2], with higher prevalence as age increases. After several years of chronic gastric infection, approximately 10%-15% of infected patients develop severe gastrointestinal diseases such as chronic gastritis, peptic ulcer and gastric carcinoma^[3,4]. In Chile, 5% of the infected population develops gastric cancer^[2] and this malignancy is the second cause of death by cancer in the country.

H. pylori carries various virulence factors, and some may have potential as vaccine antigens. These factors may be grouped as: (1) colonization factors, which allow bacterial residence; (2) persistence factors which enable bacteria to accomplish an effective and lasting survival; and (3) disease inducing factors which cause adverse pathological effects on the gastric mucosa^[5].

Based on a bioinformatics analysis of the *H. pylori* genome, a family of outer membrane proteins (OMPs) composed of 33 members has been identified^[6]. These proteins are assembled into the outer membrane exposing, on the bacterial surface, small peptide loops which may act as epitopes to induce an immune response. This feature may be useful when selecting appropriate antigens for vaccine design. All these members contain an N-terminal signal peptide (processed by signal peptidase type I or II) that allows these proteins to cross the inner membrane on their way towards the outer membrane. The *H. pylori* OMPs form 2 families: the Hop members (21 proteins) and the Hor members (12 proteins). Hor proteins lack a characteristic N-terminal Hop motif^[7]. Hop proteins have structural homology with the *Escherichia coli* (*E. coli*) outer membrane protein F (OmpF) porin^[8]. Currently, 5 *H. pylori* Hop members (HopA, HopB, HopC, HopD and HopE) from strain 26695 have been characterized as porins using planar bilayer techniques^[7,9] and some also behave as adhesins^[10,11]. These properties make them attractive

candidates as vaccine antigens. In fact, other bacterial porins from *Salmonella*, *Pseudomonas*, *Chlamydia* and *Neisseria*, have been found to be strong immunogens^[12-15]. However, in the case of *H. pylori*, it has been suggested that not all the genes encoding OMPs may be functional at a given time. Some of these genes are under a control mechanism that operates by strand slippage during DNA replication or DNA repair. DNA polymerase slippage may easily add or remove nucleotides when DNA synthesis occurs in front of a homopolymeric tract or dinucleotide repeats at the template strand (i.e. *polyG* or *polyCA* gene segments) causing mutations either at the promoter or at the coding region. This type of mutation may turn off or on some *hop* genes that may include these polynucleotide features. For instance, the *hopC* gene has been reported to carry a *polyT* tract (13 Ts in length) near the 5' end but *hopA*, *hopB* and *hopE* do not have such long *polyT* tracts either at their coding regions or 5' upstream at the promoter regions. Gene switching will produce a change in the antigenic bacterial surface, a strategy that will distract the host immune system. For this reason, whether any *H. pylori* OMP would be considered as a vaccine antigen, *omp* genes containing long homopolymeric tracts or dinucleotide repeats should be avoided.

Regarding HopV and HopW, genetic heterogeneity in orthologous members of the Hop family among *H. pylori* strains has been described^[16]. These new members were defined as part of the HopA/HopE family, because of their homology at the N-terminal sequence and the presence of 7 homologous domains in the C-terminus region. Regarding functional aspects, HopV and HopW have pore sizes similar to that of the *E. coli* OmpF porin^[16] and HopE has been defined as the homolog to the *E. coli* OmpF porin^[17].

Since the use of porins as antigens has been reported as successful^[12-15], we decided to evaluate members of the *H. pylori* Hop family as putative antigen candidates for vaccine development by determining how widely they are expressed among Chilean *H. pylori* isolates and how often Chilean patients develop antibodies against them. A brief bioinformatic survey indicated that some genes of the Hop family had homopolymeric tracts or dinucleotide repeats in their coding sequences and promoter regions, with potential capability to promote strand slippage which may affect stability of gene expression^[18]. Considering this aspect, only porin genes having single homopolymeric tracts or dinucleotide repeats no longer than 6 bases in their coding sequences were chosen as source of putative useful antigens for a vaccine. For this reason, among several OMP genes, only *hopE* and *hopV* sequences were selected for the present study.

MATERIALS AND METHODS

Bacterial strains and plasmid vectors

E. coli DH5α was used for polymerase chain reaction

Table 1 Primer sequences used for amplification and sequencing of *H. pylori* *hopE* and *hopV* genes

Name	5'→3' sequence	Restriction sites ¹
HopV1 ²	GGGCCCATATGCTCAATTTATGACAAAGAAGAAAAATAGAATGC	<i>Nde</i> I
HopV2 ²	GGATCCCATGGTTAAAAATCCCTCAAGTAAGTACTGATTG	<i>Bam</i> HI
HopE1 ²	GGCGCCATGGAATTTATGAAAAAGTTGTAGCTTTAGG	<i>Nco</i> I
HopE2 ²	CGCGAAGCTTTTAAAAAGTGTAGTTATACCCTAAATAAAG	<i>Hind</i> III
HopE11 ³	GCAAGTGGTTTGGTTTATAGAG	-
HopE22 ³	ACCATATCCAAGTGGATTTT	-
HopV11 ³	GGCGTGGGGTTAGATACGCTG	-
HopV22 ³	ACCATGTTTTCTTTATTAC	-
HopVint ³	ATGCGTTATTATGGGTTTTTGACT	-
pETT7d ⁴	TAATACGACTCACTATAGGG	-
pETT7r ⁴	GCTAGTTATTGCTCAGCGG	-

¹The restriction sites included in primer sequences and used for ligation to plasmid vectors are shown underlined;

²External primers used to clone porin genes; ³Internal primers used for gene sequencing and also for confirmatory PCR reactions for those cases in which primers derived from 5' and 3' gene ends failed to raise PCR products; ⁴Vector primers for 5' and 3' end gene sequencing.

(PCR) cloning, and *E. coli* BL21 (DE3), JM109 (DE3) and AD494(DE3)pLysS as host for porin expression assays. For cloning of PCR fragments plasmid pGEM-T Easy from Promega was used. For expression studies pET21a and pET21d (Novagen) were selected. *H. pylori* Chilean strain CHCTX-1 was used as DNA source for gene amplifications^[19]. A collection of 130 *H. pylori* strains isolated from infected patients living in different Chilean cities was already available^[20].

Bacterial cultures

E. coli cells were grown overnight in LB media at 37°C with shaking. *H. pylori* strains were grown under 50 mL/L CO₂ and 80% humidity in *Brucella* agar plates enriched with 5% horse blood cells and grown at 37°C for 2-3 d. *E. coli* strains were kept for short periods in LB plates at 4°C. Bacterial strains containing 14% glycerol were stored frozen for longer periods at -70°C.

Plasmid purification, DNA manipulation and bacterial transformation

Plasmids were usually detected by the “one step” method^[21], and purified by alkaline lysis method^[22]. Restriction digestions, DNA ligations and plasmid dephosphorylations were done according to standard procedures^[22]. Electroporation in 0.2 cm electrode separation cuvettes was performed as previously described^[23], in a Gene Pulser™ apparatus. Electrocompetent cells were prepared according to described protocols^[23] with a yield of 1×10^9 to 1×10^{10} cells/mL.

PCR assays

Primers corresponding to the 5' and 3' ends as well as the internal sequences of the *hopE* and *hopV* genes (Table 1) were designed based on *H. pylori* 26 695 GenBank sequences. As templates, chromosomal DNA from the CHCTX-1 strain^[19] and from clinical isolates was prepared according to described procedures^[24]. PCR reactions were carried out in a BioRad Mastercycler II thermocycler, with *Pfu* polymerase (Stratagene, CA,

USA) or *Taq* polymerase (Promega, Madison WI, USA). Assays were done in 25 µL final volume following the manufacturer's instructions. Gene amplicons were detected by 1% agarose gel electrophoresis. Other conditions were as previously described^[25]. *VacA* alleles were determined by using primers and assay conditions described by Atherton *et al*^[26].

Polyclonal antibodies against *H. pylori* OMPs

According to standard procedures^[27], anti-HopE and anti-HopV rabbit antibodies were prepared. Proteins were obtained from *E. coli* clones expressing the *H. pylori* porins after separation by SDS-PAGE and purification from gel slices by electroelution as previously described^[28]. Pathogen-free New Zealand adult female rabbits (approximately 1400 g in weight) were immunized with 250 µg of each porin dissolved in 2 mL of Tris-glycine buffer mixed (1:1) with complete Freund's adjuvant. Two animals were used for each porin inoculation and 100 µL aliquots were applied subcutaneously in the back. This was followed by 3 boosters every 15 d.

SDS-PAGE and Western blotting

Lysates from clones expressing HopE and HopV were separated by polyacrylamide gels (12% or 15%) and run in minichambers according to Laemmli^[29]. Western blotting were done as previously described^[30]. As first antibody, patient serum (1:100 dilution) or rabbit anti-porin antibodies against HopE or HopV (1:1000 dilution) were used. As a second antibody for patient assays, goat anti-human IgA or anti-human IgG conjugated to peroxidase, were incubated (1:1000) overnight at 4°C. For anti-porin rabbit sera, a goat anti-rabbit peroxidase-conjugated antibody (1:1000) second antibody was used. To reduce cross reactions, rabbit antisera and human antisera were adsorbed with sonicated lysates from *E. coli* AD494(DE3)pLysS/pET21d and BL21 (DE3), respectively. Human sera immunoblotting were done with lysates expressing HopE or HopV porins and products

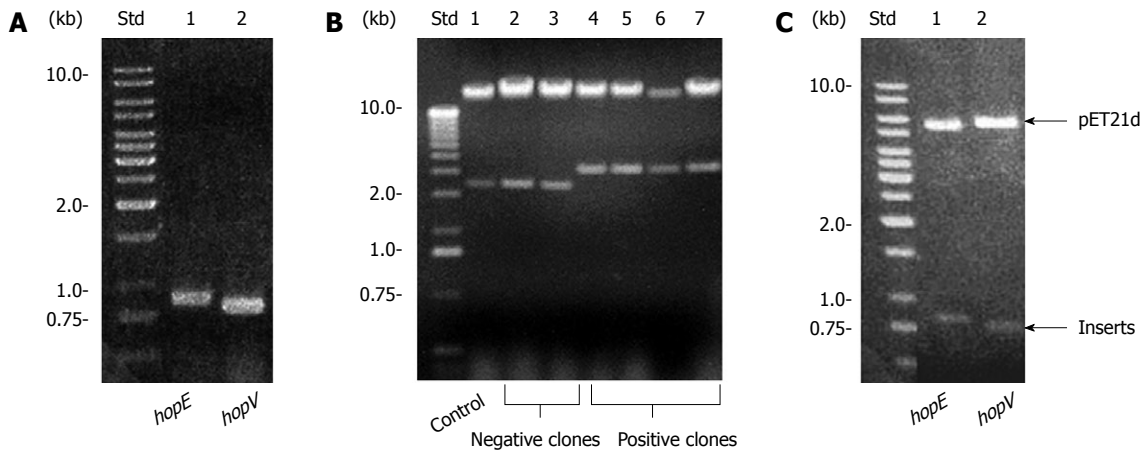


Figure 1 Cloning of *H. pylori* *hopE* and *hopV* genes. A: Polymerase chain reaction (PCR) amplification of *hopE* and *hopV* genes. Amplicons and plasmids were separated by 1% agarose gel electrophoresis. Lane Std: 1 kb DNA ladder standard; B: Detection of plasmids carrying the *hopE* gene. Lane 1: Strain AD494(DE3)pLysS with pET21d as control. Lanes 2, 3: Negative clones, lanes 4-7 plasmids carrying the *hopE* gene; C: Release of inserts carrying the *hopE* and *hopV* genes after *Nco* I / *Hind* III and *Nde* I / *Bam* H I digestions respectively. Lane Std: 1 kb DNA ladder standard (Fermentas).

derived from expression of *cagA* and *vacA* gene fragments cloned from strain CHCTX-1^[19].

Patient sera

A sera panel from 69 infected patients (63 with gastritis, 6 with ulcers) recruited from the Universidad Católica de Chile Medical Center in Santiago, with signed consent, was available. Each patient's infected condition was defined by endoscopy, positive urease rapid test and detection of hematoxylin/eosin-stained curved bacteria on gastric tissue biopsies. Also, 8 non-infected patients were included in this study. The local ethics committee approved the protocol for this study.

Immunoprecipitation of IgG from patient sera

In order to obtain a cleaner IgA reaction in Western blotting assays using patient serum antibodies, protein G-plus-Agarose (Santa Cruz Biotechnology, catalogue #sc-2002) was utilized to first remove IgG from serum by immunoprecipitation. One hundred microliters of each serum without pre-adsorption treatment were incubated overnight with 200 μ L of protein G-plus-agarose at 4°C with mild shaking. After sedimentation for 5 min at 2500 r/min and 4°C, the supernatant of each sample was used as a source of IgG-free serum.

DNA sequencing

DNA samples were previously purified by a commercial kit, and sequenced at our Sequencing Core Facility. T7 and internal primers for DNA sequencing are listed in Table 1.

RESULTS

Cloning of porin genes derived from a Chilean *H. pylori* strain as putative antigens

Selection for cloning and expression studies of the *hopE* and *hopV* genes were based on known gene

sequences from strain *H. pylori* 26 695. Since our study was focused on antigens obtained from local strains, *H. pylori* CHCTX-1 strain, a clinical isolate obtained from a Chilean patient^[19] was selected as the DNA source for gene cloning in this study.

Cloning of *HopE* and *HopV* porin genes, including their signal peptide regions, was done by PCR. Primers and assay conditions are described in Table 1 and Methods, respectively. Amplicons from *hopE*, and *hopV* genes were separated in a 1% agarose gel (Figure 1A), purified and treated with Taq polymerase and dATP to be ligated to pGEM-T. Recombinant plasmids were detected by insert release after *Eco*R I digestion and separation in 1% agarose gel electrophoresis. The *hopE* and *hopV* cloned inserts were subjected to *Nco* I - *Hind* III and *Nde* I - *Bam* H I double digestions and ligated to pET21d and pET21a, respectively. As expected, fragments with sizes corresponding to these genes were observed. For expression purposes, plasmids were transferred to *E. coli* AD494(DE3)pLysS cells and visualized by the "one step method"^[21]. Some clones containing plasmids with the *hopE* gene are displayed in Figure 1B. Purified plasmids were used for restriction digestions and also as DNA templates for PCR gene detection. *Nco* I - *Hind* III and *Nde* I - *Bam* H I double digestions of plasmid DNA isolated from single clones were analyzed by agarose gel electrophoresis, and released inserts with sizes close to the expected ones (819 bp for *hopE* and 735 bp for *hopV*) were observed (Figure 1C).

Expression of *H. pylori* *HopE* and *HopV* porin genes in *E. coli*

E. coli AD494(DE3)pLysS was able to express detectable amounts of *HopE* and *HopV* porins, as seen after SDS-PAGE and Coomassie blue staining (Figure 2A and B) and Western blotting assays (Figure 2C and D).

Expression conditions were optimized by 5 h induction with 1 mmol/L isopropyl β -D-thiogalactoside

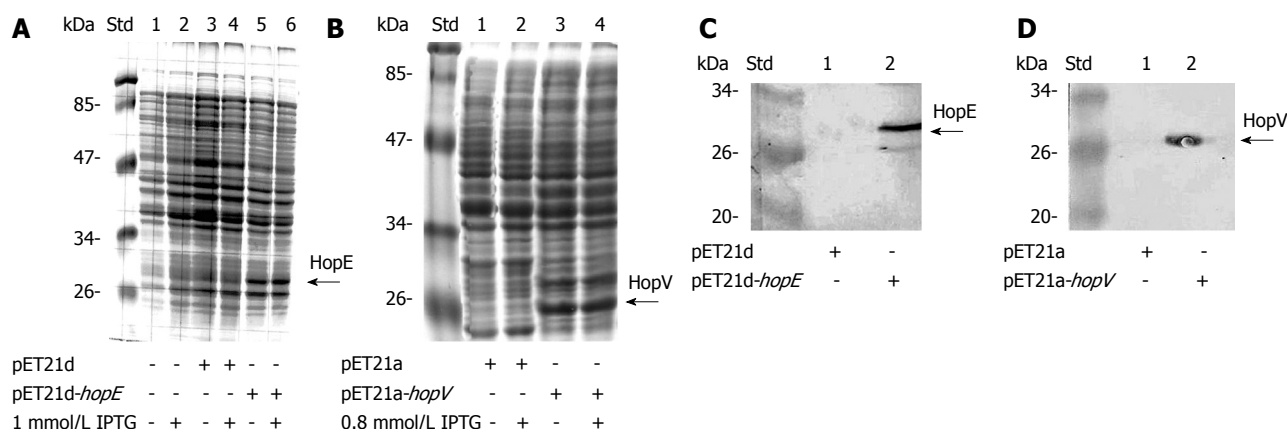


Figure 2 Expression of *H. pylori* *hopE* and *hopV* genes in *Escherichia coli* AD494(DE3)pLysS. Bacterial lysates were separated in 12%-15% PAGE-SDS gel, stained with Coomassie blue (A and B) or analyzed by Western blotting (C and D). Conditions for HopE expression are indicated below the figure (A, lanes 5 and 6; C, lane 2). Conditions for HopV expression are indicated under the figure (B, lanes 3 and 4; D, lane 2). Arrows indicate electrophoretic migration of these proteins. Lane Std: Prestained molecular weight marker in kDa (Fermentas).

(IPTG) for *hopE* and 0.8 mmol/L for *hopV* on previously saturated cultures. Protein sizes of 32 kDa for HopE and 28 kDa for HopV were observed. These porins displayed a certain amount of expression without IPTG induction, partially explained by the fact that the induction procedure was done on saturated cultures.

Sequence analysis of *H. pylori* *hopE* and *hopV* genes

The *hopE* (clone 1) and *hopV* (clone 13) gene sequences from the CHCTX-1 strain were obtained using external (T7 promoter and T7 terminator) as well as internal primers (Table 1) as described in Methods. Both *hopE* and *hopV* sequences were deposited at GenBank (accession numbers #EF635415 and #EF635416, respectively). As expected, these genes did not contain nonsense or frameshift mutations at their coding regions. Also, neither homopolymeric nor dinucleotide tracts longer than 6 nucleotides were found.

Detection of *hopE* and *hopV* genes in Chilean clinical isolates and their expression

From a collection of 240 clinical strains previously isolated^[20], we selected 130 colonies (1 to 5 isolates per patient) as representatives from 6 Chilean cities: Iquique (IQ) in the North, Valparaíso (VA) and Santiago (SA) in the central region, Los Angeles (LA) and Valdivia (VL) in the South, and Punta Arenas (PA), the Southernmost city, to evaluate the distribution of strains carrying *hopE* and *hopV* genes and their expression throughout the country.

Detection of the genes was done by standard PCR. The amplicons were almost identical in size to those expected for *hopE* and *hopV* genes from strain 26695. Representative groups of isolates carrying *hopE* and *hopV* genes are shown in Figure 3A and B, respectively.

The *hopE* and *hopV* genes were detected in 46.9% (61 out of 130 strains) and 63.1% (82 out of 130) of the studied strains, respectively, and 40% (52 out of 130) of the strains revealed the presence of both genes simultaneously (Table 2). Among different cities,

hopE and *hopV* gene contents varied between 30% and 69%. Curiously, *hopV* was frequently detected (69.2%) in strains from infected patients living in PA, the southernmost city. Patients from VL (mostly descendants from ancient aborigines) carried strains with a lower content (42.8%) of *hopV* gene. All PCR reactions were done at least twice using a pair of primers which bound to the gene ends. For those cases with negative amplification, additional assays using 2 primer combinations, including in each pair of primers one of the internal primers (Table 1), were performed. In most cases negative PCR reactions were confirmed and just a few strains showed positive PCR amplification only with pairs including internal primers, indicating that our initial estimation about the reduced presence of these genes in Chilean isolates was valid.

The positive results of HopE and HopV Western blotting expression assays in these isolates revealed no protein size variation, and selected results are displayed in Figure 3C.

Regarding porin expression, results showed that only 13.1% of the 130 strains expressed HopE and 6.9% expressed HopV. Altogether, 83.8% (109 out of 130 strains) did not express HopE or HopV porins either because of a lack of these genes, random inactivating gene mutations or gene silencing by the strand slippage mechanism (Table 2).

Recognition of HopE and HopV porins by sera from infected patients

In order to evaluate the capability of sera from Chilean *H. pylori*-infected patients to recognize recombinant *H. pylori* HopE and HopV porins, sera from 69 infected and 8 non-infected patients were tested. IgG and IgA serum antibodies against HopE and HopV antigens expressed as recombinant proteins in *E. coli* clones were tested using Western blotting assays on these bacterial lysates. VacA and CagA expressed similarly were used as immunodominant controls. The number of infected

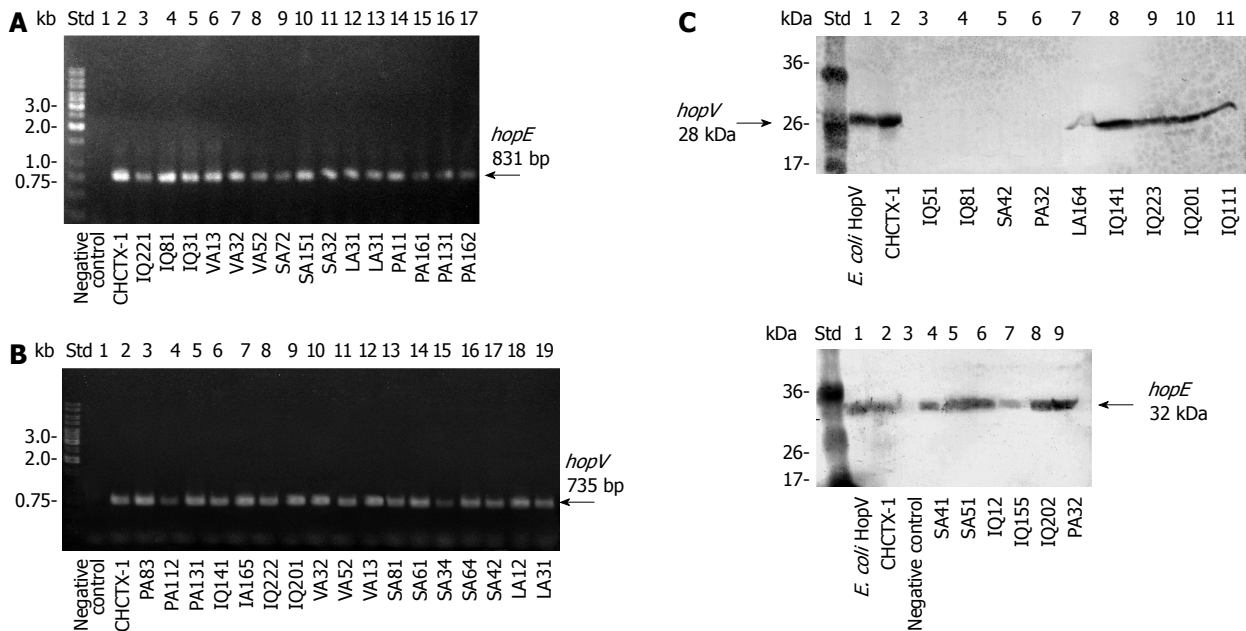


Figure 3 Detection of *hopE* and *hopV* genes and their expression in *H. pylori* clinical isolates from different Chilean cities. Amplicons were separated in 1% agarose gels. A: PCR detection of the *hopE* gene; B: PCR detection of the *hopV* gene. Arrows indicate migration of the respective gene fragments. Lane Std: 1 kb DNA ladder standard (Fermentas); Lane Std2: Lambda DNA/HindIII marker (Fermentas); C: Expression of HopV and HopE porins in *H. pylori* Chilean strains separated by 12% SDS-PAGE gels and detected by Western blots with respective polyclonal antibodies. Clinical isolates are indicated under the respective lanes. Std: Prestained molecular weight marker (Fermentas). The CHCTX-1 strain was included as a positive control.

Table 2 Number of strains presenting genotypes and corresponding phenotypes indicating the presence of *hopE* (E) and *hopV* (V) genes and their expression in 130 *H. pylori* strains isolated from infected patients from 6 Chilean cities

Genotypes ¹ (E/V)	No. of strains	Phenotypes ² (E/V)	No. of strains	No. of strains with different E/V phenotypes per city ³					
				IQ	VA	SA	LA	VL	PA
(+/+)	52	(+/+)	5	4	-	1	-	-	-
		(+/-)	12	4	-	5	1	-	2
		(-/+)	-	-	-	-	-	-	-
		(-/-)	35	15	3	3	1	7	6
(+/-)	9	(+/-)	-	-	-	-	-	-	-
		(-/-)	9	2	-	-	-	5	2
(-/+)	30	(-/+)	4	1	-	2	-	-	1
		(-/-)	26	11	-	4	-	2	9
(-/-)	39	(-/-)	39	11	2	11	2	7	6
Totals	130	Totals	130	48	5	26	4	21	26

¹Presence of *hopE* (E) and/or *hopV* (V) genes in Chilean *H. pylori* isolates determined by PCR amplifications as described in Methods using purified DNA templates from single colonies collected from 69 patients of the indicated cities. (+): gene presence; (-): no detection; ²HopE and/or HopV expression assayed by Western blotting (see Methods). (+): detection; (-): no detection; ³Total number of strains with the assigned phenotype per city. IQ: Iquique; VA: Valparaíso; SA: Santiago; LA: Los Angeles; VL: Valdivia; PA: Punta Arenas. The number of patients per city was IQ = 21, VA = 4, SA = 12, LA = 3, VL = 5, PA = 15. Number of strains isolated per patient ranged between 1 and 5.

patient sera able to recognize these antigens are shown in Figure 4A. It was found that, as expected, IgG human antibodies more frequently recognized VacA (68.1% or 47 out of 69) and CagA (59.4%), but rarely recognized HopE (8.7%) and HopV (10.1%) porins. A similar distribution for HopE (11.6%) and HopV (10.5%), but lower distribution values for VacA (42%) and CagA (17.4%) were found for IgA antibodies. The lower number of anti-HopE and anti-HopV reactive sera can be explained by the low proportion of *H. pylori* strains able to express these genes, being 13.1% (17/130) for

HopE and 6.9% (9/130) for HopV (Table 2). Taken together, these results strongly suggest that *H. pylori* possesses a mechanism to switch on/off these OMP genes as a strategy to evade the host immune response.

In addition, considering that the immune response in children^[31,32] could be quite different from that in adults^[33], the IgA (Figure 4B) and IgG (Figure 4C) immune responses of the infected patients were plotted for 2 age groups: under 18 years of age and adults. It was noted that the number of sera with IgA and IgG responses against CagA antigen was significantly higher

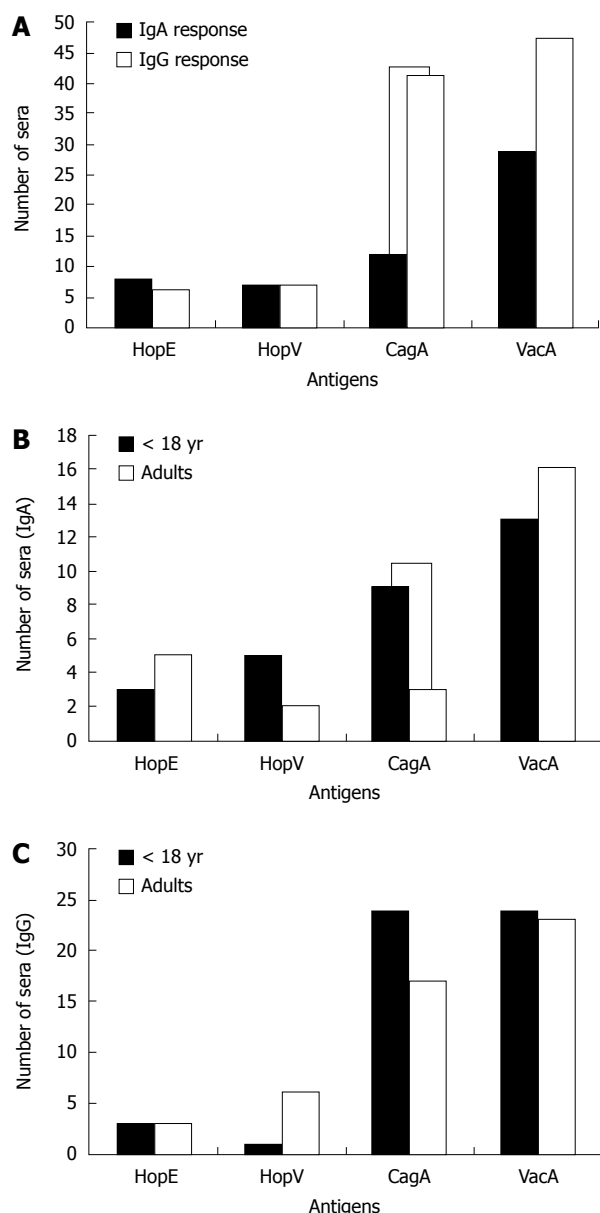


Figure 4 Frequency of recognition of 4 *H. pylori* antigens by human sera. Bars represent number of immunoreactive human sera from a panel of 69 *H. pylori*-infected patients showing IgG- and IgA-type immunoreactivity tested by Western blotting assays on *E. coli* lysates expressing separately HopE, HopV, CagA and VacA cloned antigens. A: Frequency of IgA- and IgG-type immune responses (as number of sera) which reacted with lysates containing one of these antigens; B: IgA response data taken from panel A, with patients separated by age into 2 groups: under 18 years old and adults; C: IgG response data taken from panel A, separated by age as above. As negative controls, sera from 8 non-infected patients did not display any immune response when tested with these antigens (not shown). Fisher's test was used for statistical analysis, and significance lower than 0.05 is indicated.

in patients under 18 than in adults. In contrast, IgA responses against HopE and VacA and the IgG response against HopV seemed to be more frequent in adults than in children.

DISCUSSION

H. pylori colonizes the human gastric epithelium in half

of the world's population and induces strong serological and inflammatory responses in the host which persist during the entire life of the subject, rendering the host unable to eradicate infection. Knowledge of the most frequently recognized antigens in the infected population may contribute to an understanding of the bacterium survival strategy. In addition, this could also help to select appropriate antigens for vaccine design. The most extensively studied *H. pylori* virulence factors as potential vaccine antigens are urease subunits^[34,35], VacA and CagA^[36], *H. pylori* adhesin A^[37] and neutrophil-activating protein^[38,39]. However, results indicating reduction in colonization after oral vaccination of human subjects have been rather modest^[40,41]. As new antigens are needed, and there are few studies comparing porin genes among different *H. pylori* strains, we have looked for *H. pylori* porins suitable for vaccine development. The present report provides new information, particularly about Chilean strains, regarding porin expression among clinical isolates.

Folded porins have exposed loops at the bacterial surface which may induce a strong immune response. Porins have been proposed for the design of oral vaccines to eradicate *H. pylori*^[6]. This pathogen has an extensive collection of OMPs with defined pore characteristics, such as the Hop group. However, some may display a unique on/off mechanism affecting gene expression, based on DNA strand slippage during replication. The instability of expression status makes antigen selection a very important issue. Antigens that will not be permanently expressed in most of the strains should be avoided, since they will provide a limited protection.

PCR assays carried out on 130 Chilean isolates detected *hopE* and *hopV* genes in 46.9% and 63.1%, respectively. However porin expression was infrequently detected in these strains (HopE = 13.1%, HopV = 6.9%), concurring with low seroprevalence of these porins among sera of infected patients, suggesting that these genes may be under DNA strand slippage control.

Infrequent detection of expression cannot be explained by low immunoreactivity of the rabbit antisera resulting from amino acid sequence diversity among strains, since these 2 porin sequences seem to be conserved. HopE and HopV amino acid sequence identities among those from strain CHCTX-1 and the corresponding sequences from fully sequenced *H. pylori* genomes, were in the range of 98%-99% and 94%-96%, respectively.

As a complementary approach, 69 patient sera were tested by Western blotting on *E. coli* clones expressing CagA, VacA, HopE or HopV. It was revealed that HopE and HopV porins were not often recognized within the analyzed sample. Only 6 sera (8.7%) showed IgG-type immune reaction against HopE-containing lysates and 7 (10.1%) against HopV. Similar results were obtained analyzing the IgA response. These results agree with the fact that HopE and HopV porins are sporadically expressed. In contrast, CagA (its gene is present in

about 50% of the strains) and VacA (its gene is present in almost 100% of the strains) reacted with 59.4% and 68.1% of the IgG patient sera, respectively.

Regarding nucleotide sequence features, dinucleotide repeats in *hopE* and *hopV* sequences from the CHCTX-1 strain barely reached 5 nucleotides in length. However, they contained CCCCCC and TTTTTT tracts after codons 58 and 66, respectively. These findings, taken together with the low number of strains expressing these porins, and their low seroprevalence among Chilean patients, suggest that *hopE* and *hopV* may be under strand slippage gene control. Confirmation should be done by sequencing strains carrying silenced genes.

In *H. pylori*, at least 3 porin genes from the Hop family (*hopZ*, *hopP* and *hopO*) may be subjected to this on/off switching^[11,42]. Another study^[43] showed a similar case: 3 different *H. pylori* strains re-isolated after *Maccacrus rhesus* infection lost expression of BabA adhesin which binds Lewis b antigen. These observations support the idea that *H. pylori* can modulate expression of some OMP genes. This feature provides an adaptive mechanism to avoid induction of a strong host immune response. This is supported by the large repertoire of OMPs genes in the *H. pylori* genome. Functional redundancy of porins may explain emergence of mutations in these genes without affecting bacteria viability. It has been proposed that the role of such redundancy of outer membrane proteins is to sustain antigenic variation to support pathogen survival by evasion of the host immune response^[44]. The strand slippage mechanism is not normally operating in *E. coli*, therefore, in most cases, lack of heterologous expression of *H. pylori* genes in *E. coli* should be due to mutations that previously affected the *H. pylori* genome.

In spite of the low content of homopolymeric and dinucleotide repeats found in CHCTX-1 *hopE* and *hopV* genes, some strains may have switched these genes off but, in a few cases, expression could be restored by the same mechanism. This may restrict the use of these *H. pylori* OMPs as a single source of antigens for vaccine design. However, in order to provide a wider and stronger immune response, vaccines based on a mixture of *H. pylori* antigens with the inclusion of HopE and HopV should be considered.

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COMMENTS

Background

Few studies have been done on HopE and HopV porins from *Helicobacter pylori* (*H. pylori*). These proteins have been described as part of a large family of outer membrane proteins having similar functions, mainly as gating for influx of nutrients. Compared to other enterobacteria, such as *Escherichia coli* (*E. coli*) with only 3 major porins, *H. pylori* have shown a remarkable redundancy in this kind of outer membrane protein. The explanation for such redundancy has not

arisen. A study looking at how widely these porin genes are distributed and what proportion is actually active among clinical isolates should provide some answers.

Research frontiers

Switching on/off in outer membrane genes in a few bacteria has been described as a mechanism to distract the immune system during infection by changing the proteins displayed on the surface. The authors found that *H. pylori* HopE and HopV porin genes seemed to be absent in some isolates, and about half of those who carried them did not express them. In addition, sera from infected patients do not frequently recognize these antigens. This feature may contribute to the ability of these bacteria to avoid the host immune response allowing their persistence in humans for an extended period of time.

Innovations and breakthroughs

Recent reports have shown that some outer membrane protein genes from *H. pylori* could be turned on/off by random nucleotide insertions or deletions either at the promoter or within the coding region, through a mechanism called "strand slippage" during DNA replication. This is the first report proposing that this switching may also occur in the *hopE* and *hopV* genes, explaining why around 70%-90% of these genes are shut down in Chilean clinical isolates.

Applications

Determining whether a protein is subjected to on/off switching during its expression at the bacterial surface, together with the knowledge of its immunoreactivity will be useful to select potential antigen candidates to be used in the design and development of vaccines.

Terminology

H. pylori HopE and HopV proteins are part of a large family of outer membrane proteins and are located at the bacterial surface. They are defined as porins because they form a pore structure to allow the influx of small size nutrients and other compounds. They may constitute a target for the immune system. The "strand slippage" mechanism to control gene expression is a result of random errors during strand DNA replication consisting of nucleotide insertions or deletions that alter the genetic code of the protein or the functionality of elements (i.e. promoter) that control gene expression.

Peer review

The manuscript by Lienlaf *et al* assesses the patterns of 2 porin genes in *H. pylori*. *H. pylori* remains a significant problem in developing countries around the world. Although much is now known about the pathogenesis of this bacterium and about host responses to infection, the organism remains a clinical problem. Various investigators have focused upon the establishment of a vaccine for this pathogen. An appropriate selection of a conserved and widely expressed antigen of *H. pylori* clinical isolates will assure a suitable design of a protective vaccine. This work is promising.

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Aberrant gene methylation in the peritoneal fluid is a risk factor predicting peritoneal recurrence in gastric cancer

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Abstract

AIM: To investigate whether gene methylation in the peritoneal fluid (PF) predicts peritoneal recurrence in gastric cancer patients.

METHODS: The gene methylation of *CHFR* (checkpoint with forkhead and ring finger domains), *p16*, *RUNX3* (runt-related transcription factor 3), *E-cadherin*, *hMLH1* (mutL homolog 1), *ABCG2* (ATP-binding cassette, sub-family G, member 2) and *BNIP3* (BCL2/adenovirus E1B 19 kDa interacting protein 3) were analyzed in 80 specimens of PF by quantitative methylation-specific polymerase chain reaction (PCR). Eighty patients were divided into 3 groups; Group A ($n = 35$): the depth of cancer invasion was less than the muscularis propria; Group B ($n = 31$): the depth of cancer invasion was beyond the muscularis propria. Both group A and B were diagnosed as no cancer cells in peritoneal cytol-

ogy and histology; Group C ($n = 14$): disseminated nodule was histologically diagnosed or cancer cells were cytologically defined in the peritoneal cavity.

RESULTS: The positive rates of methylation in *CHFR*, *E-cadherin* and *BNIP3* were significantly different among the 3 groups and increased in order of group A, B and C (0%, 0% and 21% in *CHFR*, $P < 0.05$; 20%, 45% and 50% in *E-cadherin*, $P < 0.05$; 26%, 35% and 71% in *BNIP3*, $P < 0.05$). In addition, the multigene methylation rate among *CHFR*, *E-cadherin* and *BNIP3* was correlated with group A, B and C (9%, 19% and 57%, $P < 0.001$). Moreover, the prognosis was analyzed in group B, excluding 3 patients who underwent a non-curative resection. Two of the 5 patients with multigene methylation showed peritoneal recurrence after surgery, while those without or with a single gene methylation did not experience recurrence ($P < 0.05$).

CONCLUSION: This study suggested that gene methylation in the PF could detect occult neoplastic cells in the peritoneum and might be a risk factor for peritoneal metastasis.

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Key words: Ascites; Dissemination; Gastric cancer; Methylation; Peritoneal fluid; Quantitative methylation-specific polymerase chain reaction

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INTRODUCTION

Peritoneal metastasis is the most frequent event in recurrent gastric cancers, and occurs in 34% of patients with recurrences, even after curative resection of the primary tumor^[1,2]. In addition, peritoneal metastasis shows resistance to various chemotherapeutic drugs and causes massive ascites and intestinal obstruction. In Japan, cytological examination of peritoneal washes using Papanicolaou staining is commonly performed during surgery to detect peritoneal metastasis. However, peritoneal metastasis sometimes occurs even in cases that show a negative cytological examination. The efficacy of immunocytology^[3,4], tumor markers^[5,6] and reverse transcriptase polymerase chain reaction (PCR) analysis of carcinoembryonic antigen (CEA), and cytokeratin (CK) mRNA^[2,7-12] in the peritoneal washes has been examined.

