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REVIEW

Iron and liver fibrosis: Mechanistic and clinical aspects

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Abstract

Liver fibrosis is characterised by excessive deposition of extracellular matrix that interrupts normal liver functionality. It is a pathological stage in several untreated chronic liver diseases such as the iron overload syndrome hereditary haemochromatosis, viral hepatitis, alcoholic liver disease, non-alcoholic fatty liver disease, non-alcoholic steatohepatitis and diabetes. Interestingly, regardless of the aetiology, iron-loading is frequently observed in chronic liver diseases. Excess iron can feed the Fenton reaction to generate unquenchable amounts of free radicals that cause grave cellular and tissue damage and thereby contribute to fibrosis. Moreover, excess iron can induce fibrosis-promoting signals in the parenchymal and non-parenchymal cells, which accelerate disease progression and exacerbate liver pathology. Fibrosis regression is achievable following treatment, but if untreated or unsuccessful, it can progress to the irreversible cirrhotic stage leading to organ failure and hepatocellular carcinoma, where resection or transplantation remain the only curative options. Therefore, understanding the role of iron in liver fibrosis is extremely essential as it can help in formulating iron-related diagnostic, prognostic and treatment strategies. These can be implemented in isolation or in combination with the current approaches to prepone detection, and halt or decelerate fibrosis progression before it reaches the irreparable stage. Thus, this review narrates the role of iron in liver fibrosis. It examines the underlying mechanisms by which excess iron can facilitate fibrotic responses. It describes the role of iron in various clinical pathologies and lastly, highlights the significance and potential of iron-related proteins in the diagnosis and therapeutics of liver fibrosis.

Key words: Iron; Liver pathologies; Liver fibrosis; Hepatic stellate cells; Cirrhosis

Core tip: Excess iron is observed in several liver pathologies, where it can accelerate the progression of liver fibrosis to cirrhosis and hepatocellular carcinoma, regardless of disease aetiology. This review narrates the role of excess iron in liver fibrosis. It examines the mechanisms by which iron enhances fibrogenic responses and describes various iron-related clinical pathologies. Furthermore, it evaluates the significance of iron and iron-related proteins in the diagnosis and therapeutics of liver fibrosis. The review is unique in that it includes both, cellular mechanisms and clinical aspects of liver fibrosis pertaining to iron. This makes it distinct from previous published reviews.

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INTRODUCTION

Liver fibrosis is a pathological state, which is attained due to an overactive wound healing response to persistent liver injury. This subsequently disrupts liver architecture and hinders its functions leading to organ failure and death[1]. Fibrotic liver is frequently observed in several untreated chronic liver diseases (CLDs) such as haemochromatosis, viral hepatitis (hepatitis B and hepatitis C infections), alcoholic liver disease (ALD), non-alcoholic fatty liver disease (NAFLD), non-alcoholic steatohepatitis (NASH) and diabetes. Elevated iron level is a common feature of all these fibrosis-promoting conditions[2], suggesting that iron loading may pose a risk for disease progression and aggravate liver pathology.

While iron is essential for normal physiology, excess iron is toxic as it can accelerate the Fenton reaction that generates noxious reactive oxygen species (ROS) and severely damage cells and tissues. Thus, maintenance of body iron homeostasis is crucial, particularly because there is no physiological pathway for removal of excess iron from the body[3]. Under normal physiological conditions, systemic iron regulation is mediated *via* the liver-secreted iron hormone hepcidin[4]. Hepcidin binds to ferroportin (transmembrane iron-exporter protein) on the iron-storing macrophages and hepatocytes, degrades ferroportin and thereby hinders iron-entry into the circulation[5]. Hepcidin also binds to ferroportin on the enterocytes and decreases the expression of divalent metal transporter (DMT)-1 protein on the apical surface of enterocytes that mediates non-haem iron uptake, and thus reduces intestinal iron absorption[6]. Lack of, or resistance to hepcidin due to mutations leads to excessive iron absorption from the duodenum, unregulated iron release from the macrophages into the circulation and excessive iron deposition in various organs. These features manifest as hereditary haemochromatosis[7]. However, in non-hereditary fibrotic CLDs, the basis for iron-loading is not fully understood and whether iron-excess is the cause, a consequence, or a mediator of pathological progression remains unknown. Therefore, it is imperative to understand the role of iron in liver fibrosis and study its mechanism of action to aid in the early diagnosis and therapeutics of myriad of non-hereditary iron-loading CLDs.

HEALTHY FIBROGENESIS TO PATHOLOGICAL FIBROSIS: LOSE CONTROL

Liver fibrogenesis is a normal process of tissue repair. It is mediated *via* a complex network of interrelated and regulated signalling interactions between the resident parenchymal cells (hepatocytes), non-parenchymal cells [hepatic stellate cells (HSCs), liver sinusoidal endothelial cells, Kupffer cells, biliary epithelial cells, liver associated lymphocytes], and the non-resident infiltrating immune cells. The HSCs located in the space of Disse between the hepatocytes and the liver sinusoids play a pivotal role in liver development and regeneration *via* fibrogenesis[1]. In addition, the quiescent HSCs

store 50%-80% of total vitamin A in the body[8].

Acute liver injury stimulates the non-parenchymal cells to secrete several pro-fibrogenic cytokines including the most potent activator of fibrosis, transforming growth factor beta (TGF-β)[9]. This signals the quiescent HSCs to differentiate into myofibroblasts-like cells to produce components of extracellular matrix (ECM) such as pro-collagen-1 α-1, alpha smooth muscle actin (α-SMA), fibronectin, laminin, elastin and proteoglycans along with mesenchymal proteins like vimentin and desmin, and cause tissue scaring. Upon removal of the stimulus (during recovery), excess ECM is degraded by matrix metalloproteinases (MMPs). In turn, MMP-activity is inhibited and modulated by tissue inhibitors of metalloproteinase (TIMPs) produced by the activated HSCs. Subsequently, the activated HSCs either undergo apoptosis and/or revert to their original quiescent phenotype, thereby terminating a well-regulated and reversible healing process[10].

Prolonged liver injury *via* chronic inflammation, infection and/or oxidative stress leads to continuous stimulation of the wound healing mechanism whereby the HSCs remain persistently activated. These activated HSCs become the main source and target of TGF-β, which greatly increases the proliferation and dedifferentiation of HSCs into ECM-producing myofibroblasts. Regulatory processes are disregarded leading to excessive deposition of ECM that can rise up to 8-fold higher than normal[11]. This, along with insufficient degradation of ECM gradually distorts the normal architecture of the liver, thereby entering the pathological fibrotic stage.

Removal of stimulus, followed by sufficient time for recovery and treatment can revert the myofibroblasts to an inactive state, reverse fibrosis and restore normal liver functionality[12-14] However, untreated fibrosis often progresses to cirrhosis, which is characterised by further deposition of collagen, nodule formations and restricted blood supply (hypoxia). This increases liver stiffness and portal hypertension, and further distorts hepatic architecture[15]. Unattended, it leads to organ failure and death. As the pathology progresses to cirrhosis, regression becomes increasingly difficult, although possible. Advanced cirrhosis may terminate in hepatocellular carcinoma, where resection or transplantation remain the only curative options.

EXCESS IRON PROMOTES LIVER FIBROSIS

The HSCs

Persistent HSC-activation is the early and key event in fibrosis, and the progression from fibrosis to cirrhosis is a crucial step in determining the fate of liver. In iron loading pathologies, HSC-activation and excessive ECM deposition are cumulative consequences of direct and indirect effect of iron on the HSCs. First, we review the direct effect of iron on HSCs. Normal liver iron concentration (LIC) is lower than 35 μmol/g of dry weight[16]. When LIC crosses a threshold of 60 μmol/g, HSC-functionality begins to derail, and when it exceeds 250 μmol/g, cirrhosis becomes inevitable[17]. Several studies have reported the fibrosis-enhancing effects of iron. For example, iron elevated collagen gene expression in HSCs and increased *TGF-β* expression in rats[18], induced collagen deposition in gerbil[19] and promoted cirrhosis in mice[20]. For the first time, Ramm *et al*[21], demonstrated a correlation between LIC and HSC-activation in humans, resulting in increased expression of α-SMA and collagen deposition in patients with haemochromatosis. Similar results were observed in rat HSCs, where iron increased HSC-cell proliferation, selectively increased collagen synthesis without affecting non-collagen proteins[22], and increased expression of *a-sma* and *col-1 a-1*[23]. Rat HSCs, when treated with ferritin, demonstrated a pro-inflammatory cascade by nuclear factor kappa-B signalling (NF-k)-B[24]. Likewise, recent studies in murine HSCs showed transferrin-induced elevations in *a-sma*, collagen secretion and vimentin[25].

Hepatocytes and macrophages

The HSCs do not function independently. Their role in fibrosis is informed by a network of events between other non-parenchymal cells and hepatocytes. Iron-loading in CLDs predominantly occurs in the hepatocytes and Kupffer cells, and this underpins the indirect effect of iron on HSCs whereby iron-damaged hepatocytes and macrophages release humoral factors that activate the HSCs.

Loading begins in the hepatocytes located in Rappaport zone 1 and progresses towards the hepatocytes in zones 2 and 3. Subsequently, when iron is co-loaded in the Kupffer cells, it is believed to trigger fibrosis[17]. The hepatocytes make majority of the liver mass, therefore, iron-loaded hepatocytes substantially affect fibrosis initiation and progression[26]. Wood *et al*[27] observed that in hereditary haemochromatosis, hepatocyte senescence positively correlated with LIC, serum ferritin and oxidative

stress. In the Kupffer cells (largest non-parenchymal cell population in liver), iron deposition causes the secretion of proinflammatory cytokines and thereby promotes fibrosis. Interestingly, phagocytosis of necrotic hepatocytes promotes a pro-inflammatory/pro-fibrotic environment, whereas phagocytosis of collagen-producing cells promotes anti-inflammatory/anti-fibrotic environment. Thus, Kupffer cells play opposing roles; in the progression and regression of liver fibrosis, likely in the early and later stages of fibrosis, respectively.

Essentially, these cells collectively produce a pool of elevated levels of proliferative, proinflammatory and profibrogenic mediators including TGF-β[1,28] (Figure 1). While TGF-β ensures a self-sustained HSC-alteration to ECM-producing myofibroblasts[17], other factors sensitize the hepatocytes to produce more proinflammatory factors causing liver inflammation, as seen in haemochromatosis patients[29]. This provokes early HSC-activation in areas of liver that are remote from regions of heavy iron-loading[21] and cause infiltration of circulating immune cells, thereby upholding an inflammatory state. Such an inflammatory liver microenvironment and overexpression of TGF-β is commonly observed in fibrotic livers[1,28].

MECHANISMS OF ACTION

The fibrotic responses are collectively mediated by multiple mechanisms involving excess-iron induced Fenton reaction, cell-signalling pathways, contribution to HSC-activation by iron-related proteins, and possibly, iron-mediated ECM remodelling (Figure 1).

Impact of Fenton chemistry on liver biology

The Fenton-Haber-Weiss reaction highlights the ability of iron to freely donate and accept electrons while altering between Fe^{2+} and Fe^{3+} states. The reactions encompass iron-catalysed generation of hydroxide ions, along with hydroperoxyl and hydroxyl radicals. Normally, limited amount of excess free-radicals are generated during cellular metabolism, which are quenched by inherent cellular antioxidant mechanisms and electron-donating moieties such as vitamins A, C and E[30]. Moreover, the tight binding of iron to cellular proteins (*e.g.*, ferritin) and circulating proteins (*e.g.*, transferrin) limits the amount of free iron available to feed the Fenton reaction. Hepcidin also offers indirect protection from excess-iron-induced toxic effects by inhibiting iron entry into the circulation[5,6,31]. However, under iron-loading conditions such as haemochromatosis, levels of non-transferrin bound iron (NTBI) (free iron circulating in plasma and iron loosely bound to moieties such as albumin, citrate and acetate) increase[32]. Here, the availability of water-soluble free Fe^{2+} iron forms the foundation for iron toxicity[33] as it accelerates the Fenton reaction to generate unquenchable levels of ROS, which can saturate the antioxidant systems. These electron-scavenging free radicals attack biomolecules and promote the formation of other free radicals such as thiyl and peroxyl radicals, thereby initiating a perpetual free radical chain reaction[34].

ROS can oxidize lipids, proteins and nucleic acids, thereby promoting fibrosis-initiation and/or fibrosis-progression. ROS-induced lipid peroxidation of cell membranes and the membranes of cellular organelles contributes to hepatocyte apoptosis and necrosis. This also enhances fibrogenic responses; for example, lipid peroxidation stimulated the expressions of *col-1 a-1* and *TGF-β* in iron-loaded rats[18]. The by-products of lipid peroxidation such as malondialdehyde (MDA), isoprostanes and 4-hydroxynonenal (4-HNE), detected in the liver of iron-loaded rats[35], act as pro-fibrogenic stimuli. Isoprostanes, the peroxidation products of arachidonic acid enhanced HSC-proliferation, HSC-collagen-production and TGF-β release from the Kupffer cells[36], while 4-HNE upregulated the expressions of *col-1 a-1* and *TIMP-1* in HSCs[37].

Cross-connection between iron-related and fibrotic pathways

TGF-β signalling is the key fibrosis-mediating pathway and its role in regulating pro-fibrogenic gene expression and ECM deposition is well established[38]. Notably, TGF-β belongs to the TGF-β super-family of molecules, which also includes the bone morphogenetic proteins (BMPs) that induce hepcidin[39], the master regulator of systemic iron homeostasis. These molecules participate in several signalling pathways and function by binding to a complex of receptors (type II and type I serine threonine kinase receptors) and induce phosphorylation of receptor-SMADs (small mothers against decapentaplegic). The phosphorylated receptor-activated SMADs bind to SMAD-4 to form a heterodimer and this complex translocates into the nucleus to modulate the transcription of several genes that determine germ-line specification, embryonic development and cellular differentiation. While TGF-β-mediated

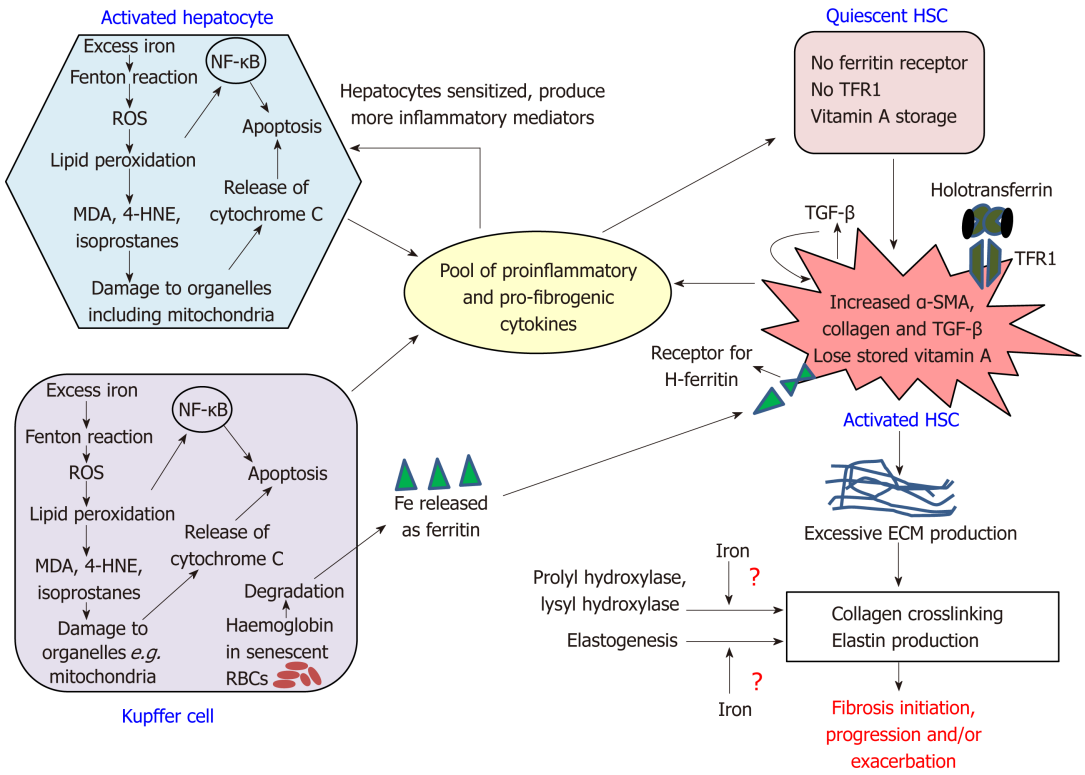

Figure 1 Intercellular network of events in fibrosis. The figure shows the interactions between hepatocytes, Kupffer cells and hepatic stellate cells that initiate and drive fibrosis progression. The pool of pro-fibrogenic and pro-inflammatory mediators include C-C motif chemokine ligand 5, macrophage inflammatory proteins 1 and 2, monocyte chemoattractant protein-1, tumor necrosis factor alpha, transforming growth factors alpha and beta, platelet-derived growth factor, interleukin (IL)-1β, IL-6, inducible nitric oxide synthase, and protein adducts of malondialdehyde and 4-hydroxynonenal. HNE: Hydroxynonenal; HSC: Hepatic stellate cell; MDA: Malondialdehyde; NF-κB: Nuclear factor kappa B; RBCs: Red blood cells; ROS: Reactive oxygen species; TFR1: Transferrin receptor 1; αSMA: Alpha smooth muscle actin; ECM: Extracellular matrix; TGF-β: Transforming growth factors beta.

activation of TGF-β receptor (R)II/RI -SMAD-2/3-SMAD-4 is the canonical fibrosis pathway, BMP (6)-mediated activation of ALK-2/3 receptor-SMAD-1/5/8-SMAD-4 is central to iron-dependent induction of hepcidin[40,41] (Table 1).