Epigenetic gene silencing through DNA methylation occurs in various cancers. DNA methylation occurs in the CpG rich promoters of tumor suppressor genes, DNA repair and cell cycle checkpoint genes, resulting in suppressed gene expression^[13,14]. Numerous studies have investigated gene methylation to assess the correlation with carcinogenesis and tumor progression in various cancers^[15-17]. Recently, several reports have demonstrated aberrant gene methylation detected in salivary rinses^[18], pleural effusion^[19], peritoneal fluid (PF)^[20,21], lymph node^[22,23], breast ductal fluid^[24], bile^[25], pancreatic juice^[26], urine^[27], stool^[28], serum and plasma^[20,29,30] from patients with various tumors and suggested the feasibility of methylation analysis in the evaluation of occult neoplastic cells or micrometastasis.

The present study investigated whether DNA methylation using PF is a possible marker for determining gastric cancer micrometastasis to the peritoneum. The DNA methylation levels of 7 genes; *CHFR* (checkpoint with forkhead and ring finger domains), *p16* (cyclin-dependent kinase inhibitor 2A), *RUNX3* (runt-related transcription factor 3), *E-cadherin*, *hMLH1* (mutL homolog 1), *ABCG2* (ATP-binding cassette, sub-family G, member 2), *BNIP3* (BCL2/adenovirus E1B 19 kDa interacting protein 3) in 80 PF specimens were analyzed by quantitative methylation-specific polymerase chain reaction (q-MSP). Furthermore, quantitative reverse transcriptase-PCR (qRT-PCR) of CEA and CK19 mRNA was examined using the same samples and the results were compared with that of q-MSP. The goal of this study was to clarify whether gene methylation in PF is feasible for determining micrometastasis to the peritoneum in gastric cancer.

MATERIALS AND METHODS

Ethics

The study protocol was approved by the Ethics Committee of Saga University Faculty of Medicine. Informed consent was obtained from all the patients before collection of the samples.

Patients and sample collection

Peritoneal lavage fluid was obtained from 80 patients

who underwent surgery at the Department of Surgery, Saga University Hospital from May 2007 to August 2008. A total volume of 200 mL of normal saline was poured into Douglas's pouch and the left subphrenic space. One hundred milliliter of PF was examined by conventional cytological diagnosis with Papanicolaou staining. The remaining PF was centrifuged at 1200 g for 10 min and the pelleted cells were stored at -80°C until the extraction of genomic DNA and RNA. A gastrectomy was subsequently performed in 72 patients. A bypass operation or exploratory laparotomy was carried out in the remaining 8 patients due to either peritoneal dissemination or cytologically positive cancer cells. The histological type, depth of tumor invasion and clinical stage were determined on the basis of the criteria of the Japanese Classification of Gastric Carcinoma guidelines^[31]. The 80 patients were further divided into 3 groups: Group A ($n = 35$): the depth of cancer invasion was less than the muscularis propria [tumor invasion of mucosa and/or muscularis mucosa (M) or submucosa (SM), tumor involved the muscularis propria (MP)]; Group B ($n = 31$): the depth of cancer invasion was beyond the muscularis propria [tumor involved the subserosa (SS), tumor penetrated the serosa (SE), tumor invasion of adjacent structures (SI)]; Group C ($n = 14$): a peritoneal metastasis was histologically diagnosed [P (+)] or cancer cells were present on peritoneal cytology [CY (+)]. No peritoneal metastasis [P (-)] and benign/ indeterminate cells on peritoneal cytology [CY (-)] were confirmed at surgery in the 66 patients in group A and group B. CY (+) or P (+) was simultaneously diagnosed at surgery in 12 of 14 patients in group C. In the remaining 2 patients, cancerous ascites were collected at the recurrence. The methylation analysis was performed using specimens obtained from all 80 patients. The mRNA analysis was done using 63 samples, because high quality RNA could not be extracted in specimens from the remaining 17 cases.

DNA extraction, sodium bisulfite modification and q-MSP

The genomic DNA was isolated from cell pellets from the abdominal fluid using an EZ1 DNA tissue kit (Qiagen, Hilden, Germany). Bisulfite modification was carried out using the EpiTet® Bisulfite Kit (Qiagen, Hilden, Germany) with 1500 ng of genomic DNA. Bisulfite-treated DNA was amplified by EpiTect® Whole Bisulfite Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The 80 DNA samples with bisulfite modification were quantitatively analyzed for the methylation levels of 8 genes (*CHFR*, *p16*, *RUNX3*, *E-cadherin*, *hMLH1*, *ABCG2*, *BNIP3* and β -actin; an internal marker). For q-MSP, a 2 μ L aliquot was amplified by PCR using a primer set along with the Taqman probe specific for methylated sequences. A q-MSP (MethyLight) was carried out with the Light-Cycler™ instrument system (Roche, Mannheim, Germany) using the Light-Cycler® TaqMan® Master (Roche, Mannheim, Germany) according to a previous report^[32]. The primer sequences are shown in Table 1^[33-37]. After a denaturing step at 95°C

Table 1 Primer and probe sequence of the LightCycler system for q-MSP

Gene		Primer sequence	Ref.
CHFR	Forward	TTTCGTGATTCGTAGGCGAC	[33]
	Reverse	CGACAATAAAACGAAACCGA	
	Probe	5' FAM-CGCGAAAATAAACGCGTAAAAAACGCTCG-3'BHQ	
p16	Forward	TGGAATTTTCGGTTGATTGGTT	[34]
	Reverse	AACAACGTCGCGACCTCCT	
	Probe	5' FAM- ACCCGACCCCGAACCGCG -3'BHQ	
RUNX3	Forward	CGTTCGATGGTGGACGTGT	[35]
	Reverse	GACGAACAACGTCCTATTACAACGC	
	Probe	5' FAM-CGCACGAACCTCGCTACGTAATCCG-3'BHQ	
E-cadherin	Forward	AATTTTAGGTTAGAGGGTTATCGCGT	[34,36]
	Reverse	TAATAAAAAATTCACCTACCGAC	
	Probe	5' FAM-CGCCCACCCGACCTCGCAT-3'BHQ	
hMLH1	Forward	CGTTATATATCGTTCGTAGTATTCGTGTTT	[34]
	Reverse	CTATCGCCGCTCATCGT	
	Probe	5' FAM-CGCGACGTCAAACGCCACTACG-3'BHQ	
ABCG2	Forward	TTGGGTAATTTGTGCGTTA	[37]
	Reverse	CTACGAAAATCACCAAACGCTC	
	Probe	5' FAM-TTAATCGCCGCTACTAACCAG-3'BHQ	
BNIP3	Forward	TAGGATTCGTTTCGCGTACG	[37]
	Reverse	ACCGCGTCGCCCATTAACCGCG	
	Probe	5' FAM-CGTAATAATACGTATAACACGTACGAC-3'BHQ	
β -actin	Forward	TGGTGATGGAGGAGGTTTAGTAAGT	[34]
	Reverse	AACCAATAAAACCTACTCCTCCCTTAA	
	Probe	5' FAM-ACCACCACCCAACACACAATAACAAACACA-3'BHQ	

Table 2 Primer and probe sequence of the LightCycler system for qRT-PCR

Gene		Primer sequence
CEA	Forward	AGTCTATGCAGAGCCACCCAA
	Reverse	GGGAGGCTCTGATTATTTACCCA
	Probe	5' FAM-ACCCTTCATCACCAGCAACAACCTCCAA-3'BHQ
CK19	Forward	GACATGCGAAGCCAATATGA
	Reverse	TCAGTAACCTCGGACCTGCT
	Probe	5' FAM-CTGGTTCACCAGCCGGACTGAAGAATT-3'BHQ
β -actin	Forward	CGAGCGCGGCTACAGCTT
	Reverse	TCCTTAATGTCACGCACGATT
	Probe	5' FAM-ACCACCACGGCCGAGCGG-3'BHQ

for 10 min, PCR amplification was performed with 45 cycles of 15 s denaturing at 95°C, 5 s annealing at 60°C and a 10 s extension at 72°C. These experiments were carried out in triplicate and the mean value was then calculated. CpGenome Universally Methylated DNA (Chemicon, Temecula, CA, USA) was used as a positive control for methylation, and CpGenome Universal Unmethylated DNA (Chemicon) was used as a negative control. The quantified value of DNA methylation of a target gene was normalized by β -actin.

RNA extraction, conversion to cDNA and qRT-PCR

Total RNA was extracted from the 80 cell pellets using the ISOGEN kit (Nippon Gene, Toyama, Japan) according to the manufacturer's instructions. Samples of RNA (100 ng) were converted into cDNA and amplified using the QuantiTect® Whole Transcriptome Kit (Qia-gen, Hilden, Germany) according to the manufacturer's instructions. In order to quantitatively estimate the expression level of CEA and CK19 mRNA, qRT-PCR was performed on a Light-Cycler™ instrument system

(Roche, Mannheim, Germany) using LightCycler® Taq-Man® Master (Roche, Mannheim, Germany) according to the manufacturer's instructions. The primer sequences are shown in Table 2. After denaturing at 95°C for 10 min, qRT-PCR amplification was performed with 45 cycles of 15 s denaturing at 95°C, 5 s annealing at 60°C and a 10 s extension at 72°C. These experiments were all carried out in triplicate and the mean value was then calculated. The quantified value of mRNA expression of a target gene was normalized by β -actin.

Comparison of gene methylation between cancer tissue and peritoneal fluid

The conventional qualitative MSP was analyzed using several cancer tissue specimens. Genomic DNA was isolated from the tissue and bisulfite treatment was carried out as described above. The methylation status in the tissue samples was determined by MSP. Amplification was performed using Takara ExTaq Hot Start Version (Takara, Shiga, Japan) according to the manufacturer's instructions. The primer se-

quences are shown in Table 1 and PCR was performed in an iCycler (Bio-Rad, Hercules, CA, USA) under the following conditions. After heating at 96°C for 3 min, 35 cycles at 96°C for 30 s, 60°C for 30 s, 72°C for 30 s and 72°C for 5 min. The PCR products were separated on 1.5% agarose gel, stained with ethidium bromide, and were then observed under ultraviolet light.

Statistical analysis

A cut-off value for distinguishing methylation status was determined using a receiver-operator characteristic (ROC) curve, which was obtained by comparing methylation values between group A and C. The cut-off value of CEA and CK19 mRNA expression was also determined by a ROC curve as described above. Differences in the frequencies were analyzed by the χ^2 test, while also applying Fisher's exact test. A statistical analysis was carried out using the SPSS 15.0j statistical software package for Windows (SPSS Japan Inc.). A *P* value of less than 0.05 was considered to be statistically significant.

RESULTS

The clinicopathological characteristics of the patients in this study are summarized in Table 3. The 80 patients included 28 (35.0%) females and 52 (65.0%) males with a mean age of 65.9 years (range, 39–88 years). The number of patients in group A, B and C was 35 (43.8%), 31 (38.8%) and 14 (17.5%) cases, respectively.

DNA hypermethylation of peritoneal fluids

Initially, q-MSP of *CHFR*, *p16*, *RUNX3*, *E-cadherin*, *hMLH1*, *ABCG2* and *BNIP3* was examined in 80 PFs. Figure 1 shows the methylation level of each gene in the 3 groups. The 80 cases were then divided into 2 groups, a methylation positive and a methylation negative group as described in the Materials & Methods section. Table 4 shows that the methylation status of *CHFR*, *E-cadherin* and *BNIP3* were significantly different among the 3 groups (*P* < 0.05). In these 3 genes, the positive rate of methylation increased in order of group A, B and C. The correlation of multigene methylation among *CHFR*, *E-cadherin* and *BNIP3* in the 3 groups was further analyzed. In group A and B, 3 of 35 cases (9%) and 6 of 31 cases (19%) showed multigene methylation in 2 or more genes, respectively (Table 5). In contrast, 8 of 14 cases (57%) were methylation positive in 2 or more genes in group C. The results showed a significant relationship between methylation status of more than 2 genes and the 3 groups (*P* < 0.001).

mRNA expression of CEA and CK19 in peritoneal fluids

The expression of CEA and CK19 mRNAs in the 63 PFs were quantitatively analyzed by qRT-PCR to detect gastric cancer cells in the peritoneal washes. Figure 2 shows the expression level of CEA and CK19 mRNAs in the 3 groups. The mRNA level was further divided into negative and positive groups and compared among the 3 groups. The results showed that CK19, but not CEA, was

Table 3 Clinicopathological factors of the patients (*n* = 80)

Clinicopathological factors	<i>n</i> (%)
Gender	
Female	28 (35.0)
Male	52 (65.0)
Age (yr)	65.9 ± 10.8
Range	39–88
Histological type	
tub	36 (45.0)
por/sig/muc	44 (55.0)
Classification	
Group A: M-MP with CY(-) and P(-)	35 (43.8)
Group B: SS-SI with CY(-) and P(-)	31 (38.8)
Group C: CY(+) or P(+)	14 (17.5)
Clinical stage at the sample collection	
I	37 (46.3)
II	8 (10.0)
III	18 (22.5)
IV	15 (18.8)
Peritoneal recurrence	2 (2.5)

tub: Tubular adenocarcinoma; por: Poorly differentiated adenocarcinoma; sig: Signet-ring cell carcinoma; muc: Mucinous adenocarcinoma. Tumor invasion of mucosa and/or muscularis mucosa (M), muscularis propria (MP) or subserosa (SS), tumor invasion of adjacent structures (SI). Cancer cells on peritoneal cytology (CY), peritoneal metastasis (P).

Table 4 Relationship between the q-MSP results and depth of cancer invasion, CY and P classification *n* (%)

Methylation status	Classification			<i>P</i> -value
	Group A: M-MP with CY(-) and P(-)	Group B: SS-SI with CY(-) and P(-)	Group C: CY(+) or P(+)	
<i>CHFR</i>				< 0.05
(-) 77 (96)	35 (100)	31 (100)	11 (79)	
(+) 3 (4)	0 (0)	0 (0)	3 (21)	
<i>p16</i>				0.821
(-) 33 (41)	14 (40)	14 (45)	5 (36)	
(+) 47 (59)	21 (60)	17 (55)	9 (64)	
<i>RUNX3</i>				0.304
(-) 36 (45)	19 (54)	11 (35)	6 (43)	
(+) 44 (55)	16 (46)	20 (65)	8 (57)	
<i>E-cadherin</i>				< 0.05
(-) 52 (65)	28 (80)	17 (55)	7 (50)	
(+) 28 (35)	7 (20)	14 (45)	7 (50)	
<i>hMLH1</i>				0.861
(-) 43 (54)	20 (57)	16 (52)	7 (50)	
(+) 37 (46)	15 (43)	15 (48)	7 (50)	
<i>ABCG2</i>				0.244
(-) 30 (38)	12 (34)	10 (32)	8 (57)	
(+) 50 (63)	23 (66)	21 (68)	6 (43)	
<i>BNIP3</i>				< 0.05
(-) 50 (63)	26 (74)	20 (65)	4 (29)	
(+) 30 (38)	9 (26)	11 (35)	10 (71)	

significantly correlated with the 3 groups (Table 6, *P* < 0.05, *P* = 0.352). The positive rate of CK19 increased in order of group A (25%), B (46%) and C (73%).

Comparison of gene methylation between the cancer tissue and ascites fluid

The gene methylation of *CHFR*, *E-cadherin* and *BNIP3* was examined in the primary or metastatic cancer tissue and was compared with those in the corresponding PF

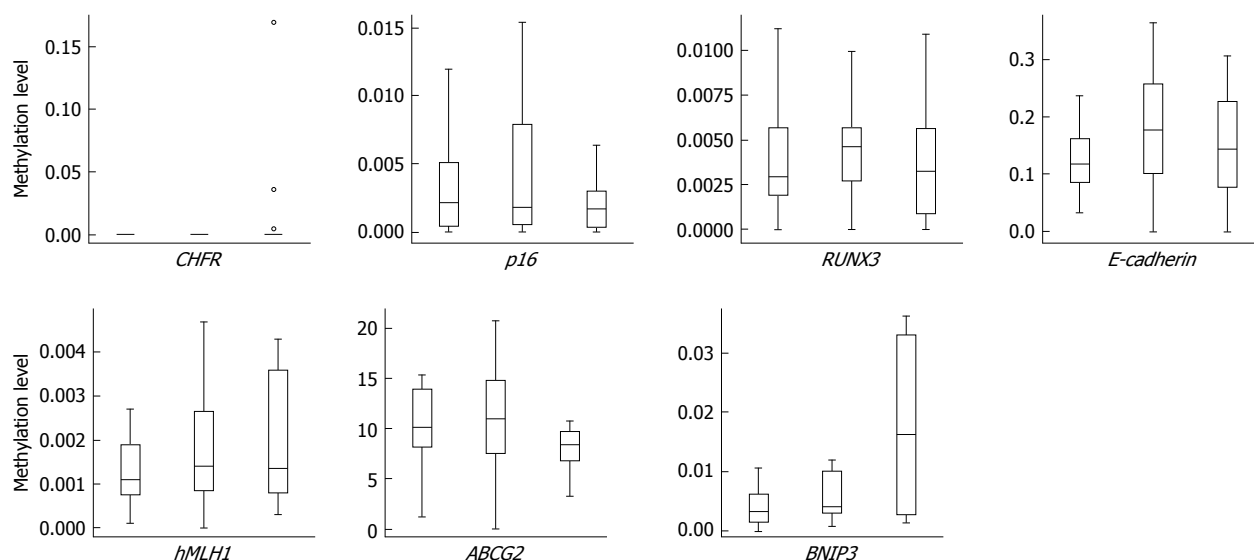


Figure 1 Methylation level of peritoneal wash specimens from gastric cancer patients measured by q-MSP according to the depth of cancer invasion and positive cancer cell findings. Left: Group A; Middle: Group B; Right: Group C.

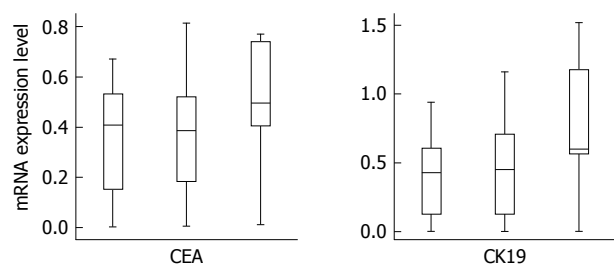


Figure 2 mRNA expression level of peritoneal wash specimens from gastric cancer patients measured by qRT-PCR according to the depth of cancer invasion and positive cancer cell findings. Left: Group A; Middle: Group B; Right: Group C.

in group C. Nine of 14 cases were analyzed because the tissues were not obtained in remaining 5 cases. The same methylation pattern was present in the cancer tissues and ascites in 100% of *CHFR*, 88.9% of *E-cadherin* and 77.8% of *BNIP3* (Table 7).

Relationship between multigene methylation or mRNA expression and peritoneal recurrence

The prognosis of patients in group A or B was followed for at least 8 mo after surgery. None of the 35 patients in group A had cancer recurrence (data not shown). The prognosis of only 28 of 31 patients in group B was followed because the remaining 3 patients were excluded from this analysis based on the results of a non-curative resection. In 28 of the patients in group B, 2 of the 5 patients with multigene methylation showed peritoneal recurrence after surgery, while patients without or with a single gene methylation did not experience recurrent cancer (Table 8). There was a significant correlation between peritoneal recurrence and multigene methylation in group B (Table 8, $P < 0.05$). Peritoneal recurrence was observed in only 1 of 10 cases that were CK19 positive, however statistical significance was not observed (Table 8, $P = 0.943$).

Table 5 Correlation between the multigene methylation of the peritoneal wash specimens from gastric cancer and the depth of cancer invasion, CY and P classification n (%)

Classification	Multigene methylation (<i>CHFR</i> , <i>E-cadherin</i> , <i>BNIP3</i>)		<i>P</i> -value
	Less than 2 genes	2 or more genes	
Group A: M-MP with CY(-) and P(-)	32 (91)	3 (9)	< 0.001
Group B: SS-SI with CY(-) and P(-)	25 (81)	6 (19)	
Group C: CY(+) or P(+)	6 (43)	8 (57)	

Table 6 Relationship between the qRT-PCR results and depth of cancer invasion, CY and P classification n (%)

Methylation status	Classification			<i>P</i> -value
	Group A: M-MP with CY(-) and P(-)	Group B: SS-SI with CY(-) and P(-)	Group C: CY(+) or P(+)	
CEA				0.352
(-) 39 (62)	17 (61)	17 (71)	5 (45)	
(+) 24 (38)	11 (39)	7 (29)	6 (55)	
CK19				< 0.05
(-) 37 (59)	21 (75)	13 (54)	3 (27)	
(+) 26 (41)	7 (25)	11 (46)	8 (73)	

DISCUSSION

Postoperative recurrence of gastric cancer usually occurs in the peritoneum, lymph node and liver^[1]. Peritoneal metastasis occurs most frequently and is highly resistant to various chemotherapies, which leads to a poor prognosis in gastric cancer patients. Peritoneal recurrence has been reported to depend on the depth of invasion, as 97.2% of recurrences occur beyond the MP. In addition, peritoneal recurrence was demonstrated in 34.9% of SS cases, 46.7% of SE cases and 60.0% of SI cases^[2].

Table 7 Comparison of the methylation status between cancerous tissue and peritoneal fluid in group C

Sample No.	Pathology	CY	P	Examined tissue	Methylation status					
					Tissue			Peritoneal fluid		
					<i>CHFR</i>	<i>E-cadherin</i>	<i>BNIP3</i>	<i>CHFR</i>	<i>E-cadherin</i>	<i>BNIP3</i>
A-03	por	+	-	Primary cancer	Negative	Positive	Negative	Negative	Positive	Negative
A-09	por	+	-	Primary cancer	Negative	Positive	Positive	Negative	Positive	Positive
A-16	por	+	+	Primary cancer	Positive	Negative	Positive	Positive	Negative	Positive
A-29	tub	+	+	Dissemination nodule	Negative	Positive	Negative	Negative	Positive	Positive
A-51	por	+	-	Metastatic lymph node	Positive	Positive	Positive	Positive	Positive	Positive
A-59	sig	-	+	Primary cancer	Negative	Positive	Positive	Negative	Negative	Negative
A-65	por	-	+	Metastatic ovarian tumor	Negative	Positive	Positive	Negative	Positive	Positive
A-69	sig	+	+	Primary cancer	Negative	Positive	Positive	Negative	Positive	Positive
A-78	tub	+	-	Primary cancer	Negative	Positive	Positive	Negative	Positive	Positive

Abbreviations as in Table 1.

Table 8 Correlation between peritoneal recurrence and multigene methylation and also the CK19 expression results in group B

	Multigene methylation (<i>CHFR</i> , <i>E-cadherin</i> , <i>BNIP3</i>)		<i>P</i> -value	CK19		<i>P</i> -value
	Less than 2 genes	2 or more genes		Negative	Positive	
Peritoneal recurrence						
Positive	0	2	< 0.05	1	1	0.943
Negative	23	3		10	9	

Another study reported that 50%-60% of gastric cancer patients with serosal invasion after a curative resection eventually developed peritoneal metastasis^[38]. Furthermore, the average survival after peritoneal recurrence is 4.9 mo^[39]. Therefore, the detection of micrometastasis in peritoneal lavage is essential, not only to make an accurate diagnosis, but also to start chemotherapy before the metastatic nodule is grossly formed in the peritoneum. The introduction of molecular technology such as RT-PCR of cancer specific genes has addressed the detection of micrometastasis in the LN^[40,41], ascites^[2,7-12,38,42,43], bone marrow and peripheral blood^[44,45] in gastric cancer. Various types of mRNA, such as CEA, CK19, and CK20 have been analyzed by RT-PCR and used as molecular markers in detecting micrometastasis in gastric cancer. Several studies have reported that the positive expression of mRNA in PF shows a significant correlation with peritoneal recurrence and survival^[2,8-12,38,43].

Recently, an analysis of cancer specific gene methylation has been utilized to detect micrometastasis in salivary rinses for head and neck cancer patients^[18], pleural effusion for lung cancer and malignant mesothelioma^[19], ductal fluid for breast cancer^[24], ascites for ovarian cancer and colorectal cancer^[21,22], bile for gallbladder cancer^[25], pancreatic juice for pancreatic cancer^[26], urine for prostate cancer^[27], stool for colorectal cancer^[28] and serum^[20,29,30]. However, few studies have addressed gene methylation for the detection of micrometastasis to PF in gastrointestinal cancer^[21].

The present study analyzed the promoter methylation of cancer related genes in 80 PFs. *CHFR*, *p16*, *RUNX3*, *E-cadherin*, *bMLH1*, *ABCG2* and *BNIP3* were chosen for the methylation analysis, because the frequent methylation of these 7 genes has been reported in several

malignancies including gastric cancer^[15,16,21,23,25-27,33-37,46-50]. Peritoneal recurrence has been reported to depend on the depth of invasion^[2]. Therefore, 80 samples from gastric cancer patients were classified into 3 groups [Group A: cancer invasion was restricted in M, SM, MP, Group B: cancer invasion deeper than MP, Group C: CY(+) or P(+)] and correlated with the gene methylation. As a result, q-MSP analysis using the 80 PFs demonstrated that the methylation status of *CHFR*, *E-cadherin* and *BNIP3* were significantly increased depending on the depth of cancer invasion. In contrast, the methylation status of the other genes was not significantly changed among the 3 groups (Table 4). These results indicate that the increasing value of the methylation of *CHFR*, *E-cadherin* and *BNIP3* from group A to group C was possibly derived from the metastatic cancer cells in the peritoneum. On the other hand, a q-MSP analysis might detect methylation from normal cells in the peritoneum at the basal level in *p16*, *RUNX3*, *bMLH1* and *ABCG2* genes. Based on these findings, *CHFR*, *E-cadherin* and *BNIP3* methylation was thus suggested to be preserved during cancer invasion, finally resulting in the occurrence of peritoneal seeding. Therefore, the methylation in more than 2 genes was compared among the 3 genes in each group. The results showed that there was a significant difference between multigene methylation and the 3 groups (Table 5). Eight of 14 patients (57%) in group C carried the multigene methylation while only 3 of 35 (9%) patients in group A exhibited multigene methylation. On the other hand, a qRT-PCR analysis examined the expression of CEA and CK19 mRNA in 63 samples (Table 6). Unexpectedly, CEA was not correlated with the classification even in group C with the highest positive rate. However, CK19 was significantly increased depending

on the depth of cancer invasion, CY and P classification. It was desirable that gene methylation should be detected in up to 100% of samples in group C with CY1 or P1. However, only 21% of gene methylation was observed in *CHFR*, 46% of *E-cadherin*, 71% of *BNIP3* and 57% of more than 2 genes were methylated in group C, indicating that gene methylation did not universally occur in all cancer cells. Thus, it is important to improve the sensitivity of multigene methylation analysis using PFs by increasing the number of genes that are specifically methylated in cancer cells. To clarify whether the methylation status of the PF originated from the cancer tissue, the methylation status of the primary or metastatic tissue in group C was compared with the methylation in the PF. The methylation status in the primary tumor was highly preserved in the PF (Table 7), thus suggesting that the methylation of the 3 genes assessed in the PF was derived from the primary tumor.

This study finally evaluated whether multigene methylation predicts peritoneal recurrence after surgery. In 21 patients in group B, peritoneal recurrence was found in 2 of 5 patients (40%) carrying multigene methylation. On the other hand, recurrence occurred in only 1 of 10 patients (10%) with positive CK19. There was a significant association between peritoneal recurrence and multigene methylation, but not CK19 (Table 8). These results suggested that multigene methylation may be a risk factor for peritoneal metastasis in the patients in group B even though the metastasis was not detected during surgery. An RT-PCR method using epithelial markers is critical in the diagnosis of micrometastasis. However, these methods only diagnose the presence of cancer cells. A methylation analysis that diagnosed micrometastasis in PFs would provide more information not only concerning the existence of cancer cells but also carcinogenesis, tumor progression and chemosensitivity, based on information on methylation status.

In conclusion, the present study investigated the methylation status in PF by both q-MSP and qRT-PCR analyses. The multigene methylation of *CHFR*, *E-cadherin* and *BNIP* in PF revealed the clinical feasibility of detecting occult neoplastic cells in the peritoneum. A methylation analysis along with a cytological examination might increase the positive detection of cancer cells in PF.

COMMENTS

Background

Postoperative recurrence of gastric cancer usually occurs in the peritoneum. Peritoneal recurrence is highly resistant to various chemotherapies, which leads to a poor prognosis in these patients. Therefore, the detection of micrometastasis in peritoneal lavage is essential, not only to make an accurate diagnosis, but also to start chemotherapy before the metastatic nodule is grossly formed in the peritoneum.

Research frontiers

Epigenetic gene silencing through DNA methylation occurs in various cancers. Recently, several reports have demonstrated aberrant gene methylation detected in various samples from cancer patients and have suggested the feasibility of methylation analysis in the evaluation of occult neoplastic cells or micrometastasis. This study clarified whether gene methylation in peritoneal fluid is feasible for determining micrometastasis to the peritoneum in gastric cancer patients.

Innovations and breakthroughs

Few studies have addressed gene methylation for the detection of micrometastasis to peritoneal fluid in gastrointestinal cancer. The authors' results indicate that gene methylation in the peritoneal fluid could detect occult neoplastic cells in the peritoneum and might be a risk factor for peritoneal metastasis.

Applications

The development of this system may improve the accurate diagnosis of peritoneal dissemination and improve the prognosis of gastric cancer patients.

Terminology

DNA methylation is an epigenetic modification in humans, and changes in methylation patterns play an important role in carcinogenesis, cancer progression and chemosensitivity.

Peer review

This paper is interesting and written well.

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Expression of p53, c-erbB-2 and Ki67 in intestinal metaplasia and gastric carcinoma

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type III IM and gastric carcinomas. The expression of p53, c-erbB-2 and Ki67 proteins in 20 SIM, 27 Atypical IM and 37 gastric carcinomas showed significant differences between SIM and gastric carcinomas while no significant differences were observed between Atypical IM and gastric carcinomas.

CONCLUSION: Atypical IM may better reveal the pre-cancerous nature of IM and could be a helpful indicator in the clinical follow up of patients.

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Key words: Simple intestinal metaplasia; p53; Atypical intestinal metaplasia; c-erbB-2; Ki67

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Abstract

AIM: To compare two types of classification of intestinal metaplasia (IM) of the stomach and to explore their relationship to gastric carcinoma.

METHODS: Forty-seven cases of gastric IM were classified into type I, type II or type III according to mucin histochemical staining and compared with a novel classification in which the specimens were classified into simple IM (SIM) or atypical IM according to polymorphism in terms of atypical changes of the metaplastic epithelium. Forty-seven IM and thirty-seven gastric carcinoma samples were stained for p53, c-erbB-2 and Ki67 proteins by Envision immunohistochemical technique.

RESULTS: There were no significant differences in the expression of p53 and c-erbB-2 among type I, type II, type III IM and gastric carcinomas. The positive expression rate of Ki67 was significantly higher in gastric carcinomas than in type I IM while no significant Ki67 expression differences were observed among type II,

INTRODUCTION

The relationship between intestinal metaplasia (IM) and gastric carcinoma has always been controversial. Correa postulated that gastric cancer develops through a complex sequence of events from normal mucosa to superficial gastritis, chronic atrophic gastritis, IM, dysplasia, and finally to intestinal type gastric carcinoma^[1]. Many investigators have given credence to the preneoplastic nature of IM^[2]. Even with common occurrence and presence in gastric biopsies, not all cases of IM may develop into gastric carcinoma. A subtype of IM which

has malignant potential should be classified for clinical follow up. Generally, IM is divided into subtypes on the basis of histochemical characteristics, and IM showing sulphomucin secretion has been considered to be a lesion with precancerous nature^[3-5], but some others considered this type of IM had no special link to gastric carcinoma^[2], and Rothery^[6] also found half of the cases with this IM showing sulphomucin secretion exist in benign lesions.

In our study, IM was divided into two subtypes according to the atypical changes of the metaplastic epithelium: simple IM (SIM) and atypical IM (AIM). We detected three tumor-associated proteins, p53, c-erbB-2 and Ki67, in different subtypes of IM in order to find which one is more associated with gastric carcinoma.

MATERIALS AND METHODS

Samples

Forty-seven formalin-fixed, paraffin-embedded samples for IM were obtained from endoscopic biopsy and thirty-seven gastric carcinoma specimens from gastrectomy at Qilu Hospital of Shandong University. Mucin histochemical staining for IM subtyping was performed.

Serial sections were cut, stained with hematoxylin-eosin and the following techniques for mucins: Alcian blue pH 2.5-periodic acid-Schiff and high iron-diamine plus Alcian blue pH 2.5 to identify neutral, sialo- and sulphomucins.

Forty-seven cases of IM were classified in accordance with the system used by Jass and Filipe^[7,8]: Type I (complete): mature absorptive and goblet cells, the latter secreting sialomucins (Figure 1); Type II (incomplete): IM cells with few or absent absorptive cells; presence of columnar “intermediate” cells in various stages of differentiation, secreting neutral and acid sialomucins; goblet cells secreting sialomucins and/or occasionally sulphomucins (Figure 1); Type III (incomplete): the cell dedifferentiation is more marked than in type II; “intermediate” cells secrete predominantly sulphomucins. A variable degree of disorganized glandular architecture is often present (Figure 1).

IM subtyping according to atypical changes

Forty-seven IM samples were classified into SIM and AIM according to atypical changes of the metaplastic epithelium.

Immunohistochemical technique

Sections 4 μ m thick were cut, deparaffinized in xylene, and then dehydrated in descending dilutions of ethanol. For the antigen retrieval regimen, all slides were micro-waved in 10 mmol/L sodium citrate buffer (pH 6.0) at 10 min intervals for a total of 20 min. The endogenous peroxidase activity was blocked by 10 min of incubation with 3% hydrogen peroxidase (reagent A) at room temperature. After washing in PBS, the sections were incubated with monoclonal mouse anti-human antibodies p53 (MAB-0364), c-erbB-2 (CB11) and Ki67 (SP6)

Table 1 The distribution of 47 IM samples within different subtypes of IM

	Type I	Type II	Type III	Total
SIM	11	7	2	20
AIM	6	11	10	27
Total	17	18	12	47

IM: Intestinal metaplasia; SIM: Simple intestinal metaplasia; AIM: Atypical intestinal metaplasia.

overnight at 4°C. The sections were washed with PBS and incubated with polymerase auxiliaries (reagent B) for 20 min. After washing in PBS, the sections were incubated with biotinylated secondary antibody (reagent C) for 30 min at room temperature and finally DAB was visualized. Tissues were counterstained with hematoxylin. A negative control was designed by using PBS instead of primary antibody.

Positive criteria of immunohistochemical staining

Sections were scored by light microscopy. The percentage of positively stained cells was calculated after 100 cells were counted in more than 5 high-power ($\times 40$) fields. The following definitions were made: p53 and Ki67: more than 10% positive staining in nuclei was defined as positive staining; c-erbB-2: more than 10% positive staining in cytoplasm was defined as positive staining in IM, and more than 10% positive staining in cell membrane was defined as positive staining in gastric carcinoma^[9].

Statistical analysis

The significance of associations was determined by the χ^2 test or the Fisher's exact test, P value < 0.05 was considered statistically significant.

RESULTS

Subtype distribution of 47 IM samples

In 47 IM samples, type I, type II and type III IM accounted for 17, 18 and 12 samples, respectively. In 20 SIM, type I, type II and type III IM accounted for 11, 7 and 2 samples, respectively. In 27 AIM, type I, type II and type III IM accounted for 6, 11 and 10 samples, respectively (Table 1).

Expression of p53 protein

The positive expression rates of p53 in type I, type II, type III IM and gastric carcinomas were 41.18%, 27.78%, 25.00% and 54.05%, respectively. The expression of p53 in gastric carcinomas was not significantly higher than in types I, II and III ($P > 0.05$).

The positive expression rates of p53 in SIM and AIM were 20.00% and 40.74%, respectively. The expression of p53 in gastric carcinomas was significantly higher than in SIM ($P < 0.05$). There was no significant difference in p53 expression between AIM and gastric carcinomas ($P > 0.05$) (Table 2, Figure 2).

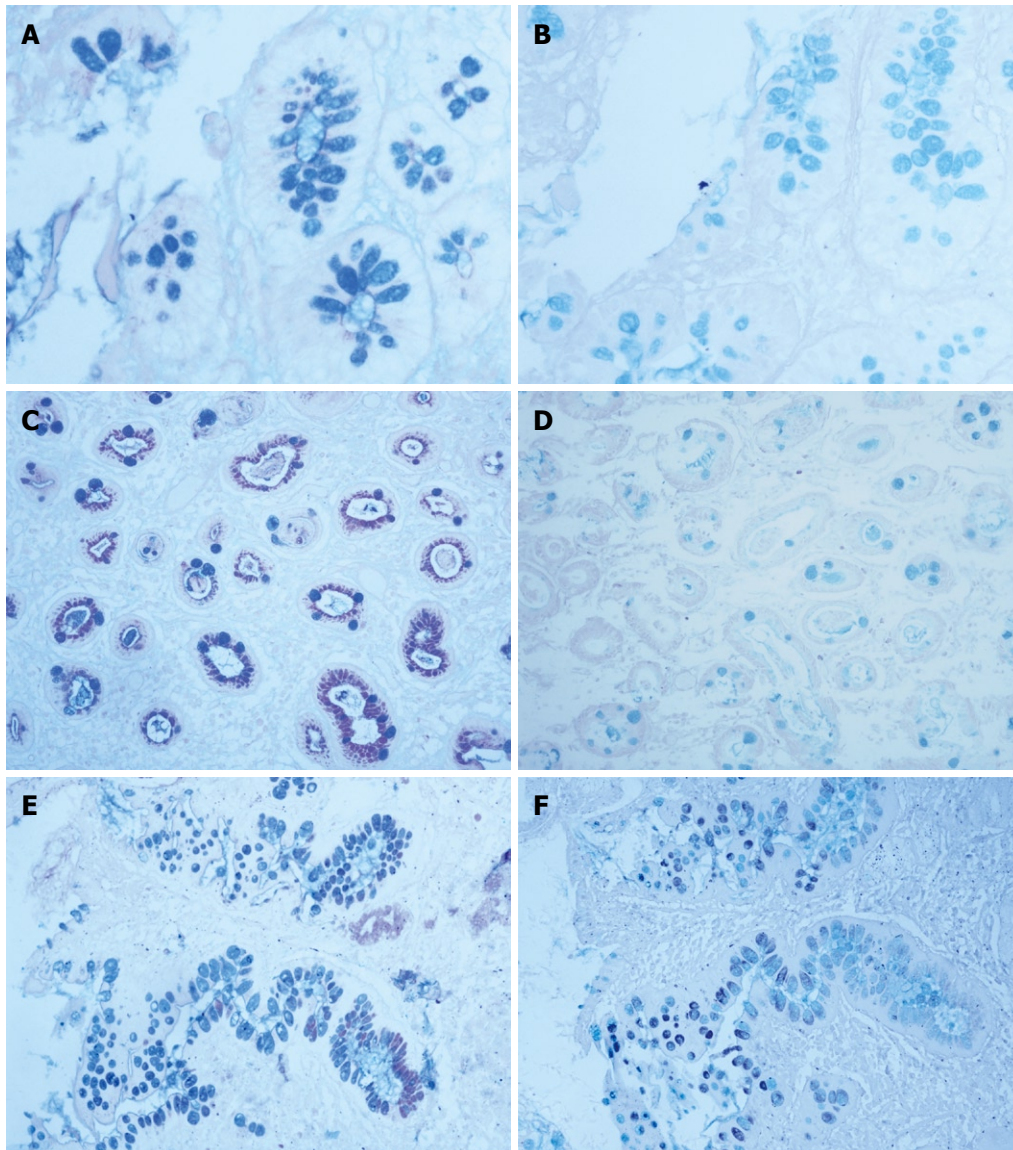


Figure 1 Three types of intestinal metaplasia (IM) according to histochemical stains. A: IM type I : AB-PAS AB positive (blue), PAS negative; B: IM type I : HID-AB AB positive (blue), HID negative; C: IM type II: AB-PAS AB positive (blue), PAS positive (red); D: IM type II: HID-AB AB positive (blue), HID negative; E: IM type III: AB-PAS AB positive (blue), PAS positive (red); F: IM type III: HID-AB AB positive (blue), HID positive (grey and black) ($\times 200$).

Table 2 The expression of p53, c-erbB-2 and Ki67 proteins in different subtypes of IM and gastric carcinoma (GC)

	Total	p53		c-erbB-2		Ki67	
		+	-	+	-	+	-
SIM	20	4 ^a	16	6 ^a	14	5 ^a	15
AIM	27	11	16	18	9	12	15
Type I	17	7	10	9	8	5 ^a	12
Type II	18	5	13	8	10	8	10
Type III	12	3	9	4	8	4	8
GC	37	20	17	22	15	28	9

^a $P < 0.05$ vs GC.

Expression of c-erbB-2 protein

The positive expression rates of c-erbB-2 in type I, type II, type III IM and gastric carcinomas were 52.94%, 44.44%, 33.33% and 59.46%, respectively. The

expression of c-erbB-2 in gastric carcinomas was not significantly higher than in types I, II and III ($P > 0.05$).

The positive expression rates of c-erbB-2 in SIM and AIM were 30.00% and 656.67%. The expression of c-erbB-2 in gastric carcinomas was significantly higher than SIM ($P < 0.05$). There was no significant difference between expression in AIM and gastric carcinomas ($P > 0.05$) (Table 2, Figure 2).

Expression of Ki67 protein

The positive expression rates of Ki67 in type I, type II, type III IM and gastric carcinomas were 29.41%, 50.00%, 41.67% and 75.68%, respectively. The expression of Ki67 in gastric carcinomas was significantly higher than in type I IM ($P < 0.05$). There was no significant difference in Ki67 expression between gastric carcinomas and type II or type III.

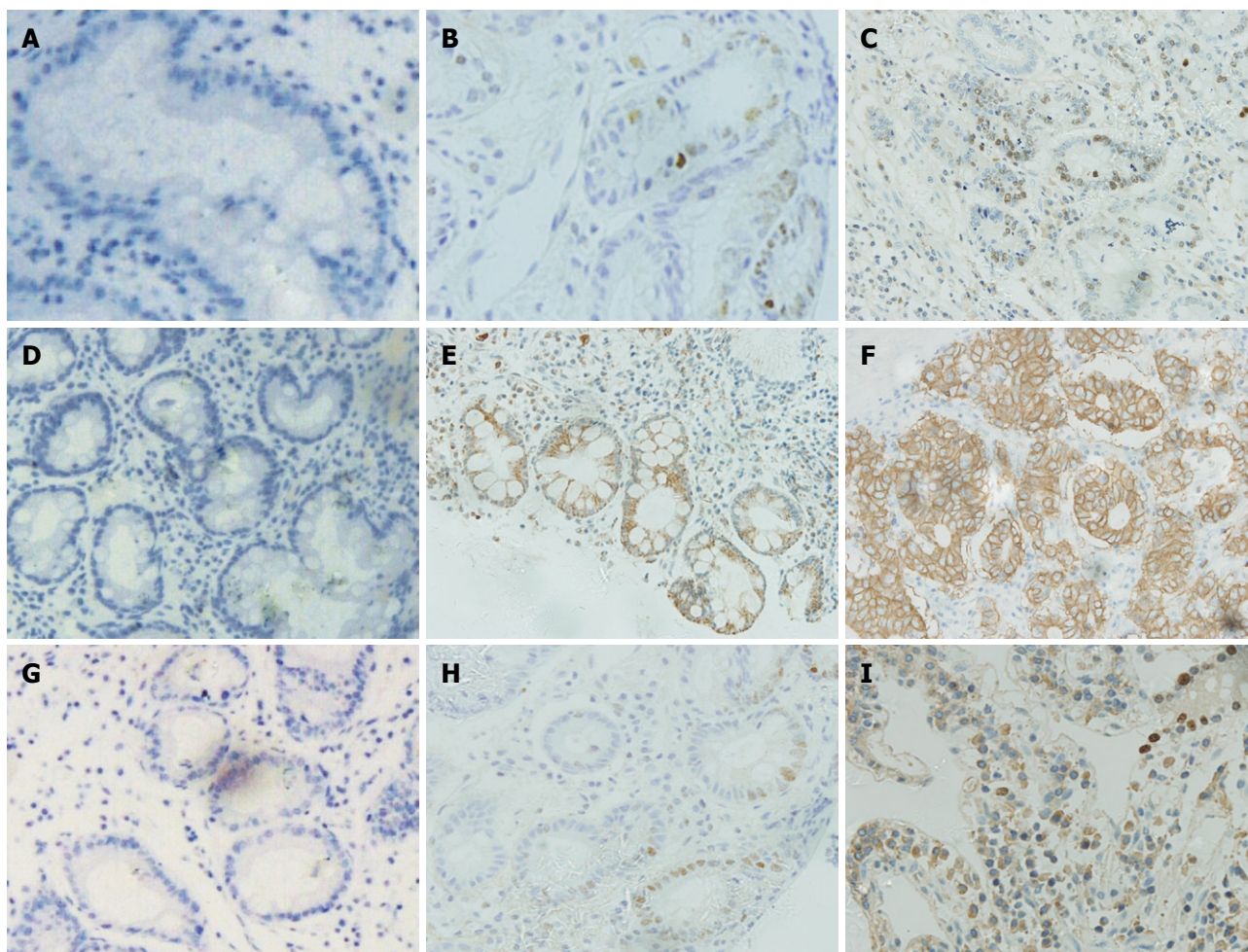


Figure 2 Expression of p53, c-erbB-2 and Ki67 proteins in simple intestinal metaplasia (SIM), atypical intestinal metaplasia (AIM) and gastric carcinoma (GC). A: p53 negative in SIM; B: p53 positive in AIM (nuclei); C: p53 expression increased in GC (nuclei); D: c-erbB-2 negative in SIM; E: c-erbB-2 positive in AIM (cytoplasm); F: c-erbB-2 expression increased in GC (membrane); G: Ki67 negative in SIM; H: Ki67 positive in AIM (nuclei); I: Ki67 expression increased in GC (Envision method, $\times 400$).

The positive expression rates of Ki67 in SIM and AIM were 25.00% and 51.85%, respectively. The expression of Ki67 in gastric carcinomas was significantly higher than in SIM ($P < 0.05$) while there was no significant difference in Ki67 expression between AIM and gastric carcinomas ($P > 0.05$) (Table 2, Figure 2).

DISCUSSION

Gastric carcinoma is the fourth most common malignancy worldwide^[10], accounting for 10% of newly diagnosed cancers^[11]. It is one of the most common causes of cancer mortality in China^[12]. Approximately 84% of patients with gastric cancer will have advanced disease and their median survival time is only 3-4 mo if they are not treated with chemotherapy^[13]. Therefore, it is necessary to diagnose at an early stage in order to improve the survival rate. Gastric carcinoma is divided into intestinal and diffuse types according to Lauren^[14] and the intestinal-type of gastric carcinoma is said to have a strong association with IM^[15]. Since Morson^[16] pointed out in 1955 that some gastric carcinomas might arise from an area

of IM, IM has been considered to be a possible precancerous state. A large number of IM patients have been found in clinical studies, and a research group reported that the detection rate of IM was 37%^[4]. Which type of IM is closely related to gastric carcinoma remains an unanswered question. Traditionally, IM is divided according to histochemical characteristics, but research concludes that sulphomucin-positive IM is of no value in surveillance for gastric cancer^[17]. In view of the inconsistency of this classification, we assigned IM into the groupings of SIM (Figure 3) and IM with atypical changes as AIM (Figure 3). The predominant difference in the two types of IM is the atypical changes of the metaplastic epithelium: SIM glands are arranged neatly and the goblet cells are in normal forms, the mucosa in the foveolae is flat; while AIM glands are crowded and the goblet cells often are associated with immature differentiation, also the mucosa in the foveolae usually become deeper than in SIM. One question should be clarified regarding the difference between AIM and gastric intraepithelial neoplasia (GIN) (Figure 3): the main distinction lies in the goblet cells. There were more goblet cells (over 10%

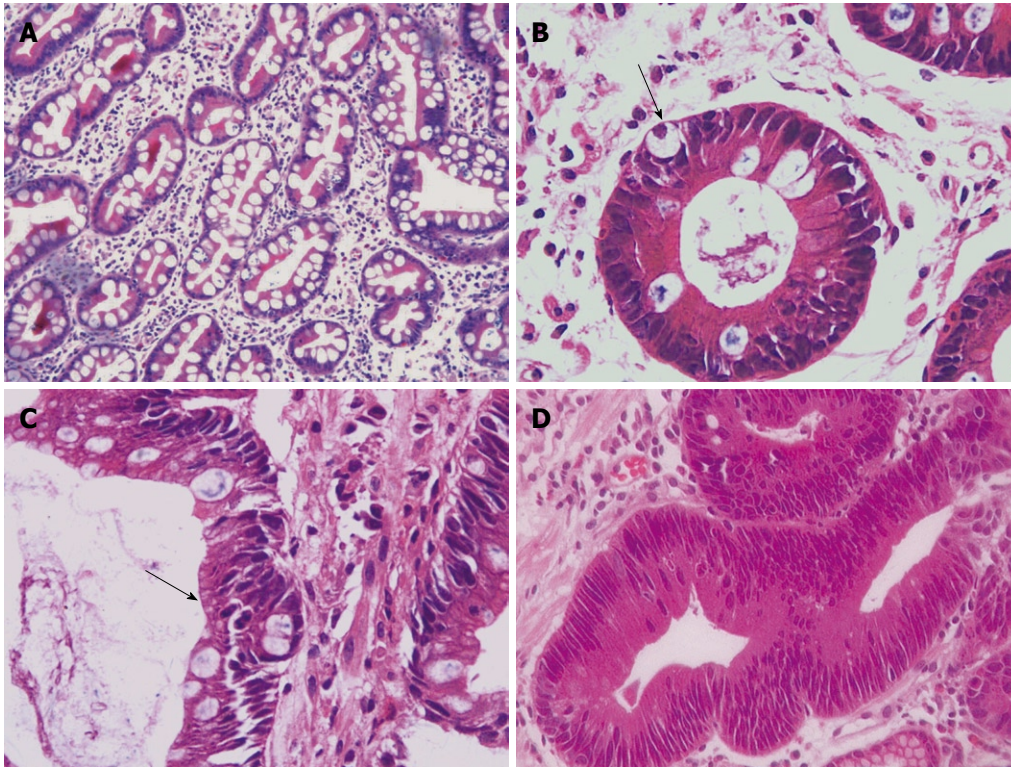


Figure 3 Morphological differences in three gastric lesions. A: SIM; B: AIM: reversed polarity of goblet cell (arrow); C: AIM: mitosis (arrow); D: Gastric intraepithelial neoplasia (GIN) (HE, $\times 400$).

of total cells) in AIM while there were less or no goblet cells in GIN.

In our study, three tumor-associated proteins, p53, c-erbB-2 and Ki67, were selected for immunohistochemical detection. The *p53* gene is localized to chromosome arm 17p13^[18]. Evidence from *in vitro* models suggests that *p53* acts as a tumor suppressor gene^[19]. The evidence accumulated so far suggests that mutant *p53* may be the commonest genetic abnormality in human cancer^[20]. *p53* gene mutation is known to play a considerable role in the carcinogenesis of colonic carcinoma and gastric carcinoma. Shiao *et al*^[21] reported that 67% of gastric carcinomas, 58% of dysplasias and 38% of metaplastic lesions stained positively for p53. *C-erbB-2* (also called *NEU* and *HER2*) is a proto-oncogene that codes for a protein product which shows considerable homology with EGFR^[22]. Several studies have reported amplification of the *c-erbB-2* gene in human neoplasms, particularly in adenocarcinomas^[23]. In recent years, researchers have revealed that c-erbB-2 plays an important role in the occurrence and development of gastric carcinoma. Observations suggested that *c-erbB-2* gene rearrangement non-randomly associated with carcinomas with glandular origin derived from the gastrointestinal tract^[24]. It is reported that over expression of the *c-erbB-2* gene is at a frequency of 8.2%-45% in gastric carcinoma^[25-27]. Ki67 is a nuclear proliferation-associated antigen expressed in the growth and synthesis phases of the cell cycle but not in the resting phase^[28]. This antigen provides information about the proportion of active cells in the cell cycle. Its expression varies

greatly during the cell cycle and is increased in many tumors^[29]. Studies have revealed that the Ki67 proliferating index increases in the transformation from IM to gastric carcinoma^[30]. For the reasons above, p53, c-erbB-2 and Ki67 proteins can be regarded as indicators of the pre-cancerous nature of IM in the gastric mucosa. Our results demonstrated that the expressions of p53 and c-erbB-2 in gastric carcinoma were not significantly higher than in types I, II and III IM. The expression of Ki67 in gastric carcinoma was significantly higher than in type I, but not significantly higher than in type II or type III.

It is difficult to determine which subtype of IM has a definite relationship with gastric carcinomas. In SIM and AIM classification, the expressions of all three proteins in gastric carcinomas were significantly higher than in SIM and no significant differences were observed between gastric carcinomas and AIM. Obviously, AIM may have a much more close relationship with gastric carcinoma.

We deduce that SIM may be merely a response to stimuli caused by the changing environment, while AIM may have malignant transformation and could be regarded as preneoplastic lesions. Clinical follow up of AIM patients may be helpful for the diagnosis of early gastric carcinomas. However, since details of the role of AIM in the multiple steps of carcinogenesis of gastric mucosa are still unknown, further study is necessary with regard to AIM, perhaps using more advanced methods. Furthermore, an adequate long term follow up is indispensable to assess the definite value of AIM in the screening for gastric carcinoma.

COMMENTS

Background

Gastric carcinoma remains a significant problem globally. The relationship between intestinal metaplasia (IM) and gastric carcinoma has always been controversial. Generally IM is divided into subtypes on the basis of histochemical characteristics; however, this classification is confusing. A new classification of IM is needed in order to follow up patients selectively.

Research frontiers

By detecting three tumor-associated proteins, p53, c-erbB-2 and Ki67, in IM and gastric carcinoma, this study compared two types of classification in IM of the stomach and explored their relationship to gastric carcinoma.

Innovations and breakthroughs

In the past, IM was classified according to histochemical characteristics. In this study, IM was first divided into simple IM (SIM) and atypical IM (AIM) was reported to better reveal the precancerous nature of IM and could be a helpful indicator in the surveillance of patients clinically.

Applications

The new classification of IM could be helpful in the surveillance of patients clinically and useful for the diagnosis of early gastric carcinomas.

Terminology

IM is defined as the appearance of intestinal epithelium in the stomach. Type I, II and III IM are subtypes of IM classified according to the histochemical characteristics of the mucin-secreting cells. SIM and AIM classification is dependent mainly on the atypical changes of the metaplastic epithelium of the stomach. p53, c-erbB-2 and Ki67 are all tumor-associated proteins that are expressed mainly in metaplastic and tumor tissues.

Peer review

The study provides important new data about the potential risk of gastric cancer in patients with IM. However, it would be important in the future to investigate the expression of p53 and/or Her2Neu in a prospective study in patients with IM, to confirm that only patients with p53/Her2Neu expression in the IM have actually a higher risk for gastric carcinomas.

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Prevalence of diverticulosis in recurrent *Clostridium difficile* infection

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Abstract

AIM: To re-evaluate the theory that colonic diverticulosis is associated with relapse of *Clostridium difficile* associated disease (CDAD) in light of data suggesting increasing rates of CDAD infection and relapse.

METHODS: Charts were reviewed for patients with recurrent CDAD who had also had a prior colonoscopy or flexible sigmoidoscopy. An age and gender matched control group was used to compare the prevalence of diverticulosis.

RESULTS: Twenty-two patients met the study criteria, and the prevalence of diverticulosis in patients with CDAD relapse was 23% compared to 32% in age and sex matched controls ($P = 0.44$). A significant proportion of patients with CDAD relapse had comorbidities associated with immune suppression.

CONCLUSION: Diverticulosis does not appear to be associated with CDAD relapse.

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INTRODUCTION

The incidence of *Clostridium difficile* (*C. difficile*) associated disease (CDAD) has reached epidemic proportions, increasing both in terms of frequency and severity. The rates of *C. difficile* associated disease have steadily increased since the 1980s, with a marked increase in infection over the past 10 years. In one region of Canada there was a four-fold increase from 2002 to 2003 alone^[1]. The cost associated with CDAD in 2008 in the U.S. has been estimated at \$32 million per day with nearly 40 000 extra hospital inpatient days due to *C. difficile* infection^[2]. The increased incidence and virulence of *C. difficile* infection are felt to be caused by a new hypervirulent strain which produces significantly more toxins A and B. In addition to increasing morbidity and mortality, this hypervirulent strain has also led to an increase in relapse rates, which have been reported to be as high as 47.2%^[3], compared to historic relapse rates of approximately 20%^[4].

The exact etiology of CDAD relapse is incompletely understood, but it is probably multifactorial. Relapse of CDAD is usually caused by the original strain, although a percentage of patients are infected with a new strain^[5]. One of the earliest theories for CDAD relapse was the presence of colonic diverticula, which were thought to

serve as reservoirs for *C. difficile* spores. This was largely based on an early study by Tedesco *et al*^[6] which found that 18 of 22 patients (82%) with recurrent *C. difficile* infection had diverticulosis. It was theorized that *C. difficile* spores in diverticula were impervious to antibiotics and so could germinate after the completion of treatment. Tapered and pulsed antibiotic regimens for recurrent *C. difficile* infection were thus aimed at treating these dormant spores^[7]. To our knowledge no paper has re-evaluated the association between diverticulosis and *C. difficile* relapse. Our study aimed to re-evaluate the association between diverticulosis and recurrent *C. difficile* infection at our medical center in light of increasing rates of CDAD infection and relapse.

MATERIALS AND METHODS

We reviewed positive ELISA-based *C. difficile* toxin assays from 2005-2007 at our tertiary level hospital. We reviewed the charts of patients with a minimum of two positive toxin assays more than 14 d apart who also had a prior colonoscopy or flexible sigmoidoscopy at our institution. Patient charts were reviewed for the presence of diverticulosis, prior antibiotic use, comorbidities, recent hospitalizations, *C. difficile* treatment course, and number of relapses.

Relapse was defined as recurrent diarrhea with a positive toxin assay or pseudomembranous colitis after completion of treatment for *C. difficile* infection within the previous 3 mo. To be included in the study, patients must have completed full initial antibiotic therapy for a minimum of 10 d with symptomatic improvement. An age and gender matched control group was used to compare the prevalence of diverticulosis at our institution. Patients in the control group had colonoscopies performed for the purpose of colorectal cancer screening. This study was approved by our medical center's institutional review board.

RESULTS

Twenty-two patients met the study criteria, with an average of 2.3 episodes of *C. difficile* infection. All patients were treated with standard metronidazole (*po* or *iv*) and/or oral vancomycin therapy. The prevalence of diverticulosis in patients with *C. difficile* relapse was 23%, with a mean patient age of 62. The prevalence of diverticulosis in the age and sex matched control group was 32% ($P = 0.44$, using χ^2 analysis, Figure 1). Table 1 shows the baseline characteristics of patients with recurrent *C. difficile* relapse. The inpatient mortality in this group was 18%.

DISCUSSION

Patients with recurrent *C. difficile* infection at our institution did not have a higher prevalence of colonic diverticulosis than age and sex matched controls. The prevalence of diverticulosis in patients with relapsing *C. difficile* and controls was 23% and 32%, respectively ($P = 0.44$), which is consistent with historical controls. To our knowledge this is the first paper to reevaluate

Table 1 Baseline characteristics of patients with recurrent *Clostridium difficile* associated disease ($n = 22$)

Baseline characteristics	
Mean age (yr)	62 (30-90)
Female	13 (59%)
Recent hospitalizations (< 3 mo)	18 (82%)
Lymphoma/malignancy	8 (36%)
HIV/AIDS	3 (14%)
Systemic steroid use	3 (14%)
Liver cirrhosis	2 (9%)

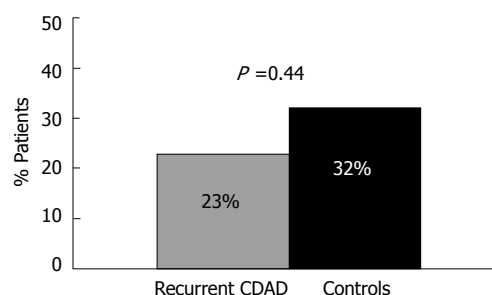


Figure 1 Prevalence of diverticulosis. CDAD: *Clostridium difficile* associated disease.

the association between diverticulosis and recurrent *C. difficile* infection since first described by Tedesco *et al*^[6].

We found that a high percentage of patients with recurrent *C. difficile* infection suffered from co-morbidities often associated with immune suppression. These results are not surprising since growing data suggest that host immunity plays a large role in promoting relapse^[8-14]. One of the earliest studies to support this theory came from Kyne *et al*^[15] where 22 patients with resolved *C. difficile* infection were compared to 22 patients with relapsing disease. Patients with relapsing *C. difficile* infection were found to have significantly lower levels of serum antibodies to toxin A. Additional independent risk factors for *C. difficile* recurrence include older age^[16] and prolonged hospitalization^[17].

Patients with impaired host immunity during chemotherapy have been found to have higher rates of CDAD^[8], even without prior antibiotic use^[9]. Higher rates of CDAD infection have been found in patients on nephrology, hematology, and organ transplantation wards^[10], as well as in patients with human immunodeficiency virus (HIV) infection^[11]. These findings have prompted research aimed at improving host immunity in patients with CDAD relapse. Intravenous immunoglobulin (IVIG) has been used in the treatment of severe, refractory, or recurrent *C. difficile* infection^[12,16]. A human monoclonal antibody against toxins A and B, MDX-1388, was shown to have efficacy in preventing relapse of *C. difficile* infection in hamsters^[13]. A small study of a toxoid vaccine against *C. difficile* showed benefit in 3 patients with recurrent *C. difficile*, allowing the cessation of antibiotic treatment^[14].

Our study had several limitations. Given the small patient size our study may have been underpowered to detect a difference in the prevalence of diverticulosis.

This study was also done retrospectively and all patients originated from a single institution. Our results did not find an association between colonic diverticulosis and recurrent *C. difficile* infection at our institution, however further studies may be needed to verify these findings. The high prevalence of co-morbidities often associated with immune suppression in patients with recurrent *C. difficile*, however, is consistent with current data that suggest host immunity may play a significant role in CDAD relapse.

COMMENTS

Background

The incidence of *Clostridium difficile* (*C. difficile*) associated disease (CDAD) has reached epidemic proportions, increasing both in terms of frequency and severity. The exact etiology of CDAD relapse is incompletely understood, and previous research has suggested an association between CDAD relapse and colonic diverticulosis.

Research frontiers

The increased incidence and virulence of *C. difficile* infection are felt to be caused by a new hypervirulent strain which produces significantly more toxins A and B. In addition to increasing morbidity and mortality, this hypervirulent strain has also led to an increase in relapse rates. In this study the authors did not find an association between colonic diverticulosis and CDAD relapse, but did find that a significant proportion of patients with CDAD relapse had co-morbidities associated with immune suppression.

Innovations and breakthroughs

Recent reports have suggested that host immunity may play a role in preventing future *C. difficile* relapse. This is the first paper to re-evaluate the association between CDAD relapse and colonic diverticulosis since the emergence of a new hypervirulent strain.

Applications

By showing a lack of association between colonic diverticulosis and CDAD relapse, this study could help guide future treatments. Previous treatments were originally aimed at treating dormant spores in diverticula with pulsed or tapering antibiotics. This study would suggest that therapy aimed at improving host immunity may be important in CDAD relapse.

Terminology

C. difficile is a gram positive bacterium that may cause a colonic infection, typically after antibiotic use. This infection may become severe and may relapse, requiring multiple antibiotic treatments. Colonic diverticula are outpouchings of the colonic wall.

Peer review

It is an interesting study that addresses a potentially important relationship between recurrence of *C. difficile* infection and diverticulosis.

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Body composition changes after transjugular intrahepatic portosystemic shunt in patients with cirrhosis

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Abstract

AIM: To investigate the effect of transjugular intrahepatic porto-systemic shunt (TIPS) on malnutrition in portal hypertensive cirrhotic patients.

METHODS: Twenty-one patients with liver cirrhosis and clinical indications for TIPS insertion were investigated before and 1, 4, 12, 52 wk after TIPS. For each patient we assayed body composition parameters [dry lean mass, fat mass, total body water (TBW)], routine liver and kidney function tests, and free fatty acids (FFA). Glucose and insulin were measured for the calculation of the homeostasis model assessment insulin resistance (HOMA-IR); liver function was measured by the galactose elimination capacity (GEC); the severity of liver disease was graded by model for end-stage liver disease (MELD).

RESULTS: Porto-systemic gradient decreased after TIPS (6.0 ± 2.1 mmHg vs 15.8 ± 4.8 mmHg, $P < 0.001$). Patients were divided in two groups according to initial body mass index. After TIPS, normal weight patients had an increase in dry lean mass (from 10.9 ± 5.9 kg to 12.7 ± 5.6 kg, $P = 0.031$) and TBW (from 34.5 ± 7.6 L to 40.2 ± 10.8 L, $P = 0.007$), as well as insulin (from 88.9 ± 49.2 pmol/L to 164.7 ± 107.0 pmol/L, $P = 0.009$) and HOMA-IR (from $3.36\% \pm 2.18\%$ to $6.18\% \pm 4.82\%$, $P = 0.023$). In overweight patients only FFA increased significantly (from 0.59 ± 0.24 mmol/L to 0.93 ± 0.34 mmol/L, $P = 0.023$).

CONCLUSION: TIPS procedure is effective in lowering portal pressure in patients with portal hypertension and improves body composition without significant changes in metabolic parameters.

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Key words: Insulin resistance; Liver cirrhosis; Malnutrition; Portal hypertension; Transjugular intrahepatic porto-systemic shunt

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INTRODUCTION

Portal hypertension and malnutrition are two important complications of cirrhosis, both affecting prognosis and risk of death^[1-3]. Portal hypertension is present in 60% of cirrhotic patients^[4]; the existence of a portal-systemic shunt modifies both fasting and post-prandial metabolism, decreasing the hepatic first-pass effect of nutrients, which become more available for peripheral tissues. Also hormone levels, like insulin, share a similar defect. The lack of a significant first-pass removal by the liver makes insulin flood the systemic circulation and promotes insulin resistance, which is further aggravated by the production of inflammatory molecules [tumor necrosis factor- α (TNF- α), lipopolysaccharide (LPS), *etc.*] which have an anti-insulin effect and contribute to systemic inflammation^[5]. The final event of the above alterations is a progressive deterioration of liver function, also resulting in poor nutritional status. Malnutrition, in turn, is a risk factor for disease progression, and is associated with the development of complications^[3], with a high risk of death^[6], as well as increased morbidity and mortality after transplantation^[7].

Several studies demonstrated that transjugular intrahepatic porto-systemic shunt (TIPS) using polytetrafluoroethylene (PTFE)-covered stents are the best rescue therapy for failures of medical and endoscopic treatment of portal hypertension^[8-10].

The effects of TIPS on metabolism and body composition are not well defined. Allard *et al.*^[11] and Plauth *et al.*^[12] found that TIPS insertion in malnourished patients with cirrhosis and hypermetabolism resulted in improved body composition. No studies, however, have clearly defined the relation between TIPS-induced metabolic changes and changes in nutritional status, and/or the underlying mechanism. In particular, no studies addressed the potential impact of pre-TIPS overweight and obesity and associated insulin resistance on the post-TIPS nutritional status and insulin levels.

We aimed to test the effects of TIPS insertion on nutritional status and insulin levels in a group of subjects with cirrhosis and complications of portal hypertension.

MATERIALS AND METHODS

Subjects

Twenty-six patients with liver cirrhosis, consecutively undergoing an elective TIPS procedure were included (Table 1). Three patients did not complete the baseline examination protocol due to variceal bleeding requiring intensive care and immediate TIPS insertion. Of the remaining 23 patients (15 males and 8 females), one received an orthotopic liver transplant after TIPS, and one died during follow-up. Three patients missed one or more follow-up evaluations; therefore, only 18 patients had all the evaluations planned by protocol. The diagnosis of cirrhosis was assessed on the basis of biochemical, clinical, and ultrasonographic findings and confirmed by liver biopsy in 8 cases. Among the 21 patients who completed the follow-up, etiology was as follows: alcohol-related, 15;

Table 1 Modification of values studied in 21 patients (mean \pm SD)

Variable	Basal Pre-TIPS	After 52 wk	P
HVPG (mmHg)	15.8 \pm 4.8	6.0 \pm 2.1	< 0.001
Bilirubin (mg/dL)	1.26 \pm 1.11	2.85 \pm 2.29	0.017
Creatinine (mg/dL)	1.02 \pm 0.56	1.14 \pm 0.61	0.18
Albumin (g/L)	31.3 \pm 7.7	33.0 \pm 7.7	0.586
GEC (mmol/min)	1.79 \pm 0.48	1.61 \pm 0.41	0.081
MELD (score) (n = 18) ¹	11 (6)	13 (7)	0.804
Blood glucose (mmol/L)	5.8 \pm 1.5	6.3 \pm 1.6	0.345
Plasma insulin (pmol/L)	128.9 \pm 84.0	182.9 \pm 100.6	0.024
HOMA- β	269 \pm 409	233 \pm 108	0.700
HOMA-IR	5.04 \pm 4.07	7.82 \pm 5.89	0.028
BMI (kg/m ²)	26.2 \pm 5.8	27.4 \pm 5.6	0.172
FFA (mmol/L)	0.54 \pm 0.23	0.89 \pm 0.44	0.013
Creatinine (mg/dL)	0.98 \pm 0.52	0.83 \pm 0.34	0.053
Fat mass (%)	29.4 \pm 7.7	27.8 \pm 8.7	0.278
Fat mass (kg)	23.4 \pm 10.5	22.9 \pm 10.0	0.812
Dry lean mass (kg)	14.0 \pm 5.8	14.8 \pm 5.0	0.115
TBW (L)	32.3 \pm 9.6	43.3 \pm 10.3	0.017

¹Median (interquartile range). Values were obtained 2 wk before (Basal) and 52 wk after TIPS insertion. *P* < 0.05 is considered statically significant. TIPS: Transjugular intrahepatic porto-systemic shunt; HVPG: Hepatic venous pressure gradient; GEC: Galactose elimination capacity; MELD: Model for end-stage liver disease; HOMA: Homeostasis model assessment; IR: Insulin resistance; BMI: Body mass index; FFA: Free fatty acids; TBW: Total body water.

autoimmune, 3; primary sclerosing cholangitis, 1; HCV-related, 1; cryptogenic, 1. Abstinence from alcohol was a goal during the study, and to our knowledge all but one adhered to that policy. All patients signed a written informed consent to take part in the study in accordance with the Helsinki II Declaration. The study was approved by the Research Ethics Committee of Aarhus County.

Experimental design

This was an observational prospective study. TIPS patients were studied approximately 2 wk before TIPS insertion, and were regularly re-evaluated at follow-up visits 1, 4, 12, and 52 wk after the procedure. All examinations were carried out after overnight fasting.

TIPS procedure

Indications for TIPS insertion were refractory ascites (12 patients), secondary prevention of variceal bleeding (seven patients) or both (two patients). None had active variceal bleeding at the time of TIPS insertion. The TIPS procedure was carried out using covered stents according to the method described by Rössle *et al.*^[13]. After insertion, a clinical and ultrasonographic control of the shunt was performed after 24 h, 4 wk, and then, at 12-wk intervals during the first year. Ascites was totally removed by paracentesis before TIPS insertion.

Diet

Each patient had a dietetic investigation and a 7-d diary report before TIPS insertion. During the study, food intake remained unchanged both for quantity and type of nutrients.

Body composition

Bio-impedance analysis (Quadscan 4000, Bodystat Ltd., Isle of Man, UK) was used to estimate body composition. The predictive equations were taken from Kushner *et al.*^[14] and Lautz *et al.*^[15]. Bio-impedance analysis was chosen because, despite some limitations in patients with ascites (not present in our patients both before and after TIPS) it is a bedside tool for the determination of body composition in cirrhotic patients with/without ascites^[16]. Dry lean body mass was calculated as body weight - fat mass - total body water (TBW). Dry lean mass was preferred to lean mass to reduce the possible interference of changes in TBW due to the fluctuating presence of ascites. According to Tsiaousi *et al.*^[17], we considered as malnourished all patients with a body mass index (BMI) lower than 23.

Liver function

The galactose elimination capacity (GEC) was used to measure quantitatively metabolic liver function, from blood concentration decay curves corrected for urinary excretion, as described by Tygstrup^[18]. The clinical status was assessed according to the model for end-stage liver disease score (MELD)^[19].

Biochemical analyses

Glucose, creatinine, bilirubin and prothrombin time were routinely assayed by automatic analyzer on fresh serum/plasma samples. Free fatty acids (FFA) were determined with a colorimetric method using a commercial kit (Wako Chemicals, Neuss, Germany). Blood aliquots for insulin concentrations were stored at -80°C and measured by a two-site immunospecific insulin enzyme-linked immunosorbent assay^[20].

Insulin secretion and sensitivity

Basal insulin secretion and sensitivity were assessed by means of the homeostasis model assessment (HOMA)^[21,22]. Secretion was estimated using the Beta index (HOMA-β), while peripheral sensitivity was measured by HOMA-IR, with the following equations: $\text{HOMA-}\beta = 20 \times \text{fasting plasma insulin (mU/L)} / [\text{fasting plasma glucose (mmol/L)} - 3.5]$; $\text{HOMA-IR} = \text{fasting plasma insulin (mU/L)} \times \text{fasting plasma glucose (mmol/L)} / 22.5$.

Statistical analysis

Data analysis was performed using STATA 10 statistical software (StataCorp LP, Texas, USA). Results are given as mean \pm SD. Changes from baseline were explored by analysis of variance (ANOVA) for repeated measurements. Changes from baseline to end-of-observation and between groups were also tested by parametric and non-parametric paired and unpaired methods, whenever appropriate. Due to non-systematic factors, blood samples were missing from 8 examinations in 6 patients. For the statistical analyses these missing values were replaced by the mean of adjacent values. A *P*-value < 0.05 was considered significant in a two-tailed test.

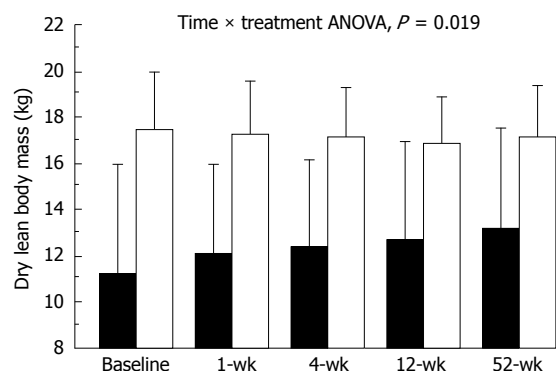


Figure 1 Time course of dry lean body mass in normal weight (black bars) and overweight subjects (white bars) following the TIPS procedure. The data are presented as means and standard error. Trend shows significant difference ($P = 0.019$).

RESULTS

TIPS was well tolerated by all patients, and there were no procedure-related complications. TIPS insertion produced a reduction in hepatic venous pressure gradient ($P < 0.001$) (Table 1). Three patients needed a stent revision during follow-up. Transient hepatic encephalopathy was observed in three patients and was reversed by diet and oral disaccharides without the need for shunt reduction.

At the end of the study period, a non-significant increase in BMI was observed in the whole population (Table 1). Patients were divided in two groups according to their BMI at enrolment [$\text{BMI} \leq 25 \text{ kg/m}^2$, normal/underweight (NW), $n = 12$; $\text{BMI} > 25$, overweight/obesity (OW), $n = 9$]. A few NW cases ($n = 7$; 58%) showed clinical evidence of malnutrition, but only 3 had a BMI below the lower cut-off of normality (18.5 kg/m^2). Analysis of variance (ANOVA) for repeated measurements did not show differences in the time trend of BMI after TIPS between the two groups. Also the non-parametric analysis of 52-wk changes failed to detect differences in BMI changes over time.

Body composition

Dry lean mass increased in NW patients by 14.5% (from $10.9 \pm 5.9 \text{ kg}$ to $12.7 \pm 5.6 \text{ kg}$, $P = 0.031$), but did not change in OW patients; the trend was significant on ANOVA test for repeated measures ($P = 0.002$) (Figure 1). The increase in dry lean body mass in normal weight patients paralleled a significant increase in TBW of 14.3%, (from $34.5 \pm 7.6 \text{ L}$ to $40.2 \pm 10.8 \text{ L}$, $P = 0.007$). Fat mass, both as absolute value or as percentage of body weight, did not vary significantly in the 2 groups (ANOVA, $P = 0.815$).

Regarding liver function, GEC did not vary over time in TIPS-treated patients; similarly MELD increased non-significantly by over 2 points during the observation period (Table 1). These changes were largely due to increased MELD values in NW patients, while no changes were observed in the OW group. Similarly, GEC remained stable in NW patients while in the OW group there was a trend toward a significant deterioration (Table 2).

Table 2 Anthropometric, pressure and laboratory values recorded in 12 normal weight (NW: BMI ≤ 25 kg/m²) and in 9 overweight (OW: BMI > 25) patients with liver cirrhosis (mean \pm SD)

Variable		Basal Pre-TIPS	After 52 wk	P
HVPG (mmHg)	NW	16.4 \pm 5.0	6.3 \pm 2.3	< 0.001
	OW	15.0 \pm 4.7	5.7 \pm 2.1	< 0.001
Albumin (g/L)	NW	30.8 \pm 8.4	32.6 \pm 8.1	0.871
	OW	31.9 \pm 7.1	33.4 \pm 7.6	0.581
Bilirubin (mg/dL)	NW	1.20 \pm 0.47	3.21 \pm 2.82	0.061
	OW	1.71 \pm 0.81	2.44 \pm 1.61	0.164
GEC (mmol/min)	NW	1.62 \pm 0.36	1.57 \pm 0.47	0.623
	OW	1.96 \pm 0.55	1.65 \pm 0.36	0.088
MELD (score) (n = 9)	NW	11 (5)	13 (4)	0.727
	OW	11 (6)	12 (8)	0.289
Blood glucose (mmol/L)	NW	5.7 \pm 1.5	5.7 \pm 0.8	0.941
	OW	5.9 \pm 1.5	6.8 \pm 1.9	0.271
Insulin (pmol/L)	NW	88.9 \pm 49.2	164.7 \pm 106.9	0.009
	OW	171.4 \pm 94.4	203.0 \pm 94.9	0.466
HOMA- β	NW	152 \pm 88	253 \pm 118	0.042
	OW	413 \pm 588	209 \pm 95	0.316
HOMA-IR	NW	3.36 \pm 2.18	6.18 \pm 4.82	0.023
	OW	6.91 \pm 4.94	9.64 \pm 6.70	0.262
BMI (kg/m ²)	NW	21.4 \pm 2.6	24.1 \pm 4.0	0.220
	OW	30.9 \pm 3.7	30.8 \pm 5.1	0.944
FFA (mmol/L)	NW	0.49 \pm 0.22	0.86 \pm 0.53	0.124
	OW	0.59 \pm 0.24	0.93 \pm 0.34	0.023
Creatinine (mg/dL)	NW	0.84 \pm 0.43	0.76 \pm 0.39	0.149
	OW	1.17 \pm 0.59	0.92 \pm 0.26	0.157
Fat mass (%)	NW	27.6 \pm 7.2	25.3 \pm 8.3	0.334
	OW	31.5 \pm 7.9	30.5 \pm 8.7	0.638
Fat mass (kg)	NW	16.9 \pm 3.7	17.5 \pm 6.7	0.782
	OW	30.5 \pm 11.0	28.8 \pm 9.9	0.649
Dry lean mass (kg)	NW	10.9 \pm 5.9	12.7 \pm 5.6	0.031
	OW	17.4 \pm 3.3	17.1 \pm 2.9	0.557
TBW (L)	NW	34.5 \pm 7.6	40.2 \pm 10.8	0.007
	OW	44.6 \pm 8.9	46.6 \pm 9.2	0.459

P < 0.05 is considered statistically significant.