Since excess iron in liver induces both, TGF-β[29] and BMP-6[40,42], a connection between the TGF-β-induced fibrosis pathway and the BMP-induced hepcidin induction was envisaged and investigated. Wang *et al*[43] showed the significance of SMAD-4 in hepcidin induction by iron, TGF-β and BMP, while liver-specific disruption of SMAD-4 abrogated the hepcidin response. This not only demonstrated positive regulation of hepcidin by SMAD-4 and its contribution to iron homeostasis, but also identified overlap between the iron-related and fibrotic pathways based on the common role of SMAD-4 in the two pathways. Moreover, Chen *et al*[44] showed that TGF-β-induced hepcidin induction occurred *via* TGF-β-RII/RI and SMAD-1/5/8 phosphorylation, the transient non-canonical TGF-β signalling response[45,46]. This further demonstrated common mediators (TGF-β receptors) between TGF-β signalling and hepcidin induction (iron-regulation). Recently, Mehta *et al*[25] (2018) demonstrated iron-induced activation of TGF-β signalling in murine HSCs. Collectively, these studies reiterate the connection between the iron-related and fibrotic pathways and highlight the contribution of TGF-β towards hepcidin synthesis, and thereby, potential regulation of iron homeostasis under iron-loaded conditions (Figure 2).

Signalling pathways such as the Wnt, Hedgehog and Notch that orchestrate the developmental processes during embryogenesis are also active during fibrogenesis to mediate survival, proliferation, differentiation and polarity of their target cells. These pathways function *via* a cross-talk with each other and with TGF-β pathway[47-49]. Their inhibition has shown to reverse liver fibrosis *in vitro* and *in vivo*[50-52]. The effect of iron-induced modulation of these pathways on liver fibrosis was examined in a few studies. Data showed that iron deficiency stimulated Notch signalling, but not TGF-β and Wnt signalling[53]. Recently, in response to iron-loading, a protective role of β-catenin (component of cadherin complex that stimulates Wnt signalling) against liver fibrosis was observed, where hepatocyte-specific β-catenin-knockout mice fed with an iron-overloaded diet developed higher degree of fibrosis and inflammation compared to controls[54]. Further studies are required to better understand the effect of iron on these pathways and how this alters fibrosis.

Table 1 Iron-related characteristics and components of transforming growth factor-β pathway and bone morphogenetic protein signalling

Stimulant	Pathway	Type II receptors	Type I receptors	Receptor-SMADs phosphorylated	Common SMAD	Significance/feature of pathway
BMPs (Belong to TGF-β superfamily)	Canonical	BMPR2, ACVR2A, ACVR2B	ALK 1,2,3,6	SMAD-1/5/8	SMAD-4	Growth, differentiation, and developmental processes
	BMP-6 induced by iron-loading (Liver specific)[133]	BMPR2, ACVR2A	ALK-2/3	SMAD-1/5/8	SMAD-4	Iron-dependent hepcidin induction, modulated by HJV, HFE and TFR2[44]
TGF-β	Canonical	TGF-β-RII	ALK-5 (TGF-β-RI)	SMAD-2/3 (Stimulation is stable over time)[44]	SMAD-4	Growth, differentiation, developmental processes and fibrotic responses.
	Non-canonical TGF-β1 induced by iron-loading[29]	TGF-β-RII	ALK-5 (TGF-β-RI)	SMAD-1/5/8 (Transient stimulation, independent of cell type)[44]	SMAD-4	Hepcidin induction, independent of modulation by HJV, HFE and TFR2[44] and independent of BMP6-mediated activation of hepcidin
Activins (Belong to TGF-β superfamily)	Canonical[134]	ACVR2A, ACVR2B	ALK-4/7	SMAD-2/3	SMAD-4	Differentiation, proliferation and determine functions of several cell types
	Non-canonical Activin B induced by inflammation[134]	ACVR2A, ACVR2B	ALK-2/3 with HJV as co-receptor	SMAD-1/5/8	SMAD-4	Hepcidin induction during inflammation[135]

ACVR: Activin receptor; ALK: Activin receptor-like kinase; BMP: Bone morphogenetic protein; BMPR: Bone morphogenetic protein receptor; HFE: High iron protein; HJV: Hemojuvelin protein; SMAD: Small mothers against decapentaplegic protein; TFR: Transferrin receptor; TFR: Transferrin receptor; TGF-β: Transforming growth factor beta; TGF-β-R: Transforming growth factor beta receptor.

Iron-related proteins modulate fibrosis

Several iron-related protein-receptor complexes either cause HSC-activation or contribute to iron movement in pre-activated HSCs. One such association is *via* the ferritin receptor. Unlike quiescent HSCs, activated HSCs express a specific receptor for H-ferritin and thereby internalise ferritin that is supposedly released from Kupffer cells following degradation of haemoglobin from senescent RBCs[55,56]. Ferritin can upregulate the genes involved in HSC-activation *via* PKCζ and p44/p42-MAP-kinase signalling resulting in activation of NF-κB, which elevates hepatic proinflammatory mediators[24]. H-ferritin from Clonorchis sinensis, which causes liver fibrosis and cholangiocarcinoma, has shown to generate free radicals that activate NF-κB-signalling by promoting nuclear translocation of NF-κB subunits p65 and p50 and increasing the expression of proinflammatory cytokines IL-6 and IL-1β in HSCs[57]. Thus, ferritin and its receptor contribute to both proinflammatory and profibrogenic effects in HSCs. Another iron-related protein-receptor association of interest is between transferrin and transferrin receptor-1 (TFR1). Transferrin is the iron carrier protein that transports iron throughout the body and binds to TFR1 present on cell surfaces to form a complex of transferrin-TFR1. This complex is then internalised into a vesicle and iron is released from this complex into the cytoplasm[58]. Interestingly, only activated HSCs express TFR1[23]. Binding of transferrin to TFR1 contributes to HSC-activation, as demonstrated *via* increased expressions of α-SMA and procollagen α1(I) mRNA in rat HSCs[23] and supported by similar studies in murine HSCs[25]. Thus, transferrin is an important factor in HSC-activation, and transferrin-bound-iron uptake may be an important route for iron acquisition by activated HSCs. Hepcidin also plays a role in fibrosis modulation, as discussed in the subsequent section.

Iron and ECM remodelling

In addition to excess ECM, fibrosis is characterised by altered composition of ECM, which includes maturation of collagen *via* crosslinking. Crosslinked collagen is more resistant to proteolytic degradation by MMP-1[59] and is therefore the most challenging therapeutic target for fibrosis resolution. Collagen cross-linking is catalysed by the

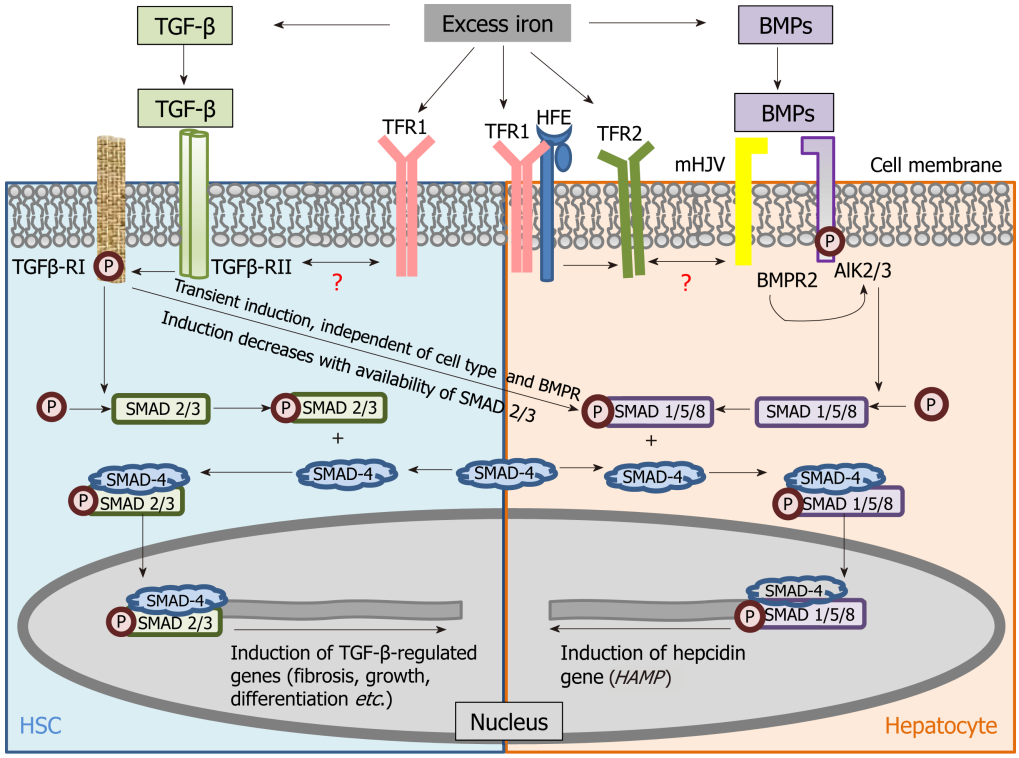

Figure 2 Schematic of mechanistic cross-connection between the transforming growth factor beta pathway and bone morphogenetic protein signaling. Shared signalling components between transforming growth factor beta (TGF-β) (fibrosis-related) and bone morphogenetic protein (iron-related) pathways have been shown in hepatic stellate cells and hepatocytes. Previous study demonstrated TGF-β-induced hepcidin expression in human macrophages, while Chen *et al*[44] showed that this occurred through TGF-β-RII/RI in mouse and human hepatocytes *via* the non-canonical pathway involving small mothers against decapentaplegic protein-1/5/8 phosphorylation. ALK: Activin receptor-like kinase; BMPR: Bone morphogenetic protein receptor; HFE: High iron protein; HSC: Hepatic stellate cell; mHJV: Membrane-bound hemojuvelin protein; P: Phosphorylation; SMAD: Small mothers against decapentaplegic protein; TFR: Transferrin receptor; TGF-β-R: Transforming growth factor receptor.

enzymes prolyl hydroxylase and lysyl hydroxylase that require vitamin C and iron as cofactors. Hence, it is possible that during iron-loading, excess iron may be channelized to promote collagen crosslinking. Along this line, a study showed increased activities of the aforementioned enzymes in rat models of carbon tetrachloride-induced liver injury[60] and in iron-deficient rats, lower levels of procollagen type I N-terminal pro-peptide and increased systemic levels of degradation products from C-terminal telopeptides of type I collagen were reported[61]. However, a previous *in vitro* study excluded iron as a major participant in collagen crosslinking since the iron chelator deferoxamine did not alter collagen modifications[62]. Thus, the exact effect of iron on collagen maturation is unclear and needs further investigation. Elastin is yet another important component of ECM. Iron appears to modulate elastogenesis in cultured human skin fibroblasts, where it increased the levels of insoluble elastin protein and elastin mRNA levels by 3-fold[63]. Further studies are required to ascertain the role of iron in elastogenesis in the HSCs, as it is a potential target for fibrosis therapy.

IRON LOADING AND FIBROSIS IN DIFFERENT LIVER PATHOLOGIES

In haemochromatosis, iron loading can be very severe. However, in ALD, NAFLD, NASH and viral hepatitis, low to moderate levels of excess iron are sufficient to support the pathological progression. Some iron-related parameters in these CLDs are summarised in Table 2.

Haemochromatosis

Pietrangelo (2010) defined haemochromatosis as a syndrome characterised by excessive deposition of iron in the parenchymal cells of several vital organs, and which is caused by mutation in single or multiple genes that regulate iron import into the circulation. It overarches the mutations in the genes *HFE*, *TFR2*, *HJV* (encoding hemojuvelin), *HAMP* (encoding hepcidin) and *SLC40A1* (encoding ferroportin)[64]. In

Table 2 Iron-related parameters in various fibrosis-promoting chronic liver diseases

	Normal	Hereditary hemochromatosis	ALD	NAFLD/NASH	Viral hepatitis	Diabetes
Iron level/accumulation	In body: 3-5 g[7]; In RBCs: about 2.5 g; In liver: 300 mg to 1 g[7]	Can be severe; Gradual increase, can reach up to 25-30 g in liver[7]	Moderate	Mild-moderate	Mild-moderate	Mild-moderate
Serum ferritin	24-300 μg/L[109]; 15-200 μg/L[101]; < 300 ng/mL in men, < 200 ng/mL in women[2]	Mostly high, but can be normal[101]	High[69,136]	High[104,105] but 1st/3rd NASH patients can be iron deficient[86]	High[116,137]	High[138], associated with pre-diabetes
Serum hepcidin	0.4-23.3 nmol/L[139]	Low[64]	Low[69,71,140]	High[80,141]; Can be low in iron deficiency[86]; High in obesity, but not in NAFLD[142]; High in obesity with NAFLD[143]; Alterations can occur without iron-overload[111]	Low in hepatitis C infections[144]; High in hepatitis B infections without cirrhosis and normal in those with cirrhosis[145]	No major alteration in type 1[146]; Low in type 2 diabetes[147,148]
Transferrin saturation	20%-45%[101]	> 45%[101,109]	High[69,136]	Slightly raised, but can be normal or sub-normal[7]	Mostly raised[88,149], but occasionally may not statistically differ from the norm[150]	Low[138], associated with pre-diabetes

Approximate values and percentages for adults have been shown. These include ranges for both genders. ALD: Alcoholic liver disease; NAFLD: Non-alcoholic fatty liver disease; NASH: Non-alcoholic steatohepatitis; RBCs: Red blood cells.

these cases, insufficient or lack of hepcidin production causes excessive duodenal iron absorption, while mutations in ferroportin reduce cellular iron export or cause hepcidin resistance. Whereas normal hepatic iron ranges from 300 mg to 1 g, haemochromatosis patients can show up to 25-30 g[7], clearly elevating the risk of fibrosis. A study in untreated haemochromatosis showed increased LIC in cirrhotic (378 ± 144 μmol/g) and fibrotic patients (331 ± 168 μmol/g) compared to non-fibrotic patients (237 ± 108 μmol/g)[65]. Interestingly, non-genetic factors like age, gender and alcoholism modulated fibrosis development in these patients. For example, those with fibrosis were significantly older than non-fibrotic patients and alcoholic males demonstrated hepatic fibrosis more frequently than non-alcoholic counterparts[65]. In a study of *HFE* gene C282Y mutation homozygotes, a higher percentage of men versus women showed increased LIC and biopsy-proven fibrosis and cirrhosis[66].

Normally, liver progenitor cells (LPCs) are activated during chronic liver injury as a backup repair mechanism to generate hepatocytes and cholangiocytes to compensate for the inability of damaged cells to replicate[67]. Activation of LPCs has also been implicated in fibrosis progression. Wood *et al*[26] suggested that in patients homozygous for the *HFE* C282Y mutation, LPCs are activated early in disease progression because excessive iron deposition in the hepatocytes hampers their ability to replicate and causes hepatocyte senescence. Reason for the iron-induced derailment of the LPC-repair-mechanism and how it contributes to predisposition to hepatocellular carcinoma in haemochromatosis patients remains unknown.

ALD

ALD exhibits liver iron loading in about half of all patients where serum iron biomarkers are raised in alcohol consumers from an early stage[68]. Alcohol-mediated suppression of hepcidin expression[69-71], upregulation of TFR1 expression in the hepatocytes by habitual alcohol drinking[72] and a concomitant increase in duodenal DMT-1 and ferroportin expression[70] collectively explain the reason for systemic and macrophage iron loading in ALD[68]. In addition, alcohol induces TGF-β expression and phosphorylates SMAD-2[73]. Such an increased availability of activated SMAD-2/3 can reduce TGF-β-induced hepcidin regulation[44]. Also, alcohol inhibits the activation of BMP receptor and SMAD-1,5, and attenuates the binding of SMAD-4 to hepcidin promoter[73]. Together, this reduces hepcidin expression and dysregulates liver iron metabolism.

Since iron and alcohol can independently cause oxidative stress, haemochromatosis patients that consume alcohol show cumulative liver damage, where alcohol-induced

damage, together with elevated intestinal iron absorption leads to more deleterious damage to the liver than either condition alone[74]. Resultantly, the pathological progression to cirrhosis is accelerated together with an increased predisposition to hepatocellular carcinoma. Haemochromatosis patients whose daily alcohol intake exceeds more than 60 g are at 9-fold higher risk of cirrhosis than those who consume lesser amount of alcohol[75]. Thus, the British Liver Trust recommends that these patients should completely refrain from alcohol consumption.

NAFLD, NASH and diabetes

While genetic polymorphisms in patatin-like phospholipase domain-containing 3 or transmembrane 6 superfamily member 2 pose a risk for NAFLD[76], high calorie intake combined with a sedentary lifestyle make NAFLD a common liver disease in affluent countries. It is characterised by insulin resistance, high serum triglyceride levels, low serum high-density lipoprotein and excessive fat deposition in the liver. The latter remains undiagnosed in the early stages and quietly progresses to the high-lipid-induced inflammatory state NASH, which can advance to cirrhosis and organ failure[77].

Elevated LIC is observed in about 33% of adult NAFLD patients[2] and it is suggested to be associated with increased fibrosis[78]. LIC can catalyse the pathological progression by causing oxidative and endoplasmic reticulum stress, activation of macrophages and HSCs, reduced export of very low density lipoprotein and increased synthesis of cholesterol[79]. NAFLD patients also exhibit elevations in serum hepcidin (typically)[80], white-adipose-tissue hepcidin and DMT-1 expression. Also, upregulated TFR1 has been observed in mice on high fat diet[79]. Overall, these factors potentiate cellular iron accumulation and can accelerate fibrosis progression. A combination of excess iron and lipids (which initiates an inflammatory cascade *via* lipid peroxidation[81]), may exacerbate fibrosis, as the excess of both, lipids and iron can distinctly cause oxidative damage. Accordingly, iron-loaded patients with NASH exhibit higher fibrosis grade and more elevated liver function test results compared to those without NASH[81]. Thus, iron has a pathogenic role in NAFLD and is amongst the many factors that determine progression from NASH to fibrosis[79].