Metabolic parameters

Plasma insulin and HOMA-IR significantly increased in TIPS-treated cirrhotic patients without any change in fasting glucose (Table 1). The changes were mainly limited to normal weight patients who also had a significant increase in HOMA- β (Table 2). However, no significant differences were demonstrated by repeated-measures ANOVA.

After TIPS insertion, changes in FFA plasma levels (Table 1) were exclusively observed in OW patients (Table 2).

No systematic changes in serum creatinine were observed (Tables 1 and 2), but patients with mild renal failure (creatinine levels ≥ 1.2 mg/dL, n = 7) slightly improved their values (from 1.75 ± 0.37 mg/dL to 1.45 ± 0.43 mg/dL, P = 0.23).

DISCUSSION

The main finding of this study is that dry lean body mass significantly increases in NW patients with cirrhosis after TIPS insertion. Advanced cirrhosis is associated with reduced lean mass^[23]; a significant protein-calorie malnutrition is present in at least 30% of patients with cirrhosis^[24]. Both parameters appear to be related to the severity of the liver disease^[25]. Considering that a large

fraction of our NW patients showed signs of malnutrition at baseline, TIPS appears to modify the natural history of malnutrition. This finding agrees with the results obtained by Plauth *et al*^[12] and Allard *et al*^[11]. Both reported an increase in dry lean mass but many questions on the metabolic mechanisms involved remain unresolved^[26]. The significant increase in dry lean mass of about 1.84 kg in the year after TIPS insertion is paralleled by an increase in TBW that, in the absence of ascites and/or regional edemas, is indicative of muscle tissue formation^[27]. Our results are in keeping with a very recent preliminary report by Camci *et al*^[28], on six TIPS-treated malnourished cirrhotic patients.

This beneficial effect of TIPS on lean body mass was not observed in overweight or obese subjects at baseline. The reasons for this different metabolic response are not easy to determine. NW cases were characterized by lower insulin resistance at baseline, but also lower insulin and lower insulin secretion. In these normal- or under-weight individuals, the additional metabolic and hormonal abnormalities already in the group with excess body weight do not increase the cirrhosis-related insulin resistance^[29]. Muscle tissue takes up 80%-85% of glucose infused during hyperinsulinemic euglycemic clamp^[30,31]. After TIPS placement, these cases were characterized by a significant increase in serum insulin, without changes in blood glucose levels, and consequently by increased HOMA-IR and HOMA- β , and stable liver function. Under these conditions, the improved hemodynamic state, in the presence of a normal intake of nutrients, could promote insulin production and insulin action, favoring the improved nutritional state. This mechanism might not be operative in the OW group, with a much higher degree of insulin-resistance than NW cases, and where insulin did not increase further after TIPS placement. In these patients, the TIPS procedure was followed by a remarkable reduction of HOMA- β , and the decreased insulin production could not sustain lean body mass formation.

After TIPS the plasma levels of FFA increased significantly only in the OW group. In agreement with the hypothesis of Yki-Järvinen *et al*^[32,34], the increase in FFA levels is likely to stimulate hepatic gluconeogenesis and competition with glucose in muscle metabolism. These metabolic changes could further promote insulin resistance.

Reduced portal hypertension might be an additional factor playing a role in the improved metabolic and nutritional status. Portal hypertension increases the permeability of enteric mucosa that promotes intestinal bacterial translocation and the systemic diffusion of LPS and other pro-inflammatory molecules, ultimately producing interleukin-1 β , interleukin-6 and TNF- α . These molecules promote insulin-resistance and have an anti-insulin, catabolic effect, leading to protein mass wasting. Any mechanism mediated by anti-insulin molecules might play a differing effect in relation to BMI and to the levels of insulin resistance, much higher in obesity. Obesity *per se* is a chronic inflammatory state that sustains insulin-resistance^[35], in the presence of obesity the multiple factors sustaining insulin resistance could not be totally

removed by TIPS; in contrast, in subjects with poor nutritional status the less severe insulin resistance might be removed by TIPS, thus explaining improved nutritional status. Larger studies are needed to explore the potential benefits of TIPS on long-term survival of underweight patients with cirrhosis, at higher risk of morbidity and mortality according to several previous studies^[36].

Finally, the presence of insulin-resistance might be related to advanced liver disease and this condition could justify the absence of any improvement in OW patients with liver cirrhosis. We used GEC, an estimate of the functional liver mass with a prognostic value in the medium term interval^[37], and there was no overall change in GEC over time, however, in OW patients a trend towards a decrease in GEC was observed. During the study, food intake remained unchanged both for quantity and type of nutrients. Further, it is our policy that alcohol has to be withdrawn in patients before TIPS insertion, as alcohol itself increases portal hypertension and may thus be involved in both the risk of variceal bleeding and ascites formation. During follow up, alcohol abstinence is important and this policy is reflected in the high number of alcohol abstainers in the present study. We therefore suggest that changes in body composition do not reflect a change in food and/or alcohol intake but are an effect of TIPS and of the reduction in portal pressure *per se*.

Finally, the study confirmed the effectiveness of TIPS on portal hypertension with a reduction in Hepatic venous pressure gradient (HVPG) to a value of 6 mmHg, lower than the 12 mmHg threshold value of increased risk for variceal bleeding, re-bleeding and mortality^[1,4]. The absence of re-bleeding episodes and the disappearance of ascites confirm this hypothesis. Furthermore, patients with functional renal failure at baseline improved their creatinine levels at follow-up. Considering that lean mass increased, this value does not reflect a worsening of malnutrition^[38] but is due to a positive effect of TIPS on the hemodynamic state^[39].

In conclusion, the TIPS procedure is effective in lowering portal pressure in patients with portal hypertension and improves body composition without significant changes in metabolic parameters.

COMMENTS

Background

Malnutrition in portal hypertensive cirrhotic patients increases the risk and the severity of clinical complications. Transjugular intrahepatic porto-systemic shunt (TIPS) is a well established therapy for complications of portal hypertension in cirrhotic patients. However, the effect of TIPS on malnutrition is unclear.

Research frontiers

The article provides insight into the beneficial effects of TIPS in regard to improvement in nutritional status. Malnutrition in liver diseases is an important area of research as it is an independent factor resulting in increased morbidity and mortality. Furthermore, the article addresses the effects of shunting of hormones/peptides *etc.* especially insulin, that relate to the possible mechanisms behind insulin resistance in patients with liver cirrhosis.

Innovations and breakthroughs

Related papers have focussed on improvement in body composition without addressing the pre-TIPS nutritional status. With a simple yet comprehensive separation into normal weight and overweight subjects, the results suggest

additional nutritional benefits in the group of malnourished patients from TIPS insertion (see next section).

Applications

TIPS treatment of the complications of portal hypertension seems to improve nutritional status in liver cirrhosis, especially in patients suffering from malnutrition. Ultimately, malnutrition may provide an additional reason/indication for TIPS insertion in patients with liver cirrhosis.

Terminology

The TIPS procedure is a minimally invasive procedure used in patients with liver cirrhosis to reduce portal hypertension and thereby ameliorating complications of portal hypertension. Using a catheter technique *via* the right jugular vein, a stent is placed within the liver connecting the portal vein and the hepatic vein and thus reducing portal hypertension.

Peer review

This is an interesting and novel study showing an amelioration of nutritional parameters after TIPS especially in lean cirrhotic patients. An improvement in the body composition (nutritional status) of liver cirrhosis patients after TIPS implementation has been studied previously. And this study is well conducted and long term follow up data are provided.

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Immunogenetic characteristics of patients with autoimmune gastritis

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interleukin (IL)-1 gene cluster, IL-2, IL-4, IL-6, IL-10, IL-12, interferon γ , transforming growth factor β , and tumor necrosis factor α . Variation in *KIR* genes was also explored. The results were compared with prevalence of the polymorphisms in Finnish or European populations.

RESULTS: All patients had pepsinogen I levels below normal (mean: 11 $\mu\text{g/L}$, range: < 5 to 25 $\mu\text{g/L}$). Three patients had elevated *H. pylori* IgG antibodies, while *H. pylori* serology was negative in the rest of the patients. AIG patients carried significantly more often HLA-DRB1*04 (58%) and DQB1*03 (83%) than the general Finnish population did (28% and 51%, respectively; $P = 0.045$ and 0.034 by the Fisher's exact test). No patient was positive for HLA-B8-DRB1*03, a well-established autoimmune marker. Neither cytokine polymorphisms nor *KIR* gene variation showed association with AIG.

CONCLUSION: As explored with modern DNA-based methods, HLA-DRB1*04 and DQB1*03 alleles, but not HLA-B8-DRB1*03, may predispose to AIG.

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Key words: Atrophic gastritis; Autoimmune diseases; Cytokines; Genetic polymorphisms; Human leukocyte antigens; Killer cell immunoglobulin-like receptor

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Abstract

AIM: To explore whether predisposition to autoimmune gastritis (AIG) is found in human leukocyte antigen (HLA), cytokine or killer cell immunoglobulin-like receptor (*KIR*) gene variations.

METHODS: Twelve Finnish patients with autoimmune-type severe atrophy of the gastric corpus were included. The patients' serum was analyzed for pepsinogen I and *Helicobacter pylori* (*H. pylori*) antibodies. DNA was separated and the patients were genotyped for HLA-A, B, Cw, DRB1 and DQB1 antigens, and studied for single nucleotide polymorphisms for the following cytokines:

INTRODUCTION

Autoimmune gastritis (AIG) is an organ-specific autoimmune disease, in which inflammation of the mucosa of the gastric corpus results in total loss of corpus-type glands, and achlorhydria. AIG patients typically have a low serum pepsinogen I (PG I) concentration, and most of them also have parietal cell antibodies (PCAs). In many, but not all patients, vitamin B12 absorption is deficient, which leads to pernicious anemia (PA)^[1].

The occurrence of AIG and PA has long been recognized to be determined strongly by genetic factors, which, however, are largely unexplored. The most important genetic association found in human AIG so far is a link with the human leukocyte antigen (HLA) region. The observed association between AIG and certain HLA antigens has, however, not been strong enough to explain the familial clustering of AIG^[2].

Polymorphisms in the genes that encode immune regulator molecules may affect the secretion or function of the corresponding proteins, and thus influence immune responses, inflammation and tissue injury. Cytokine genes have been studied widely in autoimmune diseases and associations have been found between, for instance, tumor necrosis factor α (TNF α) and interleukin (IL)-10 polymorphisms and autoimmune hepatitis and pemphigus, respectively^[3,4]. Also *Helicobacter pylori* (*H. pylori*)-associated atrophic gastritis has been shown to be more frequent in patients with proinflammatory polymorphisms of genes for *IL-1* gene cluster, and TNF α ^[5].

Killer cell immunoglobulin-like receptors (KIRs) are members of a diverse family of regulatory molecules expressed on subsets of T cells. KIRs play a role in the control of the natural killer (NK) cell immune response. The KIR receptors recognize certain HLA class I determinants and regulate NK cell activity. The number and type of *KIR* genes vary between individuals who can carry anything from seven to 12 *KIR* genes, of which, some encode activating and others inhibiting receptors^[6,7]. *KIR* genes can be divided into two main haplotype groups. Group A contains only one activating and six inhibiting *KIR* genes, whereas group B haplotypes are more variable and contain several activating *KIR* genes^[8]. In addition to the copy-number variation, individual *KIR* genes exhibit allelic variation. *KIR* genes have been shown to be associated with various diseases, including some autoimmune diseases^[9].

Recently, we sequenced the coding regions of genes for α - and β -subunits of H⁺/K⁺-ATPase, which is the main autoantigen in AIG, in AIG patients, but no disease-associated polymorphisms could be found^[10]. In the present study, a number of genes involved in immune activation were explored in patients with AIG, by modern molecular genetic methods. The aim of this study was to determine whether variations in the immune regulator genes, such as HLA, cytokine or KIR, are associated with the presence of AIG.

MATERIALS AND METHODS

Clinical information

A total of 18 patients, who had earlier undergone gastroscopy at Herttoniemi Hospital and were known to have severe atrophic corpus gastritis without any history of *H. pylori* infection, and who were under 65 years of age, were invited by letter to participate in the study. Twelve patients gave written informed consent, donated a blood sample, and completed a questionnaire about their possible vitamin B12 replacement therapy and thyroid diseases, as well as the occurrence of AIG in the family. Signs of other autoimmune diseases were looked for in the patient records. The study was approved by the Ethical Committee for Internal Medicine at Helsinki University Central Hospital.

Blood tests

EDTA blood and serum samples were kept at -20°C until analyzed. DNA was extracted from the EDTA blood sample using a DNA purification kit (PureGene®; Centralsystems, Minneapolis, MN, USA), according to the manufacturer's instructions. Serum samples were analysed for PG I, PCAs and *H. pylori* antibodies.

For serum PG I concentrations, an immunoenzymometric assay (Gastroset PG1; Orion Diagnostica, Espoo, Finland) was used. The lower normal limit of the assay was 28 µg/L. PCAs were determined by an enzyme immunoassay (Varelixa Parietal Cell Antibodies; Pharmacia Diagnostics, Freiburg, Germany), which used H⁺/K⁺-ATPase as the antigen. Concentrations < 10 U/mL were normal, according to the manufacturer. For *H. pylori* antibodies, an in-house immunoassay that measured IgG antibodies was used, and titers ≥ 700 were considered elevated^[11].

Immunogenetics

HLA genes were explored using the INNO-LiPA kit (Innogenetics, Ghent, Belgium) according to the manufacturer's instructions. The *HLA-A*, *B*, *Cw*, *DRB1* and *DQB1* genes were amplified by polymerase chain reaction (PCR), and the biotinylated PCR products were hybridized with sequence-specific oligonucleotides on membrane-based strips. Results were analyzed by the LiRAS (Innogenetics) interpretation software.

Cytokine polymorphisms in the genes of *IL-1* gene cluster, *IL-2*, *IL-4*, *IL-6*, *IL-10*, *IL-12*, interferon (IFN)- γ , transforming growth factor (TGF) β , and TNF α were genotyped using the Cytokine Genotyping Kit (Pel-Freez Clinical Systems, Brown Deer, WI, USA). Cytokine profiles (high/intermediate/low producer) based on the polymorphisms were formed according to the published phenotypes also mentioned in the product insert of the kit. *KIR* genes (*KIR2DL1*, *KIR2DL2*, *KIR2DL3*, *KIR2DL4*, *KIR2DL5*, *KIR2DS1*, *KIR2DS2*, *KIR2DS3*, *KIR2DS4*, *KIR2DS5*, *KIR3DL1*, *KIR3DL2*, *KIR3DL3*, *KIR3DS1*, *KIR2DP1* and *KIR3DP1*) were determined using the KIR Genotyping Kit (Pel-Freez Clinical Systems), following the manufacturer's instructions. Both genotyping kits were

based on PCR amplification with sequence-specific primers that were designed to detect polymorphisms of cytokine/*KIR* genes. The PCR products were separated by gel electrophoresis, and the genotype results were interpreted on the basis of specific amplification patterns.

Prevalence of the HLA genotypes was compared with HLA frequency in the Finnish population, based on the data collected in Clinical Laboratory of Finnish Red Cross blood service. The cytokine polymorphisms and *KIR* genes were compared with the frequency of polymorphisms and *KIR* genes in populations of Finnish of European ancestry published previously^[12-15].

Statistical analysis

Fisher's exact test was used to compare the prevalence of genotypes between patients and the populations used as controls.

RESULTS

Demographic and clinical characteristics of the patients are summarized in Table 1. All patients had total atrophy in the gastric corpus. The mucosa of the gastric antrum was normal in eight patients, and mild chronic inflammation or sparse intestinal metaplasia was detected in four. All patients had PG I levels below normal (mean: 11 µg/L, range: < 5 to 22 µg/L), and elevated PCAs (median: 185 U/mL, range: 20-509 U/mL). Three patients (numbers 3, 10 and 11 in Tables 1 and 2) had elevated *H. pylori* IgG antibodies (titers: 730-2200), whereas *H. pylori* serology was entirely negative in the rest of the patients (titers: 50-100). All patients but one (number 2 in Tables 1 and 2) had vitamin B12 replacement therapy.

Immunogenetics of the patients

The HLA-A, B, Cw, DRB1 and DQB1 alleles in the AIG patients are shown in Table 2. DRB1*04 was present in seven out of 12 (58%) patients, whereas 28% of the Finnish general population carry the allele ($P = 0.045$ by Fisher's exact test). Ten patients (83%) had DQB1*03; its allele frequency in the Finnish population is 51% ($P = 0.034$ by Fisher's exact test).

Only one of the 12 patients carried the DRB1*0301-DQB1*0201 haplotype, which is an established susceptibility factor for various autoimmune diseases^[16]. It is of particular note that the only DRB1*0301-positive patient did not have the classical A*01-B*08 haplotype.

The frequencies of polymorphisms in the genes of the *IL-1* gene cluster, *IL-2*, *IL-4*, *IL-6*, *IL-10*, *IL-12*, *IFNγ*, *TGFβ* and *TNFα* did not differ significantly from those found in Finnish (where data were available) or other European populations. The results for genotyping the *IL-1* gene cluster, *TNFα* and *IL-10* are shown in Table 3.

All 14 characterized *KIR* genes, *KIR2DL1*, *KIR2DL2*, *KIR2DL3*, *KIR2DL4*, *KIR2DL5*, *KIR2DS1*, *KIR2DS2*, *KIR2DS3*, *KIR2DS4*, *KIR2DS5*, *KIR3DL1*, *KIR3DL2*, *KIR3DL3* and *KIR3DS1*, were determined, as well as two *KIR* pseudogenes *KIR2DP1* and *KIR3DP1*. Ten patients

Table 1 Clinical characteristics of the 12 AIG patients

Patient	Sex	Age (yr)	Years from diagnosis	Other autoimmune diseases	AIG in family
1	F	50	7		-
2	F	56	3	RA	+
3	F	49	2		+
4	M	62	19		+
5	F	63	2	HT	+
6	F	54	3		-
7	F	38	4		+
8	F	56	5	PBC	-
9	F	52	0		-
10	F	52	0		-
11	F	47	15	T1D	-
12	F	52	0		-

AIG: Autoimmune gastritis; RA: Rheumatoid arthritis; HT: Hyperthyreosis; PBC: Primary biliary cirrhosis; T1D: Type 1 diabetes.

Table 2 HLA-A, B, Cw, DRB1 and DQB1 genotypes of the 12 AIG patients

Patient	HLA-A		HLA-B		HLA-Cw		HLA-DRB1		HLA-DQB1	
1	03	11	07	44	01	07	12	15	0301	0602
2	02	-	15	51	04	14	04	08	0302	0402
3	02	24	15	39	04	07	04	15	0302	0602
4	02	03	07	18	07	-	04	15	0302	0602
5	02	26	15	40	03	04	01	08	0402	0501
6	02	03	15	-	03	-	04	08	0302	0402
7	02	-	13	51	06	15	07	09	0202	0303
8	02	32	40	51	02	15	09	15	0303	0602
9	03	-	07	51	03	15	04	13	0301	0303
10	02	03	35	40	04	07	04	12	0301	0302
11	02	-	27	35	01	03	03	08	0201	0402
12	03	24	13	35	04	06	04	-	0302	-

carried both A and B *KIR* haplotypes; two patients were homozygotes for A haplotype. *KIR* genotype and haplotype frequencies of the patients did not differ from those reported earlier in the Finnish population^[15].

DISCUSSION

In Finnish AIG patients, the HLA-DRB1*04 and DQB1*03 alleles were more frequent than in the general population, which implies an association between certain HLA-DRB1 and DQB1 haplotypes and AIG. The well-known autoimmune markers HLA-B8, DRB1*03 and DQB1*02 were practically missing in the AIG patients. This suggests that the immunogenetics of AIG are different to that of many classical autoimmune diseases.

The co-localization of susceptibility foci in experimental AIG and type 1 diabetes (T1D) is the strongest known between two autoimmune diseases^[2], and the most prominent susceptibility locus for both diseases is located in the HLA region. Individuals with T1D also have PCAs more often than population controls do^[17]. Over 90% of Caucasians with T1D carry the DR3 or DR4 haplotype, and the DQB1*0302 allele is associated strongly with T1D^[18]. In the present study, the DRB1*04 allele was more frequent in AIG patients

Table 3 IL-1, IL-10, and TNF α polymorphisms in 12 AIG patients, and in Finnish, Italian and Czech populations

	Genotype	AIG patients	Finnish ^[11] %	Italian ^[12] %	Czech ^[13] %
TNF α -308	AA ¹	-	3	2	2
	GA	-	21	14	38
	GG	12	76	84	60
IL-1 β -511	TT ¹	-		9	10
	CT	8		41	45
	CC	4		50	45
IL1RA	CC ¹	1		3	8
mspa111100	TC	8		41	45
	TT	3		56	47
IL-10	ATA/ATA ¹	1		5	
	ATA/ACC	1		21	
	ACC/ACC	1		7	
	GCC/ATA	5		24	
	GCC/ACC	4		31	
	GCC/GCC	-		12	

¹The genotypes are the most proinflammatory ones.

than in the general population, but the DRB1*03 allele was only carried by the patient with T1D. Six of our 12 patients had DQB1*0302, the prevalence of which in the Finnish population is 13% ($P = 0.005$ by Fischer's exact test). The AIG patient with T1D was also the only one to carry the DQB1*02 haplotype, which is present in 91% of Finnish celiac disease patients^[19], and in 17% of the general Finnish population. Thus, Finnish AIG patients seem to share some of the haplotypes that are common in patients with T1D, but not those seen in patients with celiac disease.

In the 1970s, several studies were carried out to find a possible association between AIG or PA and HLA antigens. Increased frequency of HLA antigens A3, B7 or both has been found in AIG and PA patients^[20-22]; however, these findings were not confirmed by others^[23]. Subgroups of AIG patients have shown associations with different HLA antigens. Patients with a concomitant endocrine disease showed an increased frequency of the B8, B18 and BW15 antigens, and those without endocrine disease that of the B7 and B12 antigens^[24].

Of the class II HLA antigens, PA patients showed increased frequency of the DR2 and DR4 antigens and a decreased presence of the DR3 antigen, as compared to controls. PA patients with a concomitant endocrine disease showed DR3/DR4 antigens more often, and those without autoimmune endocrine disease showed DR2/DR4 and DR4/DR5 antigens, as compared to controls^[25]. Possibly because of the small number of patients in the present study, no significant difference could be found between those with and without concomitant autoimmune disease.

The role of *H. pylori* in AIG and PA is still poorly understood^[26]. On one hand, patients with *H. pylori* infection often develop atrophic gastritis and even autoimmune characteristics, such as PCAs^[27]. On the other hand, AIG patients without any signs of *H. pylori* infection, such as the majority of patients in the present study, may be found. In studies before the *Helicobacter* era, the role of *H. pylori* in atrophic gastritis was not recognized, and patients with

H. pylori-associated autoimmunity may have been included; this may have made it more difficult to detect associations between AIG and, for example, HLA antigens. Our patients were relatively young with a median age of 52 years and the majority were women, which is typical for the classic AIG^[1]. The three patients with positive *H. pylori* serology showed no clinical difference from the others.

In *H. pylori*-positive individuals, proinflammatory polymorphisms of the IL-1 β gene cluster have been found to be associated with atrophic gastritis, achlorhydria^[28,29], and even gastric cancer^[30], which often is a late sequel of atrophic gastritis. Patients carrying proinflammatory IL-1 β -511T and TNF α -308A, and who are homozygous for IL-1RN*2*2, had an OR of 5.8 for developing atrophic gastritis^[31]. In addition, patients that carried three or more of the proinflammatory polymorphisms (carriage of IL-1 β -511T+ or TNF α -308A; homozygosity for IL-1RN*2*2 or IL-10 ATA/ATA) had an OR of 26.3 for non-cardia gastric cancer^[32]. However, the association between gastric cancer and IL-1 β polymorphisms has not been seen in all studies^[33]. Despite the fact that all our patients had profound atrophy in the gastric corpus at a relatively young age, the frequencies of these particular genotypes did not differ from those found in populations with European ancestry. Even though the small number of patients and the lack of controls in the present study make it impossible to detect small or modest associations, our results suggest that these polymorphisms are not crucial for the development of AIG.

In conclusion, HLA DRB1*04 and DQB1*03 were more frequent in AIG patients than in the general Finnish population, which suggests an association between certain HLA-DRB1 and DQB1 haplotypes and AIG. Also, the well-known autoimmune markers HLA-B8, DRB1*03 and DQB1*02 were practically missing in the AIG patients. However the number of patients in the present study was small, and larger studies are needed to confirm these findings.

COMMENTS

Background

Autoimmune gastritis (AIG) is chronic inflammation in the mucosa of the gastric body, which may lead to vitamin B12 deficiency of and pernicious anemia. The cause of this inflammation is not known, but its occurrence is known to be strongly determined by genetic factors.

Research frontiers

In earlier studies using antigen determination for the detection of human leukocyte antigen (HLA) tissue determinants, an association was found between HLA tissue antigens and AIG. Before the present study, this association had not been studied by modern DNA-based methods.

Innovations and breakthroughs

The study is believed to be the first to show an association between AIG and certain HLA genotypes, as explored with modern DNA-based methods.

Applications

The study included a small number of patients. These results may in the future contribute to exploring the mechanisms of AIG and possibly other autoimmune diseases. AIG is also a risk factor for gastric cancer; thus, understanding the evolution of AIG may contribute to exploring the development of cancer.

Peer review

This is a good pilot study that indicates the need for a much bigger, longer-term study.

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***NOD2*, *IL23R* and *ATG16L1* polymorphisms in Lithuanian patients with inflammatory bowel disease**

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Abstract

AIM: To investigate the frequency of *NOD2*, *IL23R* and *ATG16L1* genetic variants in a case-control panel for inflammatory bowel disease (IBD) from Lithuania.

METHODS: One hundred and eighty unrelated IBD patients [57 Crohn's disease (CD) and 123 ulcerative colitis (UC)] and 186 healthy controls were genotyped for the following known genetic susceptibility variants: *NOD2* - Arg702Trp (rs2066844), Gly908Arg (rs2066845) and Leu1007insC (rs2066847), as well as *IL23R* - Arg381Gln (rs11209026) and *ATG16L1* - Thr300Ala (rs2241880).

RESULTS: The effect that carriership of at least one *NOD2* risk allele predisposes to CD was replicated in the

Lithuanian population (41.1% CD vs 16.9% controls, $P = 2 \times 10^{-4}$, OR = 3.48, 95% CI: 1.81-6.72). In the allelic single marker analysis, Leu1007insC was strongly associated with CD (21.4% CD vs 4.7% controls, $P = 3.687 \times 10^{-8}$, OR = 5.54, 95% CI: 2.85-10.75). Neither the other two *NOD2* variants, nor the known variants in *IL23R* and *ATG16L1* were found to be risk factors for CD, UC or IBD. However, our relatively small study population was underpowered to demonstrate such weak to moderate disease associations.

CONCLUSION: The results support a strong association between CD susceptibility and the Leu1007insC variant in *NOD2* in the Lithuanian study population.

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Key words: *NOD2*; *IL23R*; *ATG16L1*; Single nucleotide polymorphisms; Crohn's disease; Ulcerative colitis; Lithuania

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INTRODUCTION

The inflammatory bowel diseases (IBD) refer to two

clinically defined conditions, ulcerative colitis (UC) and Crohn's disease (CD) that represent major burdens of morbidity in Western countries, with prevalence rates in North America and Europe ranging from 21 to 246 per 100 000 inhabitants for UC and 8 to 214 per 100 000 inhabitants for CD^[1]. Although the exact aetiology of IBD remains unclear, accumulating data suggests that IBD occurs from the combined effects of genetic predisposition and environmental factors^[2].

Linkage, candidate gene, targeted association mapping and genome-wide association studies have identified many common variants associated with IBD and have rapidly expanded our fundamental knowledge of complex disease biology. The first and most consistently replicated genetic susceptibility variants, were found in the *NOD2* gene^[3-5], attributed to the recognition of bacterial products, along with several other genetic loci coding for cytokines involved in acquired immune responses (*IL23R*^[6]) and genes related to the autophagy pathway (*ATG16L1*^[7]).

Given the heterogeneity in allele frequencies reported for the genetic factors involved in the pathogenesis of IBD in different European populations^[8], we aimed to perform the first genetic study of IBD in a low-incidence population^[9,10] of North-Eastern Europe - Lithuania. We examined the frequencies of the previously described variants in the *NOD2*, *IL23R* and *ATG16L1* genes in a Lithuanian IBD study population.

MATERIALS AND METHODS

Patients

The study included 57 unrelated patients with CD, 123 with UC and 186 healthy, age- and gender-matched controls. All study participants were of Caucasian ethnicity. The recruitment of the study individuals was performed at the Department of Gastroenterology, Kaunas University of Medicine Hospital during the period from 2003 to 2006. Written informed consent from all participants and approval of the Kaunas Regional Biomedical Research Ethics Committee (Protocol No. 84/2003) was obtained. The diagnosis of either CD or UC was based on standard clinical, endoscopic, radiological and histological criteria^[11]. Patients' demographic and phenotypic details are summarized in Table 1. The clinical characteristics provided in the table are given according to the Montreal classification^[12].

Genotyping

Genomic DNA was isolated from EDTA peripheral blood using the Invisorb Blood Giga Kit from Invitex (Berlin, Germany). The three *NOD2* variants - Arg702Trp (rs2066844), Gly908Arg (rs2066845) and Leu1007insC (rs2066847), and the *IL23R* variant Arg381Gln (rs11209026) were genotyped using Applied Biosystem's (Foster City, CA, USA) allele-specific TaqMan™ or TaqMan-MGB assays (Table 2); *ATG16L1* variant Thr300Ala (rs2241880) detection was performed using a pre-designed TaqMan® single nucleotide polymorphism

Table 1 Summary of clinical and demographic characteristics of the IBD patients *n* (%)

Characteristics	CD	UC
Gender (male/female)	27/30	68/55
Age (years ± SD)	40.5 ± 14.9	45.4 ± 16.4
Age at diagnosis (years ± SD)	31.7 ± 16.6	34.3 ± 14.7
Familial IBD	0	0
Surgery treatment	15 (26.3)	3 (2.4)
Disease extension in UC		
Proctitis	-	26 (21.1)
Left-sided colitis	-	61 (49.5)
Extended colitis	-	36 (29.3)
Disease localization in CD		
Terminal ileum, L1	17 (29.8)	-
Colon, L2	16 (28.1)	-
Ileocolon, L3	23 (40.3)	-
Upper GI, L4	1 (1.8)	-
Disease Behavior in CD		
Non-stricturing, non-penetrating, B1	41 (71.9)	-
Stricturing, B2	5 (8.8)	-
Penetrating, B3	11 (19.3)	-
Perianal disease, B4	-	-
Extraintestinal manifestations		
Joints	6 (10.5)	13 (10.6)
Cutaneous	3 (5.3)	4 (3.3)
Ocular	1 (1.8)	0
Hepatobiliary	0	2 (1.6)

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

(SNP) genotyping assay (ID C_9095577_20). Genotyping was performed on an automated platform using the TaqMan® (Applied Biosystems, Foster City, CA, USA) technique as previously described^[13]. All genotyped markers had a call rate greater than 95% in case and healthy control samples.

Statistical analysis

Each SNP was checked for conformance with Hardy-Weinberg equilibrium in the control group using Fisher's exact test ($P_{HWE} > 0.01$). Single-marker association analyses between cases and controls were performed using χ^2 statistics or Fisher's exact genotypic test. The significance level of the tests for considering *P*-values as significant was set to < 0.05 . Data were evaluated using the web interface SISA^[14]. Carriership of mutated alleles in case and control populations was estimated by direct counting.

The population attributable risk percentage (PAR%) was calculated as the attributable risk percentage (AR%) multiplied by the proportion of exposed cases, where AR% was estimated from the odds ratio (OR), assuming that the exposure of the control population to the disease-associated variant reflects the true prevalence of the variant in the general population^[15].

RESULTS

All five SNPs were successfully genotyped in our North-Eastern European IBD case-control panel comprising 57 CD and 123 UC patients from Lithuania. The distribution of genotypes within the control group was

Table 2 TaqMan® primer and probe sequences of *NOD2* and *IL23R* assays

Marker	Primers	Probes
<i>NOD2</i>		
rs2066844	5'-TTCCTGGCAGGGCTGTGTC 5'-AGTGGAAAGTGCTTGGGAGG	TET-CCTGCTC T GGCGCCAGGCC FAM-CCTGCTC C GGCGCCAGGC
rs2066845	5'-ACTCACTGACACTGTCTGTGACTCT 5'-AGCCACCTCAAGCTCTGGTG	TET-TTCAGATTCTGG C GCAACAGAGTGGGT FAM-TTTTCAGATTCTGG G GCAACAGAGTGGGT
rs2066847	5'-CCAGGTTGTCCAATAACTGCATC 5'-CCTTACCAGACTTCCAGGATGGT	VIC-TGCAGG C CCCTTG FAM-CTGCAG G CCCTTG
<i>IL23R</i>		
rs11209026	5'-CGTCTTTGCTGTATGTTGTCAATTCTT 5'-AGAAAACAGAAATCTGCAAAAACCTACC	VIC-CAGATCATTC A AACTG FAM-ACAGATCATTC G AACTG

The examined alleles are highlighted by bold underlined typing.

Table 3 Association statistics for the *NOD2*, *ATG16L1* and *IL23R* variants in the Lithuanian IBD population

Gene marker	Minor allele	Controls (n = 186)			CD (n = 56)				UC (n = 123)			
		GT (11/12/22)	MAF	<i>P</i> _{HWE}	GT (11/12/22)	MAF	<i>P</i> _{CCA}	OR (95% CI)	GT (11/12/22)	MAF	<i>P</i> _{CCA}	OR (95% CI)
<i>NOD2</i>												
rs2066844	T	0/9/171	0.025	> 0.99	0/2/54	0.018	> 0.99	0.71 (0.15-3.33)	0/10/113	0.041	0.278	1.65 (0.66-4.13)
rs2066845	C	1/7/169	0.025	0.099	0/3/53	0.027	> 0.99	1.06 (0.28-3.97)	0/1/121	0.004	0.055	0.16 (0.02-1.25)
rs2066847	insC	2/13/166	0.047	0.048	4/16/36	0.214	3.687 × 10 ^{-8a}	5.54 (2.85-10.75)	1/8/114	0.041	0.711	0.86 (0.39-1.91)
<i>ATG16L1</i>												
rs2241880	G	44/89/53	0.476	0.560	16/28/11	0.546	0.199	1.32 (0.86-2.03)	33/61/25	0.534	0.164	1.26 (0.91-1.75)
<i>IL23R</i>												
rs11209026	A	3/16/167	0.059	0.017	0/4/52	0.036	0.335	0.59 (0.20-1.75)	0/11/109	0.045	0.477	0.76 (0.36-1.61)

Minor allele frequencies (MAF), genotype counts (GT; 11 = homozygous for minor allele; 12 = heterozygous for common allele; 22 = homozygote for common allele), allelic test *P* values (^a*P*_{CCA} < 0.05), and odds ratios (OR, shown for the minor allele) with 95% confidence intervals (CI) are depicted for both the CD and UC case-control population.

consistent with Hardy-Weinberg equilibrium (Table 3). For each of the variants studied, the risk of carrying the variant was compared between the CD, UC and healthy controls groups. The genotype and minor allele frequencies are presented in Table 3.

As expected, none of the studied individuals were carriers of all three *NOD2* risk alleles. However, two CD patients were determined as compound heterozygotes. The combined allele carriership in the group of patients with CD was much higher than in controls (41.1% *vs* 16.9%) and resulted in significant association ($P = 2 \times 10^{-4}$, OR = 3.48, 95% CI: 1.81-6.72) whereas no significant difference was observed between UC patients and controls. The PAR% in CD patients was 29.5% for possession of one or more *NOD2* variant alleles at any of the three sites.

In the allelic single marker analysis of the *NOD2* variants, a significant association was detected only between CD and Leu1007insC. For this variant, both the allelic and genotypic tests revealed *P*-values < 10⁻⁴ (OR_{allele} = 5.54, 95% CI: 2.85-10.75; OR_{carriership} = 6.12, 95% CI: 2.88-13.15), resulting from the increased minor allele frequency (MAF) in cases (21.4%) *vs* controls (4.7%). In the UC group, the risk allele frequency of 4.1% was almost identical with the frequency detected in the controls. The frequencies of the other two *NOD2* variants: Arg702Trp and Gly908Arg were low in both controls and IBD pa-

tients groups and were not statistically significant.

The allele frequencies distribution for the *IL23R* and *ATG16L1* disease associated variants were almost identical between cases and controls and did not demonstrate significant differences.

DISCUSSION

This is the first report on the prevalence of the previously defined *NOD2*, *ATG16L1* and *IL23R* disease associated variants in an IBD case-control sample from Lithuania. Baltic countries still observe low IBD incidence rates, especially for CD in their populations. In Estonia (1993-1998) the incidence rate of CD was reported to be 1.4 per 100 000 inhabitants^[16]; and in Lithuania (2006) - 2.0 per 100 000 inhabitants^[9,10]. Therefore, analysis of the genetic contribution to disease susceptibility in this region was of great interest.

Since 2001, following the identification of *NOD2* as the first gene conferring susceptibility to CD^[3-5] a significant number of studies have replicated the association of the Arg702Trp, Gly908Arg and Leu1007insC variants with the development of CD in populations of Caucasian origin from Europe and North America^[17]. However, significant heterogeneity in the frequencies of these variants has been observed not only between ethnically divergent populations^[18,19], but also within Europe^[17].

Our study results add to this pattern. The carriage of at least one *NOD2* variant was highest in the CD patients group (41.1%) compared to the control group (16.9%) and resulted in the OR = 3.48 (95% CI: 1.81-6.72). These data are in concordance with previously reported rates of 30%-50% in CD and 7%-20% in controls from other European regions^[17]. The Leu1007insC variant was responsible for the major contribution of *NOD2* to disease susceptibility in the Lithuanian CD population (MAF = 21.4%, OR = 5.54, 95% CI: 2.89-10.75). These data are consistent with previous reports from Central Europe and North America (MAF = 6.6%-16%)^[17] and contrast markedly with studies performed in Northern Europe, where carriage rates of Leu1007insC and other *NOD2* variants are relatively low, i.e. the carriage of at least one *NOD2* variant varies from 2.8% to 22%^[20,21]. However, we were not able to confirm the association between Arg702Trp, Gly908Arg and IBD susceptibility in our study group. These findings are in contrast with previous reports from Southern and Central European populations, where a positive association between Arg702Trp, Gly908Arg and CD was detected. The reported alleles frequency rates in these European countries vary from 6.7% to 12.5% for Arg702Trp, from 3.3% to 6.1% for Gly908Arg, respectively, in CD patients and from 3.5% to 6.9% and from 0.6% to 3.0%, respectively, in controls^[17].