However, in some NAFLD/NASH cases, LIC may not be associated with increased fibrosis[82]. Along the same line, in haemochromatosis patients heterozygous for the compound C282Y/H63D *HFE* mutation, fatty liver and metabolic syndrome were not directly associated with hepatic fibrosis[83]. Such observations are confounded by conflicting opinions on the significance of hepatocellular and macrophagic iron in NAFLD-related fibrosis. Some studies suggest that increased macrophagic iron cause macrophage and HSC activation, and it is primarily responsible for increased risk of advanced fibrosis in NAFLD[2,84]. Others suggest that hepatocellular iron, rather than macrophagic iron, poses a higher risk of fibrosis[85].

Nonetheless, a link between increased iron stores (ferritin), insulin resistance, diabetes and NAFLD is well established, where insulin resistance is central to NAFLD pathogenesis. Iron may promote insulin resistance in the adipose tissue, which in turn may trigger lipolysis of triglycerides; a process that produces most of the free fatty acid influx into the liver[79]. Probably, increased dietary iron in the form of red meat causes predisposition to insulin resistance and type II diabetes[79]. Predictably, type II diabetes is prevalent in iron loading pathologies like HFE-related hereditary haemochromatosis and β-thalassemia major[79]. This partly explains why glucose intolerant patients demonstrate more severe fibrosis than glucose tolerant ones, indicating that glucose intolerance is a risk factor for hepatic fibrosis in C282Y/H63D patients[83]. Also, iron deficiency has been associated with obesity and NAFLD. About 33% of NAFLD patients show transferrin saturation below 20%[79,86]. Thus, the role of iron in NAFLD is multi-dimensional and can differ between NAFLD cases.

Viral hepatitis

Unlike the aforementioned conditions, increased liver iron in viral hepatitis may be a combined consequence of dysregulated liver iron homeostasis and normal defensive processes adopted during infections, which involves sequestration of iron by hepatic cells to limit access to pathogens to inhibit their proliferation. This may explain the differences in LIC during the early and late phases of infection; low in the early stage and gradual increase after 2 wk[87].

About 30%-40% of chronic hepatitis C patients demonstrate elevated levels of serum iron, transferrin saturation and ferritin[88]. In these patients, rapid progression of tissue scarring is observed in those with excess iron, compared to those without[2]. Here, LIC correlates positively with HSC number, where iron could play a crucial role in HSC-activation and fibrosis progression[89]. Although the reason for iron loading in these patients has been attributed to the reduction in hepcidin due to virus-induced oxidative stress, there have been some discrepancies in clinical studies, where no

causal relationship between iron overload and hepcidin inhibition was noted[90]. Interestingly, in a case-control study, patients with chronic hepatitis C infection showed lower expression of hepcidin mRNA and more frequent hepatocyte iron deposition than hepatitis B infected patients[91]. Hepatitis B infected patients also show elevated LIC, where high iron is speculated to increase disease severity[92]. Iron can increase hepatitis B virus mRNA expression in HepG2 cells[93], which may contribute to sustenance of infection and inflammation, thereby potentiating fibrosis.

IRON-ASSISTED ASSESSMENT OF LIVER FIBROSIS

Early diagnosis of liver fibrosis is crucial for preventative, prognostic and therapeutic purposes. Liver biopsy is often considered a gold standard for definitive diagnosis, but it presents limitations such as sampling errors variability, invasive nature of the procedure and risk of life-threatening complications[94]. Recent advent of reliable serum-based markers and tools have drastically reduced the need for liver biopsy; for example magnetic resonance imaging (MRI) that accurately measures LIC, and transient elastography and MRI elastography that assess liver stiffness[94,95]. Note that in haemochromatosis, elevated LIC is the main driver of pathology, but in non-hereditary low-moderate iron-loaded CLDs, neither elevated LIC nor the altered iron-related markers are necessarily the main drivers of pathology *per se*, though these alterations are believed to accelerate the pathological progression to and through fibrosis. Thus, assessment of hepatic iron is not a routine part of CLD evaluation, except for haemochromatosis. However, it is useful to review the iron-related parameters that aid /may aid in prediction, diagnosis, staging and prognosis of liver fibrosis, when used in combination with the routine markers of liver dysfunctionality. Here, we specifically discuss LIC, ferritin, hepcidin and transferrin.

In haemochromatosis patients, LIC correlates significantly with the risk of fibrosis and cirrhosis[66]. Similarly, in chronic hepatitis C infections, hepatic iron accumulation increases with fibrosis stage[96,97]. In NAFLD, hepatocellular siderosis has been associated with higher risk of fibrosis than the absence of siderosis[85]. Thus, regardless of disease aetiology, hepatic iron is considered as a surrogate marker of fibrosis severity and not only a fibrogenic factor[98]. Historically, liver iron was assessed by histological staining of iron granules on samples from liver biopsy. However, presently, serum-based markers are used in combination with MRI, which not only detects and quantifies liver iron, but also helps in the staging of high degree fibrosis (F3-F4)[95]. Although LIC determination is important as it correlates with total body iron, it may not reflect iron deposition in extra-hepatic organs. Likewise, low LIC does not exclude the probability of iron loading in extra-hepatic organs[99].

An iron-related protein of immense clinical significance is ferritin. Serum ferritin is shown to be derived primarily from macrophages in mice models[100]. In C282Y homozygotes, serum ferritin > 1000 μg/L with elevated alanine transaminase (ALT) or aspartate transaminase (AST) predicted cirrhosis[101], and with transient elastography, it accurately classified the severity of fibrosis in more than 50% of patients[102]. Thus, in C282Y homozygotes, serum ferritin proved to be a better predictor of hepatic fibrosis than LIC[103]. In NAFLD, elevated serum ferritin not only acted as an independent predictor of advanced fibrosis, but it was also associated with disease severity. Essentially, serum ferritin greater than 1.5 times the upper limit of normal (> 300 ng/mL in women and > 450 ng/mL in men) was associated with hepatic iron deposition and proved to be a useful marker in identifying NAFLD patients with increased risk for NASH and fibrosis[104]. Also, increased serum ferritin was associated with advanced fibrosis, high NAFLD activity scores and increased mortality in NAFLD patients[105], while it also predicted early mortality in patients with decompensated cirrhosis[106]. Moreover, elevated serum ferritin has been strongly associated with the development of diabetes and increased risk of the metabolic syndrome. It is a marker of histologic damage and has been used in a clinical scoring system for NAFLD patients[79]. However, a few studies could not observe a clear association between serum ferritin and fibrosis. Groups such as Valenti *et al*[85], Chandok *et al*[107] and Chitturi *et al*[108] noted that serum ferritin could not effectively predict fibrosis stage and could not independently predict advanced fibrosis in NAFLD/NASH. Another discrepancy is related to the cell-specific accumulation of iron. While serum ferritin levels were 2-fold higher in NAFLD patients with non-parenchymal iron loading than those with parenchymal iron loading, non-parenchymal siderosis was not found to be associated with moderate-severe fibrosis[85]. Notably, elevated ferritin marks inflammation and can be observed in the absence of iron overload[109].

Like ferritin, hepcidin is also affected by both, inflammation and iron excess[110] and

holds diagnostic significance in fibrosis assessment. Its levels decreased in CLD patients and were the lowest in cirrhosis patients[111]. Moreover, the hepcidin:ferritin ratio was lower in CLD patients and further decreased as fibrosis progressed[112]. Similarly, another study showed that in children with CLD, as the severity of fibrosis increased, hepcidin:ferritin ratio decreased, while serum ferritin and transferrin saturation remained high[113]. These studies present hepcidin as a valuable marker of fibrosis progression. Serum hepcidin:ferritin ratio is a potential marker for cirrhosis too[112], where, in addition to the primary insult, oxidative stress may further supress hepcidin synthesis[114].

Yet another iron-related protein of significance in fibrosis evaluation is transferrin. In hepatitis C infection, while ferritin was the only independent predictive factor of severity, transferrin saturation was found to be associated with advanced fibrosis[96]. Also, since the survival estimates were low in patients with transferrin< 180 mg/dL[115], transferrin could act as a predictor of survival in cirrhosis patients. This is in line with observations in chronic hepatitis B infection where serum transferrin reduced as fibrosis progressed from mild to advanced stage and was lower in cirrhotic patients than non-cirrhotic patients[116]. With regards to TFR1, no relationship was observed between its expression and the degree of fibrosis in hepatitis C patients. Levels were upregulated regardless of the degree of liver iron deposition, which suggest that elevated TFR1 may contribute to hepatic iron accumulation in chronic hepatitis C infection[97].

Whether the exclusive usage of such iron-related proteins would be sufficient to predict, diagnose and stage fibrosis/cirrhosis in all liver pathologies remains to be fully answered. However, based on studies hitherto, serum ferritin and hepcidin-ferritin ratio appear to be able to significantly and sufficiently contribute to fibrosis evaluation.

IRON-RELATED THERAPEUTICS FOR LIVER FIBROSIS

Presently, there are no clinically-approved treatments for fibrosis[117]. For decades, several studies have been conducted on animal models and *via* human clinical trials that evaluated the anti-fibrotic efficacy of herbal and pharmacological agents, but none have translated into established protocols for human use till date. While in clinical settings, fibrosis management is considered holistically, here, we specifically review the iron-related strategies.

Phlebotomy is commonly used as a treatment for haemochromatosis. It not only removes excess systemic iron, but also triggers haematopoiesis that utilises the ongoing high absorption of iron for synthesis of new RBCs, thereby controlling the excess-iron-induced pathology. Thus, it controls the excess iron-induced liver damage in haemochromatosis patients, and has shown to effectively reverse liver fibrosis[14,66], reduce the complications of portal hypertension and restore normal life expectancy in these patients[2]. Long-term phlebotomy along with subsequent maintenance of low iron levels can reverse even cirrhosis, but this data needs to be supported by randomized trials[2]. Similarly, in NASH patients, phlebotomy significantly reduced the staining for 7,8-dihydro-8-oxo-2′ deoxyguanosine, a product of oxidative damage to DNA due to iron excess[118]. It improved glucose tolerance, insulin sensitivity in type II diabetics with hyperferritinemia, and liver histology in majority of NAFLD patients in a randomised controlled trial[79]. It also improved ALT levels and glucose-induced-insulin-response in carbohydrate-intolerant non-iron-overloaded NAFLD patients[119], insulin resistance in NAFLD patients without impaired glucose tolerance[120] and ALT, AST and liver histology in NAFLD with hyperferritinemia[121]. In contrast, phlebotomy showed no effect on liver enzymes, hepatic fat or insulin resistance in a study in NAFLD patients[122]. Also, it was not fully successful in dysmetabolic iron overload syndrome, where there was a subtle increase in iron stores (ferritin) in insulin resistant patients[79]. Here, it did not improve the metabolic features, but improved insulin resistance[123].

Iron chelation has been considered for haemochromatosis patients that could not undergo phlebotomy, where the iron chelator deferoxamine (DFO) successfully removed liver iron[124] and thereby contributed to fibrosis control. However, to tackle fibrosis in non-hereditary mild-moderately iron-loaded CLDs, where phlebotomy is not the norm, the iron chelation strategy is not fully developed yet, although promising results are in sight. Studies in various cell lines and animal models revealed that iron chelation decreased the stability of procollagen mRNA in human foetal fibroblasts[125], and reduced elastin mRNA and elastin deposition in human skin fibroblasts[63]. In another study, DFO inhibited and reversed HSC-activation, induced apoptosis of activated rat HSCs, and reduced the expressions of *a-sma*, procollagen

and TIMPs[126]. More recently, a combination of DFO with pegylated interferon-α showed better antifibrotic effects than either treatment alone and increased hepcidin expression in concanavalin A-induced liver fibrosis in rats[127]. This shows the potential for combining iron-chelation with antifibrotic agents to accelerate fibrosis recovery. Oxidative stress degrades apolipoprotein B100 (apoB100), a major component of very-low density lipo-protein (VLDL) that transports cholesterol throughout the body. This hinders the secretion of VLDL and thereby enhances steatosis. In primary rodent hepatocytes, DFO could restore ApoB100 and increase VLDL secretion[128]. In contrast, deferasirox (another iron chelator) showed some inconsistent anti-fibrotic effects in cell lines and animal models[129]. Thus, the benefits need to be ascertained *via* clinical trials before drawing final conclusions.

In addition to phlebotomy and iron-chelation, attempts have been made to modulate iron-related proteins to ameliorate fibrosis. Wang *et al*[130] (2013) demonstrated that inhibition of haem oxygenase-1 (the rate-limiting enzyme in haem catabolism) reduced hepatic iron accumulation, improved portal vein pressure and attenuated rat liver fibrosis. Hepcidin is yet another promising therapeutic agent. Previously, intraperitoneal injections of mini-hepcidin to mice models of hereditary haemochromatosis showed reduced iron loading[131]. Later, Han *et al*[132] conducted elaborate studies and demonstrated that hepcidin expression inversely corelated with the fibrosis severity in human and rodent models. Also, over-expression of hepcidin in rodents attenuated fibrosis, as demonstrated *via* reduced expressions of α-SMA, collagen type 1 and other markers. Cell based assays showed a mechanism whereby exogenous hepcidin hindered TGF-β1-induced SMAD-3 phosphorylation in HSCs and inhibited HSC-activation[132]. Thus, hepcidin therapy may be capable of modulating liver fibrosis in the future.

The significance of formulating novel iron-related therapies emerges from the iron-imposed acceleration of fibrosis progression. Even after liver transplantation in CLD patients, iron loading can increase the probability of post-operative infections and can show poor survival, as demonstrated by the HFE-related hereditary haemochromatosis patients following transplantation[2]. Thus, targeting iron metabolism for fibrosis resolution is a valuable and promising adjunctive strategy. Note that all CLDs do not necessarily trigger fibrosis, so fibrosis may not be present in all patients. Likewise, the levels of iron loading and other iron related parameters may differ between patients and between stages of the disease[111].

CONCLUSION

Excess iron is toxic. It is frequently observed in CLDs and can accelerate the progression of liver fibrosis to cirrhosis and hepatocellular carcinoma, regardless of disease aetiology. From an iron-perspective, mechanisms that promote liver fibrosis include the free-radical generating Fenton reaction, direct or indirect HSC-activation by iron or iron-related protein-receptor complexes, iron-induced intercellular interactions that provide an inflammatory milieu, cross-connection between iron and TGF-β signalling, and a putative role of iron in ECM remodelling. Iron-related proteins such as ferritin, hepcidin (hepcidin:ferritin ratio) and transferrin have successfully contributed to disease prognosis and acted as markers of fibrosis severity and progression in certain liver pathologies. Presently, there are no approved antifibrotic protocols for CLDs with mid-moderate iron-loading. Although iron-chelation and modulation of iron-related proteins show potential therapeutic benefits, these need to be tested rigorously in clinical trials before drawing definitive conclusions on their anti-fibrotic effects. The aim would be to design adjunctive strategies to halt, decelerate and/or reverse fibrosis progression, before it reaches the irreversible stages of advanced cirrhosis and hepatocellular carcinoma.

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MINIREVIEWS

Contribution of ghrelin to functional gastrointestinal disorders' pathogenesis

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Abstract

Functional gastrointestinal disorders (FGID) are heterogeneous disorders with a variety of clinical manifestations, primarily defined by signs and symptoms rather than a definite underlying cause. Their pathophysiology remains obscure and, although it is expected to differ according to the specific FGID, disruptions in the brain-gut axis are now thought to be a common denominator in their pathogenesis. The hormone ghrelin is an important component of this axis, exerting a wide repertoire of physiological actions, including regulation of gastrointestinal motility and protection of mucosal tissue. Ghrelin's gene shows genetic polymorphism, while its protein product undergoes complex regulation and metabolism in the human body. Numerous studies have studied ghrelin's relation to the emergence of FGIDs, its potential value as an index of disease severity and as a predictive marker for symptom relief during attempted treatment. Despite the mixed results currently available in scientific literature, the plethora of statistically significant findings shows that disruptions in ghrelin genetics and expression are plausibly related to FGID pathogenesis. The aim of this paper is to review current literature studying these associations, in an effort to uncover certain patterns of alterations in both genetics and expression, which could delineate its true contribution to FGID emergence, either as a causative agent or as a pathogenetic intermediate.

Key words: Functional gastrointestinal disorders; Functional colonic diseases; Irritable bowel syndrome; Cyclic vomiting syndrome; Infantile colic; Gastrointestinal disease; Ghrelin; Genetics; Epigenetic processes

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Core tip: Functional gastrointestinal disorders are diverse clinical entities whose pathogenesis and phenotype are thought to stem from both genetic and environmental factors. Many reviews have attempted to summarize general pathogenetic mechanisms related to functional gastrointestinal disorders (FGIDs), but more specific knowledge is currently limited. Studies on the brain-gut axis peptide ghrelin have chiefly concentrated on its association with obesity and eating disorders. This review focuses on the possible role of ghrelin in FGID pathogenesis, in an attempt to elucidate the contribution of certain genetic alterations to the emergence of disease.

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INTRODUCTION

Functional gastrointestinal disorders (FGIDs) comprise a variety of disorders stemming from disruptions of the brain-gut axis. These disorders are classified by gastrointestinal symptoms linked to any combination of motility disruption, visceral hypersensitivity, malformed mucosal and immune function, altered gut microbiota and altered central nervous system processing[1,2]. Some FGIDs are quite prevalent, for instance, functional dyspepsia (FD) and irritable bowel syndrome (IBS), which affect roughly 20% and 10% of the general population[3]. It should be noted that the prevalence of FGIDs varies significantly among different countries worldwide; for instance, IBS has lowest estimated prevalence of 3% in the United States and 6.1% in Japan whereas FD has a prevalence of 26% in the United States and 18.4% in Hong Kong[4,5]. It is postulated that many differences in genetic background, diet, physical environment, socioeconomic status, and microbiome may contribute to this observed variation[4,6-8]; thus, for these mainly symptom-defined disorders, the need for a deeper understanding of their pathogenesis and potential treatment strategies is clinically crucial[2].