Moreover, the PAR%, an indication of the contribution of a mutation to the disease in a specific area, was 29.5% in the present study and contrasts with the other Northern European populations reporting lowest PAR% (range: 1.88%-11%)^[20,21]. The PAR% measured in the Central European populations and North America was around 30%^[3,5,17]. Therefore, the results of our study indicate that CD in Lithuania has a strong genetic background that is related partially to *NOD2* susceptibility variants. Interestingly, the relatively high carriership frequency of any of the three *NOD2* alleles in the healthy controls (16.9%) in our study is in contrast with data of low CD incidence in Lithuania^[9]. This indicates the importance of environmental factors (e.g. diet, lifestyle) in disease development.

The first two genome wide association studies identified genetic alterations within *IL23R*^[6] a component of the adaptive immune system - and *ATG16L1*^[7] - a protein involved in autophagic processes - to be associated with IBD and CD. These findings broadened the understanding of the different pathways that are involved in IBD susceptibility and/or pathogenesis. In addition, the *IL23R* and *ATG16L1* findings were confirmed in large independent Caucasian samples^[22-34]. A study in Japan^[35] failed to replicate these results, supporting the previously reported distinct ethnic difference of the genetic background of CD. Upon analysis of the Lithuanian IBD population we were not able to confirm any of these findings. We were just able to observe trends for possible associations with *ATG16L1* risk allele. The frequencies and contributable risk of the *ATG16L1* G allele reported in our study (55% CD and 48% controls, OR: 1.32) were similar to the published studies performed in Germany (59% CD and 52% controls, OR: 1.35)^[22], UK

(57% CD and 51% controls, OR: 1.30)^[23], Hungary (58% CD and 50% controls, OR: 1.39)^[26] and pooled study of German, Dutch and Hungarian cohorts (57% CD and 51% controls, OR: 1.32)^[24]. The allele frequency distribution of the protective *IL23R* variant in our control samples (5.9%) was similar to previous reports in Caucasian populations (approx. 6%), whereas the allele frequencies in our both IBD cases groups were higher (3.6% CD and 4.5% UC) compared to the results of other studies (1.87%-2.85% CD and 1.9%-2.68% UC)^[26-34].

It must be noted that our relatively small study population was underpowered to demonstrate such weak to moderate disease associations. The panel had a power of 80% to detect an odds ratio of 1.8 or higher at the 5% significance level, assuming a frequency of the disease-associated allele of at least 30% in the controls. Therefore, larger-sized case-control panels will be needed in order to further evaluate the importance of the herein tested loci.

In summary, our study provides clear evidence that the *NOD2* Leu1007insC variant increases susceptibility to CD in the Lithuanian study population, whereas associations of *IL23R* and *ATG16L1* variants with any of the distinct IBD subtypes need to be further evaluated in larger North-Eastern European IBD sample collections.

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COMMENTS

Background

Numerous genome-wide and linkage studies have identified and replicated significant association between inflammatory bowel disease (IBD) development and polymorphisms of genes attributed to recognition of bacterial products (*CARD15*), adaptive immune responses (*IL23R*), and autophagosome pathways (*ATG16L1*). However, there has been reported a heterogeneity in allele frequencies of genetic factors involved in the pathogenesis of IBD in different European populations. The genetic association with IBD susceptibility has never been investigated in Lithuania previously.

Research frontiers

The research was performed to obtain data about the frequency of *NOD2*, *IL23R* and *ATG16L1* genetic variants in a case-control study group for IBD from Lithuania.

Innovations and breakthroughs

The results of the authors' study indicate that Crohn's disease (CD) in Lithuania has a strong genetic background that relates partially to *NOD2* susceptibility variants, especially Leu1007insC. The relatively high carriership frequency of any of the three *NOD2* alleles in the healthy controls (16.9%) in this study is in contrast with the data of low CD incidence in Lithuania. This indicates the importance of environmental factors (e.g. diet, lifestyle) in disease development.

Applications

This is one of the first studies investigating the genetic association with IBD in a North-Eastern European country. The results of this study confirm that the heterogeneity of variants might be observed within Europe and will further help to understand the role of interplay between genetic and environmental factors in the development of complex diseases. Future studies in larger study groups

and further analysis of the biological functions of the identified variants are required to understand their role in determining the risk of CD and ulcerative colitis in ethnically divergent populations.

Terminology

NOD2 is a member of the NACHT-LRR receptor (NLR) protein family, which is known to be involved in recognition of microbial structures. *ATG16L1* encodes a protein which is part of a larger family of proteins that are required for the intracellular degradation system - autophagy process. *IL23R* encodes a protein which is a subunit of the receptor for IL23A/IL23 and participates in JAK-STAT3 signaling pathway.

Peer review

The authors concluded that the *NOD2* Leu1007insC variant increases susceptibility to CD in the Lithuanian study population, whereas associations of *IL23R* and *ATG16L1* variants with any of the distinct IBD subtypes need to be further evaluated in larger Eastern European IBD sample collections. The study was conducted with good design and convincing analysis, and the manuscript has been well written and solid conclusions have been drawn.

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Leukocyte-technetium-99m uptake in Crohn's disease: Does it show subclinical disease?

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C-reactive protein (CRP) of each patient were performed 7 d before the scintigraphic images. The leukocytes were labeled according to the International Society of Radiolabeled Blood Elements (ISORBE) consensus protocol and the scintigraphic images, including single photon emission computed tomography, were obtained 30 min and 2 h after injection of the radiolabeled leukocytes.

RESULTS: The labeling yield of the leukocytes with the lipophilic complex 99mTc-HMPAO was 55.0% ± 10%. Six of the 20 patients (30%) presented congruent results for the three parameters investigated (CDAI, Scintigraphic Index and CRP). On the other hand, 14 patients (70%) did not show congruent results. There was no significant correlation between the indices analyzed according to the Spearman test ($P > 0.05$, $n = 20$).

CONCLUSION: The results suggest that 99mTc-HMPAO-labeled leukocyte scintigraphy could be important for determining inflammatory activity in CD even in the absence of clinical symptoms.

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Key words: Technetium-99m; Hexamethylpropyleneamine oxime; Leukocytes; Inflammatory bowel disease; Scintigraphy

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Abstract

AIM: To evaluate inflammatory activity in patients with Crohn's disease (CD) using technetium-99m-hexamethylpropyleneamine oxime (99mTc-HMPAO) granulocyte scintigraphy.

METHODS: Twenty patients (7 male and 13 female) with CD and five healthy volunteers were selected for 99mTc-HMPAO granulocyte scintigraphy. The Crohn's Disease Activity Index (CDAI), blood tests and

Mota LG, Coelho LGV, Simal CJR, Ferrari MLA, Toledo C, Martin-Comin J, Diniz SOF, Cardoso VN. Leukocyte-technetium-99m uptake in Crohn's disease: Does it show subclinical disease? *World J Gastroenterol* 2010; 16(3): 365-371 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v16/i3/365.htm> DOI: <http://dx.doi.org/10.3748/wjg.v16.i3.365>

INTRODUCTION

Crohn's disease (CD) is characterized by chronic intestinal inflammation of unknown etiology that can affect any segment of the digestive tract from the mouth to the rectum^[1]. The diagnosis of this disease is based on clinical manifestations, radiological, endoscopic, surgical and anatomical pathological observations. However, none of these findings is considered pathognomonic of the disease^[2-4].

The initial clinical symptoms of CD may be subtle, variable, nonspecific and easily overlooked. Recurrent episodes of inflammation in the gastrointestinal tract are typical. This inflammation underlies many of the symptoms and signs of the disease, thus its detection and monitoring are of the utmost importance in clinical management^[5]. The follow-up of patients in clinical remission is based currently on the calculate of clinical activity indexes, including the Crohn's Disease Activity Index (CDAI) that is based on clinical and laboratory parameters, and measures CD activity as a sum of points^[6]. CDAI < 150 is characteristic of remission of the disease. Values between 150 and 250 are associated with mild inflammatory activity. Inflammatory activity is considered moderate when the values lie between 250 and 350, and CDAI > 350 characterizes intense activity^[7]. Another parameter used in the CD is the Vienna Classification that considers data constant such as age (A), location (L) and behavior (B) of the disease. It has as its aim phenotype standardization to evolutionary studies and works involving genetic, biological and environment factors^[3].

Gastrointestinal inflammation is not directly observable by patients or physicians, therefore, many methods have been developed to quantify the severity and extent of this inflammation. Therefore, a simple, rapid, sensitive, specific, inexpensive, noninvasive method to detect and monitor intestinal inflammation in CD is needed. According to Annovazzi *et al*^[6], if relapse or subclinical inflammation can be predicted in CD, it is likely to change the approach to treatment^[6]. In this case, the use of a functional imaging method such as technetium-99m-hexamethylpropyleneamine oxime (99mTc-HMPAO) granulocyte scintigraphy could be more important to elucidate the location of the inflammatory site in the bowel. Scintigraphic images are based on functional alterations of the tissue, which permit an early diagnosis of the inflammation and infection when the anatomical alterations are not visible^[4,8]. Among the scintigraphic methods used in the identification of the inflammatory and infectious foci, the use of radiolabeled leukocytes has been employed as a specialized technique that explores the natural migratory behavior of the white blood cells^[9].

Arndt *et al*^[10] have demonstrated that 99mTc-HMPAO-labeled leukocyte scintigraphy is better than the Van Hees activity index and laboratory parameters for the evaluation of the inflammatory activity of intestinal diseases. Other authors have reported that autologous radiolabeled leukocyte scintigraphy can be utilized in

the monitoring of patients to evaluate the efficiency of therapy, differentiation between fibrotic and inflammatory stenosis, and the recurrence of the disease after surgery^[11,12]. Among the treatment options for CD, the following stand out: aminosalicylates, corticosteroids, immunomodulators and biological therapy for control of inflammatory activity^[2].

The aim of the present study was to evaluate the presence of the inflammatory activity in patients with CD, who were subjected to usual treatment using 99mTc-HMPAO-labeled leukocyte scintigraphy.

MATERIALS AND METHODS

Materials

HMPAO (Ceretek) was supplied by Amersham Health (UK). Technetium-99m was obtained from a molybdenum generator (IPEN/Brazil). All other chemicals and reagents used were of analytical grade.

Subjects

Twenty patients (mean age 38.7 years, 7 male and 13 female) with previous diagnosis of CD were selected at the Gastroenterology Alfa Institute of the Clinical Hospital at Federal University of Minas Gerais in the period between September 2007 and June 2008. The diagnoses were based on the patient's clinical history and physical examination, as well as the results of radiological and endoscopic examinations. The patients were being treated with corticosteroids, aminosalicylates, antibiotics, immunomodulators and biological therapy (Table 1). Informed consent was obtained from all patients admitted to the study. This study was approved by the Ethical Committee at Federal University of Minas Gerais. Seven days before 99mTc-HMPAO-labeled leukocyte scintigraphy, patients were subjected to determination of complete hemography, erythrocyte sedimentation rate and C-reactive protein (CRP) level. The CRP reference value was considered < 8 mg/L. In this same period, all patients filled in the card to calculate CDAI^[6]. Five healthy volunteers were invited to participate in this study as controls.

Cell labeling with 99mTc-HMPAO

The labeling of leukocytes with 99mTc-HMPAO was performed in accordance with the method described by Martin-Comin *et al*^[13]. Briefly, blood samples (45 mL) were withdrawn from patients and healthy volunteers with a syringe that contained 6.0 mL anticoagulant [citric acid-citrate-dextrose (ACD)]. The leukocyte-rich pellet was obtained according to the established protocol of the International Society of Radiolabeled Blood Elements (ISORBE)^[14]. The leukocyte-rich pellet was gently resuspended in 0.5 mL cell-free plasma using a polypropylene Pasteur pipette. The HMPAO was labeled with a solution of sodium pertechnetate ($\text{Na}^{99\text{m}}\text{TcO}_4$) with 1480 MBq of activity. After labeling, the radiochemical purity of the 99mTc-HMPAO was determined by partition between 0.9% saline and chloroform^[15]. Freshly prepared 99mTc-

Table 1 Summary of data from patients with CD

Patients (sex, yr)	CDAI	Vienna classification	SI	CRP (mg/L)	Treatment
I.M.F. (M, 41)	330.0	A1L1B2	11	30.0	Methotrexate
A.A.A. (M, 40)	53.4	A1L1B2	4	2.1	Prednisone
J.A.N. (M, 44)	2.0	A1L1B3	0	4.0	Prednisone, mesalazine, azathioprine
L.M.T.(F, 54)	59.3	A2L1B1	5	7.3	Prednisone, azathioprine
L.J.O. (F, 37)	126.6	A1L4B1	0	12.0	Mesalazine, azathioprine
T.F.C. (M, 24)	56.3	A1L3B2	0	9.2	Without medication
F.R.F. (F, 44)	52.6	A1L1B1	2	2.5	Prednisone, mesalazine, azathioprine
A.A.S. (F, 24)	1.7	A1L1B1	3	6.0	Mesalazine
L.D. (F, 24)	47.1	A1L1B1	8	34.1	Azathioprine
A.F.S. (F, 35)	62.9	A1L3B3	5	3.0	Prednisone, mesalazine, ciprofloxacin, azathioprine, infliximab
L.P.M. (M, 38)	146.6	A1L1B1	3	17.5	Prednisone, mesalazine
M.O.R. (F, 30)	197.4	A1L3B3	4	6.0	Hydrocortisone, mesalazine, ceftriaxone, azathioprine
G.E.S.F.(M, 37)	162.1	A1L1B3	5	16.0	Sulfasalazine, ciprofloxacin/metronidazole, thalidomide
E.C.S. (F, 31)	122.1	A1L1B3	3	8.0	Prednisone, ciprofloxacin, azathioprine
J.G.R. (M, 41)	97.5	A1L1B3	3	6.0	Prednisone, mesalazine, azathioprine
J.D.G. (F, 52)	126.2	A1L2B2	3	14.0	Prednisone, mesalazine
R.M.M.(F, 2)	73.4	A1L1B1	0	6.1	Azathioprine
A.B.O. (F, 58)	81.3	A2L3B1	2	2.5	Mesalazine
M.F.L. (F, 46)	84.3	A1L1B2	3	48.0	Prednisone
M.J.A. (F, 51)	179.9	A2L1B1	2	3.2	Mesalazine, azathioprine

CD: Crohn's disease; CDAI: Crohn's Disease Activity Index; SI: Scintigraphic index; CRP: C-reactive protein.

HMPAO (0.7 mL, approximate 600 MBq) was added to the leukocyte-rich pellet. This preparation was incubated at 37°C for 15 min. Aliquots of 4 mL of cell-free plasma were added to the test tube. The tube was centrifuged (150 g) for 5 min. The plasma supernatant that contained unbound 99mTc-HMPAO was removed, and the 99mTc-HMPAO-labeled leukocyte pellet was suspended in 4.0 mL cell-free plasma. The labeling yield was calculated from: Labeling yield = {[cpm (precipitate)]/[cpm (precipitate) + cpm (supernatant)]} × 100; cpm = counts per minute or disintegrations per minute.

Scintigraphic imaging

Images were obtained at 30 min and 2 h after injecting patients and healthy volunteers with the labeled leukocytes (mean activity approximate 273 MBq). Abdominal scans were obtained in the anterior and caudal views (patients sat on the camera bed with the detector head positioned below the bed) using a wide field gamma camera (Orbiter, Siemens, Germany and Millennium MG, General Electric Company, Milwaukee, WI, USA)^[16]. The time of each image was approximately 10 min or one million counts^[17].

The single photon emission computed tomography (SPECT) study was performed just after completing the 30-min and 2-h planar images^[16]. SPECT was acquired using the following parameters: a matrix size of 64 × 64, 360° circular rotation, and a 5° step angle with a 20-s time frame.

Scintigraphic index (SI)

SI was calculated according to the method of Ybern *et al.*^[18]. Briefly, regions of interest (ROIs) were outlined over the liver, spleen, iliac crest and abnormal accumulations when present (Figure 1). The processing program of the gamma camera furnished the number of counts/area proportional

to the radioactivity and the average value of the activity per pixel present in each region. The abdomen was divided into five zones: right, top, left, bottom and center, which corresponded approximately to the ascending colon, transverse colon, descending colon, sigmoid colon/rectum and small bowel. The SI was calculated in all scans in the anterior view: $SI = (\sum A_i) + B$. "A_i" represents the degree of activity of the accumulations in each zone (1 = activity less than bone activity; 2 = activity greater than bone activity; 3 = activity greater than liver activity; 4 = activity greater than spleen activity). "B" indicates the number of zone with abnormal accumulations of labeled leukocytes (1 = one or more accumulations in one zone; 2 = accumulations in two or three zones; 3 = abnormal accumulations in four or five zones). SI > 2 was considered as active disease^[18].

Statistical analysis

CDAI, SI and CRP were compared using the Spearman's rank correlation.

RESULTS

The radiochemical purity of the lipophilic complex 99mTc-HMPAO presented a mean labeling percentage of the order of 85.0% ± 9.0% for the 25 samples. The mean yield for labeling of the autologous leukocytes with the lipophilic complex 99mTc-HMPAO was 55.0% ± 10.0%.

Six of the 20 patients (30%) presented congruent results for the three parameters investigated (CDAI, SI and CRP), which were two patients (I.M.F.; G.E.S.F.) with inflammatory activity and four (J.A.N.; F.R.F.; R.M.M.; A.B.O.) with disease in remission. On the other hand, 14 patients (70%) did not show congruent results for CDAI, SI and CRP (Table 1). Twelve patients showed results that were congruent with the Vienna Classification

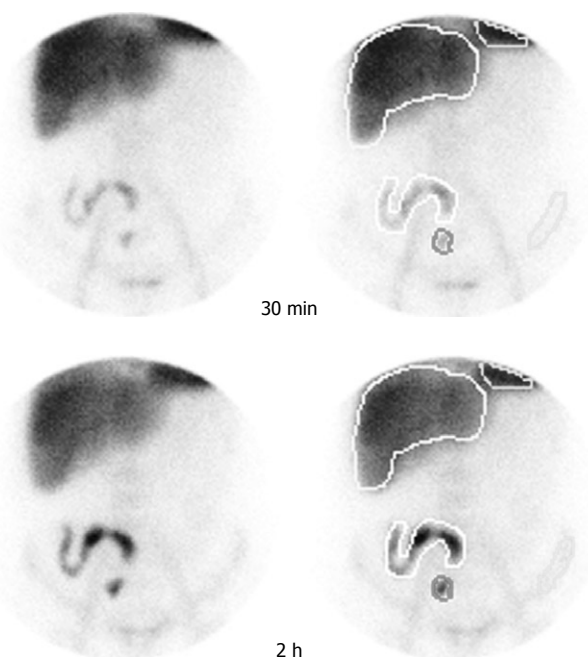


Figure 1 Regions of interest (ROIs).

(Table 1) and technetium-99m-HMPAO-labeled leukocyte scintigraphy with regard to disease location. Besides, there was no correlation for both parameters that were described above in five patients; since scintigraphy images showed positive areas while these regions were not included in the Vienna Classification. In three other patients, the scintigraphy results did not show radioactivity uptake in the intestinal segments.

The images from a healthy volunteer in the anterior and caudal projections at 30 min and 2 h after injection of ^{99m}Tc-HMPAO-labeled leukocytes are presented in Figure 2. Intense accumulation in the liver and the spleen could be seen at 30 min post-injection, as well as accumulation in the bone marrow. Because it was a control case, there was no abnormal accumulation in the digestive tract.

Accumulation of ^{99m}Tc-HMPAO-labeled leukocytes in the region of the terminal ileum, which suggested the presence of an inflammatory process at this location (anterior view), is shown in Figure 3A. At 2 h, uptake of labeled leukocytes increased, which showed the concentration of radiotracer in the indicated region. No pathological accumulation of ^{99m}Tc-HMPAO-labeled leukocytes could be seen in the caudal view, which indicated that there was no inflammation in the sigmoid and/or rectum.

The presence of inflammatory foci in the terminal ileum, descending colon, sigmoid and rectum, revealed by the intense accumulation of radiolabeled leukocytes, is shown in Figure 3B. Regions of the sigmoid and rectum affected by inflammation can be seen in the caudal projection. An increase in the radioactivity with time can also be seen.

According to the Spearman test, there was no significant correlation between the CDAI, SI and CRP in any of the investigated cases (Table 2).

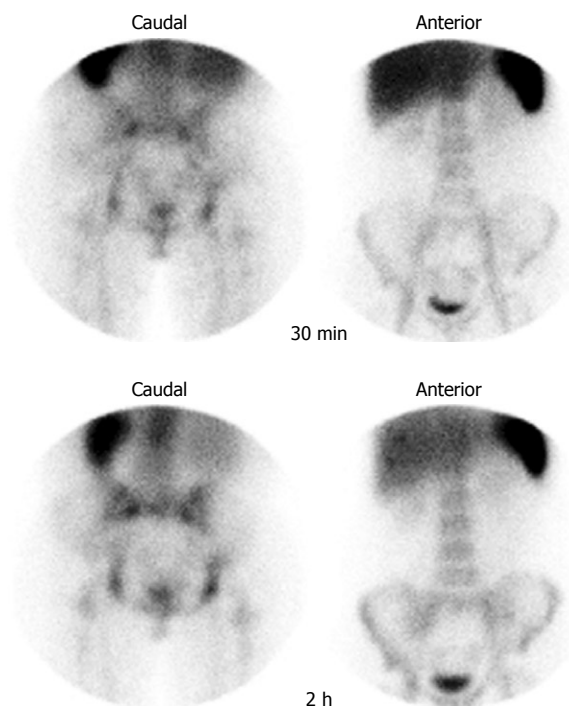


Figure 2 Images of a healthy volunteer (control) in the anterior and caudal projections at 30 min and at 2 h after injection of radiolabeled leukocytes.

Table 2 Spearman's rank correlation ($n = 20$)

	Spearman's rank	Correlation coefficient (ρ)	
		t	P
CDAI and SI	0.1835	0.7918	0.4388
CDAI and CRP	0.3373	1.5204	0.1457
SI and CRP	0.2783	1.2293	0.2347

DISCUSSION

The radiochemical purity of the lipophilic complex (HMPAO) labeled with technetium-99m was $85.0\% \pm 9.0\%$. The data suggest that 85% of the technetium-99m atoms were bound to HMPAO molecules. This result is in agreement with other data described in the literature^[15]. On the other hand, the labeling yield for leukocytes was $55.0\% \pm 10\%$. Labeling yields of 46% and 65.5% have been reported previously^[19,20]. Thus, the value obtained in the present work is supported by published data, which suggests that the manipulation process utilized in the preparation of the radiolabeled cells was adequate.

^{99m}Tc-HMPAO granulocyte scintigraphy of a healthy volunteer (Figure 2) showed uptake of radiolabeled leukocytes by the liver, spleen and bone marrow, which reflected physiological retention of labeled white blood cells^[11].

The scintigraphic images were based on physiological alterations such as an increase in blood flow, vasodilatation, increased permeability and cellular leakage, which permit the precocious detection of inflammatory foci. These findings support the use of ^{99m}Tc-HMPAO granulocyte scintigraphy as an important examination for

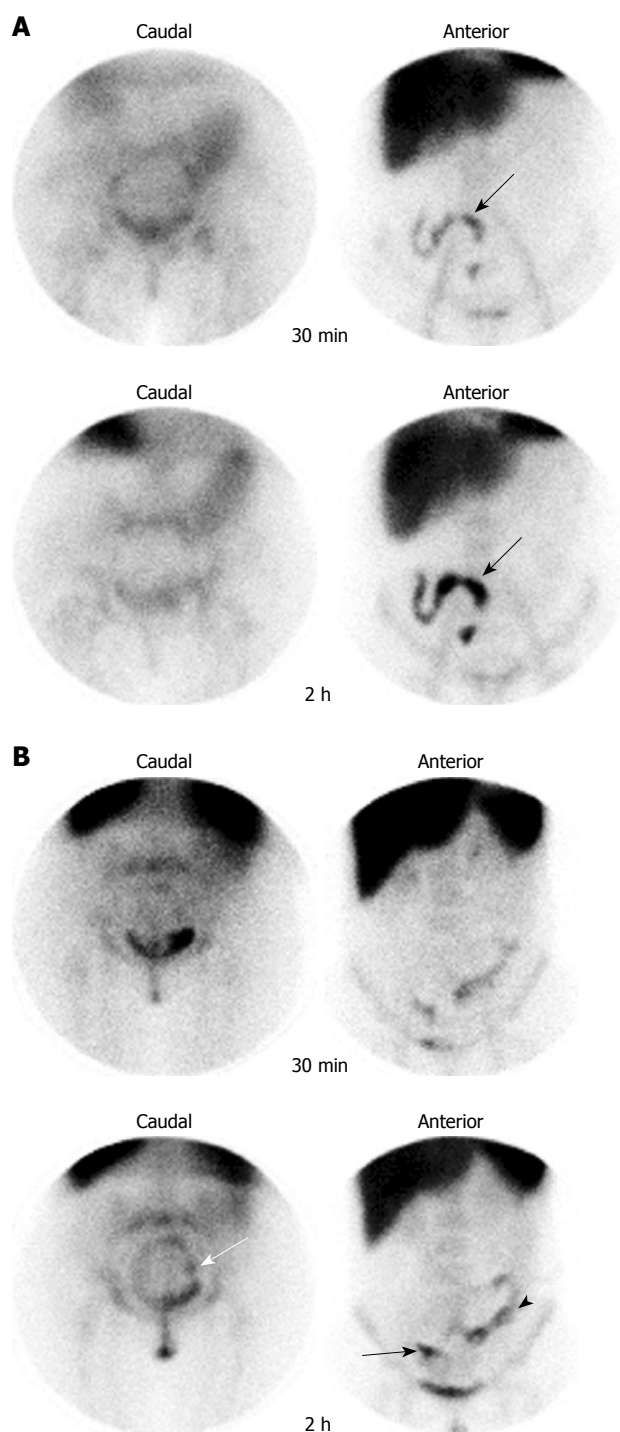


Figure 3 Images obtained 30 min and 2 h post-injection of ^{99m}Tc -HMPAO-labeled leukocytes in the caudal and anterior views. A: The arrows indicate the region of the intestine affected by the inflammation (L.M.T., female, 54 years); B: The arrows indicate ^{99m}Tc -HMPAO-labeled leukocyte uptake in the terminal ileum, descending colon and rectum-sigmoid (L.D., female, 24 years). The black arrows indicate terminal ileum; the arrowhead indicates descending colon; the white arrow indicates rectum-sigmoid.

diagnostic screening of inflammatory diseases^[4]. Cardoso *et al*^[19] have observed sensitivity, specificity and accuracy values of $> 87.5\%$ in studies performed in patients highly suspected of having inflammatory bowel disease.

The data obtained in the present study showed that, in 70% of investigated cases, the results did not show correspondence with CDAI, CRP and SI (Table 1). It

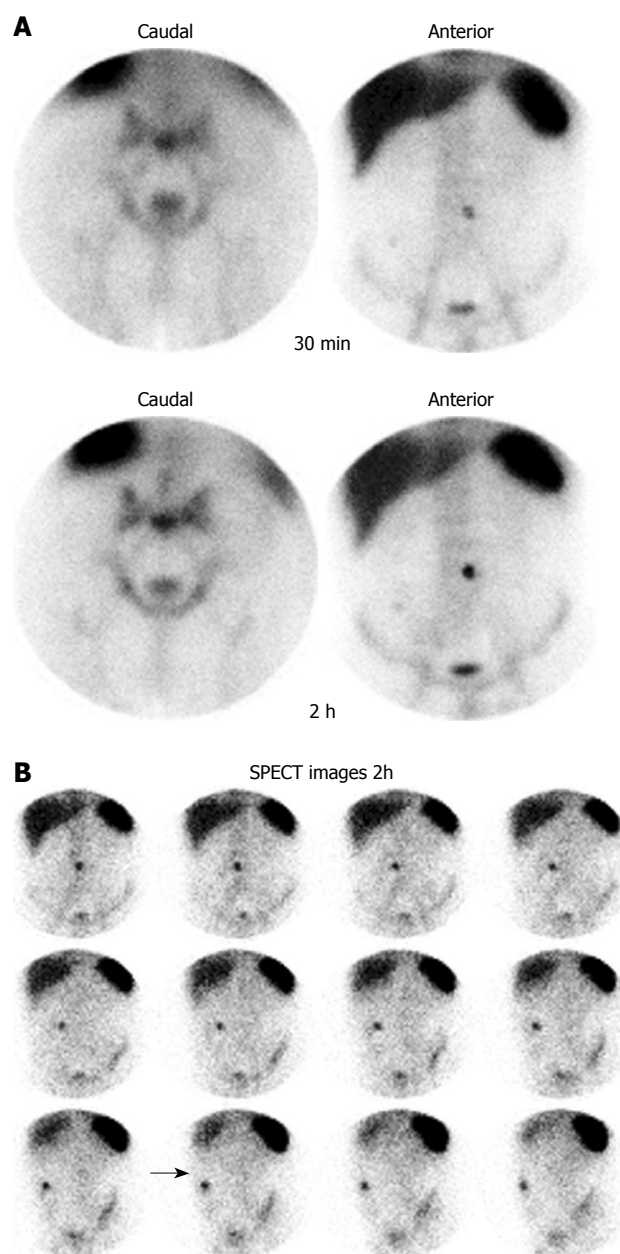


Figure 4 ^{99m}Tc -HMPAO granulocyte scintigraphy of patient J.A.N., male, 44 years. A: Images obtained at 30 min and at 2 h after injection of the radiolabeled leukocytes, in the caudal and anterior projections; B: SPECT images 2 h after injection of the radiolabeled leukocytes. The arrow indicates the fistula region of the patient's abdomen.

is known that CDAI is quite subjective and is based on a group of signs and clinical symptoms associated with erythrocyte sedimentation rate. This method was able to indicate inflammatory activity in four patients, while the SI and CRP indicated inflammatory activity in 13 and nine patients, respectively. Therefore, it is reasonable to suppose that the intestines of patients may have inflammatory foci that attract radiolabeled leukocytes in the specific case of CD, which results in a positive SI, although the signs and symptoms may still be absent.

As example of this observation, the patient L.M.T. (female, 54 years, Figure 3A) showed retention of radiolabeled leukocytes in the terminal ileum. On the other hand, the CDAI index (59.3) indicated disease remission

and the CRP value (7.3 mg/L) suggested the absence of inflammatory activity. This patient was receiving treatment with prednisone and azathioprine, but inflammatory activity was present, which justified the uptake of ^{99m}Tc-HMPAO-labeled leukocytes in the region, however, the patient was asymptomatic.

A case that deserves special attention is the pathological accumulation of radiolabeled leukocytes in the terminal ileum, descending colon, sigmoid and rectum, which resulted in an SI of 8 (disease activity) as illustrated in Figure 3B (patient L.D., female, 24 years). Before performing ^{99m}Tc-HMPAO-labeled leukocyte scintigraphy, it was known that the CD in this patient was located only in the region of the terminal ileum, with absence of clinical signs, and the patient was receiving immunomodulator treatment. Seven days before ^{99m}Tc-HMPAO granulocyte scintigraphy, CRP level was elevated (34.1 mg/L). Despite all the segments that were identified by scintigraphy, the disease was considered to be in remission according to the CDAI (47.1). Thus, this result showed the importance of ^{99m}Tc-HMPAO-labeled leukocyte scintigraphy as a early diagnosis method for identifying affected regions not previously known. In addition, the therapeutic response should be also evaluated using this method, since the dose of immunomodulator administered to the patient probably was not sufficient to control the disease^[11,12].

^{99m}Tc-HMPAO-labeled leukocyte scintigraphy performed in the patient J.A.N. (male, 44 years; Figure 4A) showed uptake of radiolabeled leukocytes in the central region of the abdomen, which indicated the presence of enterocutaneous fistula. On the other hand, the results did not show uptake of ^{99m}Tc-HMPAO-labeled leukocytes in segments of the intestine. SPECT showed that this radioactive uptake in the planar images corresponded to the external surface of the patient's enterocutaneous fistula (Figure 4B). According to Arndt *et al.*^[10], the capture of leukocytes in areas outside the intestinal segments, such as abscesses and fistulas, should be analyzed separately, but not considered for the calculation of SI^[10]. In our opinion, this uptake should be considered for SI calculation, because a fistula is a clinical feature that is suggestive of inflammatory activity and possible complications of CD.

The data obtained in the present work show that ^{99m}Tc-HMPAO-labeled leukocyte scintigraphy of the intestine could be useful for the evaluation of CD inflammatory activity, even in the absence of clinical signs and symptoms (disease remission). A functional method (scintigraphy) was compared with subjective (CDAI) and nonspecific (CRP) tests. Therefore, further studies will be necessary to prove the real utility of radiolabeled leukocytes to diagnosis and monitor patients with CD. Thus, it will be interesting to compare the scintigraphic method with another examination such as CT enterography.

scintigraphy was able to identify inflammatory activity in patients with Crohn's disease (CD) even in the absence of signs and clinical symptoms.

Research frontier

Scintigraphic images are based on functional alterations of the tissues, and permit the early diagnosis of inflammatory bowel disease, when the anatomical alterations are not visible.

Innovations and breakthroughs

In the authors' clinical routine, the Crohn's disease activity index is used normally to evaluate the presence of inflammatory activity in patients. However, this index is subjective and sometimes is not able to detect the presence of inflammatory activity in subclinical disease. Thus, ^{99m}Tc-HMPAO granulocyte scintigraphy could contribute to follow-up of these patients.

Applications

The results obtained suggest that ^{99m}Tc-HMPAO-labeled leukocyte scintigraphy could be used to monitor inflammatory bowel disease and evaluate the efficiency of therapy.

Terminology

Scintigraphic images are obtained using compounds or cells labeled with radioactive isotopes such as technetium-99m, iodine-131 and gallium-67. CD is a chronic intestinal inflammation of unknown etiology that can affect any segments of the digestive tract.

Peer review

The number of patients is small and therefore their positive experience with the technique may decrease with time and number of patients. The figures are of high quality and indicate the expertise of the authors.

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COMMENTS

Background

technetium-99m-hexamethylpropyleneamine oxime (HMPAO)-labeled leukocyte

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Genetic variants involved in gallstone formation and capsaicin metabolism, and the risk of gallbladder cancer in Chilean women

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patients with gallstones, and 70 controls were enrolled in this study. DNA was extracted from their blood or paraffin block sample using standard commercial kits. The statuses of the genetic variants were assayed using Taqman® SNP Genotyping Assays or Custom Taqman® SNP Genotyping Assays.

RESULTS: The non-ancestral T/T genotype of apolipoprotein B rs693 polymorphism was associated with a decreased risk of GBC (OR: 0.14, 95% CI: 0.03-0.63). The T/T genotype of cholesteryl ester transfer protein (CETP) rs708272 polymorphism was associated with an increased risk of GBC (OR: 5.04, 95% CI: 1.43-17.8).

CONCLUSION: Genetic variants involved in gallstone formation such as the apolipoprotein B rs693 and CETP rs708272 polymorphisms may be related to the risk of developing GBC in Chilean women.

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Key words: Genetic risk factor; Gallbladder cancer; Gallstone; Genetic polymorphism; Apolipoprotein B; Cholesteryl ester transfer protein

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Abstract

AIM: To determine the effects of genetic variants associated with gallstone formation and capsaicin (a pungent component of chili pepper) metabolism on the risk of gallbladder cancer (GBC).

METHODS: A total of 57 patients with GBC, 119

Báez S, Tsuchiya Y, Calvo A, Pruyas M, Nakamura K, Kiyohara C, Oyama M, Yamamoto M. Genetic variants involved in gallstone formation and capsaicin metabolism, and the risk of gallbladder cancer in Chilean women. *World J Gastroenterol* 2010; 16(3): 372-378 Available from: URL: <http://www.wjg-net.com/1007-9327/full/v16/i3/372.htm> DOI: <http://dx.doi.org/10.3748/wjg.v16.i3.372>

INTRODUCTION

There is a prominent worldwide geographical and racial variability in the incidence of gallbladder cancer (GBC), which correlates with the prevalence of cholelithiasis^[1]. High incidences of GBC are observed in specific countries and in confined areas. For example, the incidence of GBC is very high in northern Indian cities (5-7 per 100 000 women) and low (0-0.7 per 100 000 women) in southern India, possibly reflecting the different ethnic origins of these populations^[2]. The evidence suggests that the incidence of GBC is associated with the presence of both geographically-specific environmental factors and environmental factor-related genetic factors.

Recent studies have shown that the incidence rate for GBC is higher in Chile than in other countries^[3-5]. According to a previous epidemiological study, the consumption of high levels of red chili pepper has been identified as an important risk factor for GBC in Chilean women who carry gallstones (GS)^[6]. However, the pathogenic mechanism by which GBC occurs *via* chili pepper consumption in the presence of GS remains uncertain.

Although GS is the main cause of GBC, not all patients with GS develop GBC. While the standard mortality rates for GBC between 1985 and 2002 remained unchanged at 11.3 per 100 000 (0.0113%) in Chile^[7], 38.8% of adult women and 14.9% of adult men, which are staggeringly high rates, were GS carriers between 1972 and 1995^[8]. Red chili pepper is a widely consumed spice among the Chilean population. Therefore, the development of GBC in Chilean women cannot be completely explained by the presence of GS and chili pepper consumption alone.

In some but not all studies, lipid metabolism-related gene variants have been associated with GS formation. Apolipoprotein (apo) B, apo E, and cholesteryl ester transfer protein (CETP) polymorphisms have been associated with increased risk for cholelithiasis^[9-11]. On the other hand, capsaicin (8-methyl-N-vanillyl-6-nonenamide) is the active ingredient that makes chili peppers pungent. Although previous findings regarding the potential genotoxicity of capsaicin are inconsistent^[12-15], it is possible that GBC can be caused by high consumption of red chili pepper that contains capsaicin. Therefore, genes involved in the metabolism of capsaicin, such as cytochrome P450 (CYP) 2E1, CYP2C9, and CYP3A4, may be related to increased risk for GBC^[16-18]. However, no study has examined the association between the risk of GBC in Chile and the genetic variants involved in GS formation and capsaicin metabolism.

We hypothesized that individuals with a genotype promoting greater lipid metabolism/capsaicin metabolism would be more prevalent among the GS and GBC patients than among healthy subjects. We conducted a hospital-based case-control study in a Chilean population with special reference to polymorphism-polymorphism combination.

MATERIALS AND METHODS

Study subjects

A total of 57 female patients with GBC who had been diagnosed by histological examination of tissue at Sótero del Río Hospital in Santiago, Chile, between January 2007 and February 2008 were enrolled in this case-control study. A total of 119 female patients with GS who were diagnosed by an ultrasonic diagnostic method were also enrolled in the study. Seventy controls, who were patients with hernia or varicose veins of the legs who had no history of GS or any cancer and who were diagnosed by an ultrasonic diagnostic method, were selected randomly at the same hospital over the same period.

All patients gave their written informed consent, and our study protocol was approved by the Ethics Committee at Sótero del Río Hospital.