The Rome Committee for the Classification of Functional Gastrointestinal Disorders has developed diagnostic criteria for FGIDs, and as of 2018, the Rome IV classification recognizes 33 adult and 20 pediatric FGIDs grouped into 8 categories: esophageal disorders, gastroduodenal disorders, bowel disorders, centrally mediated disorders of gastrointestinal pain, gallbladder and sphincter of Oddi disorders, anorectal disorders, childhood functional gastrointestinal (GI) disorders of neonates/toddlers and childhood functional GI disorders of children/adolescents[2,9].

Many pathophysiological mechanisms have been proposed in search of FGID etiologic factors, including genetics, immune contribution, disorders of the GI serotoninergic (5-hydroxytryptamine, 5-HT) innervation, infections, inflammation, increased intestinal permeability, disrupted bile salt metabolism, changes in the microbiota, the impact of diet, and disturbances in the brain-gut axis.

Ghrelin is a brain-gut axis peptide that was isolated from stomach cells and was found to be the endogenous ligand for growth hormone (GH) secretagogue receptor[10,11]. It is known to have growth hormone inducing and appetite-stimulating effects, while also being of importance in gut motility regulation[12].

As noted earlier, brain-gut axis dysfunctions, namely disruptions of enteric nervous system (ENS) and CNS interconnections, are considered to be critical in FGID pathogenesis[13]. This review explores the role of the brain-gut peptide ghrelin in certain of these disorders, based on current scientific data.

LITERATURE SEARCH

Scientific literature search was conducted using the PubMed and Google Scholar databases with the keywords "ghrelin", "obestatin", "ghrelin opposite strand", "GHSR", "GOAT", "functional GI disorders", "IBS", "irritable bowel syndrome", "esophageal disorders", "gastroduodenal disorders", "functional dyspepsia", "bowel disorders", "gastrointestinal pain", "gallbladder disorders", "sphincter of Oddi

disorders", "anorectal disorders", "CVS" and combinations of the aforementioned.

GHRELIN BIOCHEMISTRY, PHYSIOLOGY, AND PATHOPHYSIOLOGY

Ghrelin is a 28 amino acid peptide hormone secreted mainly by the stomach endocrine cells[14]. In humans, the ghrelin gene (GHRL) is located on the short arm of chromosome 3 and comprises five exons coding for the precursor preproghrelin, the first two coding for the functional 28-amino acid region[14,15]. Opposite to the coding strand is an antisense gene called GHRLOS (ghrelin opposite strand) spanning 44kb, whose RNA products undergo alternative splicing and are likely to serve as non-coding regulatory RNAs in the ghrelin axis[16]. The GHRL gene is greatly polymorphic, having more than 300 coding or non-coding region single nucleotide polymorphisms (SNPs) currently validated by 1000 genomes on dbSNP, 4 of which are listed as pathogenic[17].

Ghrelin is found in plasma in two major forms: one that has undergone n-octanoylation at Ser3, called n-octanoyl- or acyl- ghrelin, and one that lacks this modification, referred to as des-acyl ghrelin[14]. The enzyme ghrelin-O-acyltransferase acts on des-acyl ghrelin to produce n-octanoyl ghrelin in ghrelin-producing cells[14,18]. Des-acyl ghrelin is the predominant circulating form of ghrelin[19].

The n-octanoylation at the N-terminal serine is necessary for ghrelin to exert its whole range of physiological actions, such as GH induction, although des-acyl ghrelin can also have certain physiologic effects, for instance, anti-apoptotic and hypotensive actions on the cardiovascular system[14,18,20]. Des-acyl ghrelin and acyl-ghrelin can have both similar and opposing physiological actions (Table 1)[19].

Ghrelin is mainly produced by P/D1 oxyntic cells (similar to mouse X/A cells) mainly in the fundus of the stomach[18,21,22]. Its mRNA is also found in small and large intestine cells, in pancreatic alpha and beta islet cells, in kidney glomeruli and, to a certain degree, in most human tissues[14,19,21-23]. In secretory granules of endocrine cells in the upper small intestine, ghrelin is colocalized with motilin, a peptide hormone involved in phase III of the migrating motor complex[24,25].

Ghrelin's receptor is known as growth hormone secretagogue receptor (GHSR) and is a rhodopsin-like G-protein coupled receptor (GPCR) with seven membrane-spanning segments[26,27]. The receptor's gene is located on the large arm of chromosome 3, and its mRNA product can be alternatively spliced to two different transcripts, 1a and 1b; however, it is only the 1a mRNA protein product, GHSR1a, which can bind ghrelin[26]. GHSR1a also displays constitutive activity[24]. Type 1a mRNA has been found in the arcuate and ventromedial nuclei of the hypothalamus, in the pituitary, thyroid gland, and adrenals, whereas the type 1b mRNA is ubiquitously present in human tissue[19,22]. It has been argued that GHSR1b, the 1b type mRNA protein product, may modulate the constitutive activity displayed by GHSR1a[26]. When bound to ligand, be that ghrelin or the 36% homologous protein motilin, the GHSR1a receptor has been found to activate many downstream signaling cascades, including the MAPK, mTOR, AMPK, PI3K/Akt and PLC pathways, while displaying functional agonism[14,18,24,26].

Many factors are thought to regulate ghrelin secretion by the stomach P/D1 like cells. These include positive regulators such as fasting, muscarinic stimulation by the vagus nerve, beta-adrenergic stimulation, estrogen, cholecystokinin, glucagon, and deep sleep as well as inhibitory regulators, including alpha-adrenergic stimulation, insulin, glucose, leptin, long chain fatty acids and somatostatin[18,24]. Total plasma ghrelin concentration peaks before meals and drops to a minimum within an hour postprandially[18,21]. Protein and fatty meals have been found to reduce total and acyl-ghrelin plasma levels, whereas carbohydrate meals cause an initial elevation of both levels followed by suppression, thought to be mediated by insulin[28]. Women have been found to have higher levels of total plasma ghrelin[29].

Ghrelin displays a large repertoire of physiological actions (Figure 1). It directly acts on pituitary somatotrophs to increase growth hormone (GH) secretion synergistically with growth hormone-releasing hormone (GHRH), being the more potent GH secretagogue[19,27,30]. Regarding appetite stimulation, ghrelin acts on vagal afferent neurons in the stomach which project to the nucleus tractus solitarius (NTS) of the brainstem, a CNS region which participates in visceral reflexes and connects to hypothalamic feeding centers[24,31,32]. Acting on arcuate nucleus cells of the hypothalamus, it upregulates production of the orexigenic peptides neuropeptide Y (NPY) and agouti-related peptide (AgRP)[19,33,34]; therefore, it increases appetite. The same effect could also be explained by ghrelin's action in the area postrema (AP), a brainstem region poorly shielded by the blood-brain barrier that is responsible for

Table 1 Comparison of physiological actions of acyl- and des-acyl ghrelin

Parameter	Acyl-ghrelin	Des-acyl ghrelin	Ref.
Appetite[1]	Increase	Decrease	[87]
Metabolism[1]	Positive energy balance	Negative energy balance	[87]
Adipogenesis	Increase	Increase	[88]
Cardiomyocyte and endothelial cell death	Inhibition	Inhibition	[20]
Papillary muscle contractility	Negative inotropy	Negative inotropy	[89]
Blood pressure	Decrease	Decrease	[90]
Insulin release[1]	Decrease	Increase	[91]
Insulin sensitivity[1]	Decrease	Increase	[92]
Muscle wasting	Decrease	Decrease	[93]
Gastric emptying[1]	Increase	Decrease	[94]
Gastric motility[1]	Increase	Decrease	[95]

[1]Opposing actions.

humoral emesis initiation[35]. Acting on AP neurons, ghrelin could decrease nausea and increase feeding[24]. In general metabolism, ghrelin decreases fat utilization[36].

In the gastrointestinal system, ghrelin is considered to be a component of the brain-gut axis[37]. Its effects include gastric acid secretion and generation of nitric oxide and prostaglandins, vasodilators which protect the gastric mucosa from ischemic stress and alcoholic damage[18,21]. Similar to the structurally related protein motilin, it can induce migrating motor complexes, causing premature phase III activity in the stomach and duodenum[18] and, moreover, accelerates gastric emptying[14,21,37,38]. It also has been found to increase muscle tone in the proximal stomach[38]; furthermore, ghrelin decreases the small intestine transit time but has no such effect on the colon[21]; however, ghrelin agonists which, unlike ghrelin, penetrate the blood-spinal cord barrier have been found to increase colonic contractility and propulsion, activating the defecation reflex[39]. It should be noted that, low ghrelin levels have been detected in obese individuals or individuals after bariatric surgery, and administration of physiological doses of exogenous ghrelin to these individuals does not appreciably affect gastric motility[40].

Pathophysiologically, ghrelin serves as a marker for chronic gastritis and its levels are indicative of the histopathological severity of *H. pylori* infection; furthermore, it has been reported that in gastric adenocarcinoma and advanced grade colorectal cancer, plasma ghrelin levels are significantly lower, possibly due to inadequate ghrelin production by the affected tissue[41]. Ghrelin receptor type 1b (GSHR1b), although formerly considered physiologically inert, has been found to promote proliferative and invasive activity in colorectal malignancies[26,41]. Regarding inflammatory GI disorders, ulcerative colitis and ileal Crohn disease patients have significantly elevated plasma ghrelin levels. Ghrelin is also positively correlated with serum inflammatory markers in active inflammatory bowel disease patients[42]. Ghrelin could exert antinociceptive effects in GI disease, acting on opioid receptors and downregulating TRPV1, an ion channel expressed chiefly on nociceptive sensory neurons, as found in patients with IBS[43].

GHRELIN GENETICS, EXPRESSION, AND FGIDs

FD

FD is a clinical disorder whose symptoms originate in the gastroduodenal region[44]. According to Rome criteria, the symptom complex is often related to ingestion of a meal and includes epigastric pain, postprandial fullness, bloating, early satiety, belching, nausea, vomiting and epigastric burning. Attempts have been made to simplify the intricate heterogeneity of the dyspepsia symptom complex and a subdivision based on the predominant symptoms of either pain or discomfort has been proposed; thus, there are two clinically distinct FD syndromes, although these often overlap clinically: postprandial distress syndrome (PDS; comprising early satiety or meal-related fullness) and epigastric pain syndrome[44]. In a recent meta-analysis, the overall pooled prevalence of uninvestigated dyspepsia was 20.8% (95%CI: 17.8% to 23.9%)[45,46]; over 75% of those patients will not have a structrural cause for their symptoms after a thorough work-up and are thought to have FD[45,46].

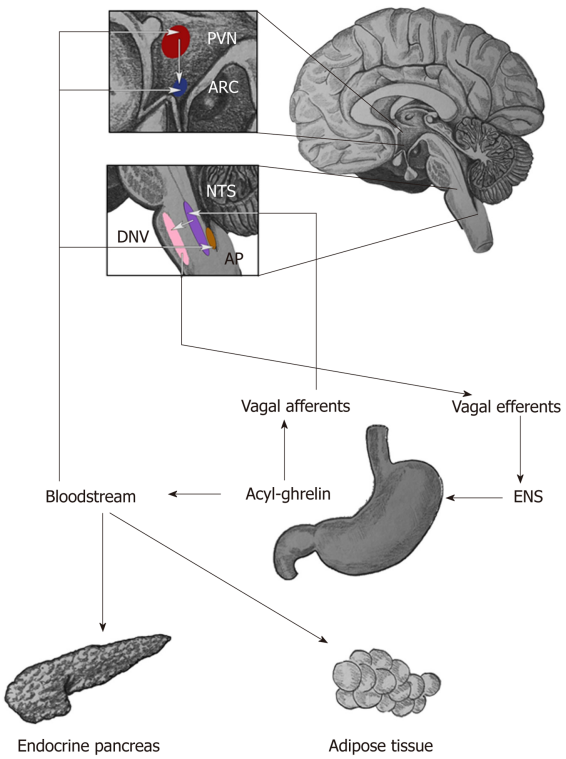

Figure 1 Acyl-ghrelin is produced by stomach P/D1 cells. It enters the bloodstream, reaching the pancreas, where it inhibits insulin release, and adipose tissue, where it inhibits adipogenesis. Through the circulation, it also acts on the paraventricular and arcuate nuclei of the hypothalamus, increasing growth hormone releasing hormone release and appetite. It also affects neurons in the area postrema, where it acts to decrease nausea. Ghrelin acts directly through the GHSR receptor on vagal afferents, which project to the nucleus tractus solitaries of the brainstem. This region in turn connects to the dorsal motor nucleus of the vagus nerve, which gives rise to vagal efferent that increase gastric contractions, acid secretion, and GI motility through the enteric nervous system. Ghrelin does not affect defecation, as it cannot penetrate and act on the defecation center of the spinal cord[11,18,24,37,39,96]. PVN: Paraventricular; ARC: Arcuate; AP: Area postrema; NTS: Nucleus tractus solitaries; DNV: Dorsal motor nucleus of the vagus nerve; ENS: Enteric nervous system.

Although multiple mechanisms of FD pathogenesis (including abnormal gastric motility, visceral hypersensitivity, low grade mucosal inflammation and cellular changes in enteric nerves, muscle or interstitial cells of Cajal) have been suggested, its underlying etiology and pharmacological therapy remain unclear. As we have already mentioned, ghrelin, a gut-derived peptide found in the stomach, plays a role in the regulation of gastric motility[42,47].

Several papers have been published studying associations between plasma ghrelin levels and FD (Table 2 includes a summary of the literature on changing serum ghrelin levels in patients with FD, which remains controversial).

Studies on the relationship between FD and ghrelin reported that circulating levels of total ghrelin were significantly higher in patients with FD than in controls[48,49]. The higher levels of ghrelin occurred at 30 min. after the meal which is the time when most of the dyspeptic symptoms usually occur, supporting the idea of a putative role for ghrelin in the etiology of the dyspeptic symptoms in FD patients.

In contrast, Lee *et al*[50] found that dysmotility-like FD patients had lower total ghrelin levels in the fasting state than controls and that these patients displayed no postprandial drop in total ghrelin levels. In addition, in the same group of FD patients, Takamori *et al*[51] showed lower fasting total ghrelin levels than in controls and no difference between fasting and postprandial levels in FD patients. Interestingly, a study on a rat model for chronic stress showed that stress initially decreases gastric emptying, due to sympathetic nervous system activation, but eventually gastric emptying accelerates and plasma ghrelin levels are found elevated. This was considered an indication that alterations in ghrelin physiology may in part be responsible for gastric motility disorders which are triggered by stress, such as FD[52,53].

Keeping in mind that the acylated form of ghrelin exhibits physiologic activity (*i.e.* appetite, adipogenesis *etc.*), many studies have focused on this subtype (Table 1). Shinomiya *et al*[54] found that although fasting levels of acylated ghrelin were not

Table 2 Ghrelin genetics and expression in functional dyspepsia patients compared to controls

Ref.	Participants	Parameters	FD patients compared to Controls	*P* value
Shindo *et al*[57]	PDS (*n* = 76)/Controls (*n* = 20)	Acyl-ghrelin, plasma levels	Lower	< 0.05[a]
Shinomiya *et al*[54]	FD (*n* = 18)/Controls (*n* = 18)	Acyl-ghrelin, plasma levels	Higher	> 0.05
		Des-acyl ghrelin, plasma levels	Lower	> 0.05
Nishizawa *et al*[49]	FD (*n* = 47)/Controls (*n* = 17)	Total ghrelin, plasma levels	Higher	< 0.05[a]
		Acyl-ghrelin, plasma levels	Higher	< 0.05[a]
Lanzini *et al*[48]	FD (*n* = 39)/Controls (*n* = 53)	Total ghrelin, plasma levels	Higher	< 0.01[a]
Takamori *et al*[51]	FD (*n* = 16)/Controls (*n* = 19)	Des-acyl ghrelin, fasting state, plasma levels	Lower	0.0019[a]
		Acyl-ghrelin, fasting state, plasma levels	Lower	0.4191
		Des-acyl ghrelin to acyl-ghrelin ratio, fasting state, plasma levels	Lower	0.0154[a]
Lee *et al*[50]	FD (*n* = 42)/Controls (*n* = 14)	Total ghrelin, fasting state, plasma levels	Lower	< 0.05[a]
Choi *et al*[56]	FD (*n* = 39)/Controls (*n* = 38)	Male patients, acyl-ghrelin, plasma levels	Lower	0.017[a]
	FD (*n* = 65)/Controls (*n* = 49)	Female patients, acyl-ghrelin, plasma levels	Lower	0.348
Lee *et al*[65]	FD (*n* = 167)/Controls (*n* = 434)	rs42451, A allele	More frequent (PDS subgroup)	< 0.05[a]
		rs42451, AA genotype	More frequent (PDS subgroup)	< 0.05[a]

[a]Statistical significance. PDS: Postprandial distress syndrome; FD: Functional dyspepsia.

significantly different between healthy controls and FD patients, its plasma levels correlated with the subjective symptom score in patients with FD, especially females. In accordance to this study, Takamori *et al*[51] reported that fasting and postprandial levels of acylated ghrelin were similar in FD patients and healthy controls. Authors suggested that in those patients with dysmotility-like FD, secretion and metabolism of ghrelin are the pathologically affected factors and not the step of acylation. A recent study by Kim *et al*[55], confirmed the lack of difference in plasma acylated ghrelin levels between healthy controls and FD patients; however, abnormal plasma acylated ghrelin levels before or after a meal related to specific symptoms seen in patients with FD; remarkably, FD patients with higher fasting plasma levels of acylated ghrelin suffered from less epigastric pain, whereas post prandial decrease of the high fasting levels of acylated ghrelin were associated with higher early satiety scores. Apparently, higher fasting plasma levels of acylated ghrelin yield a gastroprotective effect on the gastric mucosa, while postprandial levels interfere with the gastric accommodation reflex.

In contrast, other studies measured lower acylated ghrelin levels in samples from FD patients. Significantly lower plasma acylated ghrelin levels in FD patients compared to controls were reported by Choi *et al*[56], however only for male patients, suggesting that FD pathogenesis in females may employ additional mechanisms. Shindo *et al*[57] also reported that acylated ghrelin levels were significantly lower in PDS patients than in controls, while des-acyl ghrelin levels did not differ significantly among EPS, PDS patients, and healthy volunteers. They suggested that lower acylated ghrelin levels may reflect the gastric atrophy and ghrelin-producing cell loss in PDS patients; furthermore, they found that in PDS patients, plasma acyl-ghrelin levels are inversely correlated with the diagnostic marker *Tmax*, the time of maximal gastric excretion, to a significant degree. They suggested that the study of gastric emptying and plasma ghrelin levels could be useful in the diagnosis of PDS[57,58]; indeed, plasma acylated ghrelin levels were lower in the PDS group than in controls, EPS, and combination of PDS and EPS groups[59]. Interestingly, *H. pylori* eradication increased plasma acylated ghrelin levels, in contrast to a systematic review which reported that *H. pylori* eradication had no influence in circulating ghrelin levels[60]. Evidently, it was not just the presence of *H. pylori*, but also the histological changes associated with it,

such as intestinal metaplasia, that affected plasma ghrelin levels[61].

Ghrelin's genetic polymorphisms and FD

There have also been studies linking certain genetic polymorphisms of the GHRL gene with certain parameters of the FD phenotype (Table 3). Ando *et al*[62] found that the Leu72Met genotype that is a SNP outside the region coding for mature ghrelin was significantly and independently correlated with low plasma acylated ghrelin levels; however, this correlation, as well as a correlation with the Gln90Leu genotype, could not be confirmed in a later study on *H. pylori* negative FD patients[63]. In contrast, this study found a significant correlation between the preproghrelin 3056TT genotype and the plasma levels of acylated ghrelin, although it did not find a correlation between any of the Arg51Gln, Preproghrelin3056TC, Leu72Met and Gln90Leu polymorphisms and FD phenotypes[63]. Trying to clarify the putative associations of Gln90Leu and Leu72Met genotypes with ghrelin activity, Yamawaki *et al*[64] showed that Leu72Met SNP was associated with early phase of gastric emptying but not with entire gastric emptying in FD patients, while there was no significant association between this SNP and early phase of gastric emptying in healthy volunteers; moreover, the Gln90Leu SNP has been associated with depression severity in FD patients[64]. The GHRL rs42451 AA genotype, as well as the A allele, have been reported to be more frequent among PDS patients, compared to controls (Table 2)[65].

Ghrelin modulators as treatment for FD

Regarding treatment outlooks, FD has been considered as a possible indication for ghrelin and ghrelin agonists, such as the synthetic pentapeptide relamorelin, given ghrelin's prokinetic effects on the stomach[39,53]. Ghrelin's use is limited due to short plasma half-life and bioavailability, so mainly ghrelin agonists are currently being tested in clinical trials for the treatment of diabetic gastroparesis and chronic constipation[39,53,66,67]. Akamizu *et al*[47] postulated the possible usefulness of ghrelin in the treatment of weight loss in FD patients, after a 2009 trial in which intravenous ghrelin was administered to patients with FD, causing increased appetite (approx. by 30% before and after treatment) in 4 out of 5. This trial did not, however, yield statistically significant results[47].

Recently, rikkunshito, a standardized Japanese herbal medicine with ghrelin signal-enhancing actions, has been studied as a candidate for the treatment of FD[68,69]. A 2014 double-blind randomized control trial showed that treatment with rikkunshito significantly reduced epigastric pain in patients with the PDS subtype of FD, an effect which was not accompanied by an elevation in plasma ghrelin levels[69]. A later study reported that a low baseline level of plasma des-acyl ghrelin was significantly and independently associated with stronger symptom relief following treatment with rikkunshito in *H. pylori* negative patients, which was also not accompanied by elevated post-treatment plasma ghrelin levels[68]. These findings suggest that the clinical value of ghrelin, ghrelin agonists and ghrelin-enhancing drugs in treating FD is probably greater in patients with certain patterns of alteration in ghrelin physiology.

IBS

IBS is characterized by the presence of recurrent abdominal pain that is associated with defecation or a change in bowel habits (*i.e.* constipation, diarrhea, or an alteration between constipation and diarrhea). Bloating could often be present, but it is not mandatory for diagnosis. Symptom onset should occur at least 6 mo before diagnosis and symptoms should be present during the last 3 mo[70]. Diagnosis focuses on symptom assessment, as biochemical, histopathological and radiological tests are currently of little clinical value[71]. Based on its clinical features, IBS can be subdivided into constipation predominant (IBS-C), diarrhea predominant (IBS-D) and alternating stool pattern (IBS-A) IBS. IBS is the most common gastrointestinal disorder encountered in primary care with a world-wide prevalence of around 11.2% (95%CI: 9.8%-12.8%) based on a recent meta-analysis[45,72].

IBS is a disorder whose pathophysiology has yet to be completely elucidated. Many pathophysiological mechanisms have been correlated with the emergence of IBS symptoms, including visceral hypersensitivity, abnormal gut motility, dysregulated intestinal secretions, autonomous dysfunction, increased intestinal permeability, neurotransmitters, behavioral changes, immune activation, endocrine dysfunction and altered gut microbiota, and previous infection[73-75]; moreover, up to 33% patients with IBS had a positive for IBS family history compared to 2% of the controls, a finding which suggests the contribution of a strong genetic component[71,76].

Focusing on endocrine dysfunction, ghrelin is one of the gut hormones which are

Table 3 *GHRL* genetic polymorphisms and associations with laboratory/clinical parameters related to functional dyspepsia

Ref.	Participants	Genotype	Phenotype	*P* value
Ando *et al*[62]	Healthy (n = 264)	Leu72Met, AA+CA compared to CC	Higher acyl-ghrelin plasma levels	0.015[a]
		3056T C, CC+TC compared to TT	Higher acyl-ghrelin plasma levels	0.021[a]
Futagami *et al*[63]	FD (n = 74)/PDS (n = 51)/EPS (n = 23)/Controls	3056TC, TC+CC compared to TT	Higher acyl-ghrelin plasma levels	0.025[a]
		Leu72Met, CA+AA compared to CC	Higher acyl-ghrelin plasma levels	0.347
Yamawaki *et al*[64]	FD (n = 74)	Leu72Met, GG compared to GT+TT	Early phase of gastric emptying, 10 minutes after meal	0.038[a]
	FD (n = 74)	Leu72Met, GT+TT compared to GG	Higher SRQ-D scores (GT+TT compared to GG genotype)	0.0097[a]
	Controls (n = 64)	Leu72Met, GG compared to GT+TT	Early phase of gastric emptying, 10 minutes after meal	> 0.05

[a]Statistical significance. FD: Functional dyspepsia; PDS: Postprandial distress syndrome; SRQ-D: Self-rating Questionnaire for Depression Score (an index of depression severity).

thought to be involved in IBS pathogenesis; therefore, its expression patterns have been studied in IBS patients compared to controls (Table 4). In a 2008 study, El-Salhy *et al*[77] reported that the density of ghrelin-immunoreactive cells in stomach oxyntic mucosa was significantly lower in IBS-C and significantly higher in IBS-D patients than in healthy controls, with the plasma and tissue extract concentrations of total and acyl ghrelin showing no significant difference. They argued that the latter finding may suggest the existence of compensation in ghrelin secretion, and it is the occasional instability of this mechanism that may result in intermittent IBS symptoms[77]. Sjlund *et al*[78] were later able to confirm that circulating ghrelin levels covaried with motilin in IBS patients but not in healthy volunteers. Authors suggested that the two peptides' synergy together with low vagal activity may result in typical IBS dysmotility[78]. Şahin-Eryılmaz *et al*[79] later reported higher plasma ghrelin levels in IBS-D patients and higher ghrelin staining in the antral mucosal glands of IBS-C patients, compared to controls. No studies have reported different patterns of ghrelin alterations among IBS patients according to gender, in spite of IBS predominantly affecting women, which more often complain of abdominal pain, compared to men who primarily complain of diarrhea, findings which indicate different pathophysiological basis for the disorder between genders[80].

Regarding ghrelin polymorphisms (Table 4), Lee *et al*[65] found that the G allele of rs3755777 was significantly associated with IBS-D, as the GG and CG genotypes were significantly less frequent in IBS-D patients than in controls. Russo *et al*[36] later found the Leu72Met T allele and GT genotype to be significantly reduced in IBS-D patients compared to controls.

Considering other alterations in ghrelin expression, a 2017 study found that GHRLOS, the GHRL opposite strand transcript assumed to have a regulatory function in the ghrelin expression (see above), was downregulated in sigmoid biopsy samples from IBS patients compared to controls[81]. This finding underlines the contribution of DNA epigenetics - regulatory RNA, DNA methylation, histone covalent modification- in the emergence of many pathophysiologically obscure diseases, possibly guiding future IBS research towards new directions[82].

Unlike FD, ghrelin and ghrelin receptor agonists are not thought to be of as great therapeutic value in IBS, because ghrelin weakly affects the distal GI tract, which is more involved in IBS pathophysiology[53].

CYCLIC VOMITING SYNDROME

Cyclic vomiting syndrome (CVS) is a functional gastrointestinal disorder characterized by recurrent episodes of severe nausea and vomiting, separated by relatively asymptomatic periods. It was previously thought to be a pediatric disorder, but current knowledge recognizes its incidence in adults. However, it is still underdiagnosed[83]. CVS pathophysiology includes brain-gut axis dysfunction,

Table 4 Ghrelin genetics and expression in irritable bowel syndrome patients compared to controls

Ref.	Participants	Parameters	IBS patients compared to Controls	*P* value
Salhy *et al*[77]	IBS-C (n = 19)/Controls (n = 10)	Ghrelin cell density in gastric mucosa	Higher	< 0.0001[a]
	IBS-D (n = 18)/Controls (n = 10)	Ghrelin cell density in gastric mucosa	Lower	< 0.0001[a]
Şahin-Eryılmaz *et al*[79]	IBS-C (n = 30)/Controls (n = 30)	Ghrelin staining, antral mucosal glands	Higher	0.038[a]
	IBS-D (n = 30)/Controls (n = 30)	Plasma ghrelin levels	Higher	0.001[a]
Sjölund *et al*[78]	IBS (n = 9)/Controls (n = 9)	Acyl-ghrelin and motilin plasma levels covariation	Covariation present in IBS patients only	< 0.02[a]
		Des-acyl ghrelin and motilin plasma levels covariation	Covariation present in IBS patients only	< 0.04[a]
		Total ghrelin and motilin plasma levels covariation	Covariation present in IBS patients only	< 0.004[a]
Lee *et al*[65]	IBS (n = 60)/Controls (n = 434)	rs3755777, G allele	Less frequent (IBS-D subgroup)	< 0.05[a]
		rs3755777, GG+CG genotypes	Less frequent (IBS-D subgroup)	< 0.05[a]
Russo *et al*[36]	IBS-D (n = 28)/Controls (n = 19)	Leu72Met, T allele	Less frequent	0.027[a]
		Leu72Met, GT genotype	Less frequent	0.041[a]
Videlock *et al*[81]	IBS (n = 20)/Controls (n = 10)	GHRLOS lncRNA levels	Down-regulated, IBS	< 0.05[a]
			Down-regulated, IBS-D	< 0.05[a]

[a]Statistical significance. IBS-C: Irritable bowel syndrome, constipation predominant; IBS-D: Irritable bowel syndrome, diarrhea predominant; GHRLOS: Ghrelin opposite strand transcript; lncRNA: Long non-coding RNA.

mitochondrial mutations, autonomic involvement, cannabinoid, and opioid receptor mutations and dysregulation of the hypothalamic-pituitary-adrenal axis[83].

A 2018 study by Hejazi *et al*[84] found that in adult patients with CVS, fasting serum ghrelin levels were significantly elevated in comparison with controls. It was speculated that elevated ghrelin levels could be related to the rapid gastric emptying found during the recovery phase of CVS[84].

INFANT COLIC

Infant colic is a functional gastrointestinal disorder of early infancy defined as "paroxysms of irritability, fussing or crying lasting more than 3 h per day and occurring more than 3 d each week"[9,85]. Many organic factors are thought to participate in its pathogenesis, including diet, gas, intestinal hypermotility and hormones, although it has been argued that these explain only a few of infant colic cases[85].

Savino *et al*[86] reported in a 2006 paper that infants with colic have significantly higher serum ghrelin levels compared to controls; however, they could not clarify whether this was a cause or effect of the condition. One proposed hypothesis was that ghrelin induces hypermotility in the physiologically immature infant intestine[86].

CONCLUSION

It is evident that no clear pattern of ghrelin genetic alterations has been established which can be integrated into a model for FGID pathogenesis. Even for certain types of FGIDs, statistically significant results often contradict previously published data or later studies fail to reproduce previous significant findings. The role of ghrelin in the emergence, progress, or treatment of FGIDs could be important, depending on certain disease subtype, patient gender, age, or other related physiological factors. Ghrelin's wide range of physiological effects, although, shows that a pathophysiological involvement is certainly plausible.

The complicated nature of these clinical entities suggests that ghrelin disruptions

could account for a small percentage of cases or that they only weakly affect the disease phenotype. This indicates that further studies should employ more specific strategies in order to elucidate its role in pathogenesis, to control for confounding factors and to clarify whether ghrelin is an independent causal factor or an intermediate in disease mechanisms

The plethora of statistically significant data, despite displaying occasional contradiction, does encourage further research into the genetic or epigenetic alterations of the GHRL gene, as these may possibly serve as substrate for the different pathophysiological pathways which give rise to the variety of FGID phenotypes. Further studies should focus on thoroughly applying official diagnostic criteria among patient groups, on recognizing FGID subtypes as distinct pathophysiological entities, on controlling for factors (*e.g*. BMI and gender) which also affect ghrelin physiology, on studying specific clinical parameters (*e.g*. gastric emptying times) in relation to certain ghrelin alterations, and on exploring other polymorphisms related to the hormone's physiology, such as those referring to the ghrelin receptor gene or regulatory lncRNAs and miRNAs. Comparable results between studies are also imperative for meta-analysis, and few currently exist as such.

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MINIREVIEWS

Functional gastrointestinal disorders and gut-brain axis: What does the future hold?

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Abstract

Despite their high prevalence, lack of understanding of the exact pathophysiology of the functional gastrointestinal disorders has restricted us to symptomatic diagnostic tools and therapies. Complex mechanisms underlying the disturbances in the bidirectional communication between the gastrointestinal tract and the brain have a vital role in the pathogenesis and are key to our understanding of the disease phenomenon. Although we have come a long way in our understanding of these complex disorders with the help of studies on animals especially rodents, there need to be more studies in humans, especially to identify the therapeutic targets. This review study looks at the anatomical features of the gut-brain axis in order to discuss the different factors and underlying molecular mechanisms that may have a role in the pathogenesis of functional gastrointestinal disorders. These molecules and their receptors can be targeted in future for further studies and possible therapeutic interventions. The article also discusses the potential role of artificial intelligence and machine learning and its possible role in our understanding of these scientifically challenging disorders.

Key words: Functional gastrointestinal disorders; Idiopathic bowel syndrome; Gut-brain axis; Microbiome-gut-brain axis; Machine learning; Artificial intelligence

Core tip: The multifactorial nature of functional gastrointestinal disorders makes the diagnosis challenging. The identification of pathogenic microbiome signatures, combined with demographical, immunologic and neuroimaging findings can be encoded into machine learning algorithms which may help identify trends and patterns that can be studied to further our understanding of these disorders. These patterns can help

determine the causality or can guide further research.

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INTRODUCTION

Functional gastrointestinal disorders (FGIDs) are a highly prevalent group of disorders diagnosed solely by symptomatology as there is a lack of understanding of the underlying structural or chemical abnormalities. The main symptoms described by patients with FGIDs include abdominal pain, dyspepsia, regurgitation, bloating, constipation, diarrhea, incontinence, problems in the passage of food or stool, or any combination of these symptoms. Different mechanisms have been understood to play a role in pathogenesis including disturbance in motility, altered mucosal and immune function, visceral hypersensitivity, disturbance in gut microbiota, and altered processing of visceral signals in the central nervous system (CNS). Common FGIDs include gastroesophageal reflux disease (GERD), functional dysphagia, functional dyspepsia, gastroparesis, irritable bowel syndrome (IBS), functional constipation, diarrhea, and fecal Incontinence.

It has been well established that patients with FGIDs, along with having symptoms related to the gastrointestinal tract, have co-existing psychosocial symptoms such as stress, anxiety and depression and thus a biopsychosocial model has been proposed for FGIDs as depicted in Figure 1[1]. The bidirectional communication pathways between the gut and the brain, involved in the pathogenesis of FGIDs, are collectively known as the gut-brain axis[2]. This communication occurs through a number of neuronal pathways and is modified by environmental and anatomical factors such as hypothalamus-pituitary axis, limbic system, autonomic nervous system, and endocrine system. The gut microbiota has recently emerged as a possible influencer of the axis and has seized the much-needed attention of researchers.

Although it has been a widely held belief that human cells are outnumbered by microorganisms by a ratio of 1:10 recent literature shows that the ratio is closer to1:1[3]. This, however, does not diminish the important role of microbiota in our bodies. The microbiota, living in harmony with the human tissues, has a number of synergistic roles. Although the exact composition of the microbiota may differ among individuals as each individual has their own microbiome signature, its functional role in homeostasis and development is ubiquitous to all humans. From helping in digestion, to protecting against pathogenic microorganisms, the gut microbiota has played an important role in maintaining immunity and homeostasis. Recently, studies have shown that one of the main inputs to the gut-brain axis comes from microbiota, leading to the coining of the term 'microbiome-gut-brain axis'[4].