DNA extraction and storage

Samples collected in the hospital were sent to Niigata University, Japan, for DNA extraction and genotyping assay. Genomic DNA was extracted from the blood or paraffin block samples using standard commercial kits for blood samples (DNA Extractor WB-rapid, WAKO Pure Chemicals Industries, Ltd., Osaka, Japan) and for paraffin block samples (Dexpat, Takara Bio Co. Ltd., Tokyo, Japan). The extracted DNA samples were stored in a freezer at -80°C until genetic polymorphism analyses were performed.

Genotyping assay

The statuses of the genetic variants of apo B rs693, apo E rs7412, rs429358, CETP rs708272, CYP2C9 rs1057910, CYP2C9 rs1799853, and CYP3A4 rs12721627 were assayed using TaqMan® SNP Genotyping Assays purchased from Applied Biosystems (Foster City, CA, USA). The assay IDs were C_7615420_20 for rs693, C_904973_10 for rs7412, C_3084793_20 for rs429358, C_9615318_10 for rs708272, C_27104892_10 for rs1057910, C_25625805_10 for rs1799853, and C_30634207_10 for rs12721627. Genotyping for the presence of CYP2E1 polymorphisms (rs2031920, rs6413432) was performed using the Custom Taqman® SNP Genotyping Assays purchased from Applied Biosystems. The reaction components for a single 10 µL reaction (using a 96-well plate) included sample genomic DNA, TaqMan® Genotyping Master Mix (Applied Biosystems), SNP Genotyping Assay-Mix (Applied Biosystems), DNase free water was used. The thermal cycle (PE 9700, Applied Biosystems) conditions were as follows: 95°C for 10 min, followed by 40 cycles of 92°C for 15 s and 60°C for 1 min. After the PCR reaction, the plate read-out and allele discrimination were analyzed using a multiplex real-time QPCR system (Mx3000P, Stratagene Japan, Tokyo).

For quality control, all genotyping assays were reconfirmed according to the PCR-restriction fragment length polymorphism method and the replicates were 100% concordant.

Table 1 Demographic characteristics and food intake frequency of study subjects (mean \pm SD)

	Controls <i>n</i> = 70	GS patients <i>n</i> = 119	GBC patients <i>n</i> = 57	<i>P</i> value		
				Control vs GS	GS vs GBC	Control vs GBC
Age (yr)	45.8 \pm 14.1	42.7 \pm 9.2	56.5 \pm 11.2	NS	<i>P</i> < 0.001	<i>P</i> < 0.001
Height (m)	1.56 \pm 0.07	1.57 \pm 0.07	1.54 \pm 0.06	NS	NS	NS
Weight (kg)	72.1 \pm 15.5	69.3 \pm 12.8	64.1 \pm 12.8	NS	NS	NS
BMI (kg/m ²)	29.5 \pm 6.1	28.2 \pm 4.8	27.3 \pm 5.6	NS	NS	NS
Chili pepper	0.8 \pm 1.0	0.9 \pm 1.1	1.7 \pm 1.4	NS	<i>P</i> < 0.05	<i>P</i> < 0.01
Beef	1.2 \pm 0.6	1.5 \pm 0.7	1.8 \pm 0.5	NS	NS	<i>P</i> < 0.01
Pork	0.8 \pm 0.6	1.0 \pm 0.8	1.4 \pm 0.8	NS	<i>P</i> < 0.05	<i>P</i> < 0.01
Chicken	1.6 \pm 0.6	2.0 \pm 0.8	2.0 \pm 0.5	<i>P</i> < 0.01	NS	NS
Fish	1.0 \pm 0.6	1.1 \pm 0.6	1.5 \pm 0.7	NS	<i>P</i> < 0.05	<i>P</i> < 0.01
Fried food	1.0 \pm 0.9	1.3 \pm 1.1	2.2 \pm 1.1	NS	<i>P</i> < 0.001	<i>P</i> < 0.001
Butter	2.3 \pm 1.5	2.6 \pm 1.6	3.2 \pm 1.1	NS	NS	<i>P</i> < 0.05
Cheese	1.1 \pm 1.0	1.1 \pm 0.9	2.0 \pm 1.0	NS	<i>P</i> < 0.001	<i>P</i> < 0.001

Food intake was grouped into the following 5 categories, and a sequence number was assigned to each category: (0) never; (1) 1-3 times/mo or less; (2) 1-3 times/wk; (3) 4-6 times/wk; and (4) everyday. Data on height, weight, and food intake was collected from 70 controls, 119 GS patients, and 26 GBC patients. Data were evaluated by one-way analysis of variance (ANOVA) followed by Scheffe's multiple comparison test. GS: Gallstone; GBC: Gallbladder cancer; BMI: Body mass index; NS: No significant difference.

Measurement of dietary intake

An interviewer-administrated food-frequency questionnaire was used in the present study. Our subjects were asked to report the frequencies of their long-term intake of red chili pepper, vegetables, fruits, beef, pork, chicken, fish, milk, butter, cheese, and fried foods. Food intake was grouped into the following 5 categories, and a sequence number was assigned to each category: (0) never; (1) 1-3 times/mo or less; (2) 1-3 times/wk; (3) 4-6 times/wk; and (4) every day. Each dietary intake assessment was made according to the score obtained from each subject.

Statistical evaluation

Statistical analyses were performed using SAS software (Release 6.12, SAS Institute Inc., Cary, NC, USA) and STATA software (SE 8.0, STATA Corporation, TX, USA). Fisher's exact probability test was used to assess the association between the genotypes or alleles and GBC risk. The age-adjusted odds ratios (ORs) and 95% confidence intervals (95% CI) were calculated from logistic regression coefficients. Data on demographic characteristics and food intake frequencies were evaluated by one-way analysis of variance (ANOVA) followed by Scheffe's multiple comparison test. *P* values of less than 0.05 were considered to indicate statistical significance. The genotypic distribution of the polymorphisms in the controls was compared with that expected based on Hardy-Weinberg equilibrium (HWE) by the χ^2 (Pearson) test. When the *P* values exceeded 0.05, we estimated that the sample was under the HWE.

RESULTS

Table 1 shows the demographic characteristics and food intake frequencies of our subjects. The GBC patients had the highest mean age among the three groups, showing significant differences from the other two groups. No significant differences were found in the height, weight, or body mass index (BMI) among the three groups.

Significantly higher consumption of red chili pepper was observed in the GBC patients compared with the controls (*P* < 0.01) and the GS patients (*P* < 0.05). This difference in the consumption of red chili pepper between the GBC and GS patients was in agreement with the result of a previous study^[6]. In the GBC patients, the consumption of pork, fish, fried foods, and cheese were significantly higher than in the controls and the GS patients. Overall, higher consumption of the foods we asked about was observed in the GBC patients.

Table 2 shows the GBC risk associated with the apo B rs693 polymorphism. The genotype distributions were consistent with HWE in the controls (*P* = 0.44). The frequency of the T/T genotype was significantly lower in the GBC patients than in the controls; the age-adjusted OR for the GBC risk was 0.14 (95% CI: 0.03-0.63, *P* = 0.0099). The T allele was associated with a decreased risk of GBC (OR: 0.49, 95% CI: 0.29-0.84, *P* = 0.010). Based on these results, we designated the C allele that is presumed to increase the risk of GBC as the "at-risk" allele. No significant differences in the genotypic and allelic distribution were detected between the controls and the GS patients.

Table 3 shows the risk of GBC associated with the CETP rs708272 polymorphism. In the GBC patients, the frequencies of the C/C, C/T and T/T genotypes were 28.1%, 40.3% and 31.6%, respectively. The frequencies of the C/C, C/T, and T/T genotypes in the controls were 25.7%, 57.2% and 17.1%, respectively, and in the patients with GS they were 35.3%, 47.9% and 16.8%, respectively. The distribution of the genotypes of the rs708272 polymorphism agreed with HWE in the controls (*P* = 0.20). The frequency of the T/T genotype was significantly higher in the GBC patients than in the GS patients (*P* = 0.012), though no significant differences were found between the controls and the GS patients, or between the controls and the GBC patients. Based on these results, we designated the T allele that is presumed to increase the risk of GBC as the "at-risk" allele.

Table 2 Association of the apo B rs693 polymorphism with gallbladder cancer risk

Genotypes and alleles	Frequency (%)		Age-adjusted OR	95% CI	P value
	Controls	GS patients			
C/C	25 (35.7)	41 (34.5)	1.0		
C/T	31 (44.3)	65 (54.6)	1.27	0.66-2.47	NS
T/T	14 (20.0)	13 (10.9)	0.59	0.24-1.45	NS
C	81 (57.9)	147 (61.8)	1.0		
T	59 (42.1)	91 (38.2)	0.85	0.56-1.30	NS
	$P_{HWE} = 0.442$ $P_{HWE} = 0.088$				
	Controls	GBC patients			
C/C	25 (35.7)	30 (52.6)	1.0		
C/T	31 (44.3)	24 (42.1)	0.67	0.30-1.51	NS
T/T	14 (20.0)	3 (5.3)	0.14	0.03-0.63	0.010
C	81 (57.9)	84 (73.7)	1.0		
T	59 (42.1)	30 (26.3)	0.49	0.29-0.84	0.010
	$P_{HWE} = 0.516$				
	GS patients	GBC patients			
C/C	41 (34.5)	30 (52.6)	1.0		
C/T	65 (54.6)	24 (42.1)	0.70	0.31-1.59	NS
T/T	13 (10.9)	3 (5.3)	0.14	0.02-1.05	NS
C	147 (61.8)	84 (73.7)	1.0		
T	91 (38.2)	30 (26.3)	0.58	0.35-0.94	0.028

NS: No significant difference; P_{HWE} : P for Hardy-Weinberg equilibrium test.

The apo E and capsaicin metabolism-related gene variants were not associated with either GBC or cholelithiasis risk.

Table 4 shows the risk of GBC associated with the combined “at-risk” genotypes of the apo B rs693 and CETP rs708272 polymorphisms. The frequencies of the combined “at-risk” genotypes of the C/C genotype of the apo B rs693 polymorphism and the T/T genotype of the CETP rs708272 polymorphism were 4.3% in the controls, 6.7% in the GS patients, and 19.3% in the GBC patients. Compared with all remaining combinations combined, the frequency of the C/C plus T/T genotypes was significantly higher in the GBC patients than in the controls; the age-adjusted OR for the GBC risk was 4.75 (95% CI: 1.16 -19.4, $P = 0.030$).

DISCUSSION

In this hospital-based case-control study, the T allele carriers of the apo B rs693 polymorphism were associated with a decreased risk of GBC. In contrast, the T/T genotype of the CETP rs708272 polymorphism was associated with an increased risk of GBC. However, the capsaicin metabolism-related gene variants were not associated with GBC risk.

Singh *et al.*^[19] found that the frequency of the C allele of the apoB rs693 polymorphism was significantly higher in GBC patients than in GS patients or healthy subjects. Their data also showed that the C/T and T/T genotypes are associated with a lower risk for GBC compared with the C/C genotype (ORs: 0.28 and 0.34, 95% CI: 0.17-0.46, and 0.13-0.89, respectively). They suggested that the apo B rs693 polymorphism confers susceptibility to GBC under specific environmental conditions. Our results were

Table 3 Association of the CETP rs708272 polymorphism with gallbladder cancer risk

Genotypes and alleles	Frequency (%)		Age-adjusted OR	95% CI	P value
	Controls	GS patients			
C/C	18 (25.7)	42 (35.3)	1.0		
C/T	40 (57.2)	57 (47.9)	0.60	0.30-1.19	NS
T/T	12 (17.1)	20 (16.8)	0.68	0.27-1.70	NS
C	76 (54.3)	141 (59.2)	1.0		
T	64 (45.7)	97 (40.8)	0.82	0.54-1.24	NS
	$P_{HWE} = 0.204$ $P_{HWE} = 0.930$				
	Controls	GBC patients			
C/C	18 (25.7)	16 (28.1)	1.0		
C/T	40 (57.2)	23 (40.3)	0.75	0.30-1.93	NS
T/T	12 (17.1)	18 (31.6)	1.80	0.62-5.26	NS
C	76 (54.3)	55 (48.2)	1.0		
T	64 (45.7)	59 (51.8)	1.27	0.78-2.09	NS
	$P_{HWE} = 0.147$				
	GS patients	GBC patients			
C/C	42 (35.3)	16 (28.1)	1.0		
C/T	57 (47.9)	23 (40.3)	1.31	0.52-3.28	NS
T/T	20 (16.8)	18 (31.6)	5.04	1.43-17.8	0.012
C	141 (59.2)	55 (48.2)	1.0		
T	97 (40.8)	59 (51.8)	1.56	0.99-2.44	NS

CETP: Cholesteryl ester transfer protein.

in agreement with their findings, showing an association between the T allele and the lower risk of GBC. Since apo B is a key protein in lipid metabolism^[20], the apo B variant may be related to a higher incidence of GS and subsequently GBC. Generally, the T/T genotype has been reported to have significantly higher serum total cholesterol, low density lipoprotein cholesterol (LDL), and apo B levels compared with the C/C genotype^[21,22]. Therefore the T/T genotype or the T allele appears to relate to a higher risk of GS or GBC. However, our findings showed that the T allele may work as a preventive factor for GBC. The inverse association between the T/T genotype of the apo B polymorphism and the GBC risk may be explained by structural changes of apo B as proposed by Boekholdt *et al.*^[23]. Their hypothesis about the inverse association between the T/T genotype and ischemic heart disease (IHD) is as follows: the apo B variant causes hypercholesterolemia, modifies LDL to become a less atherogenic particle, and causes IHD. We could not clarify by what mechanism the T allele decreases the risk of GBC, and the mechanism research will be the topic of our next trial.

Some genetic variants may exert population-specific effects that are independent of the other genetic profiles of the individual and of environmental exposures, while other population-specific effects may be generated under differential gene-gene interactions in different populations, differential gene-environment interactions, or both^[24].

As reported by some researchers, GS which is a main risk factor for GBC, has been associated with both decreased levels of high-density lipoprotein (HDL) cholesterol and increased levels of LDL cholesterol and triglyceride^[25-27]. CETP has a central role in the metabolism of HDL and therefore might relate to the susceptibility to

Table 4 Effects of the combined genotypes of the apo B rs693 and CETP rs708272 polymorphisms on the risk of gallbladder cancer

Genotypes apo B-CETP	Frequency (%)		OR	95% CI	P value
	Controls	GS patients			
Others	70 (95.7)	111 (93.3)	1.0		NS
C/C-T/T	3 (4.3)	8 (6.7)	1.64	0.42-6.46	
	Controls	GBC patients			
Others	70 (95.7)	46 (80.7)	1.0		
C/C-T/T	3 (4.3)	11 (19.3)	4.75	1.16-19.4	0.030
	GS patients	GBC patients			
Others	111 (93.3)	46 (80.7)	1.0		
C/C-T/T	8 (6.7)	11 (19.3)	2.77	0.85-9.04	NS

Others: All remaining combinations combined.

cholelithiasis^[28]. The CETP variant has been reported to be associated with higher plasma CETP levels and lower HDL cholesterol levels^[29]. For this reason, we examined the association between the genotypic frequencies of the CETP variant and GBC risk. No significant difference in the CETP variant was found between the controls and the patients with GS or between the patients with GS and those with GBC. However, the frequency of the T/T genotype of CETP rs708272 polymorphism was significantly higher in the GBC patients than in the patients with GS (OR = 5.04, $P = 0.012$). Hassanzadeh *et al*^[30] reported that the C allele is associated with higher HDL cholesterol levels and lower CETP activity. Since the C allele is the major allele in GS patients^[31], the frequency of the C allele or that of the C/C genotype may have been higher in the GS patients than in the GBC patients. Obesity is one of the risk factors for GBC, and an association between obesity and low HDL cholesterol level has been found^[32,33]. Since the CETP variant has been associated with lower HDL cholesterol levels^[29], the risk of progression from cholelithiasis to GBC may be increased by obesity through an abnormality in the lipid metabolism of HDL cholesterol. However, the difference in the frequency of the T/T genotype may be caused by the small sample size of the controls and the GBC patients in our study, because the 95% CI was quite broad, ranging from 1.43 through 17.8. Further study in which the numbers of cases and controls are increased is needed to demonstrate our finding more clearly.

We also examined the combined effects of the apo B rs693 and CETP rs708272 polymorphisms on the risk of GBC. As shown in Table 4, the frequency of the combined C/C genotype of the apo B rs 693 polymorphism and the T/T genotype of the CETP rs708272 polymorphism was significantly higher in the GBC patients (19.3%) than in the controls (4.3%, $P = 0.030$). The OR for the “at-risk” T/T genotype of the rs708272 polymorphism alone was 5.04 (95% CI: 1.43-17.8), and that for the combined “at-risk” genotype of the C/C and T/T was 4.75 (95% CI: 1.16-19.4); their 95% CIs were widely overlapping. Thus, the T/T genotype of the CETP polymorphism appeared to be a

good candidate gene for the genetic factor independently.

The other genetic variants involved in GS formation that we evaluated in this study did not reach conventional levels of statistical significance. As patients with hernia or varicose veins of the legs who had no history of GS or cancer were used as controls in this study, the association may be attenuated. Since both disease incidences might affect the genotype distribution, healthy subjects having no GS or cancer may be needed to detect significant differences between the controls and cases.

The genotypic and allelic frequencies in the capsaicin metabolism-related gene polymorphisms were not significantly different among the three groups. Previous studies indicated that pure capsaicin was a non-mutagenic substance when tested using the Ames test^[12,13], but other studies showed that capsaicin and chili extract both acted as tumor promoters, carcinogens, and potential mutagens^[14,15]. Capsaicin is catalyzed by CYP 2E1, CYP2C9, and CYP3A4 to reactive species^[16-18]. On the basis of this evidence, we examined the effects of the CYP2E1, CYP2C9, and CYP3A4 variants on the GBC risk. No significant differences in the genotypic and allelic frequencies were found between the three groups. Some other constituents of the chili pepper, e.g., aflatoxin contamination, may be associated with the GBC risk rather than capsaicin itself.

Identification of the high-risk group characterized in terms of genetic measures is important for GBC screening studies. The high-risk group also should be a target of chemoprevention and treatment trials. In addition to genetic association studies of apo B, CETP, CYP2C9 and CYP3A4, further genetic association studies of inflammatory (cyclooxygenase and ATP-binding cassette half-transporters, interleukin-1 beta) genes are needed to help illuminate the complex landscape of GBC risk and genetic variations. We also anticipate that in future genetic association studies of GBC, new approaches will facilitate the evaluation of haplotype effects, either for selected polymorphisms that are physically close to each other or for multiple genes in the overall gallbladder carcinogenesis pathway.

This study had the following limitation. Our sample size was small, and the GBC patients had a bias in age distribution with respect to the controls and the GS patients. Thus, our results may have reduced statistical power to detect a possible association between genetic variants and GBC risk, or they may have failed to reflect precisely the genetic risk factors for the development of GBC. Nonetheless, our finding of the apo B rs693 polymorphism was in agreement with the result of the Indian study^[19]. To the best of our knowledge, the present study is the first to demonstrate that the T/T genotype of the CETP rs708272 polymorphism was associated with an increased risk of GBC. An additional study including a greater number of controls and cases is required to clarify the association between these genetic factors and the GBC risk.

In conclusion, the C/C genotype of the apo B rs693 polymorphism and the T/T genotype of the CETP

rs708272 polymorphism were associated with increased risk of GBC in Chilean women. While our findings require further confirmation, they provide evidence that the apo B and CETP genes are associated with a higher risk of GBC in Chilean women.

COMMENTS

Background

Gallbladder cancer (GBC) is the most common type of biliary tract cancer which results from a complex interplay of genetic and environmental risk factors, like other common multifactorial diseases such as cardiovascular disease, diabetes mellitus and autoimmune disease. Whether genetic variants involved in gallstone formation and capsaicin metabolism affect the risk of GBC in Chilean women is unknown.

Research frontiers

In this study, the frequency of the cholesteryl ester transfer protein (CETP) rs708272 T/T genotype was significantly higher in the GBC patients than in the gallstone patients. This is the first analysis of the association between genetic predisposition and GBC risk in Chilean women.

Innovations and breakthroughs

Variants of gallstone formation-related genes such as apolipoprotein B and CETP were associated with an increased risk of GBC in Chilean women. However, capsaicin metabolism-related gene variants were not associated with GBC risk. To clarify the role of genetic predisposition in the development of GBC, we may have to pay more attention to other genes such as inflammatory genes.

Applications

The apolipoprotein B rs693 T/T and CETP rs708272 T/T genotypes can be used as biomarkers for selecting patients from the group of individuals at high risk for GBC in Chile. Identifying such susceptibility polymorphisms may lead to the development of tests that allow more focused follow-ups of high-risk groups.

Terminology

CETP is a protein that facilitates the exchange of cholesteryl esters for triglycerides between high-density lipoproteins (HDL) and triglyceride-rich lipoproteins. Therefore, CETP has a central role in the metabolism of both of these types of lipoproteins. Although the C/C genotype was associated with higher plasma CETP and lower HDL cholesterol levels, there is no consistent result regarding the role of the C/C genotype in the development of gallstones.

Peer review

This study provided some useful data on genetic predisposition and risk of GBC. Methods used in this study are generally reliable, the results are reasonable and convincing. The manuscript is also well written. Authors also pointed out the shortcoming of this study-small sample size.

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HBV genotype C is independently associated with cirrhosis in community-based population

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Abstract

AIM: To determine the association of hepatitis B virus (HBV) genotypes with probable cirrhosis and fatty liver in community-based populations.

METHODS: A multi-stage cluster probability sampling method was applied to recruit 10 167 subjects aged between 6 and 72 years from our epidemiological bases in Eastern China. After excluding the subjects co-infected with hepatitis C or hepatitis D viruses, the hepatitis B surface antigen (HBsAg)-positive subjects were examined for HBV genotype, serum viral load, alanine aminotransferase (ALT), hepatitis B e antigen (HBeAg) status, and ultrasonographic changes. Logistic regression models were used to determine the factors associated with probable cirrhosis and fatty liver.

RESULTS: Of 634 HBsAg-positive subjects with HBV genotype determined, 82 had probable cirrhosis (ultrasonographic score ≥ 5), 42 had ultrasonographic fatty liver. Probable cirrhosis was only found in the HBeAg-negative subjects, and more frequently found in the subjects with genotype C than in those with genotype B (14.8% vs 8.0%, $P = 0.018$). In HBeAg-negative subjects, high viral load was frequently associated with abnormal ALT level, while ALT abnormality was more frequent in those with probable cirrhosis than those without (19.5% vs 7.8%, $P = 0.001$). Univariate analysis showed that age, sex, HBV genotypes, and viral load were not significantly associated with ultrasonographic fatty liver, whereas ALT abnormality was significantly related to ultrasonographic fatty liver (OR = 4.54, 95% CI: 2.11-9.75, $P < 0.001$). Multivariate analysis demonstrated that HBV genotype C, age (≥ 45 years), male sex, and ALT abnormality were independently associated with probable cirrhosis (AOR = 2.30, 95% CI: 1.26-4.19; AOR = 1.81, 95% CI: 1.10-2.99; AOR = 1.74, 95% CI: 1.03-2.95; AOR = 2.98, 95% CI: 1.48-5.99, respectively).

CONCLUSION: A crude prevalence of probable cirrhosis is 12.9% in the community-based HBV-infected subjects. HBV genotype C is independently associated with probable cirrhosis in the HBeAg-negative subjects.

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Key words: Hepatitis B virus; Genotype; Viral load; Alanine aminotransferase; Probable cirrhosis; Ultrasonography

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INTRODUCTION

Hepatitis B virus (HBV) infection is a serious public health problem. Approximately 2 billion people have been exposed to HBV, and more than 300 million are chronically infected with HBV. Chronic HBV infection is the most important risk factor of liver cirrhosis and hepatocellular carcinoma (HCC) in HBV endemic areas^[1]. Liver fibrosis, which is the natural wound healing process to necroinflammation frequently caused by chronic HBV infection, is the essential pathogenic process that leads to cirrhosis. Metabolic syndrome is also an independent risk factor of liver cirrhosis in the patients with chronic hepatitis B^[2]. Subclinical liver cirrhosis diagnosed by ultrasonography is significantly associated with the risk for HCC^[3].

HBV genotypes have distinct geographical distributions, and have been shown to differ with regard to clinical outcome and prognosis^[4]. Genotypes B and C are endemic in most parts of Asia^[5]. Genotype C is associated with HCC in the aged^[6,7]. Genotype B is associated with HCC in the young, relapse of HCC, and acute hepatitis B in adults^[8-10]. However, the relationship between HBV genotypes and liver cirrhosis remains controversial. Some studies suggested that genotype C had a higher risk of cirrhosis, whereas other studies indicated that the progression to cirrhosis did not differ among genotypes B- and C-related chronic liver diseases^[11-13]. In addition, the association between HBV genotypes and subclinical cirrhosis has not been evaluated in community-based studies in the HBV endemic areas.

Our objective was to determine the prevalence of probable liver cirrhosis in community-based subjects who were seropositive for hepatitis B surface antigen (HBsAg), and to evaluate the viral and demographic factors contributing to subclinical cirrhosis.

MATERIALS AND METHODS

Study population and epidemiological survey

The study was carried out at our epidemiological bases in Eastern China, from February to July 2009. A multistage cluster probability sampling method was applied to select the study population. A total of 10167 residents aged between 6 and 72 years were involved in this study. The participants were interviewed by the trained research assistants using a standard questionnaire requesting information about sociodemographic characteristics. Fasting blood samples (4 mL) were collected with vacuum blood collection tube (BD Diagnostics, Plymouth, UK) without anticoagulant. The serum was separated by centrifugation at 4°C at the Centers for Disease Control and Prevention, transported on dry ice and stored at -40°C in the Department of Epidemiology, Second Military Medical University. Informed consent in writing was obtained from each participant or guardian. Each resident who agreed to participate in the study completed a questionnaire and provided blood samples. The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki, and was approved by the Institutional Review Board of this university.

Examination of HBV serological markers, serum viral load, and serum alanine aminotransferase level

All participants received HBsAg examination. Those positive for HBsAg were examined for hepatitis B e antigen (HBeAg), serum viral load, and alanine aminotransferase (ALT). HBsAg was examined using enzyme linked immunosorbent assay (Kehua, Shanghai, China) according to the manufacturer's instructions. Serological testing for HBeAg, antibody to hepatitis C virus (HCV), and antibody to hepatitis D virus (HDV), liver function tests, and α -fetoprotein examination were performed as previously described^[9]. Upper limit of normal ALT was 45 U/L. Viral load was measured in the LightCyclerTM 480 (Roche, Basel, Switzerland) using quantitative HBV PCR fluorescence diagnostic kits (Fosun Diagnostics, Shanghai, China). The kit has a certified lower limit of detection of 500 copies/mL, which was standardized using the Abbott reagents (Abbott Laboratories, North Chicago, IL).

HBV genotyping

HBV DNA was extracted from 200 μ L HBsAg-positive serum using High Pure Viral Nucleic Acid Kits (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instruction. HBV genotype was determined using a multiplex PCR assay^[9,14]. HBV genotypes of samples with low level of HBV DNA were identified by nested multiplex PCR. Outer primers were 5'-TTTGCGGGTCACCATAATTCTTGG-3' and 5'-CGA ACCACTGAACAAATGGCACTAG-3'. An Autorisierter Thermocycler (Eppendorf AG, Hamburg, Germany) was programmed to initially denature the samples for 3 min at 95°C, followed by 35 cycles consisting of 94°C for 60 s, 58°C for 60 s, 72°C for 60 s, followed by a final elongation step at 72°C for 10 min. The products (2 μ L) were used as templates for multiplex PCR^[14].

Ultrasonographic examination of liver cirrhosis and fatty liver

With the use of a Philips iU22 scanner (Philips Medical Systems, Best, the Netherlands) equipped with a 2-4 MHz variable convex probe or Toshiba systems (SSA-340; Toshiba, Tokyo, Japan) with a 3.75 MHz convex probe, probable liver cirrhosis and fatty liver were determined. Each subject was examined by two independent operators who were blinded to the clinical details. Discrepancies were resolved by consensus. The ultrasonographic scoring system consisting of liver surface, parenchyma, vascular structure, and splenic size was used to describe the existence and the severity of cirrhosis. The scores ranged from 4 for a normal liver to 11 for advanced cirrhosis^[15]. A score of 8 or more was used as the cutoff point for ultrasonographic cirrhosis. The subjects with the score from 5 to 7 were diagnosed as having cirrhosis-like ultrasonographic abnormality. A score of 5 or more was defined as probable cirrhosis. The subject with an ultrasonographic steatosis score of 2 or more was diagnosed as having fatty liver^[16].

Statistical analysis

χ^2 test was used to determine the differences in categorical variables, such as HBeAg positivity and the percentage of HBV genotypes. Continuous variables, like serum viral load and ALT level with skewed distribution, were adjusted to normal distribution by transformation into logarithmic function, and then tested by Student's *t* test. Univariate and multivariate regression analyses were performed to obtain the odds ratio (OR) and adjusted odds ratio (AOR) of factors for the risk of probable liver cirrhosis or ultrasonographic fatty liver and their 95% confidence intervals (CI). All statistical tests were two-sided, and performed using the Statistical Program for Social Sciences (SPSS15.0 for Windows, SPSS, Chicago, IL). A *P* value of < 0.05 was considered as statistically significant.

RESULTS

Of the 10167 participants, 793 were HBsAg positive; 745 of the 793 subjects were free of antibodies to HCV or HDV; and 634 of 745 subjects had HBV genotyped. Ten of the 634 subjects (8 with genotype C, one with genotype D, and one with genotype B) were diagnosed as having ultrasonographic cirrhosis (score 8 or higher), while 72 had cirrhosis-like ultrasonographic abnormalities (scores 5-7). Of the 634 subjects, one was diagnosed as having HCC. A crude prevalence of probable cirrhosis (ultrasonographic cirrhosis and cirrhosis-like ultrasonographic abnormalities) was 12.9%. Table 1 shows the demographic and viral characteristics and liver abnormalities of the 634 subjects. There were no significant differences in proportions of age, sex, ALT level, HBeAg positivity, and fatty liver between the subjects infected with genotype B and those with genotype C. Compared with genotype C, HBV genotype B was more frequently seen in those with a high viral load (\log_{10} copies/mL ≥ 4). Of the 634 subjects, 39 were positive for HBeAg. The HBeAg-positive subjects were significantly younger than the HBeAg-negative

Table 1 Demographic and viral characteristics and liver abnormalities of 634 subjects seropositive for hepatitis B surface antigen *n* (%)

Variables	Genotype B (<i>n</i> = 199)	Genotype C (<i>n</i> = 411)	Others (<i>n</i> = 24) ¹	<i>P</i> value ²
Age (yr) ³	42.1 \pm 13.0	41.3 \pm 12.8	43.8 \pm 12.8	0.493
Gender				
Male	116 (58.3)	228 (55.5)	16 (66.7)	0.511
Female	83 (41.7)	183 (44.5)	8 (33.3)	
ALT (\log_{10} U/L) ³	1.4 \pm 0.3	1.4 \pm 0.3	1.4 \pm 0.2	0.238
≤ 45 U/L	174 (87.4)	373 (90.8)	23 (95.8)	0.207
> 45 U/L	25 (12.6)	38 (9.2)	1 (4.2)	
HBeAg				
Negative	186 (93.5)	385 (93.7)	24 (100.0)	0.922
Positive	13 (6.5)	26 (6.3)	0	
HBV DNA levels (\log_{10} copies/mL) ³	3.7 \pm 1.7	3.5 \pm 1.7	3.1 \pm 1.1	0.194
< 4	151 (75.9)	347 (84.4)	23 (95.8)	0.011
≥ 4	48 (24.1)	64 (15.6)	1 (4.2)	
Fatty liver				
Yes	16 (8.0)	24 (5.8)	2 (8.3)	0.303
No	183 (92.0)	387 (94.2)	22 (91.7)	
Cirrhosis status				
Normal	183 (92.0)	350 (85.2)	19 (79.2)	0.018
Probable cirrhosis ⁴	16 (8.0)	61 (14.8)	5 (20.8)	

¹23 cases for genotype mixture, 1 case for genotype D; ²Genotype C *vs* genotype B; ³mean \pm SD; ⁴Ultrasonographic cirrhosis and cirrhosis-like ultrasonographic abnormalities (ultrasonographic score ≥ 5). ALT: Alanine aminotransferase; HBeAg: Hepatitis B e antigen; HBV: Hepatitis B virus.

subjects (25.4 \pm 11.2 years *vs* 42.7 \pm 12.4 years, *P* < 0.001). The subjects with probable cirrhosis were significantly older than those without probable cirrhosis (45.3 \pm 10.5 years *vs* 41.1 \pm 13.3 years, *P* = 0.001). Probable cirrhosis was only found in the HBeAg-negative subjects, and more frequently in the subjects with genotype C than in those with genotype B (14.8% *vs* 8.0%, *P* = 0.018). Serum viral load was significantly higher in the HBeAg-negative subjects with abnormal ALT levels than in the HBeAg-negative subjects with normal ALT levels (4.54 \pm 2.16 \log_{10} copies/mL *vs* 3.31 \pm 1.36 \log_{10} copies/mL; *P* < 0.001). However, this association was not found in the HBeAg-positive subjects. Serum ALT level was significantly higher in the subjects with high viral load ($\geq 1 \times 10^4$ copies/mL) than in those with low viral load ($< 1 \times 10^4$ copies/mL) (33.9 \pm 2.4 U/L *vs* 22.4 \pm 1.9 U/L, *P* < 0.001). Serum ALT level was significantly higher in the subjects with ultrasonographic cirrhosis (score ≥ 8) than in the HBeAg-negative subjects with ultrasonographic score less than 7 (41.1 \pm 1.6 U/L *vs* 24.1 \pm 2.0 U/L, *P* = 0.026). ALT abnormality was more frequent in HBeAg-negative subjects with probable cirrhosis than those without probable cirrhosis (19.5% *vs* 7.8%, *P* = 0.001).

Table 2 shows the factors associated with probable liver cirrhosis in the HBeAg-negative subjects by univariate and multivariate regression analyses. Age (≥ 45 years *vs* < 45 years), sex (male *vs* female), HBV DNA ($\geq 4 \log_{10}$ copies/mL *vs* < 4 \log_{10} copies/mL), ALT (> 45 U/L *vs* ≤ 45 U/L), and HBV genotypes (genotype C *vs* genotype B) were included in the models. It was found that age (≥ 45 years), male sex, genotype C, and ALT abnormality were independently associated with

Table 2 Univariate and multivariate regression analyses for the risk factors of probable liver cirrhosis in the 595 HBeAg negative subjects infected with HBV

Variables	Controls (<i>n</i> = 513)	Cases (<i>n</i> = 82)	OR (95% CI)	AOR (95% CI)
Age (yr)				
< 45	284 (55.4)	31 (37.8)		
≥ 45	229 (44.6)	51 (62.2)	2.04 (1.26-3.29)	1.81 (1.10-2.99)
Sex				
Female	235 (45.8)	25 (30.5)		
Male	278 (54.2)	57 (69.5)	1.93 (1.17-3.18)	1.74 (1.03-2.95)
ALT (U/L)				
≤ 45	473 (92.2)	66 (80.5)		
> 45	40 (7.8)	16 (12.9)	2.87 (1.52-5.41)	2.98 (1.48-5.99)
Viral load (Log ₁₀ copies/mL)				
< 4	432 (84.2)	67 (81.7)		
≥ 4	81 (15.8)	15 (18.3)	1.19 (0.65-2.19)	1.06 (0.54-2.08)
Genotype				
B	170 (34.4)	16 (20.8)		
C	324 (65.6)	61 (79.2)	2.00 (1.12-3.58)	2.30 (1.26-4.19)

AOR: Adjusted odds ratio; OR: Odds ratio.

probable cirrhosis (AOR = 1.81, 95% CI: 1.10-2.99; AOR = 1.74, 95% CI: 1.03-2.95; AOR = 2.30, 95% CI: 1.26-4.19; AOR = 2.98, 95% CI: 1.48-5.99, respectively).

Forty-two (6.6%) of the 634 subjects had ultrasonographic fatty liver, including 11 with abnormal ALT levels. Ultrasonographic fatty liver was not found in the subjects with probable cirrhosis. In the subjects with high viral load (log₁₀ copies/mL ≥ 4), ultrasonographic fatty liver was more frequently found in those with genotype B than in those with genotype C (12.5% *vs* 0.0%, *P* = 0.005). Univariate analysis showed that age, sex, HBV genotypes, and viral load were not significantly associated with ultrasonographic fatty liver, whereas ALT abnormality was significantly associated with ultrasonographic fatty liver (OR = 4.54, 95% CI: 2.11-9.75, *P* < 0.001).

DISCUSSION

This large epidemiological study for the first time described the prevalence of probable liver cirrhosis in community-based, HBV-infected subjects who were free of HCV or HDV infection. About 13% of HBV-infected subjects had probable cirrhosis. The subjects with probable cirrhosis were significantly older than the subjects without cirrhosis. Probable cirrhosis was only found in the HBeAg-negative subjects. The HBeAg-positive subjects were significantly younger than the HBeAg-negative subjects. These results indicate that age is an important determinant for the development of probable liver cirrhosis. High viral load and ALT abnormality are associated with liver fibrosis in the HBeAg-negative patients^[17]. We further demonstrated that high viral load was associated with increased serum ALT levels in the HBeAg-negative subjects and high ALT levels were frequently found in the subjects with probable cirrhosis, indicating that continuing HBV replication and hepatocyte damage contribute to the development of liver cirrhosis.

Importantly, the occurrence of probable liver cirrhosis

was significantly higher in the subjects with genotype C than in those with genotype B. Multivariate analysis indicated that genotype C was significantly associated with an increased risk of probable liver cirrhosis. This was probably related to the prolonged immune clearance and delayed HBeAg seroconversion^[18,19]. Although genotype B is associated with acute hepatitis^[10], it tends to be self-limiting and short-living. However, genotype C was associated with the longer duration of liver damage in the HBeAg-negative subjects^[12,20], which may be the main reason for the development of liver cirrhosis. In addition, genotype C-specific viral mutations are associated with probable cirrhosis^[11,21,22]. Our recent meta-analysis has shown that PreS deletion, C1653T, T1753V, and A1762T/G1764A are increasingly more prevalent as chronic HBV infection progressed from the asymptomatic HBsAg carrier to cirrhosis or HCC^[23]. Further studies are needed to probe into the different mutation patterns between genotypes B and C and their roles in the development of liver cirrhosis.

Since metabolic syndrome increased the risk of liver cirrhosis in the patients infected with HBV^[2], we evaluated the prevalence and possible risk factors of ultrasonographic fatty liver in the 634 HBV-infected subjects. Interestingly, ultrasonographic fatty liver was not found in those with probable cirrhosis, while ultrasonographic fatty liver was more frequently found in those with genotype B than in those with genotype C at high viral load levels. This suggests that ultrasonographic fatty liver is unlikely to be a late event during the development of probable cirrhosis.

In conclusion, this study found that HBV genotype C, age (≥ 45 years), ALT abnormality, and male sex are independently associated with an increased risk of probable cirrhosis. Ultrasonographic fatty liver is not found in the subjects with probable cirrhosis. Although cirrhosis-like ultrasonographic abnormalities are not clinical liver cirrhosis, it is an early event during the development of clinical cirrhosis. Genotype C HBV-infected male residents at the age of 45 years or older should be routinely examined for active hepatitis and early cirrhosis. Early intervention to the HBV-infected subjects with high risks of cirrhosis might be effective for decreasing the overall mortality from liver cirrhosis and subsequent HCC.