This article reviews the important anatomical aspects of the gut-brain axis in order to discuss the underlying molecular mechanisms that may have a role in the pathogenesis of FGIDs. It further discusses the different factors that may influence the gut-brain axis and can be used in the future as possible therapeutic targets. The article also looks at the potential new approaches such as the use of artificial intelligence and machine learning to help advance our understanding of these complex disorders.

ANATOMICAL CORRELATES OF GUT AND BRAIN CONNECTIONS IN RELATION TO FGIDS

The brain and gut communicate continuously through a number of complex pathways involving the enteric nervous system (ENS), the autonomic nervous system (ANS), the hypothalamus-pituitary axis (HPA), and the central nervous system (CNS) as shown in Figure 2[2]. Each pathway is highly integrated and regulated by interrelational neuronal and neurohumoral factors.

FGIDS: Enteric nervous system and central nervous system interaction

The ENS is responsible for the intrinsic innervation of the GI tract and consists of two plexuses. The outer plexus, called myenteric plexus, is involved in the regulation of

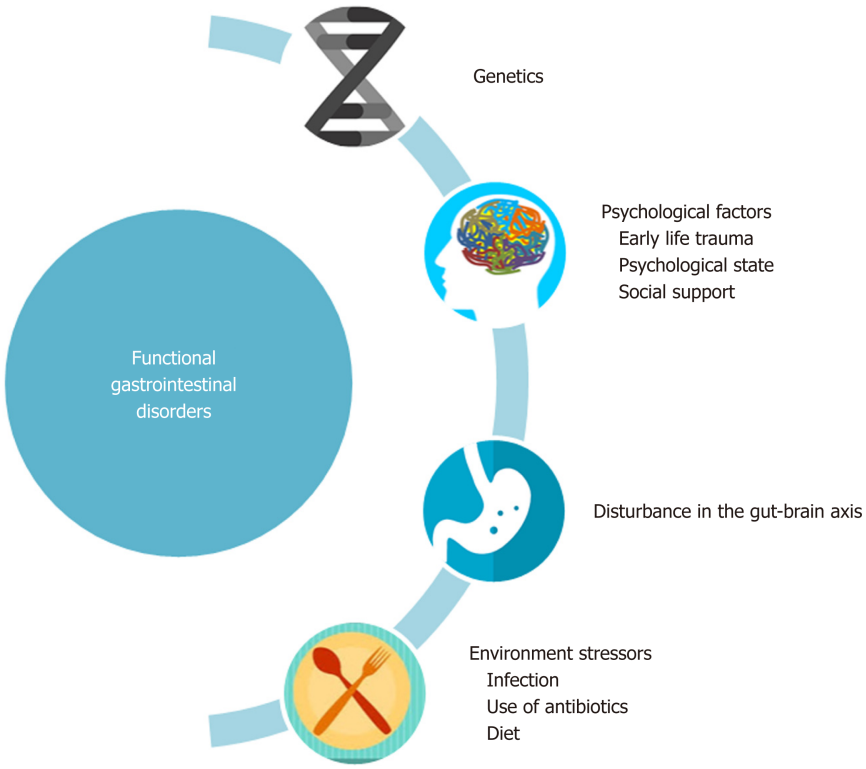

Figure 1 The biopsychosocial model for functional gastrointestinal disorder. The figure illustrates the interaction of psychosocial factors, environmental factors and disturbances in gut-brain axis with functional GI disorders. Early life stress events combined with psychosocial state of an individual determines the symptomatology and quality of life of individuals. Adopted from ROME IV[1].

smooth muscles controlling the gut movements such as peristalsis. The inner, submucosal, plexus is responsible for secretion and absorption[5]. The ENS is modulated by the extrinsic innervation coming from the ANS and the CNS. The ANS consists of sympathetic (splanchnic) nerves and parasympathetic (vagal-sacral) nerves. The somatic nervous system controls the striated muscles of the proximal esophagus and the external anal sphincter. In CNS, the highly integrated gut-brain communication axis is mainly controlled by the limbic system which receives input from the ENS through the ANS and is modulated by higher cortical areas[2].

The limbic system consists of the amygdala, hypothalamus, medial thalamus and the anterior cingulate cortex (ACC). It is primarily concerned with the regulation of behavior, emotions, arousal, memory and motivation. It is also partly responsible for the modulation of the visceral organs by providing input through a number of hemostatic mechanisms. The visceral region, also known as 'visceral brain', lies specifically in the hypothalamus which is a central component of the limbic system[6]. The amygdala is responsible for emotional and stressful responses and drives other areas such as the prefrontal cortex for execution of complex functions. Functional brain imaging using magnetic resonance imaging (MRI) has shown that damage to the amygdala results in an alteration in stress responses such as flat affect seen in schizophrenia patients[7]. The damage may also lead to inhibition of social interaction and emotional conditioning[7]. The amygdala also consolidates memories with the help of emotional stimuli[6].

There are two pathways involved in the processing of the emotional input. A direct pathway, which processes crude information, is called the thalamo-amygdala pathway. It is chiefly responsible for rapid, unconditioned fear response without the intervention of the cortex. The thalamo-cortico-amygdala pathway, on the other hand, allows for a slower, conditioned response by more complex processing of the emotional stimuli (Figure 3)[8].

The anterior cingulate cortex (ACC) has a central role in the thalamo-cortico-amygdala pathway. The ACC detects the conflict between the current emotional state and any new stimulus that can incite a new affective or motivational response. It relays information to the pre-frontal cortex (PFC) where further processing of the input takes place and the decision is made about how to respond to the stimuli. Functional Magnetic Resonance Imaging (fMRI) has highlighted that the perigenual

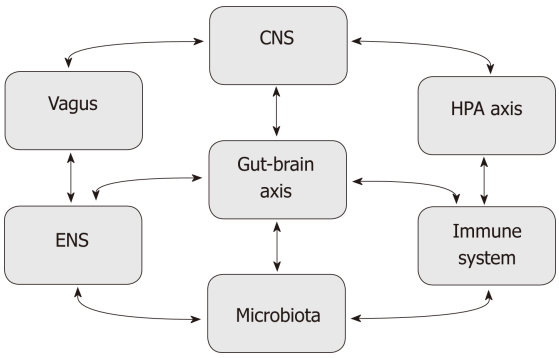

Figure 2 Schematic representation of different factors modulating the gut-brain axis. The microbiota and central nervous system interact in a bidirectional relationship bridged by the gut-brain axis. This axis is also influenced by Immune system, enteric nervous system, hypothalamic-pituitary axis, and vagus nerve. CNS: Central nervous system; ENS: Enteric nervous system; HPA: Hypothalamic-pituitary.

ACC becomes activated in response to an emotional input. In situations of conflict, the PFC cortex further activates different regions of the cortex which helps in the decision-making process for deriving the appropriate response to the presented stimuli. Patients with depression have been often shown to have abnormalities in the left PFC and may also express difficulties in executive functioning.

Interaction between ANS and the Vagus nerve in FGIDs

Disturbance in the ANS has often shown to correlate with flare-up of IBS symptoms[9], however, no definite pattern of ANS activation has emerged. Some studies suggest activation or inhibition of parasympathetic innervation while others suggest increased or decreased sympathetic activity. Some authors suggest that there is a specific pattern in IBS depending on diarrhea or constipation-predominant symptoms but the reproducibility of such patterns has been inconsistent due to methodological limitations or limited power to allow for a specific pattern to emerge[2,10,11]. The role of ANS in psychiatric disorders, acute stress and pain is relatively more evident[2,12].

The Vagus nerve is responsible for relaying visceral information to the brain via parasympathetic pathways. It contains both sensory and motor pathways and provides innervation to the entire gut except the distal colon, rectum and internal anal sphincter which are innervated by the sacral (parasympathetic) ganglion. The afferent fibers carry sensations to the nucleus of solitary tract (NTS) and mediate both noxious stimuli, such as dull pain, non-noxious sensations such as hunger and nausea[13]. The motor fibers travel from nucleus ambiguus (NA) and the dorsal motor nucleus (DMN) in the brain stem to the ENS and regulate physiological functions such as motility and secretion. A number of vasovagal reflexes such as enterogastric and gastrocolic also modulate GI function[14].

The higher cortex influences the vagus nerve as well by modulating the vagus nerve nuclei in the brainstem. The NTS receives sensory information from the abdominal viscera and relays it to the higher brain regions[15]. Projections from NTS also terminate into the hypothalamus and limbic system which may explain why there is an altered perception of visceral pain in individuals with psychiatric symptoms. Vagal motor nuclei receive input from brain regions such as the hypothalamus, area postremia, and inferior-anterior cingulate cortex. These networks assimilate sensory input coming from NTS and formulate appropriate downstream responses via nucleus ambiguus[14].

The HPA axis in FGIDs

The Corticotropin-releasing hormone (CRH) seems to affect the motility and sensitivity of the gut, however, it is not completely understood if it is a primary response or occurs due to another stimulus[16]. Studies have reported increased levels of CRH in IBS patients and increased baseline levels in patients with anxiety disorders, however, the replication of these results has often been inconsistent.

The role of different emotional states in FGID has been well established. For example, volunteers who were subjected to different stressors showed alterations in their GI function[17-19]. Studies on rodents have shown that when these animals are put under stress, their brain undergoes neurochemical changes[20,21] along with augmented visceral sensitivity[22].

In one study, women with IBS showed altered cellular immune response compared to control subjects. This in part is thought to be mediated by adrenergic pathways but

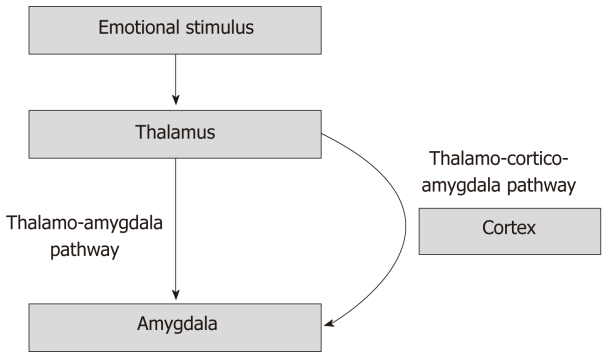

Figure 3 The pathways involved in emotional response. The thalamo-amygdala pathway is responsible for unconditioned fast response without the input from the cortex. The thalamo-cortico amygdala pathway provides input for a complex, conditioned response due to input from the cerebral cortex.

does not essentially include the activation of the HPA axis[23]. Dinan *et al*[24] showed that patients with IBS had increased response to CRH and expressed increased levels of inflammatory hormone Interleukin-6 and Interleukin-8. Another study involving twenty-one IBS patients and 18 controls, showed that IBS patients had increased HPA axis responsiveness, however, this overstimulation was more likely due to traumatic events early in life which are common in patients with IBS[25].

Neuroimmune interactions in FGIDs

The ENS, also called the 'second brain', is capable of independent functioning without intermediation from the ANS. It consists of an estimated 10^8 neurons arranged in two ganglionic plexuses[26]. There is a complex interdependent relationship between the gut immune system and the ENS. Physiological functions of the gut such as motility, absorption and secretion are all very sensitive to subtle changes in this fine balance between the immune system and the nervous system[27]. For example, immune activation due to local inflammation can have a diffuse effect on GI motility[28].

Patients with post-infectious IBS (PI-IBS) have been studied for understanding the role of the immune system in FGIDs. The incidence of PI-IBS is reported between 5% and 32%[29]. The risk of developing PI-IBS increases many times if the presenting illness is predominantly diarrheal and lasts for more than 3 wk[30]. Additionally, hypochondriasis and stressful life event at the initial illness doubles the risk of PI-IBS, providing further evidence for the involvement of the immune system[31].

Ascending and descending pain pathways and FGIDs

Diffuse, visceral pain is the most common symptom and hallmark feature in a number of patients with FGIDs. Significant pain or abdominal disturbance leads to impairment in social functioning which may prompt hospital visits.

While the neurological pathways and underlying molecular mechanisms for somatic pain have been well studied, the understanding of the visceral pain remains a daunting challenge. The altered perception of pain can occur due to an abnormality in the visceral pain pathways and can occur at the level of the nociceptors, neuronal pathways, thalamus, and corticolimbic signaling pathway[32].

Pain receptors in the viscera mainly respond to chemical stimulation, mechanical stimulation such as distension or stretching, and ischemia or infection[33]. Initial insult leads to a release of inflammatory mediators such as prostaglandins, adenosine triphosphate, histamine, serotonin, and bradykinin. These inflammatory mediators cause pain sensitization by acting on a number of receptors, prominent among which are transient receptor potential vallinoid (TRPV) receptors 1 and 4, voltage-gated sodium calcium channels (VGSCs) and protease-activated receptors 2 (PAR(2)).

TRPV1 and 4 receptors mainly sense mechanical stimulus. TRPV1 is particularly implicated in patients with FGIDs as it has been shown to play a role in visceral pain hypersensitization. In rat models, Akbar *et al*[34] have shown that there was a 3.5-times increase in the concentration of TRPV1 receptors in IBS patients compared to healthy individuals. Reciprocally, TRPV1 antagonists have been shown to decrease the visceral pain sensitivity in rat models and may present a possible therapeutic target[35]. The TRPV4 receptors and PAR (2) receptors work closely and may become activated by serine proteases. These proteases are found in increased concentration in patients with FGIDs. Antagonizing these receptors reduces nerve discharge possibly preventing the sensitization[36].

The afferent pain fibers, which originate in viscera, relay to the dorsal horn of the

spinal cord and then ascend through the spinal cord to the midbrain and cortex. Biochemically active agents such as substance P, glutamate, aspartate and vasoactive intestinal peptides are released in nerve terminals of the dorsal column of the spinal cord. These nociceptive signals ascend in two major ascending pathways: the spinothalamic and the spinoparabrachial tracts. Studies using positron emission tomography (PET) have helped in outlining the functioning of these pathways. The mid-cingulate portion of the ACC has often been stimulated in patients with visceral hypersensitivity compared to healthy individuals. This is also the same area which is involved in the perception of fear and obnoxiousness[37].

The afferent signal is processed and modulated by the higher cortex which then transmits it to the perigenual ACC. This signal further travels down to the spinal cord *via* opioidergic, serotoninergic and noradrenergic systems to dorsal column of the spinal cord and modulates the afferent pain signals[38]. As the same limbic areas control emotional and cognitive signals, it is thought that the downstream inhibitory pain signals may be altered by the attentional and emotional state of an individual.

ROLE OF MICROBIOTA-GUT-BRAIN AXIS IN FGIDs

Microbiota has recently emerged as a key player in the gut-brain axis. This interaction between the brain and the microbiota has led to the recognition of a new term called 'microbiota-gut-brain axis'[39]. This interaction is bidirectional, meaning that the disturbance in the complex community of microbiota (dysbiosis) can affect the brain and vice-versa. The underlying signaling mechanisms for these communicating networks between gut flora and the gut-brain axis have been of special interest to researchers and pharmacists who are seeking potential therapeutic interventions.

The microbiota-gut-brain axis has been mainly studied in rodent models and two approaches have been used. In the first one, germ-free mice are compared with healthy mice with normal gut-flora to look for changes in desired characteristics or behaviors. Although the germ-free model has certain limitations, it is an excellent tool that has been used over the years to help advance our understanding of the axis. In the other approach, wide-spectrum antibiotics are used to induce changes in the composition of microbiota and then these treated mice are compared with untreated mice to look for the desired characteristics.

The evidence for bidirectional communication comes from studies that have shown that early life stress can alter the composition of gut microbiota, highlighting the role of the brain as an influencer on the gut through the gut-brain axis[22]. Reciprocally, healthy mice showed anxiety-like behaviors after the administration of pathogenic bacteria such *campylobacter jejni* or *Citrobacter rodentium*[40,41], suggesting an inverse role of the role microbiome on the brain.

Dysbiosis

Every human has a unique and subject specific community of microorganisms. This fingerprint of microorganisms, which is an ecosystem in homeostasis, develops early in life and may undergo some modifications but by and large, it remains stable throughout life[42]. The modifications may be due to competition from other microorganisms or pressure from the host[43]. Microbiota participates in the modulation of intestinal motility, blood flow, secretions, immunity and perception of visceral signals[44]. Therefore it is speculated that dysbiosis plays an important role in the pathogenesis and symptoms perception of FGID.

Studies demonstrate that the germ-free mice show developmental changes from mice with normal microbiota. These changes may resemble features of functional GI diseases[45,46]. Conversely, recolonization of these mice can restore some functions such as mucosal immunity[47].

On the contrary, when these mice are infected with pathogenic microorganisms, they may produce features that may resemble symptoms of IBS and in some cases IBD[48]. Other mechanisms that have been suggested include gut distention and alterations in secretion and motility of the GI tract. These changes seem to arise from the production of gas and fatty acids by the bacterial flora influencing the microbiota to host signaling[49].

Dysbiosis is seen in different GI diseases including celiac disease, IBS and IBD[50,51]. Although dysbiosis seems to be an important denominator in patients with FGIDs, no specific symbiotic signature has been identified. This could be due, in part, to different sites used for sampling and varying techniques used to analyze the cultures[52-54].

Dysbiosis can also upset the HPA axis; for example, compared to mice with standard microbiota, germ-free mice produce increased levels of the

adrenecorticotrophin (ACTH) and other stress hormones[55]. Conversely, on restoring the intestinal flora of germ-free mice, there is decreased production of stress hormones and a partial reversal of anxiety-like behaviors[55]. Germ-free mice also have decreased levels of brain-derived neurotrophic factor (BDNF) due to decreased expression of NMDA receptors[56]. These proteins are involved in the differentiation and growth of new neurons[55,57].

Other studies have also highlighted the role of microbiota in the development of the brain and gut through a number of complex pathways. Thus it is thought that dysbiosis could be one of the causes of behavioral traits associated with anxiety disorders[56]. Table 1 represents the different factors that that modify gut-brain-axis and play a role in the pathophysiology of FGIDs.

MODULATION OF THE GUT-BRAIN AXIS IN FGIDS

Our understanding of the pathophysiology of FGIDs is still developing, as currently there is a dearth of studies on human subjects and the challenge is to test whether findings in animals are translatable to humans. Only a few controlled studies have been done in human subjects which have highlighted the modifying role of probiotics, antibiotics, diet and fecal microbiota transplantation.

Role of probiotics in FGIDs

Probiotics contain live microorganisms that, when ingested, can have a beneficial effect on the host. Probiotics have found application in a number of gastrointestinal and immune system disorders[58] and their evaluation as a possible therapeutic target in FGIDs is one trending in the research community. One thing is certain that the role of probiotics cannot be overlooked as has been shown in numerous studies on animals and humans as highlighted below.

Studies on rodents have shown that the administration of probiotics leads to a reduction in visceral pain sensitization[59,60]. Mckernan *et al*[60] demonstrated that 14 d oral gavage of *Bifidobacterium infantis* showed reduced colorectal distension, increased pain threshold and delayed first pain behavior. Bercik *et al*[61] infected mice with *Trichuris muris*, a noninvasive parasite, and treated with etanercept, budesonide, or specific probiotics. They found that infected mice showed anxiety-like behaviors secondary to colonic inflammation. There was decreased BDNF messenger RNA (mRNA) in the hippocampus. The mice also showed immune system changes with increased Circulating tumor necrosis factor-α and interferon-γ. The administration of *Bifidobacterium longum* restored the normal behavior to some extent and normalized BDNF mRNA levels but did not affect immune system changes[61]. The study shows that the role of probiotics may be selective in that they may affect the gastrointestinal system but spare other systems.

Studies in human subjects are relatively limited. In a randomized controlled study in patients with IBS, the intake of Bifidobacterium lactis significantly reduced abdominal distension and resulted in an overall improvement of symptoms[62]. O'Mahony *et al*[63] demonstrated that 8 weeks intake of *Bifidobacterium infantis* led to an improvement of IBS symptoms and restoration of normal interleukin10:interleukin 12 ratio in blood.

The evidence for the use of probiotics has been mounting but specific strains and molecular targets remain to be determined.

FGIDs and role of antibiotics

Antibiotics can rapidly reduce the diversity of the organisms allowing certain pathogenic bacteria to wreak havoc. Antibiotics have been shown to influence psychiatric behaviors in individuals being treated for different reasons. Mohle *et al* observed that in comparison to non-treated mice, stressful behavior and poor memory and poor hippocampal functioning was evident in those who were treated with antibiotics. The same mice, when treated with probiotics, showed improvement in these cognitive symptoms[64]. Verdú *et al*[59] showed that the administration of oral antimicrobials in mice led to mucosal inflammation and visceral hypersensitivity due to increased substance P expression in the enteric nervous system (ENS). This inflammatory response was alleviated by the administration of *Lactobacillus paracasei*[59].

FGIDs and role of diet

Diet can improve the symptoms of FGID in two ways by modulating the gut-brain axis: improving psychological symptoms and by having a probiotic-like effect[65]. Magnusson *et al*[66] studied changes in the behavior of mice and their gut flora composition following the administration of different diets. Analysis showed that

Table 1 Different factors that influence the brain - Gut interaction in functional gastrointestinal disorders

Nature of link	Evidence	Comments
Dysbiosis	Kassinen *et al*[82]; Tojo *et al*[83]; Chassard *et al*[84]; Cryan *et al*[85]	Disturbance in the complex community of microbiota seems to influence gut-brain axis by modulating neuroendocrine, neuroimmunal and visceral sensory system.
Altered mucosal secretions	Mazmanian *et al*[86]; Xue *et al*[87]	Secretion is modulated by complex interaction of intrinsic and extrinsic factors acting on gut mucosa. Dysregulation of the epithelial cells due to autonomic reactivity may lead to 5-HT release contributing to altered secretion
Disturbance in motility	Randich *et al*[13]; Dass *et al*[88]; Barbara *et al*[89]	Products of metabolism of gut bacteria, such as short-chain fatty acids modulate enteric system and influence the rate of gut transit
Visceral hypersensitivity	O'Mahony *et al*[22]; Akbar *et al*[34]	Patients with IBS have been found to have an increased concentration of pain-sensing receptors such as TPRV1 compared to the controls.
Altered processing of visceral signals	Lemann *et al*[90]; Mertz *et al*[91]	There is increased activation of certain cerebral areas in IBS patients compared to the controls. Altered processing of the visceral pain in the central nervous system has been a recurring theme in many studies.
Immune dysfunction	Chadwick *et al*[92]; Dinan *et al*[24]; Keely *et al*[93]	Patients with prolonged Infectious diarrhea are much more prone to developing IBS. Also, biopsies of patients with IBS have shown increased immune cells in the mucosa[92].
Psychological disturbances	Creed *et al*[17]; Gwee *et al*[94]; Drossman *et al*[95]; Monnikes *et al*[2,12]	Patients with FGIDs have co-existing psychosocial symptoms such as stress, anxiety and depression and thus a biopsychosocial model has been proposed for FGIDs
Early life stress	O'Mahony *et al*[22]; Bailey *et al*[96]	Early life-stress can alter the composition of gut microbiota

5-HT: 5-hydroxytryptamine; FGID: Functional Gastrointestinal diseases; IBS: Inflammatory bowel disease; TRPV1: Transient receptor potential vallinoid 1.

Erysipelotrichales was increased in the gut of mice which were fed high fat diet and Lactobacillus was increased in the mice which were given high sucrose diet. The diet influenced the cognitive behavior of the mice and these changes correlated with the composition of different bacteria in the gut of these mice[66]. Li *et al*[67] observed that compared to control mice, the mice which were fed a meat-containing diet showed better microbial diversity, improved memory and less stressful behavior. Similar findings were reported by a number of other studies suggesting that diet can have a profound effect on the composition of microbiota in mice and can improve or worsen anxiety-like behaviors[68,69]. However, due to difficulties in analyzing the gut microbiota, the reproducibility of these results in human subjects requires continued efforts with new research approaches.

POTENTIAL USEFULNESS OF FUNCTIONAL AND STRUCTURAL NEUROIMAGING IN FGIDS

The complex nature of the psychosocial interactions that underlie the pathophysiology of the FGID has always eluded researchers. The plethora of studies highlighting the role of gut-brain axis in the development of symptoms in FGID has not been met with effective strategies that can use this relationship to develop key diagnostic tools and therapeutic agents[70]. Studies have mainly focused on the biochemical interactions that play a role in the gut-brain axis but in vivo studies have been limited so far due to lack of noninvasive neurophysiological techniques. Recently, the utilization of new techniques such as functional brain imaging has allowed an objective assessment of the axis. Table 2 summarises the different techniques that have been used to study the gut-brain axis.

The field of neuroimaging has rapidly evolved recently and provides a promising new approach to the complex interactions of peripheral nerves and the brain. Since pain and discomfort are the main symptoms in people with FGID, studies have been limited to peripheral pain reflex pathways and ANS responses. It is thought that the treatment models that have worked on rodents and failed in human subjects might be

Table 2 Methods used to study brain - Gut interaction in functional gastrointestinal disorders

Link to be tested	Name of the test	Evidence	Comments
Microbiota-gut-brain axis			
	Germ-free mice	Abrams *et al*[45]	This has been the most widely used technique to study the gut-brain axis. Germ-free mice are compared with healthy to look for changes in desired characteristics or behaviors
	Antibiotic-treated mice	Verdú EF *et al*[59]	Antibiotics are used to induce changes in the composition of microbiota and then these treated mice are compared with untreated mice to look for the desired characteristics. Antibiotics are useful for selectively eliminating certain bacteria from the gut, allowing the growth of other strains.
	Mice treated with probiotics	Mohle *et al*[64]	Once germ-free mice have been studied, they can be injected with probiotics to establish the reciprocity of the relationship that has been studied.
The interactions between visceral, peripheral and central pathways			
	Functional MRI (fMRI)	Tillisch *et al*[72]; Aziz *et al*[73]; Mayer *et al*[75], and Labus *et al*[76]	fMRI measures the changes in oxygenated and deoxygenated hemoglobin where oxygenated hemoglobin denotes the group of neurons that have increased activity. They are useful in studying the complex relationship between visceral stimuli and brain response.
	PET imaging	Tillisch[72]	PET imaging has the advantage of probing a particular receptor by developing a radiolabeled ligand. This important feature can be used to assess specific receptor activities during pain and stress response in control and FGID patients.
	Structural MRI (sMRI)	Seminowicz *et al*[77]	Whole and regional brain images using sMRIs have been used to study differences between individuals with FGIDs and control groups

FGIDs: Functional gastrointestinal diseases; MRI: Magnetic resonance imaging; PET: Positron emission tomography.

due to the increased CNS modulation of the subcortical pathways[71]. Neuroimaging offers a non-invasive method to evaluate the interactions between the visceral and central pathways and the effect of psychological symptoms on these pathways.

Functional brain imaging in FGIDs

Functional brain imaging assesses brain function in response to different visceral stimuli. There are two main modalities that are used for imaging brain function namely, functional magnetic resonance imaging (fMRI) and positron emission tomography (PET)[72].

MRI has been the focus of attention for experts from a number of fields. For example, psychiatrists have used fMRI to gain insight into complex psychological disorders such as obsessive-compulsive disorders and schizophrenia. Functional MRI (fMRI) measures the changes in oxygenated and deoxygenated hemoglobin where oxygenated hemoglobin denotes the group of neurons that have increased activity[73].

Studies that have looked at the complex relationship between visceral stimuli and brain response have frequently produced inconsistent results which are often difficult to interpret. The only consistent finding that has been elicited so far after a meta-analysis of a number of studies has shown that the rectal distension in IBS patients is linked to the stimulation of anterior cingulate gyrus. Compared to the controls, the areas of the brain for emotional arousal in anterior cingulate gyrus were differentially activated in patients with IBS. The same meta-analysis also showed that the history of abuse and stressful life events synergistically correlated with the activation of these same brain areas[74]. The anterior cingulate gyrus has also been implicated in the

modulation of the autonomic nervous system, maintenance of homeostasis and has been shown to have been affected by disturbances in emotions and the gut-brain axis[75]. Despite the limited results so far, fMRI remains an important modality and ongoing research promises to understand the functional neural networks. Studies are aiming to examine the entire functioning networks rather than focusing on particular regions. Two main functioning networks that have been the focus of scientists are the emotional arousal network (including amygdala and anterior cingulate cortical subregions) and hemostatic afferent network (anterior mid cingulate cortex, posterior insula, thalamus, dorsal pons) as previous studies have suggested an increased activity in these networks in patients with FGIDs[75,76].

The use of positron emission tomography (PET) imaging in FGIDs has been limited due to high costs, difficulty in engineering relevant ligands and the widespread availability of fMRIs. However, PET imaging has the advantage of probing a particular receptor by developing a radiolabeled ligand. This important feature can be used to assess specific receptor activities during pain and stress response in control and FGID patients. The technique may also attract pharmacists who want to study the distribution and response of a particular therapeutic agent targeted at a receptor and comparisons can be made between the placebo and intervention groups[72].

Structural brain imaging in FGIDs

Whole and regional brain images have been used to study differences between individuals with FGIDs and control groups. It has also been useful in baseline imaging and response to treatment. Structural brain images use high resolution structural MRI (sMRI) to measure cortical thickness and gray matter density which can be compared between different groups. The role of structural brain imaging in FGID has been not explored in great detail compared to psychiatric disorders. Structural changes in the brain have been seen in individuals with a history of childhood trauma. Although the structural changes may seem 'fixed', dynamic changes have been seen in patients under conditions such as learning, illness and stress. The few studies that have been carried out on FGID patients using structural images have indicated that there is decreased grey matter density in certain areas of the brain such as the medial prefrontal and ventrolateral prefrontal cortex, striatum and thalamus; while there may be increased density in the anterior cingulate and orbitofrontal cortex. Interestingly, controlling for stressors showed that there was no significant difference in the grey matter density between the patients with FGIDs and controls[77].

WHAT DOES THE FUTURE HOLD?

There is still a long way to go to understand the exact role of the neural, immunologic, biochemical and other pathways in the gut-brain axis and the FGIDs. There is also a need to these translate findings of animal studies to human subjects using multi-population randomized controlled trials. The role of microbiota has opened exciting new avenues and its exact role needs to be explored with the identification of the individual specific strains that may help to tailor the probiotic therapy. The neuroimaging combined with immunologic and biochemical findings can be used to develop patterns of pathogenesis and for guiding further research.

The application of machine learning to medical diagnosis

The multifactorial nature of FGID can make diagnosis a challenging and time-consuming task. Machine learning and artificial intelligence (AI) are important tools that can potentially be employed to help with the diagnosis of FGID and aid healthcare professionals and researchers in combing through the plethora of available patient data to identify trends and investigate causal relationships between the trends. Once causality has been established it could shed light on the mechanisms behind the pathogenesis of FGID and help researchers understand how the gut-brain-microbiome axis functions.

The use of machine learning processes such as inductive learning to define rules that govern diagnostic algorithms in different plant species has been shown to be effective and at times superior to rules obtained by human experts. It has often been proven to outperform rules defined by human experts even as early as 1980[78]. Inductive learning algorithms can learn from examples or prototypes and come up with diagnostic rules for the examples provided[79].

In recent times, the availability of large databases of digitized patient data provides a unique opportunity for machine learning algorithms to analyze and interpret databases to identify correlations between different symptoms, imaging findings and biochemical findings. The algorithms then develop classifiers by pattern recognition

which can be used as a framework to develop a diagnostic algorithm or improve existing algorithms by incorporating new classifiers into them or testing the reliability of established classifiers. A machine learning program can be used in such a way to diagnose conditions based on clinical findings and investigations[80]. For example, Kukar *et al* successfully used a machine learning approach to successfully diagnose patients with ischemic heart disease using an algorithm employing a step-wise diagnostic approach. The algorithms improved the detection of positive and negative cases by 6% compared to manual detection by the clinicians[81].

The advantages of machine learning algorithms

Autonomy and automation: Most machine learning algorithms are autonomous to variable degrees and the process of obtaining knowledge is largely automated which makes these methods of learning and data analyses highly efficient. The algorithms also look for trends in the data that the researchers may not have considered and therefore provides more knowledge than conventional methods of data analysis over a significantly smaller amount of time[80].

Dealing with incomplete or noisy data: Machine learning algorithms are highly adaptable and consequently very good at dealing with and accounting for missing and/or noisy data which is a very common occurrence in electronic patient records[80].

Explanation ability: Most algorithms have the ability to explain the trends and qualifiers that they identify. These explanations can be very valuable for guiding further research[79,80].

Transparency: The knowledge generated and the explanations behind the decisions made by most algorithms is transparent to the physicians and therefore the reasoning of the algorithm is easy to understand.

Future studies on the gut-brain-microbiome axis need to evaluate the feasibility of using machine learning programs to study patient data to identify correlations between the clinical findings, demographic information, neuroimaging findings, lab tests and gut microbiome analysis to develop newer and more reliable diagnostic criteria.

Role of gut microbiota

An aspect of the gut-brain-microbiome axis which requires further investigation is the gut microbiota and its effects on the pathogenesis of FGID. Future studies need to look at both the bacterial and fungal parts of the microbiome to ascertain the extent to which the gut microbiota plays a role (if at all) in FGID as there is a dearth of literature on this topic.

CONCLUDING

The gastrointestinal tract and nervous system are constantly communicating with each other in a bidirectional relationship which is influenced by the autonomic nervous system, immune system, hypothalamic-pituitary axis and gut microbiota. Understanding the molecular and biochemical mechanisms disturbing this complex network of communication is key to our understanding of the pathophysiology of the functional GI diseases. Studies in rodents have provided us with substantial evidence about the underlying mechanisms, however, there is a need to translate these findings in human subjects to successfully identify the therapeutic targets.

The identification of pathogenic microbiome signatures in individuals combined with demographical data, serological findings and neuroimaging findings can be encoded into machine learning algorithm which may help identify trends and patterns that can be potentially overlooked by humans.

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ORIGINAL ARTICLE

Basic Study

Effect of adipose-derived mesenchymal stem cells on hepatocellular carcinoma: *In vitro* inhibition of carcinogenesis

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Institutional review board statement: This research was conducted with ethical approval from the Saint-Joseph University ethical committee and was carried out in accordance with the approved guidelines. Adipose tissues were obtained from healthy donors undergoing an elective liposuction procedure from abdominal, hip or thigh regions after written consent in the Department of Plastic Surgery, Hotel Dieu De France Hospital, Beirut, Lebanon.

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Abstract

AIM

To investigate the effect of adipose-derived mesenchymal stem cells (ADMSCs) and their conditioned media (CM) on hepatocellular carcinoma (HCC) cell tumorigenesis.

METHODS

The proliferation rate of HepG2 and PLC-PRF-5 HCC cancer cells was measured using the trypan blue exclusion method and confirmed using the cell-counting kit 8 (commonly known as CCK-8) assay. Apoptosis was detected by flow cytometry using annexin V-FITC. Protein and mRNA expression was quantified by ELISA and real time PCR, respectively. Migration and invasion rates were performed by Transwell migration and invasion assays. Wound healing was examined to confirm the data obtained from the migration assays.

RESULTS

Our data demonstrated that when co-culturing HCC cell lines with ADMSCs or treating them with ADMSC CM, the HCC cell proliferation rate was significantly inhibited and the apoptosis rate increased. The decreased proliferation rate was accompanied by an upregulation of P53 and Retinoblastoma mRNA and a downregulation of c-Myc and hTERT mRNA levels. More notably, ADMSCs and their CM suppressed the expression of the two important markers of HCC carcinogenicity, alpha-fetoprotein and Des-gamma-carboxyprothrombin. In

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addition, the migration and invasion levels of HepG2 and PLC-PRF-5 cells significantly decreased, potentially through increased expression of the tissue inhibitor metalloproteinases TIMP-1, TIMP-2 and TIMP-3.