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COMMENTS

Background

Chronic hepatitis B virus (HBV) infection is the most important risk factor of liver cirrhosis and hepatocellular carcinoma (HCC) in HBV endemic areas. Metabolic syndrome has been found to be an independent risk factor of liver cirrhosis in the

patients with chronic hepatitis B. The relationship between HBV genotypes and liver cirrhosis remains controversial. Furthermore, the association between HBV genotypes and subclinical cirrhosis has not been evaluated in community-based population.

Research frontiers

HBV genotypes have distinct geographical distributions and differ with regard to clinical outcome, prognosis, and response to interferon treatment. The role of genotype B and C, the two major HBV genotypes endemic in East Asia, in the development of liver cirrhosis has not been unequivocally addressed. In this study, the authors demonstrate that infection with HBV genotype C is closely associated with subclinical cirrhosis in the community-based subjects with increasing age.

Innovations and breakthroughs

Recent reports have highlighted the importance of HBV genotypes, alanine aminotransferase (ALT), age, and sex in hepatocarcinogenesis and the development of clinical liver cirrhosis. Metabolic syndrome has been found to be independently associated with liver cirrhosis in the patients with chronic hepatitis B. This is the first study to report that HBV genotype C, age (≥ 45 years), male sex, and ALT abnormality are independently associated with probable cirrhosis in community-based HBV-infected subjects, especially with the subclinical liver cirrhosis. Furthermore, this study suggested that fatty liver may not be associated with probable liver cirrhosis.

Applications

This study suggests that genotype C HBV-infected male residents at the age of 45 years or older should be routinely examined for active hepatitis and early cirrhosis. Early intervention to the HBV-infected subjects with high risks of cirrhosis might be effective for decreasing the overall mortality from liver cirrhosis and subsequent HCC.

Terminology

Probable cirrhosis is referred to ultrasonographic cirrhosis (ultrasonographic score ≥ 8) and cirrhosis-like ultrasonographic abnormalities (ultrasonographic score from 5 to 7). Probable cirrhosis is not histologically confirmed liver cirrhosis. Ultrasonography is an imaging examination which is widely accepted by the community-based HBV-infected subjects without apparent clinical manifestations.

Peer review

The results of this study provide sufficient experimental evidences or data from which scientific conclusions can be drawn. The discussion is well organized and an overall theoretical analysis is given.

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***Gentiana manshurica* Kitagawa prevents acetaminophen-induced acute hepatic injury in mice *via* inhibiting JNK/ERK MAPK pathway**

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Abstract

AIM: To investigate the *in vivo* hepatoprotective effects and mechanisms of *Gentiana manshurica* Kitagawa (GM) in acetaminophen (APAP)-induced liver injury in mice.

METHODS: GM (200, 150 or 50 mg/kg body weight) or N-acetyl-L-cysteine (NAC; 300 mg/kg body weight) was administrated orally with a single dose 2 h prior to APAP (300 mg/kg body weight) injection in mice.

RESULTS: APAP treatment significantly depleted hepatic glutathione (GSH), increased serum aspartate aminotransferase (AST), alanine aminotransferase (ALT) and malonyldialdehyde (MDA) and 4-hydroxynonenal levels, and decreased hepatic activity of glutathione peroxidase (GSH-px) and superoxide dismutase (SOD). However, the pretreatment of GM significantly alleviated APAP-induced oxidative stress by increasing

GSH content, decreasing serum ALT, AST and MDA, and retaining the activity of GSH-px and SOD in the liver. Furthermore, GM pretreatment can inhibit caspase-3 activation and phosphorylation of c-Jun-NH₂-terminal protein kinase 2 (JNK1/2) and extracellular signal-regulated kinase (ERK). GM also remarkably attenuated hepatocyte apoptosis confirmed by the terminal deoxynucleotidyl transferase mediated dUTP nick end-labeling method.

CONCLUSION: Hepatoprotective effects of GM against APAP-induced acute toxicity are mediated either by preventing the decline of hepatic antioxidant status or its direct anti-apoptosis capacity. These results support that GM is a potent hepatoprotective agent.

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Key words: *Gentiana manshurica* Kitagawa; Acetaminophen; Oxidative stress; Caspase-3; JNK/ERK MAPK

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INTRODUCTION

Acetaminophen (APAP) is a widely used analgesic and antipyretic drug. It is safe at therapeutic doses, however,

when taken at high doses, APAP can precipitate severe liver injury that may develop into a liver failure^[1,2]. Overdoses of APAP lead to the generation of high amounts of the toxic metabolite N-acetyl-quinoneimine (NAPQI) by the cytochrome P-450 isoenzyme mixed-function oxidase system, which is immediately conjugated with glutathione (GSH), forming the nontoxic metabolites cysteine and mercapturic acid conjugates. Although a number of P450 enzymes can metabolize APAP, the most relevant isoenzyme is CYP2E1^[3]. There is an alternative view that oxidative stress plays a role in hepatotoxicity. Oxidative stress in APAP hepatotoxicity is characterized by several features, including lipid peroxidation, mitochondrial damage and ATP depletion in proteins^[4]. NAPQI reacts with GSH spontaneously or catalyzed by glutathione-S-transferases to form a GSH-adduct. Thus, GSH depletion and formation of protein adducts are key mechanisms of APAP-induced cell death^[5-7].

Many antioxidant agents have been studied in experimental and clinical studies to reduce or prevent APAP-induced hepatotoxicity. The most popular antioxidant for APAP hepatotoxicity is N-acetyl-L-cysteine (NAC)^[8]. Protection by NAC is believed to be attributable to its ability to regenerate GSH stores because of its capacity to provide cysteine residues^[9].

In recent years, plant-derived natural products have received considerable attention due to their diverse pharmacological properties. A growing interest has been observed in the analysis of these natural entities for their potential benefits to human health on hepatoprotective effects. *Gentiana manshurica* Kitagawa (GM) is distributed in northeastern China and reputed “Dongbei longdan”, which is one of the most common herbal medicines used by Chinese people suffering from chronic liver diseases. As an iridoid containing plant, GM has various pharmacological activities. Previous phytochemical studies reported that GM includes loganic acid, 6-O- β -d-glucopyranosylgentiopicroside, swertiamarin, gentiopicroside, sweroside and 2-(o,m-dihydroxybenzyl)-sweroside^[10]. It was reported that gentiopicroside involves down-regulation of NR2B receptors in the anterior cingulate cortex to persistent inflammatory pain^[11]. Animal experiments have revealed adaptogenic^[12] and anti-inflammatory^[13] activities. GM has also been used traditionally as a folk remedy for healing wound^[14,15]. However, no research has been conducted about its hepatoprotective patterns.

Since a high dose APAP-induced hepatotoxicity resulted from the generation of free radicals during its metabolism at liver, the possible protection by GM was evaluated and the results are presented in this paper.

MATERIALS AND METHODS

Chemicals

APAP and NAC were purchased from Sigma Chemicals (St. Louis, MO, USA). Detection kit for GSH and malondialdehyde (MDA), glutathione peroxidase (GSH-px) and superoxide dismutase (SOD) were purchased

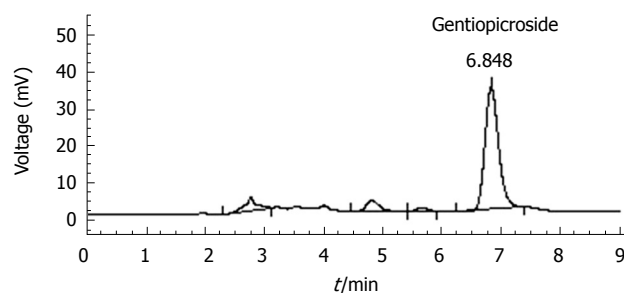


Figure 1 HPLC chromatograms of *Gentiana manshurica* Kitagawa (GM). Column: Diamonsil C18 (250 mm \times 4.6 mm); Flow rate: 1 mL/min; Mobile phase: Methanol and H₂O (35:65).

from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). All other chemicals used were of analytical grade.

Preparation of GM

The rhizomes and roots GM were purchased from Yanbian Puhe, China in March 2006 and authenticated by Prof. Hui-Zi Lv of College of Pharmacy, Yanbian University. The rhizomes and roots of GM (1 kg) were extracted three times with methanol (10 L) and boiled under reflux for 4 h at 40°C, and then the percolated was concentrated in a rotary vacuum evaporator followed by lyophilization. The yield (w/w) of extract was about 22.35%. The freeze-dried extract was used in both chemical analysis and pharmacological studies. GM extract was analyzed on HPLC to confirm the presence of gentiopicroside (Figure 1). The content of gentiopicroside was 2.48% in GM extract. The extract was pre-solubilized in distilled saline for the *in vivo* studies.

Animals and treatment

Male Kunming mice (20-22 g) were provided by Yanbian University Laboratory Animal Center, fed with a standard chow diet and given tap water *ad libitum*. Animals were housed in plastic cages and maintained at 22 \pm 2°C and 50%-60% relative humidity, with a 12-h light-dark cycle throughout the experiment. Animal experiments were performed under the latest edition of “Guiding Principles in the Use of Animals in Toxicology” adopted by the Society of Toxicology (USA).

The mice were fasted overnight (16 h) prior to administration of a single intraperitoneal dose (300 mg/kg) of APAP dissolved in sterile phosphate buffered saline (PBS, pH 7.4) warmed to 40°C^[16]. Animals were divided into 7 groups of ten animals each. Animals of group 1 received vehicle only and served as normal and group 2 treated intraperitoneally with a single dose of APAP (300 mg/kg) was kept as control. Groups 3, 4 and 5 were administered orally with GM extract (50, 100 or 200 mg/kg) 2 h before APAP injection, and served as GM *per se*. Group 6 was treated with NAC (300 mg/kg) 2 h before APAP injection, and served as positive control. Group 7 received only GM extract (200 mg/kg) 2 h after saline injection, instead of APAP injection. Animals were sacrificed and blood was collected from the carotid artery 12 h after administration of APAP. Serum was then separated by

centrifugation at 4°C, 3000 r/min for 30 min. The liver was removed immediately from each mouse, and kept at -80°C until analyzed.

Blood biochemistry

Blood was collected at 12 h after APAP administration. Serum levels of AST and ALT were detected using an Autodry Chemistry Analyzer (SPOTCHEM™ SP4410, Arkray, Japan).

GSH, SOD, GSH-px activities and hepatic lipid peroxidation assay

The removed liver tissue was homogenized in 9 volumes of cold buffer (0.01 mol/L Tris-HCl, 0.0001 mol/L EDTA-2Na, 0.01 mol/L sucrose, and 0.8% saline, pH 7.4) on ice. The homogenate was centrifuged at 4°C (3000 r/min, 15 min) and the supernatant was used for the determination of GSH and MDA, GSH-px and SOD following the manufacturer's instructions. Briefly, the MDA was detected using the thiobarbituric acid reactive substances (TBARS) methods; 4-hydroxynonenal (4-HNE) was detected as a fluorimetric derivative^[17]; the GSH activity was detected through yellow tetramethyl-benzidine and oxidised GSH produced by the combination of GSH and dithio-nitrobenzene; SOD activity was examined through nitroblue tetrazolium coloration; and GSH-px activity was determined through detecting selenium cysteine, the active centre of GSH-px. Protein content was determined with a Bio-Rad Protein Assay Kit (Bio-Rad, USA).

Histopathological analysis

Liver samples obtained at different time points after the APAP injection were fixed in 10% phosphate-buffered formalin and embedded in paraffin. Sections were stained with hematoxylin and eosin for histopathological analysis. The sections were examined under light microscopy and graded for the presence and intensity of lesions using a scale from 0 to 5 (0 = no lesions; 1 = minimal lesions involving a single or few necrotic cells; 2 = mild lesions, 10%-25% necrotic cells or mild diffuse degenerative changes; 3 = moderate lesions, 25%-40% necrotic or degenerative cells; 4 = marked lesions, 40%-50% necrotic or degenerative cells; and 5 = severe lesions, more than 50% necrotic or degenerative cells)^[18,19].

Transferase-mediated dUTP nick end-labeling (TUNEL) assay

Apoptotic cells were detected by the terminal deoxynucleotidyl TUNEL method using an *in situ* cell detection kit (Roche, Mannheim, Germany) for the detection and quantification of apoptosis at a single-cell level. Staining of tissue sections was performed according to the manufacturer's protocol, as follows. Paraffin-embedded sections were dewaxed in xylene and rehydrated by passing through a graded series of ethanol solutions, ending with phosphate-buffered saline. Sections were permeabilized with proteinase K (20 µg/mL in 10 mmol/L Tris-HCl,

pH 7.4-8.0) at 37°C for 15 min. After washing, sections were stained with fluorescent anti-TdT. Sections were viewed and photographed using standard fluorescent microscopic techniques.

Western blotting analysis

The total protein extracts were made by pulverization in a grinder with liquid nitrogen, then using a ratio of 1 mL lysis buffer (150 mmol/L NaCl, 1.0% NP-40, 0.5% NaVO₄, 0.1% SDS, 50 mmol/L Tris, pH 7.5) containing 1 mmol/L PMSF for each 100 mg powdered liver sample. Liver lysates (40 µg) were electroblotted onto a PVDF membrane following separation on 8%-12% SDS-polyacrylamide gel electrophoresis. Blotted membranes were blocked with 5% skim milk in incubation buffer at room temperature, followed by incubation overnight at 4°C with 1:1000 dilution of caspase-3, JNK, ERK (Santa Cruz biotechnology, CA, USA) and phospho-JNK, phospho-ERK (Cell Signaling Technology, MA, USA) primary antibody. Bound antibody was detected with horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology, CA, USA) and immunodetected proteins were visualized using WEST-ZOL™ (plus) Western blotting detection system (iNtRON Biotechnology, Gyeonggi, Korea). Loading accuracy was evaluated by membrane rehybridization with monoclonal antibodies against α -tubulin (Sigma, St. Louis, MO, USA). Densities of the immunoreactive bands were analyzed with the Image Master 1D Elite software (Amersham Pharmacia Biotech, Piscataway, NJ, USA).

Statistical analysis

All values were expressed as mean \pm SD. All other results, except pathological findings, were evaluated by one-way ANOVA and Tukey's multiple comparison tests. Liver histopathological examination data were analyzed by the Kruskal-Wallis nonparametric test, followed by a Mann-Whitney test. Statistically significant differences between groups were defined as *P* values less than 0.05. Calculations were performed with the GraphPad Prism program (Graphpad Software, Inc, San Diego, USA).

RESULTS

Effects of GM on serum AST and ALT levels

Serum activities of ALT and AST are shown in Figure 2. A single dose of APAP significantly elevated the serum ALT (*P* < 0.05) and AST (*P* < 0.01) activities when compared with the normal animals. Pretreatment with GM 2 h prior to APAP administration lowered markedly both serum ALT and AST levels. Serum ALT levels were significantly decreased to 43%, 26%, 13% or 13% in GM groups (200, 100 and 50 mg/kg) or NAC group (300 mg/kg) compared with APAP alone group, respectively. Serum AST levels were significantly decreased to 58%, 14%, 10% or 8% in GM groups (200, 100 and 50 mg/kg) or NAC group (300 mg/kg) compared with APAP alone group, respectively.

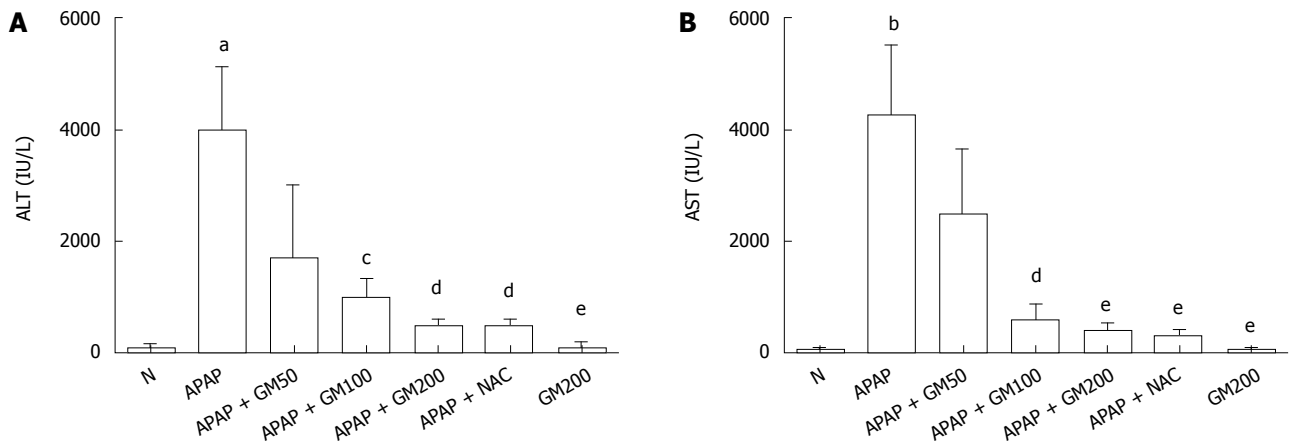


Figure 2 Protective effects of GM on serum biochemical parameters after acetaminophen (APAP) administration. Mice were intraperitoneally injected with APAP (300 mg/kg body weight). GM (200, 100 or 50 mg/kg body weight) was orally administered at 2 h before APAP injection. All data are presented as mean \pm SD, $n = 10$ /group. ^a $P < 0.05$, ^b $P < 0.01$, significantly different when compared with normal controls. ^c $P < 0.05$, ^d $P < 0.01$, ^e $P < 0.001$ significantly different when compared with APAP alone group.

Effects of GM on GSH, SOD, GSH-px activities, MDA and 4-HNE contents

Twelve hours after APAP administration, GSH, GSH-px and SOD concentrations were significantly decreased to 44% ($P < 0.05$), 35% ($P < 0.05$) and 55% ($P < 0.05$) respectively in APAP group compared with the normal group. However, pretreatment with a high dose of GM (200 mg/kg) significantly alleviated subsequent APAP-induced GSH depletion to 1561 ± 186 ($P < 0.05$). SOD ($P < 0.01$) and GSH-px activities ($P < 0.05$) were significantly enhanced in GM 200 mg/kg plus APAP treated group. MDA levels increased by 252% in mice treated with APAP, indicating that APAP administration significantly increased lipid peroxidation in liver ($P < 0.01$). Briefly, in mice receiving GM (200 or 100 mg/kg) plus APAP, the MDA levels were significantly reduced to 42% ($P < 0.01$) or 43% ($P < 0.01$) as compared with the APAP treated mice. In mice receiving GM (200 mg/kg) plus APAP, the 4-HNE levels were significantly reduced to 59% ($P < 0.05$) compared with the APAP treated mice (Figure 3).

Effects of GM on histopathology

Histopathological analysis of the APAP alone treated animal showed severe centrilobular necrosis, fatty infiltration and lymphocytes infiltration (data not shown), which were significantly less in the GM plus APAP treated groups with mild sinusoidal congestion, less inflammatory cell infiltration, and well preserved hepatocytes with less area of necrosis (Table 1 and Figure 4).

GM protects against APAP-induced hepatocyte apoptosis in mice via inhibiting caspase-3 and JNK/ERK MAPK pathway

Apoptotic hepatocytes were detected by TUNEL staining. A large number of TUNEL-positive hepatocytes were seen in the livers of APAP-treated mice 12 h after injection, however, a few TUNEL-positive hepatocytes were found in livers from animals pretreated with GM (Figure 4). The protease activity of caspase-3 was measured in APAP-induced liver injury mice treated with

and without GM. Caspase-3 was proteolytically processed to the active p17 fragment 12 h after APAP treatment in mice (Figure 5). However, GM significantly inhibited caspase-3 activity. It has recently been reported that c-Jun-NH2-terminal protein kinase 2 (JNK1/2) plays a critical role in mediating APAP hepatotoxicity in mice^[20]. JNK1/2 activation is an early key signal in mediating mitochondria-mediated lethal cell injury triggered by toxicants in hepatocytes^[21]. Therefore, we investigated whether APAP-induced JNK activation was attenuated by GM. Our data revealed that phosphorylated JNK and phosphorylated ERK, significantly increased after treatment with APAP when oxidative stress in the liver had been significantly enhanced as described above, while the JNK and ERK total protein level were almost normal 12 h after APAP treatment (Figure 5). After administrated with various doses of GM 2 h *via* APAP injection, phosphorylated JNK and phosphorylated ERK levels were declined (Figure 5). These data are consistent with our hypothesis that GM inhibits JNK/ERK MAPK signaling pathway.

DISCUSSION

Progress has been achieved in research of the chemical constituents and pharmacological activities of genus *Gentiana*. It is reported that gentianine from *Gentiana Macrophylla* Radix, one of *Gentiana* species, might express anti-inflammatory activities at least partly through preventing the immune cells, including macrophages, from producing TNF- α and IL-6, pro-inflammatory cytokines in male *Sprague Dawley* rats treated with LPS^[22]. Hepatoprotective effects of *Gentiana olivieri* Griseb, flowering herbs on subacute administration were studied using *in vivo* models in rats, and the remarkable hepatoprotective activity of *Gentiana olivieri* might be due to the potent antioxidant activity of iso-orientin^[23]. However, this is the first study to report the hepatoprotective effects of GM against APAP-related liver toxicity.

APAP is a safe and effective analgesics when used at therapeutic doses, an overdose of APAP, however, can

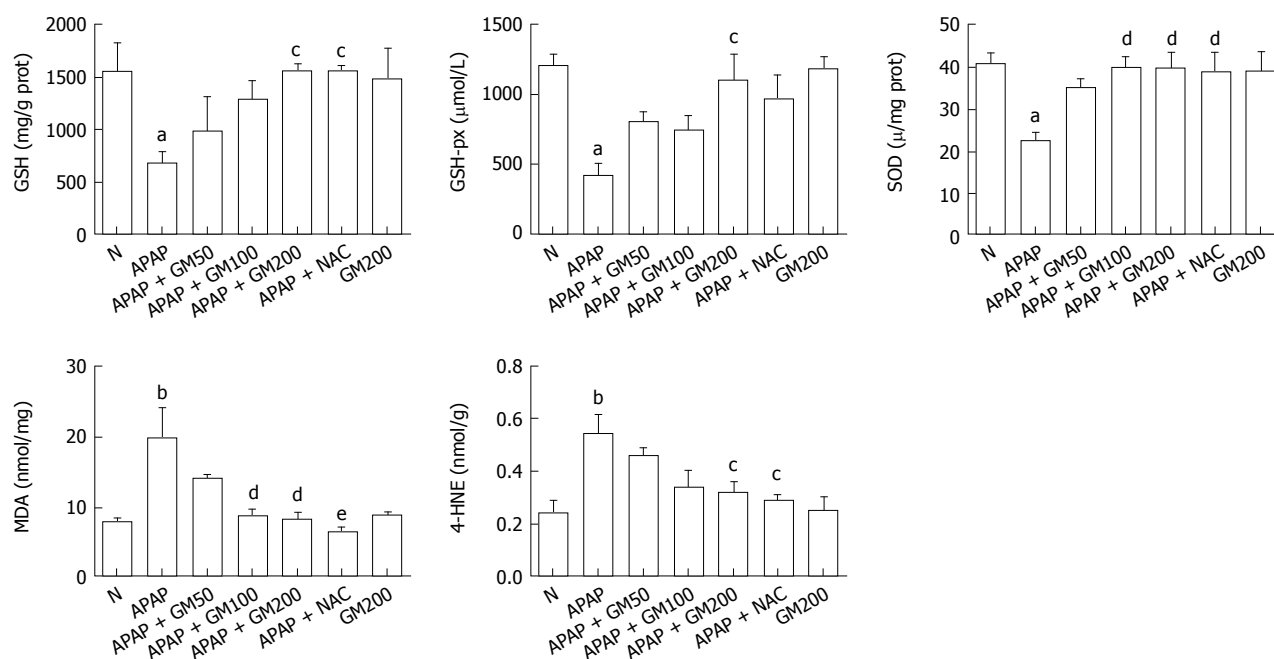


Figure 3 Protective effects of pretreatment with GM against APAP-induced glutathione (GSH) depletion, malonyldialdehyde (MDA) levels, 4-hydroxynonenal (4-HNE) levels and superoxide dismutase (SOD), glutathione peroxidase (GSH-px) activity in liver of mice. APAP was given intraperitoneally with a single dose of 300 mg/kg. GM was given orally with a single dose of 200, 100 or 50 mg/kg. All data are presented as mean \pm SD, $n = 10/\text{group}$. ^a $P < 0.05$, ^b $P < 0.01$, significantly different when compared with normal controls. ^c $P < 0.05$, ^d $P < 0.01$, ^e $P < 0.001$ significantly different when compared with APAP alone group.

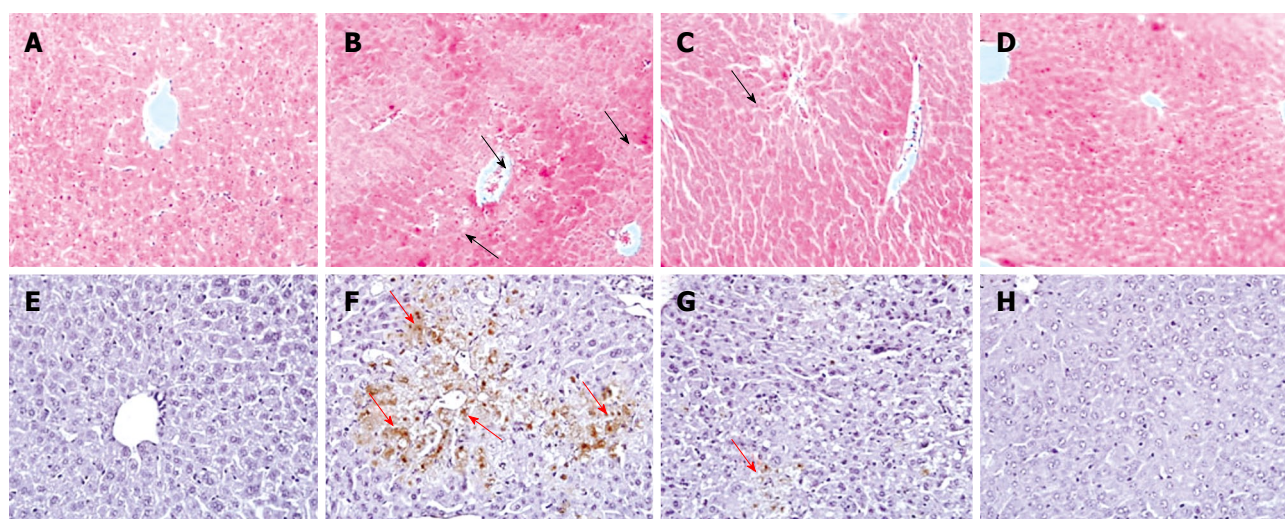


Figure 4 Liver histopathology and transferase-mediated dUTP nick end-labeling (TUNEL) assay. APAP was given intraperitoneally with a single dose of 300 mg/kg. GM was given orally with a single dose of 200 mg/kg. A and E: Normal group; B and F: APAP group; C and G: APAP plus GM 200 mg/kg; D and H: Only GM 200 mg/kg. Upper panels stand for HE staining and the lower panels for TUNEL assay. The regions by black arrows in the upper figures indicate the hepatocyte necrosis or hepatocyte degeneration; the regions by red arrows in the lower figures indicate the TUNEL-positive apoptotic cells ($\times 100$ magnification).

induce severe hepatotoxicity in experimental animals and in human^[24,25]. Liver injury induced by APAP is commonly used for the screening of hepatoprotective drugs^[16]. NAC is used currently in clinical treatment for APAP overdose. Hereby, we used NAC as positive control, to compare with GM on hepatoprotective effects.

Administration of a single high dose of APAP significantly ($P < 0.01$) elevated the serum transaminase activities compared with the normal controls (Figure 2). The significantly decreased serum transaminases activities in the GM administered groups prior to APAP demonstrated

its hepatoprotective effects. Thus, a single dose of methanol extract of GM could render a complete protection.

Cytochrome P-450 enzymes are the major catalysts involved in the metabolism of drugs. APAP is mainly metabolized by cytochrome P-450 to form an electrophilic metabolite, N-acetyl-p-benzoquinonimine, which is primarily inactivated by conjugation with GSH^[26,27]. A large number of the metabolites produced by APAP are found to generate superoxide anion and other free radicals in the biological systems^[28]. However, at a higher dose of APAP, intermediate metabolites accumulate and cause

Table 1 Effects of GM on hepatic damage induced by APAP in mice

Treatment	Dose (mg/kg)	Histopathological scores						Average
		0	1	2	3	4	5	
Saline + Saline	-	10	0	0	0	0	0	0.0
APAP + Saline	-	0	0	1	3	4	2	3.7
APAP + GM	200	0	4	3	2	1	0	2.0 ^b
	100	0	3	3	2	2	0	2.3 ^a
	50	0	2	3	2	2	1	2.7
APAP + NAC	300	0	3	4	2	1	0	2.1 ^b

APAP was given intraperitoneally with a single dose of 300 mg/kg. GM was given orally with a single dose of 200, 100 or 50 mg/kg. Livers were graded from 0 to 5 as described in Materials and Methods. Each value is the number of animals with grading changes. ^a*P* < 0.05, ^b*P* < 0.01 significantly different when compared with APAP alone group. GM: *Gentiana manshurica* Kitagawa; APAP: Acetaminophen.

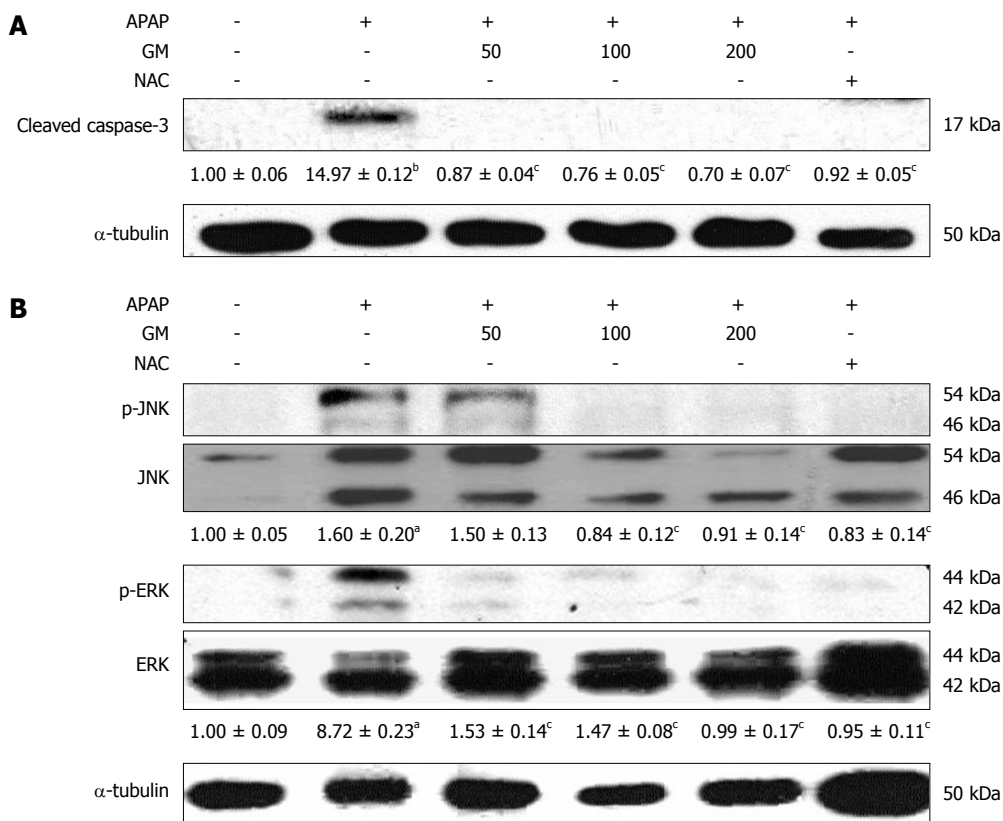


Figure 5 Western blotting analysis of caspase-3 and JNK/ERK MAPK. GM (200, 100 or 50 mg/kg), NAC (300 mg/kg) or saline was orally administered 2 h before APAP injection. Active form of caspase-3, phospho-JNK and phospho-ERK were detected by Western blot. The active form of caspase-3 levels corresponding to each immunoreactive band were digitized and expressed as a percentage of the α-tubulin levels. Densitometric scanning data of phospho-JNK and phospho-ERK levels were expressed as the ratio of JNK or ERK, respectively. The ratio of the normal group band was set to 1.00. Data of three independent experiments are expressed as mean ± SD. ^a*P* < 0.01, ^b*P* < 0.001, significantly different when compared with the normal group. ^c*P* < 0.001 significantly different when compared with APAP alone group.

liver damage. Depletion of GSH beyond certain critical level can lead to oxidative stress and development of overt hepatotoxicity^[29].

The decreased hepatic antioxidant status is related to oxidative stress and elevation of lipid peroxidation that lead to the leakage of hepatic enzymes to serum in APAP alone treated animals. To determine whether GM could inhibit APAP-induced GSH depletion, we measured the hepatic GSH levels. Our results showed that co-treatment with GM and APAP inhibited APAP-induced GSH depletion (Figure 3). NAC reduced APAP hepatic toxicity

by increasing hepatic GSH levels. The increasing GSH levels had no significant differences between the group pretreated with GM (200 mg/kg) and NAC. The elevated hepatic reduced GSH level could partially explain the hepatoprotective mechanism of GM. Reduced GSH can function as a reductant in the metabolism of hydrogen peroxide and various organic peroxides. The GSH-px present in the cells can catalyze this reaction^[30]. It is reported that depletion of GSH below a threshold value was associated with a significant conversion of xanthine dehydrogenase to reversible xanthine oxidase, a superoxide

radical generation reaction catalyzing enzyme. Therefore, the enhanced hepatic GSH-px and SOD activities in the GM plus APAP treated group further support its hepatoprotective effects (Figure 3). MDA and HNE are major end-products of oxidation of polyunsaturated fatty acids, and are frequently measured as indicators of lipid peroxidation and oxidative stress *in vivo*. Thereby, the elevated antioxidant status in the liver of GM plus APAP treated group is related to the decreased MDA level and 4-HNE level, could maintain the membrane integrity and prevented lipid peroxidation and was comparable to NAC (Figure 3). The histopathological analysis of liver section indicates a moderate centrilobular necrosis, fatty infiltration and lymphocytic infiltration in the GM plus APAP treated animals with respect to the APAP alone treated animals (Table 1 and Figure 4).

APAP was believed to induce apoptosis based on the observation that APAP treatment results in the activation of caspase-3. In this study, pretreatment with GM prior to APAP inhibited caspase-3 cleavage (Figure 5). Thus, the active form of caspase-3 was not observed in GM or NAC pretreated groups. Microscopic observation on TUNEL-stained sections demonstrated that GM significantly decreased the TUNEL-positive apoptotic hepatocytes. Furthermore, it was reported that p38 MAPK, JNK, and ERK were activated by APAP^[31]. JNK2 plays a protective role against APAP-induced liver injury in mice, in part, by modulating hepatocellular regeneration and repair, which further suggests the use of JNK inhibitors as a potential treatment for APAP-induced liver injury^[32]. In both cultured hepatocytes and *in vivo* livers, treatment with APAP induced a sustained activation of JNK as reflected in increased phospho-c-jun levels^[33]. A number of studies have suggested that oxidative stress leads to JNK activation, either through redox alteration of the sequestration of JNK or through inhibition of JNK phosphatase^[34,35]. Our data showed that phospho-JNK1/2 expression was greatly increased 12 h after APAP administration (Figure 5). Coinciding with the expression of phospho-JNK1/2, APAP also increased the level of phospho-ERK1/2. However, GM pretreatment can effectively inhibit phosphorylation of ERK1/2 (Figure 5). These data suggest that GM inhibits the JNK/ERK signaling pathway at a more proximal regulatory step, resulting in inhibition of its downstream effector mechanisms.

In conclusion, in this study, GM can significantly prevent the APAP-induced acute hepatotoxicity by enhancing the hepatic antioxidant activity, and inhibiting the caspase-3 cleavage and JNK/ERK MAPK activation. GM exerts some effects which resemble those of an antidote of acetaminophen such as NAC. However, further detailed studies are required to confirm its clinical application.

COMMENTS

Background

Acetaminophen (APAP) is a widely used analgesic and antipyretic drug that is safe at therapeutic doses, however, when taken at high doses, APAP can precipitate severe liver injury that can develop into a liver failure. Overdoses of APAP lead to the generation of high amounts of the toxic metabolite N-acetyl-

quinoneimine (NAPQI). NAPQI reacts with glutathione (GSH) spontaneously or catalyzed by glutathione-S-transferases to form a GSH-adduct. Thus, GSH depletion and formation of protein adducts are key mechanisms of APAP-induced cell death.

Research frontiers

In recent years, plant-derived natural products have received considerable attention due to their diverse pharmacological properties. Further studies on hepatoprotective effect of these natural entities and its possible mechanism are important for understanding the mechanism of APAP-induced liver injury.

Innovations and breakthroughs

The authors investigated the effects of *Gentiana manshurica* Kitagawa (GM) on APAP-induced liver injury in mice and whether GM prevents APAP-induced hepatocyte apoptosis *in vivo*. The present study concluded that GM can significantly prevent the APAP-induced acute hepatotoxicity by enhancing the hepatic antioxidant activity, and inhibiting the caspase-3 cleavage and JNK/ERK MAPK activation, and GM exerts some effects which resemble those of an antidote of acetaminophen such as NAC.

Applications

The results provide significant evidence illustrating the key feature of recovery from APAP-induced acute liver injury.

Peer review

This is an interesting paper that, although descriptive, points out to the potential hepatoprotective effect of a herbal compound (*Gentiana manshurica* Kitagawa: GM) on the acetaminophen-induced acute hepatotoxicity. Results are clear and manuscript is well written.

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A laterally-spreading tumor in a colonic interposition treated by endoscopic submucosal dissection

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Abstract

Herein we describe an early colonic carcinoma which developed in a colonic interposition 14 years after surgery for esophageal cancer, which was successfully treated by endoscopic submucosal dissection (ESD). An 80-year-old man underwent colonic interposition between the upper esophagus and stomach after surgery for an early esophageal squamous cell carcinoma in 1994. He received a surveillance endoscopy, and a laterally-spreading tumor of granular type, approximately 20 mm in size, was identified in the colonic interposition. An endoscopic biopsy revealed moderately differentiated adenocarcinoma histologically, however, we diagnosed the lesion as an intramucosal carcinoma based on the endoscopic findings. The lesion was safely and completely removed *en bloc* by ESD using a bipolar knife. Histologically, the lesion was an intramucosal moderately differentiated adenocarcinoma in a tubular adenoma.

INTRODUCTION

Although rarely reported, adenoma and adenocarcinoma can occur as a late complication in colon segments used to replace the esophagus. Herein, we describe an early colonic carcinoma which developed in a colonic interposition 14 years after surgery for esophageal cancer, which was successfully treated by endoscopic submucosal dissection (ESD).

CASE REPORT

An 80-year-old man underwent colonic interposition between the upper esophagus and stomach after surgery for an early esophageal squamous cell carcinoma (T1, N0, M0, stage I according to the TNM classification) in 1994. He received an esophagogastroduodenoscopy for surveillance and a laterally-spreading tumor of granular type (LST-G), approximately 20 mm in size, was identified in the colonic interposition. On conventional view, a

Table 1 Summary of reported cases of neoplasia arising in a colonic interposition

Case	Authors	Age	Gender	Size (mm)	Histology	Period after surgery (yr)	Follow up	Therapy	Course
1	Goldsmith <i>et al</i> ^[5] , 1968	48	F	50	Adenocarcinoma	2	+	Surgery	Follow up
2	Szántó <i>et al</i> ^[6] , 1981	65	M	5	Adenomatous polyp	1	-	Polypectomy	Follow up
3	Haerr <i>et al</i> ^[7] , 1987	72	M	NI	Adenocarcinoma	9	+	Radiation chemotherapy	Death
4	Houghton <i>et al</i> ^[8] , 1989	64	M	NI	Adenocarcinoma	20	-	Surgery	Follow up
5	Theile <i>et al</i> ^[9] , 1992	68	M	29	Adenocarcinoma	12	NI	Surgery	Follow up
6	Lee <i>et al</i> ^[10] , 1994	75	F	NI	Adenocarcinoma	20	+	Surgery	Follow up
7	Altörjay <i>et al</i> ^[11] , 1995	NI	M	60	Adenomatoid polyp	6	+	Surgery	Death
				NI	Carcinoma				
8	Kovacs <i>et al</i> ^[12] , 1997	8	M	9	Tubular adenoma	13	+	Polypectomy	Follow up
				11	Tubular adenoma				
9	Altomare <i>et al</i> ^[13] , 2006	64	M	6	Tubular adenoma	7	+	Polypectomy	Follow up
10	Present case, 2008	80	M	25	Adenocarcinoma in tubular adenoma	14	-	ESD	Follow up

ESD: Endoscopic submucosal dissection; NI: No information.

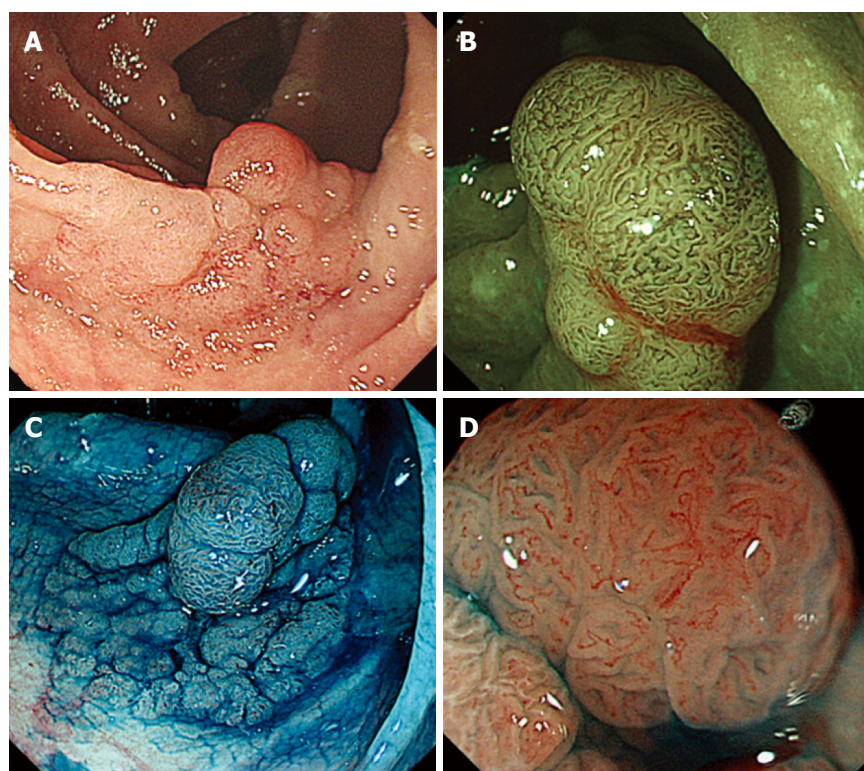


Figure 1 A laterally-spreading tumor of granular type (LST-G) in the colonic interposition was shown at colonoscopy. Narrow-band imaging with magnification revealed a capillary pattern type II. Magnifying chromoendoscopy using 0.4% indigo carmine revealed a type IV pit pattern. A: Conventional view; B: Narrow-band imaging with magnification; C: Chromoendoscopy with 0.4% indigo carmine; D: Magnifying chromoendoscopy using 0.4% indigo carmine dye spraying.

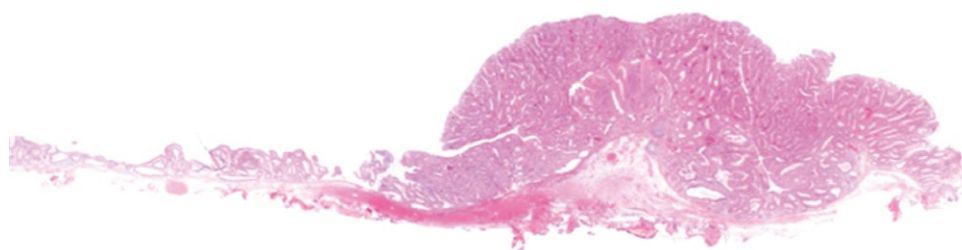


Figure 2 Histologically, the resected specimen showed an intramucosal adenocarcinoma in a tubular adenoma. Cross sectional view (HE, magnification $\times 5$).

large, reddish nodule was detected in the lesion. With magnifying narrow-band imaging (NBI) observation, the lesion revealed a capillary pattern type II according to Sano's classification^[1], and a type IV pit pattern according to Kudo's classification was detected under magnifying chromoendoscopy using 0.4% Indigo carmine dye

spraying^[2]. An endoscopic biopsy was taken from the large nodule and a histological diagnosis of moderately differentiated adenocarcinoma was established, however, we diagnosed the lesion as an intramucosal carcinoma based on the above endoscopic findings (Figure 1). Thus, the lesion was considered a good candidate for endoscopic

resection. The lesion was safely and completely removed *en bloc* by ESD using a bipolar knife (B-knife® XEMEX Co. Ltd. Tokyo, Japan)^[3,4]. Histologically, the lesion was an intramucosal moderately differentiated adenocarcinoma in a tubular adenoma. Lateral and vertical margins of the specimen were negative. There was no lymphatic and venous invasion (Figure 2). The patient was hospitalized for 6 d after ESD to confirm the absence of complications such as delayed perforation, and was then discharged.

DISCUSSION

Despite the fact that many interposition grafts are performed for malignant esophageal disease, to the best of our knowledge, there have only been 10 reported cases, including four adenomatous polyps and six adenocarcinomas, arising in a colonic interposition (Table 1)^[5-13]. Because the sizes of the adenomatous polyps in the reported cases were small, they were treated with polypectomy. Reoperation or chemoradiotherapy was performed in patients with cancers. Therefore, this is the first case of an early adenocarcinoma in a colonic interposition resected by ESD.

We performed ESD instead of endoscopic mucosal resection (EMR) in this case, as the lesion was not well-elevated even after submucosal injection of glycerol. This phenomenon is the so-called “non-lifting sign positive” as determined by Uno *et al.*^[14]. As our endoscopic diagnosis of an intramucosal carcinoma was established with magnifying NBI and chromoendoscopy, submucosal benign fibrosis rather than desmoplastic reaction created by invasive cancer was considered to cause the non-lifting sign positive. EMR for the lesion with the non-lifting sign positive may result in incomplete resection or unfavorable complications such as colonic perforation. During ESD, hyaluronic acid was additionally injected into the submucosal layer and a transparent hood was attached to the tip of the scope for better submucosal dissection^[15]. To reduce deep burn to the muscle layer, we used a bipolar knife instead of a monopolar knife. To reduce operating time, we used a bipolar snare to remove the lesion after adequate dissection. These efforts enabled us to completely and safely remove the lesion *en bloc* without complication. Furthermore, the patient's colonic interposition was reconstructed using the subcutaneous route, and thus the risk of mediastinitis even if perforation occurred was lower than that if reconstructed substantially.

Despite the fact that many interposition grafts are performed for malignant esophageal disease, few reports of adenocarcinoma arising in a colonic interposition have been reported. It is commonly thought that patients who have esophageal malignancy carry a dismal prognosis, and few of these patients will survive long enough to develop colonic adenocarcinoma. However, with recent progress in chemotherapy, many patients have long-term survival. Almost all case reports presenting with adenoma or adenocarcinoma arise five or more years after colonic interposition surgery, and there are only two case reports where adenoma or adenocarcinoma in the

colonic interposition has arisen 1 or 2 years after surgery (Table 1). In our case, adenocarcinoma in a tubular adenoma was detected 14 years postoperatively. Colonoscopic screening is usually performed before colonic interposition. However, Heresbach *et al.*^[16] reported an overall miss rate of 23.4% in the colonoscopic detection of neoplasia including both adenomas and colorectal cancers. Therefore, we recommend upper endoscopic screening within 1 year of colonic interposition and periodic surveillance, as lesions may be detected early and removed safely by endoscopy.

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Actinomyces of the appendix mimicking appendiceal tumor: A case report

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Abstract

Actinomyces is an uncommon chronic infectious disease. Common sites of involvement include the cervicofacial, thoracic and abdominopelvic regions. In abdominopelvic actinomyces, the ileocecal region, including the appendix, is the most commonly involved site. In some reports, limited appendiceal actinomyces has revealed a thickened appendiceal wall with peri-appendiceal inflammation as acute appendicitis or perforated appendicitis. We experienced pathologically confirmed intraluminal limited appendiceal actinomyces without peri-appendiceal infiltration. Here, we report the computed tomography and ultrasound findings.

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Key words: Actinomyces; Actinomyces; Appendiceal neoplasms; Appendicitis

INTRODUCTION

Actinomyces is a chronic progressive suppurative disease caused by *Actinomyces israelii*, and is characterized by the formation of multiple abscesses, draining sinus, abundant granulation, and dense fibrous tissue^[1]. Several reports have described the radiological findings of abdominopelvic actinomyces. The infiltrative mass with unusual aggressiveness is the one of important radiological findings^[1-3]. Also, some reports of appendiceal actinomyces have described wall thickening and peri-appendiceal inflammation, with contrast enhancement^[4,5]. We experienced pathologically confirmed appendiceal actinomyces that presented as a small intraluminal mass without peri-appendiceal infiltration. Here, we report the computed tomography (CT) and ultrasound (US) findings.

CASE REPORT

A 50-year-old woman was found incidentally to have a small appendiceal mass during routine screening. Past clinical history, physical examination, and laboratory examination were all unremarkable. Contrast-enhanced abdominal CT showed a well-defined small mass at the origin of the appendix. The length of the mass on CT



Figure 1 Contrast-enhanced CT revealed a well-defined solid mass with strong enhancement in the base of the appendix (arrow). Peri-appendiceal infiltration was not seen.

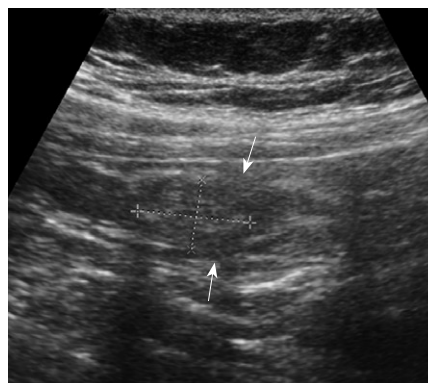


Figure 2 US showed a heterogeneous, hyperechoic, intraluminal mass at the base of the appendix, without peri-appendiceal infiltration. We also noted focal defects at the echogenic inner mucosal layer (arrows).

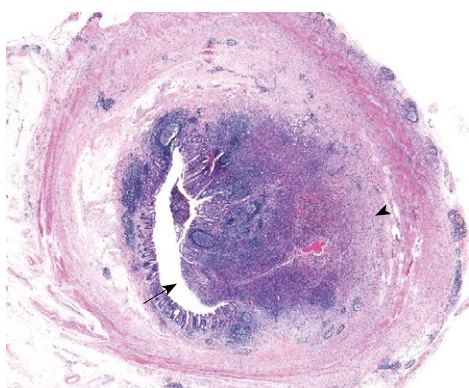


Figure 3 Microscopy of appendiceal actinomycosis. An abscess composed of chronic and acute inflammatory cells was observed in a mass-like lesion (arrow), from the mucosal surface to the superficial submucosa (arrowhead) (HE, $\times 10$).

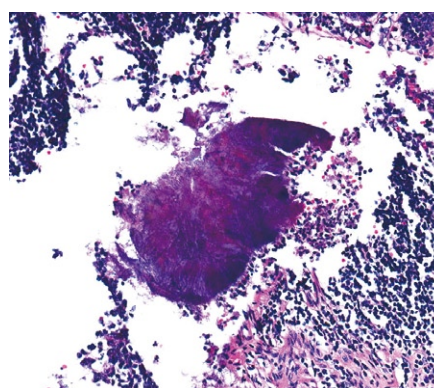


Figure 4 Higher magnification showed a typical sulfur granule surrounded by neutrophils in the clefted abscess center (HE, $\times 200$).

images was 2 cm. This mass showed dense enhancement of 100 HU at the arterial phase and 125 HU at the portal phase. Infiltration around the meso-appendix and peri-appendiceal area was not demonstrated by CT, nor was regional lymph node enlargement detected (Figure 1). Positron emission tomography (PET) with [F18]-2-fluoro-2-deoxy-D-glucose (FDG) showed a high metabolic rate, with a maximum standardized uptake value (SUV) of 3.64.

US images obtained with a 9.4-MHz linear probe showed a well-defined intraluminal mass with heterogeneous echogenicity in the base of the appendix. The diameter of the mass was 1.6 cm. Peri-appendiceal infiltration was not demonstrated by US. US revealed focal defects at the echogenic inner mucosal layer. However, relatively well preserved mural echogenicity was noted (Figure 2).

From these findings, appendiceal neoplasms of mucosal origin, such as mucinous adenocarcinoma and carcinoid, were diagnosed, and a laparoscopic partial cecectomy was performed. Surgical findings revealed an intraluminally growing small mass with no sign of macroscopic serosal invasion. Also, there was no sign of infiltration or invasion of the meso-appendix and other surrounding organs.

Gross pathology revealed an ill-defined, yellowish

white, protruding mass lesion, which originated from the basal portion of the appendix. Adjacent appendiceal and cecal wall showed edema, and the serosal surface was relatively clear. Upon microscopy, the appendix showed a localized, mass-like abscess formation of the appendiceal wall (Figure 3). With higher magnification, a typical sulfur granule surrounded by neutrophils was found (Figure 4), which was confirmed as appendiceal actinomycosis. At the submucosa, the outer portion of the abscess showed evidence of organization, with vascularized granulation tissue and fibrosis in addition to chronic inflammatory cell infiltration.

DISCUSSION

Actinomycosis has a worldwide distribution and is found with equal frequency in urban and rural dwellers. In humans, *Actinomyces israelii* is the most common cause of the disease. These organisms are indigenous in the oral cavity, gastrointestinal tract, and genital tract, with opportunistic infection occurring when the mucosal barrier is broken, which leads to multiple abscess formation, fistula, or mass lesions^[6,7]. Actinomycosis commonly occurs in three distinct forms that may occasionally overlap; most clinical disease is cervicofacial (55%), with only 20% occurring in the abdominopelvic

form and 15% in the thoracic form^[8]. Although the clinical features depend on which organs are involved, common symptoms and signs include fever and leukocytosis^[3,6].

Abdominopelvic actinomycosis is associated with abdominal surgery (such as appendectomy), bowel perforation, or trauma^[9]. In addition, the presence of a long-standing intrauterine device (IUD) is a reported risk factor in young women^[10]. Although the pathogenesis of abdominopelvic actinomycosis is not well understood, the appendix is the most commonly involved intra-abdominal organ, the colon, stomach, liver, gallbladder, pancreas, small intestine, pelvis, and abdominal wall may also be involved^[6]. However, development of abdominopelvic actinomycosis after acute appendicitis has decreased because of early diagnosis, a lower incidence of perforated appendicitis, and improved antibiotic therapy^[8].

As a result of its resemblance to other diseases such as appendicitis, diverticulitis, colon carcinoma, Crohn's disease, ulcerative colitis, and tubo-ovarian abscess, the diagnosis of abdominopelvic actinomycosis is difficult^[11]. A definite diagnosis is generally based on histological identification of actinomycotic granules or culture of the *Actinomyces* species, or both. High-dose intravenous penicillin injection is the treatment of choice and has a favorable response^[11]. Therefore, early diagnosis is important to minimize morbidity of the disease and prevent unnecessary surgery.

Direct spread into adjacent tissue is the most common primary route of propagation after penetration of the organism through the mucosal barrier. Therefore, infiltration has been well described as an important radiological characteristic^[1,3]. CT is an important imaging modality for suggesting the diagnosis and determining the anatomical location and extent of the disease, as well as monitoring the effectiveness of treatment^[2,3,6]. Important CT features are an infiltrative mass (predominantly cystic or solid) adjacent to the other involved organs or anatomical structures, and the main CT feature when the gastrointestinal tract is involved is bowel wall thickening^[1-3]. After infusion of contrast material, dense contrast enhancement in the mass or involved bowel has been reported, which may be caused by abundant granulation and dense fibrous tissue^[1,3,10]. Although these findings are nonspecific, actinomycosis should be included in the differential diagnosis, especially in patients with abdominal pain, fever, leukocytosis, or

long-term IUD use^[6,9]. CT features of abdominopelvic actinomycosis closely resemble complicated gastrointestinal malignancy or other chronic inflammatory disease (such as intestinal tuberculosis or Crohn's disease). However, because of the size of the bacterium, it usually does not spread via the lymphatic system; therefore, regional lymphadenopathy is uncommon or develops late^[8,9].

In our case, appendiceal actinomycosis showed dense contrast enhancement, especially at the portal phase, and vascularized granulation tissue with fibrosis in the submucosa. There was no sign of regional lymphadenopathy. However, appendiceal actinomycosis presented as an intraluminal limited mass without peri-appendiceal infiltration, which is an unusual finding. A review of the literature about abdominopelvic actinomycosis did not reveal any similar cases.

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Cerebral lipiodol embolism following transcatheter arterial chemoembolization for hepatocellular carcinoma

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Abstract

Cerebral lipiodol embolism (CLE) is an extremely rare complication of transcatheter arterial chemoembolization for hepatocellular carcinoma (HCC). The authors present a case of CLE that occurred after the second hepatic arterial chemoembolization for HCC, and attempt to introduce several plausible mechanisms of CLE, after reporting the clinical and radiological findings and reviewing the medical literature.

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Key words: Intracranial embolism; Lipiodol; Chemo-therapeutic embolization; Hepatocellular carcinoma

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INTRODUCTION

Transcatheter arterial chemoembolization (TACE) is utilized worldwide in the treatment of patients with unresectable hepatocellular carcinoma (HCC). The documented complications of TACE include post-embolization syndrome, septicemia, acute hepatic failure, liver infarction or abscess, intrahepatic biloma, embolization of extrahepatic organs, pseudoaneurysm formation, cholecystitis, tumor rupture, splenic infarction, gastritis, duodenitis, gastroduodenal ulceration, variceal bleeding, and iatrogenic dissection^[1-5]. Cerebral lipiodol embolism (CLE) is a rare complication of TACE. The authors report a case of CLE that occurred after the second TACE, and present its clinical and imaging findings, as well as a review of the literature.

CASE REPORT

A 41-year-old man with multiple HCC accompanied by portal vein invasion (Figures 1 and 2) underwent a second course of TACE via the proper hepatic artery by using a mixture of 30 mL lipiodol, 10 mg hydroxyl camptothecin and 40 mg pirarubicin, as well as gelatin sponge particles (1400-2000 μ m) and coils (5 mm \times 8 mm) (Figure 3). The tumor marker test showed 136.9 μ g/L alpha fetoprotein (AFP), 0.7 μ g/L carcinoembryonic antigen (CEA), and of 28.2 μ g/mL carbohydrate antigen 19-9 (CA19-9). The liver function was ranked A in Child-Pugh classification. During the procedure, the patient experienced cough, visual loss, headache and motor weakness of the left upper limb and left lower limb. Upon physical examination, blood pressure was 158/93 mmHg, and pulse rate, respiratory rate and body temperature were normal. The muscle strength of the left upper limb was III+, while the left lower limb was IV+. No neurological pathological reflex was observed. The peripheral oxygen saturation was 94%, and with a nasal catheter, the arterial oxygen saturation was elevated and maintained higher than 97%. The patient was given neurotrophins, cerebrovascular dilators and supportive therapy immediately. Functional exercise was added in the following days.



Figure 1 Computed tomography (CT) scan obtained before the first Transcatheter arterial chemoembolization (TACE) procedure. Portal vein was shown during the arterial period, which suggested that the tumor embolus invaded the portal vein (arrow).

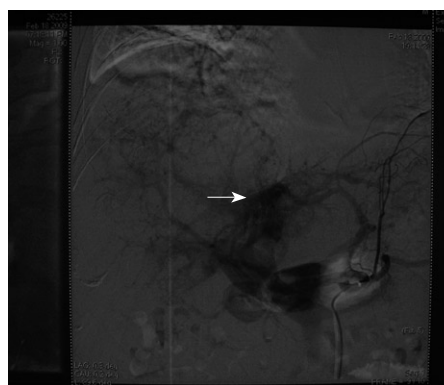


Figure 2 First TACE procedure. Arterial-portal fistula (arrow).

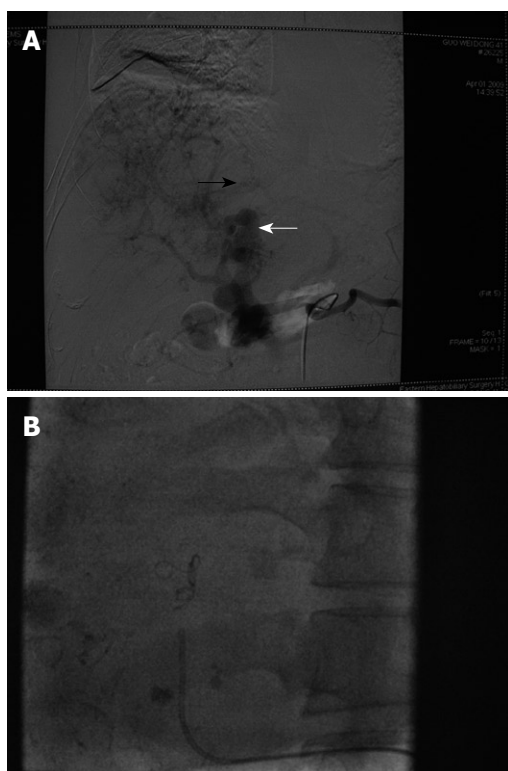


Figure 3 Second TACE procedure. A: Hepatic arteriovenous fistula (black arrow), arterial-portal fistula (white arrow); B: Coils (5-8 mm).

The laboratory data after TACE revealed a white blood cell count of $13.51 \times 10^9/L$ (normal: 4×10^9 to $10 \times 10^9/L$), platelet count of $83 \times 10^9/L$ (normal: 100×10^9 to $300 \times 10^9/L$), total bilirubin of $28.6 \mu\text{mol/L}$, direct bilirubin of $11.0 \mu\text{mol/L}$, alanine aminotransferase of 187.4 U/L , aspartate aminotransferase of 429.7 U/L , and normal renal function. Consultation with a neurologist suggested a diagnosis of cerebral embolism induced by the introduction of iodized oil. Magnetic resonance imaging (MRI) was performed 19 h after the TACE procedure, which revealed multiple abnormal signals that indicated ischemic foci of the centrum semiovale, and both parietal and occipital lobes (Figure 4). On the 10th day, with the

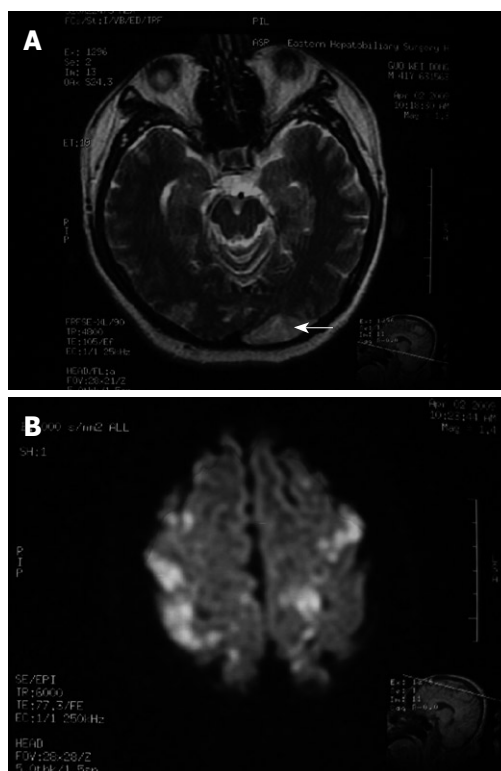


Figure 4 Cranial magnetic resonance imaging (MRI) obtained after the second TACE procedure. A: cerebral lipiodol embolism (arrow); B: cerebral lipiodol embolism (multiple high signals).

appearance of melena, erythrocyte suspension and plasma were transfused along with hemostatic agents. Melena disappeared 2 d later. Vision and muscle strength of the affected extremities also improved gradually. Six weeks after CLE, the patient completely recovered without any neurological sequelae.

Two months after the second TACE procedure, the patient came back for follow-up. The tumor markers were tested: AFP $11.0 \mu\text{g/L}$, CEA $0.8 \mu\text{g/L}$, CA-19-9 12.5 U/mL , and digital subtraction angiography (DSA) revealed that an arterial-portal fistula was still present but improved, meanwhile, hepatic arteriovenous fistula was not seen. Therefore, we performed a third course of TACE. A mixture of 8 mL lipiodol, 10 mg hydroxyl

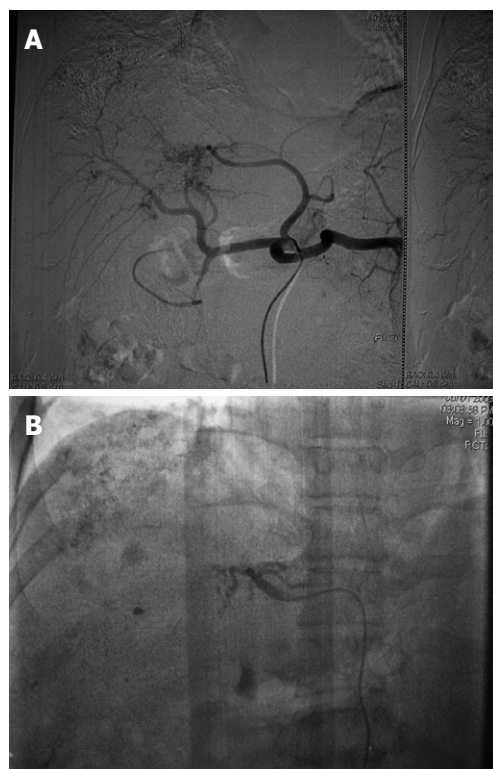


Figure 5 Third TACE procedure. A: Hepatic arteriovenous fistula was not seen, and the arterial-portal fistula was still present but improved; B: Lipiodol and gelatin sponge were injected through the left gastric artery and the arterial-portal fistula was embolized.

camptothecin and 40 mg pirarubicin, as well as gelatin sponge particles (760-1000 μm) was injected through the right hepatic artery, left hepatic artery and left gastric artery (Figure 5). The patient did not have any respiratory or neurological complaints and was discharged 2 d later, and was confirmed to be in good condition until the present follow-up.

DISCUSSION

CLE is an extremely rare complication of the invasive procedure TACE^[1-3,5]. Only 11 papers have reported 16 cases of lipiodol-associated embolic brain damage following TACE. In China, the first case report of CLE was by Li *et al*^[6] in 2001, while the first overseas was by Yoo *et al*^[11] in 2004.

The symptoms of CLE are nonspecific, including visual loss, headache, motor weakness, and change of mental status. These symptoms vary in severity according to the site of iodized oil deposition. In addition, if the lipiodol enters the lungs as well, the patient complains of chest pain and dyspnea^[7]. Actually, among the twelve prior cases presented, 10 were confirmed with pulmonary embolism after initial diagnosis of respiratory manifestations. Most CLE occurs during or immediately after TACE and is identified initially by one or more symptoms mentioned above, however, a delayed type of CLE also has been reported^[8]. Although some of the patients who suffered from CLE after TACE die, despite

Table 1 Analysis of 13 cases of CLE following TACE ($n = 12$) [1-3,5-8,11,13,19]

	<i>n</i>
Sex	
Male	8
Female	4
Age (yr)	
≥ 50	10
< 50	2
Course of TACE	
First	3
Second	6
Third	2
More than third	1
Doses of lipiodol (mL)	
≥ 20	6
< 20	3
ND	3
Gelatin sponge particles	
Yes	5
No	7
Embolization through extra-hepatic collateral artery	
Yes	6
No	4
ND	2
Tumor site including right lobe	
Yes	8
No	0
ND	4
Size of tumor/invasion of diaphragm	
Large/multiple	9
Minor	1
ND	2
Vascular invasion	
Yes	3
No	2
ND	7
Arteriovenous fistula	
Yes	2
No	5
ND	5
Right-to-left shunt	
Yes	0
No	6
ND	6
Pulmonary embolism	
Yes	10
No	1
ND	1
Time of neurologic symptoms	
During or shortly after TACE	11
69 h after TACE	1
Recovery time (All ≤ 6 wk)	
≥ 4 wk	3
< 4 wk	5
ND	1
Death	3

CLE: Cerebral lipiodol embolism; TACE: Transcatheter arterial chemo-embolization; ND: Not described.

positive interventions, most of them recover completely without any neurological sequelae^[5] (Table 1). In our case, the patient developed CLE during the TACE procedure, and after 6 wk of supportive therapy, he recovered, leaving no neurological symptoms.

The radiological findings of CLE on computed tomography (CT) and MRI in the previously reported

cases are similar. The site of lipiodol deposition includes the basal ganglia, thalamus, gray-white matter junction, and both parietal and frontal cortices. Cranial CT/MRI taken after the neurological symptoms disappeared is usually clear, which infers that the lipiodol in the brain could have been cleared entirely^[5,8]. In our case, the sites of iodized oil deposition were mainly on the centrum semiovale, and both parietal and occipital lobes.

Ten papers covering 12 cases (the present case included) have described in detail the clinical and radiological data of CLE following TACE, and have been selected to make an analysis (Table 1) (in fact, 12 papers in total were searched, and because Zhao *et al*^[9] and Li *et al*^[10] provided no detailed information, their two cases were excluded). In six TACE procedures that were complicated by CLE, lipiodol was infused through extra-hepatic collateral arteries, and mostly the inferior phrenic arteries. In all 12 cases, only eight of them described the tumor sites, all of which involved the right liver lobes. This implies that invasion of the diaphragm is probably common in patients with CLE after TACE, although it was confirmed only in three cases^[2,5,11].

Six authors have analyzed the mechanisms of CLE following TACE treatment of HCC. Wu *et al*^[3] have shown that the most probable cause of lipiodol-induced brain embolism after TACE is a combination of a right-to-left shunt and the dose-dependent effect of the drug^[3,12]. The communication between the systemic and pulmonary vessels might develop via adhesive pleurae or tumor invasion of the diaphragm, thus a right-left shunt is formed. When injecting iodized oil via the inferior phrenic artery, some oil droplets might enter the brain, thus bypassing the right-left shunt. Combined with an increased dose of lipiodol during the second course of TACE, the patient has a greater risk of CLE^[3]. Wu *et al*^[11] have added that an intra-pulmonary arteriovenous shunt might appear during the pulmonary lipiodol embolism because of increasing pulmonary artery pressure or hypoxia^[1,11]. Cui *et al*^[13] have observed that the contrast medium injected into the hepatic artery enters the pulmonary veins directly during angiography in patients with CLE after TACE. This phenomenon supports the mechanism of intra-pulmonary arteriovenous shunting suggested by Wu *et al*^[3]. Choi *et al*^[7] have performed echocardiography and DSA to exclude intra-cardiac and intra-tumoral shunts, to support the mechanism that the communication between the inferior phrenic artery and pulmonary vessels occurs via adherent pleura, and tumor recurrence causes the lipiodol-induced brain embolism after TACE. Li *et al*^[6] and Matsumoto *et al*^[5] have made similar speculation.

Yoo *et al*^[1] have presented three cases of CLE following TACE; all of which had evidence of pulmonary involvement but without a demonstrable intra-cardiac shunt. Yoo *et al*^[1] have speculated that since it has been verified that fat globules < 7 μm in diameter can pass directly through the pulmonary arteriolar network (i.e. trans-pulmonary shunt) and cause cerebral injury^[14], the presence of an intra-cardiac right-to-left shunt might not be necessary^[1].

In another case reported by Wu *et al*^[8], pulmonary and cerebral embolism occurred 34 and 69 h, respectively, after TACE treatment of HCC. Wu *et al*^[8] have concluded that the rapid-flow, tumor-feeding artery washes out the iodized oil, which leads to embolic damage of the lungs. Then, the lipiodol that is deposited in the lungs is washed out again and enters the systemic circulation, thus causing embolism of the brain.

In the present case, the most probable mechanism of pulmonary and cerebral embolism is attributed to a hepatic venous-arterial shunt and an intra-pulmonary or intra-cardiac shunt. Lipiodol entered the pulmonary circulation through a hepatic arteriovenous fistula (Figure 3), which caused pulmonary embolism that increased pulmonary artery pressure, and produced a temporary pulmonary arteriovenous fistula. Lipiodol entered the brain through this temporary fistula. In addition, the use of a large dose of lipiodol also promotes CLE.

It is well known that CLE after TACE might be associated with intra-cardiac shunt, intra-pulmonary shunt and infusion of large doses of lipiodol^[7]. Intra-cardiac right-to-left shunt can occur in some congenital heart diseases, such as patent foramen ovale. Research has revealed that among 1100 non-selected autopsies, 386 cases showed patent foramen ovale and 83% of them were < 0.2 cm in diameter^[15]. The small foramen ovale, being often undetectable on routine echocardiography, might allow transient right-to-left shunting when the right heart pressure increases following oil trapping in the lungs^[3]. Pulmonary arteriovenous shunts usually are associated with congenital pulmonary vascular malformations, acute or chronic pulmonary diseases, pulmonary tumors and advanced liver diseases^[3,16]. The communication that can develop between the inferior phrenic artery and the pulmonary vessels also suggests a possible route for right-to-left shunting^[4] (Table 1). Large dose of lipiodol infusion might increase the risk of extra-hepatic embolism. Some authors have suggested that a lipiodol dose less than 15 or 20 mL can prevent ectopic embolism^[17,18]. However, in most prior cases of CLE, including the one described in this study, the HCCs were single large or multiple tumors (Table 1). As a result, limiting the dose of lipiodol to < 20 or 15 mL routinely might not be reasonable, especially when there is insufficient evidence to confirm that a large dose of lipiodol is a determinant factor for CLE following TACE. Three previous cases have revealed that CLE also occurs when the lipiodol dose is < 15 mL^[1,3,5]. Therefore, all procedures must be individualized.

It is not necessary to search routinely for shunts prior to TACE, especially when right-to-left shunting is often undetectable in routine examination, as in chest or abdominal CT, DSA, and echocardiography^[7]. In the present case, the patient was subjected to echocardiography and no positive evidence of right-to-left shunting was revealed. The patient had a history of bleeding gastroesophageal varices, therefore, transesophageal echocardiography was not performed. Direct evidence of a shunt has not yet been reported in previous studies (Table 1). Kim *et al*^[19] have presented one case of CLE

following TACE during which the presence of a right-to-left shunt (was demonstrated by the presence of microbubbles in the left middle cerebral artery and left atrium, while trans-cranial Doppler and transesophageal echocardiography were performed during the intravenous injection of agitated saline.

The mechanism of CLE following TACE has not yet been elucidated. Intracardiac or intrapulmonary right-to-left shunts and infusion of large doses of lipiodol might contribute to the increased risk of CLE following TACE. An individualized plan of therapy, including lipiodol dose determination, shunting detection, as well as selecting vessels for the lipiodol infusion prior to TACE is of great importance to achieve an efficient overall result^[11].

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2nd Middle East Gastroenterology
Conference

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February 26-28
Carolina, United States
First Symposium of GI Oncology at
The Caribbean

March 04-06
Bethesda, MD, United States
8th International Symposium on
Targeted Anticancer Therapies

March 05-07
Peshawar, Pakistan
26th Pakistan Society of
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Meeting

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Medicine

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Bhubaneswar, India
18th Annual Meeting of Indian
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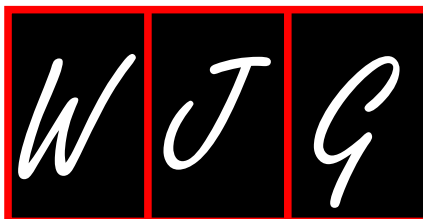
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Books

Personal author(s)

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- 11 **Lam SK**. Academic investigator's perspectives of medical treatment for peptic ulcer. In: Swabb EA, Azabo S. Ulcer disease: investigation and basis for therapy. New York: Marcel Dekker, 1991: 431-450

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- 12 **Breedlove GK**, Schorfheide AM. Adolescent pregnancy. 2nd ed. Wiczorek RR, editor. White Plains (NY): March of Dimes Education Services, 2001: 20-34

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- 13 **Harnden P**, Joffe JK, Jones WG, editors. Germ cell tumours V. Proceedings of the 5th Germ cell tumours Conference; 2001 Sep 13-15; Leeds, UK. New York: Springer, 2002: 30-56

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- 14 **Christensen S**, Oppacher F. An analysis of Koza's computational effort statistic for genetic programming. In: Foster JA, Lutton E, Miller J, Ryan C, Tettamanzi AG, editors. Genetic programming. EuroGP 2002: Proceedings of the 5th European Conference on Genetic Programming; 2002 Apr 3-5; Kinsdale, Ireland. Berlin: Springer, 2002: 182-191

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- 15 Morse SS. Factors in the emergence of infectious diseases. Emerg Infect Dis serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: <http://www.cdc.gov/ncidod/EID/eid.htm>

Patent (list all authors)

- 16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

Statistical data

Write as mean \pm SD or mean \pm SE.

Statistical expression

Express *t* test as *t* (in italics), *F* test as *F* (in italics), chi square test as χ^2 (in Greek), related coefficient as *r* (in italics), degree of freedom as *v* (in Greek), sample number as *n* (in italics), and probability as *P* (in italics).

Units

Use SI units. For example: body mass, *m* (B) = 78 kg; blood pressure, *p* (B) = 16.2/12.3 kPa; incubation time, *t* (incubation) = 96 h, blood glucose concentration, *c* (glucose) 6.4 ± 2.1 mmol/L; blood CEA mass concentration, *p* (CEA) = 8.6 ± 24.5 μ g/L; CO₂ volume fraction, 50 mL/L CO₂, not 5% CO₂; likewise for 40 g/L formaldehyde, not 10% formalin; and mass fraction, 8 ng/g, etc. Arabic numerals such as 23, 243, 641 should be read 23 243 641.

The format for how to accurately write common units and quantums can be found at: <http://www.wjgnet.com/wjg/help/15.doc>.

Abbreviations

Standard abbreviations should be defined in the abstract and on first mention in the text. In general, terms should not be abbreviated unless they are used repeatedly and the abbreviation is helpful to the reader. Permissible abbreviations are listed in Units, Symbols and Abbreviations: A Guide for Biological and Medical Editors and Authors (Ed. Baron DN, 1988) published by The Royal Society of Medicine, London. Certain commonly used

abbreviations, such as DNA, RNA, HIV, LD50, PCR, HBV, ECG, WBC, RBC, CT, ESR, CSF, IgG, ELISA, PBS, ATP, EDTA, mAb, can be used directly without further explanation.

Italics

Quantities: *t* time or temperature, *c* concentration, *A* area, *l* length, *m* mass, *V* volume.

Genotypes: *gyrA*, *arg 1*, *c myc*, *c fos*, etc.

Restriction enzymes: *EcoRI*, *HindI*, *BamHI*, *Kho I*, *Kpn I*, etc.

Biology: *H. pylori*, *E. coli*, etc.

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