CONCLUSION
These findings shed new light on a protective and therapeutic role for ADMSCs and their CM in controlling HCC invasiveness and carcinogenesis.

Key words: Hepatocellular carcinoma; Adipose-derived mesenchymal stem cells; Adipose-derived mesenchymal stem cell conditioned media; Proliferation; Apoptosis; Migration; Invasion

Core tip: In this study, we report the *in vitro* effect of adipose derived mesenchymal stem cells (ADMSCs) on HepG2 and PLC-PRF-5 liver cell lines. It is the first study to demonstrate that ADMSCs and their respective conditioned media inhibited the expression of hepatocellular carcinoma markers alpha-fetoprotein and Des-gamma-carboxy-prothrombin and decreased cancer cell invasiveness by increasing the mRNA expression of tissue inhibitor metalloproteinases TIMP-1, TIMP-2 and TIMP-3. In addition, ADMSCs significantly reduced the proliferation rate, the invasiveness and the migration of the cancer cells while inducing their apoptosis.

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the most common primary hepatic cancer that accounts for approximately 70%-80% of all primary liver cancers[1]. It is now considered the second cause of cancer related mortality worldwide[2]. HCC development results from an imbalance between excessive cell growth and apoptosis, which is mainly regulated by P53, a tumor suppressor gene. Alterations in the expression or activation of P53 have been extensively reported in HCC and are related to hepatocarcinogenesis[3,4].

Early detection of HCC is crucial but difficult due to the presence of inflammation and liver damage. Several markers, such as Lens culinaris agglutinin-reactive fraction of alpha-fetoprotein (AFP) (AFP-L3), Des-gamma-carboxy-prothrombin (DCP), Dickkopf-1, Midkine and microRNA, have been suggested as biochemical indicators in the diagnosis of different phases of primary liver cancer[5]. However, AFP is used for monitoring liver cancer recurrence after treatment[6]. Late stages of HCC, more specifically HCC metastasis, is associated with upregulation of matrix metalloproteinases (MMPs)[7,8], as these proteins are implicated in matrix degradation that allows for malignant growth and cancer cell invasion.

HCC treatment entails liver transplantation and/or other palliative modalities such as liver resection, local ablation, transarterial chemoembolization, and systemic cytotoxic chemotherapy. These treatments are limited by their toxicity towards normal tissues, by multifocal development and tumor[9]. Hence, the development of new targeted therapies is necessary to prevent HCC in cirrhotic liver or to restrain metastasis and abolish cancer invasiveness.

Recent accomplishments in stem cell (SC) research provide a new prospective in cell-based therapy and tissue regeneration. Indeed, the interaction between mesenchymal SCs (MSCs) and cancer has been extensively studied. MSCs are adult, multipotent, non-hematopoietic cells that have auto-renewing capacity and a multilineage potential. MSCs can be isolated from different sources such as bone marrow[10], umbilical cord[11], peripheral blood[12], placenta[13], and adipose tissue[14]. Adipose tissue remains the most abundant source. SCs are called intrinsic drug stores, not only because of their differentiation capacity but because of their paracrine and

trophic effects. Indeed, the exact role(s) that MSCs play in tumor modulation remains controversial. It has been reported that MSCs promote cancer via immune suppression[15,16], the promotion of vasculature or angiogenesis[16,17], the stimulation of epithelial-mesenchymal transition[18], and their contribution to the tumor microenvironment[19,20]. The use of bone marrow-derived MSCs in a model of Kaposi sarcoma has been shown to exert anti-tumorigenic and pro-apoptotic effects via the suppression of Akt activity upon direct cell-cell contact[21]. In addition, it has been demonstrated that co-culturing of glioma cancer cells with cord blood MSCs induced cancer cell apoptosis[22]. Emerging evidence has established that MSCs may serve as vehicles to deliver therapeutic agents, such as cytokines, apoptosis inducers and prodrugs, and that they can be genetically engineered to produce antitumor molecules such as interferon β (INF β) and tumor necrosis factor-related apoptosis inducing ligand (TRAIL)[23]. However, the antitumor properties of MSCs and their secretions are not yet clear. The role of MSCs on HCC remains controversial, and few reports have studied the effects of adipose-derived MSCs (ADMSCs) on HCC.

The present work aims to investigate the effect of human ADMSCs and their conditioned medium on HCC cell line carcinogenesis through the modulation of proliferation, apoptosis, tumor marker expression, migration and invasion.

MATERIALS AND METHODS

Cell lines and culture conditions

The human HCC cell lines (HepG2/C3A/HB-8065, PLC-PRF-5/CRL-8024) were purchased from American Type Culture Collection (ATCC, Manassas, VA, United States) in 2015. All cells were cultured in cancer cell media as suggested by ATCC at 37 ˚C in low glucose DMEM media (1 g/L glucose) (Sigma Aldrich, Steinheim, Germany) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (PS) (Sigma Aldrich) in a humidified atmosphere containing 5% CO_2 at passages 1 to 5.

MSC isolation and primary culture

Adipose tissues were obtained from healthy donors undergoing an elective liposuction procedure from abdominal, hip or thigh regions after consent in the Department of Plastic Surgery, Hotel Dieu De France Hospital, Beirut, Lebanon. Briefly, lipoaspirates were washed extensively with a saline solution then digested with type I collagenase solution (Sigma Aldrich) for 1-2 h at 37 ˚C. After centrifugation, the cell pellet was washed and filtered to remove debris. The synovial vascular fraction was plated into tissue culture flasks in DMEM nutrient mix F12 (Sigma Aldrich) containing 10% FBS (Sigma Aldrich) and 1% PS-amphotericin then incubated at 37 ˚C and 5% CO_2. After 48 h, non-adherent cells were removed and fresh DMEM was added and replaced every 2-3 d. The isolated ADMSCs at passage 1 were used for subsequent experiments.

Morphological observation

The morphology of ADMSCs and HCC cell lines before and after coculture was observed under an inverted microscope. Photos were taken at a magnification of 100 ×.

Characterization of MSCs

Morphology and immunophenotyping: ADMSCs at passage 1 had a fibroblast-like morphology and were characterized by immunophenotyping using flow cytometry analysis. The following PE-conjugated mAbs were used: anti CD73, anti CD29, anti CD44, anti CD45, anti CD31, anti CD106, anti CD34, anti CD90, and anti CD105 (BD Biosciences, San Jose, CA, United States). Appropriate isotype controls were used at the same concentrations to determine non- specific staining.

Multilineage differentiation of ADMSCs: ADMSCs showed a differentiation capacity to become adipocytes, osteocytes, and chondrocytes. Adipogenic differentiation, osteogenic differentiation and chondrogenic differentiation was performed as previously described[24].

Coculture of ADMSCs with HCC cell lines

To determine the effect of ADMSCs on HCC cell lines, the HepG2 and PLC-PRF-5 were cultured directly in six-well plates with ADMSCs and indirectly in an inverted Transwell cell culture insert for six-well plates (1-μm pore poly (ethylene terephthalate) (Corning, Corning, NY, United States) at 1:1 and 2:1. In the case of indirect coculture, ADMSCs were seeded in the apical compartment, and the cancer

cells were seeded in the basal compartment. In the direct co-culture, the number of cells seeded are mentioned in each specific experiment. In all experiments, cells were grown in DMEM F12 supplemented with 10% FBS in a humidified atmosphere at 37 ˚C and 5% CO_2. After 48 h, the media were removed and replaced with fresh DMEM F12. Finally, the supernatant was collected after 48 h and stored at -80 ˚C for subsequent ELISA analysis.

Preparation of ADMSC conditioned media

ADMSCs were grown in 75 cm^2 flasks (Sarstedt, Newton, NC, United States) with 10% FBS DMEM F12 (Sigma Aldrich) at 37 ˚C with serum free media. After 24 h, the conditioned media (CM) was collected, centrifuged, filtered and conserved at -80 ˚C until used.

Treatment of cancer cells with ADMSC CM

In all experiments where CM was used, the HepG2 and PLC-PRF-5 cancer cell lines were seeded in six-well plates in CCM as described earlier [low glucose DMEM media (1g/L glucose) supplemented with 10% FBS and 1% PS]. After adherence, the HepG2 and PLC-PRF-5 cell supernatants were removed and replaced with prepared ADMSC CM at different dilutions (1:1, 1:2, 1:4, 1:5, 1:10, 1:25, 1:50, 1:100, 1:200, 1:400) for 48 h. All dilutions were significant in respect to cancer markers and morphology. After 1:25 dilution, no differences in results were observed (data not shown). Thus, in all experiments, ADMSC CM was diluted at 1:1, 1:5 or 1:25.

Cell count assay

HCC cells were harvested and counted with the trypan blue exclusion method using a hemocytometer.

Proliferation test

The effect of ADMSCs and ADMSC CM on HCC cell proliferation was evaluated using a cell-counting kit 8 (CCK-8) (Sigma Aldrich) according to the manufacturer recommendations. The tetrazolium salt or WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2-H-tetrazolium, monosodium salt] is cleaved into formazan by succinate-tetrazolium reductase, an enzyme that exists only in the mitochondrial respiratory chain and is active only in viable cells. The formazan production is proportional to the number of living cells in the culture. Briefly, HCC cells were directly cocultured with ADMSCs in six-well plates, indirectly in six-well plates and 24-mm Transwells (Corning) or treated with ADMSC CM for 48 h. The WST-8 was then added into the wells after removal of Transwells in case of indirect coculture. Finally, the absorbance was measured in triplicates at 450 nm.

Flow cytometry analysis of apoptosis

To study the effect of ADMSCs on inducing apoptosis of HCC cells, an annexin V-FITC kit (MiltenyiBiotec, Bergisch Gladbach, Germany) was used according to the manufacturer's instructions. Briefly, HCC cells were indirectly cocultured with ADMSCs in six-well plates and 24-mm Transwells (Corning) or were treated with ADMSC CM for 48 h. Next, the Transwell was removed and 10^6 of freshly obtained HCC cells were washed and resuspended in binding buffer. The cells were stained with annexin V-FITC, incubated in the dark for 15 min, then binding buffer and propidium iodide solution were added. For each sample, 10^6 cells were analyzed by flow cytometry using a MACSQuant analyzer device. Apoptosis was analyzed using the MACSQuant software, and the percentage of apoptosis was determined and plotted.

AFP and DCP ELISA

To test the level of AFP and DCP in cell supernatants, quantitative enzyme linked immunosorbent assay kit (HUMAN, Germany and CUSABIO, Houston, TX, United States, respectively) were performed as per manual instructions. The optical density was determined using an ELISA plate reader at 450 nm.

Wound healing assay

For monolayer wound healing assay, ADMSCs (negative control), HCC cells (positive control), and HCC cells directly cocultured with ADMSCs or treated with ADMSC CM were are seeded and cultured until > 90% confluence in 10% FBS DMEM F12 in six-well plates (Corning). By scratching the cells with a 20 μL plastic pipette tip, three wounds were stimulated per well. After gently washing the wells with PBS, the cells that migrated into the wounded areas were monitored and photographed at 0 h, 6 h, 12 h, 24 h and 48 h. The distance migrated was measured using image J 1.48v software (Wayne Rasband, National Institutes of Health, United States) by comparing the

images from time 0 to the last time point. The relative migration distance of cells was measured by the distance of cell migration/the distance measured at 0 h.

Transwell migration assay

The HepG2/C3A and PLC/PRF/5 cell migration assay was performed using a Boyden chamber in a 24-well plate designed by Cell Biolabs Inc. (San Diego, CA, United States) according to the manufacturer's recommendations. Briefly, for each condition, 10^6 cells were suspended in 1 mL serum-free DMEM. Then, 3×10^6 cells were added in the upper chamber of each well. The same medium supplemented with 10% serum was added to the lower chamber of each well as a chemo-attractant solution. After 24 h, the cells that migrated to the lower chamber of the wells were stained using crystal violet cell staining solution. The stain was instantly dissolved once the kit extraction solution was added. The solution was then transferred to a 96-well microtiter plate, and the absorbance was measured at 560 nm using a plate reader.

Transwell invasion assay

The invasion ability of HepG2/C3A and PLC/PRF/5 cells was assayed using a Boyden chamber in a 24-well plate designed by Cell Biolabs Inc. According to the manufacturer's recommendations, all cells were incubated in serum-free DMEM overnight. For each condition, 10^6 cells were suspended in 1 ml serum-free DMEM. Then, 3×10^6 cells were added in the upper chamber of each well. The same medium supplemented with 10% serum was added to the lower chamber of each well as a chemo-attractant solution. After 48 h, the cells that invaded the bottom of the membrane were stained using crystal violet cell staining solution. An extraction solution was then added, and the mixture was transferred to a 96-well microtiter plate. Finally, the absorbance was measured at 560 nm using a plate reader.

RNA extraction and real time PCR

Total RNA from cell cultures was extracted using QIAamp RNA extraction kit (Qiagen, Valencia, CA, United States). cDNA was generated from 500 ng of total RNA with iScript Reverse Transcription Kit (Bio-Rad Laboratories, Hercules, CA, United States). Quantification of gene expression was conducted using iQ SYBR Green Supermix (Bio-Rad Laboratories). The reverse transcription (RT) product was used to measure the expression of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as a positive housekeeping gene, AFP, TIMP-1, TIMP-2, TIMP-3, P53, RB, hTERT and c-Myc using real time PCR (qPCR) and specific primer sequences (Table 1). The reaction conditions were as follows: pre-denaturation at 95 ˚C for 3 min; 40 cycles of denaturation at 94 ˚C for 20 s, annealing and elongation at 60 ˚C for 60 s. The threshold cycle (Ct) value for triplicate reactions was averaged, and the relative genomic expression was calculated by the $2^{-\Delta\Delta Ct}$ method [ΔCt= Ct (gene) - Ct (GAPDH)][25]. Melting curves were performed to ensure that only a single product was amplified.

Statistical analysis

For immunophenotyping experiments, the values are presented as the mean ± standard error mean of the mean (SEM). For other experiments, data were expressed as mean ± standard deviation (SD). The differences between the groups were analyzed by student's *t*-test using GraphPad prism online software, and $P < 0.05$ was considered significant.

RESULTS

Characterization and differentiation of ADMSCs

Isolated ADMSCs at passage 1 showed a fibroblast-like morphology (Figure 1A). Flow cytometry analysis showed a high expression of the fibroblast markers CD73 (82.02% ± 4.84%), CD29 (90.28% ± 2.24%), CD44 (88.3% ± 1.78%), CD90 (93.19% ± 1.65%), CD105 (59.63% ± 8.13%), and a lack of expression of the endothelial and hematopoietic markers CD34 (2.17% ± 0.34%), CD31 (2.27% ± 0.43%), CD45 (2.18% ± 0.31%), and CD106 (2.31% ± 0.11%) (Figure 1B). Furthermore, our cells were able to differentiate into adipocytes, chondrocytes and osteocytes (Figure 1C).

ADMSCs inhibit HCC cell line proliferation

Unregulated cell proliferation is a fundamental abnormality in cancer development. Previous studies have been controversial and have demonstrated that MSCs either suppress or induce cell growth[26,27]. Here, we aimed to determine the effect of our ADMSCs and their secreted soluble factors on cancer cell proliferation and growth.

Table 1 List of primer sequences for real time PCR

Primer	Sequence
GAPDH F	5'- GCACCACCAACTGCTTAGCA -3'
GAPDH R	5'- CTTCCACGATACCAAAGTTGTCAT -3'
AFP F	5'- CAGCCACTTGTTGCCAACTC -3'
AFP R	5'- GGCCAACACCAGGGTTTACT -3'
TIMP-1 F	5'- GACCAAGATGTATAAAGGGTTCCAA -3'
TIMP-1 R	5'- GAAGTATCCGCAGACACTCTCCAT -3'
TIMP-2 F	5'- AGGCGTTTTGCAATGCAGAT -3'
TIMP-2 R	5'- TCCAGAGTCCACTTCCTTCTCACT -3'
TIMP-3 F	5'- CAGGACGCCTTCTGCAACTC -3'
TIMP-3 R	5'- AGCTTCTTCCCCACCACCTT -3'
P53 F	5'- CAAGCAATGGATGATTTGATGCT -3'
P53 R	5'- TGGGTCTTCAGTGAACCATTGT -3'
RB F	5'- GCAAATTGGAAAGGACATGTGA -3'
RB R	5'- GAAACTTTTAGCACCAATGCAGAA -3'
C-Myc F	5'- CACCACCAGCAGCGACTCT -3'
C-Myc R	5'- TTCCACAGAAACAACATCGATTTC -3'
hTERT F	5'- GACGTAGTCCATGTTCACAATCG -3'
hTERT R	5'- CGTCCAGACTCCGCTTCATC -3'

Therefore, we cultured HCC cell lines under the following conditions: HepG2 and PLC-PRF-5 alone (control) or directly or indirectly with ADMSCs (at a ratio HCC: ADMSCs of 1:1 or 2:1) or treatment with different dilutions of ADMSC CM for 48 h. Morphological observation revealed a considerable inhibition in the cell numbers of the two cancer cell lines in the presence of ADMSCs, either in direct or indirect cocultures. This inhibition was less remarkable when cancer cells were treated only with ADMSC CM (Supplementary Figure 1). Using cell count assay and WST-8 proliferation tests, our results showed that ADMSCs in indirect coculture reduced the number of HCC cells (Figure 2A) and inhibited their proliferation (Figure 2B) compared to control cells ($P < 0.001$).

In direct coculture, we could not discriminate between the proliferation of HCC cells and ADMSCs (data not shown), knowing that the microscopic observation showed strong inhibition of cancer cell number (Supplementary Figure 1). Similarly, the ADMSC CM, undiluted or diluted 5 × or 25 ×, significantly reduced the number of HepG2 and PLC-PRF-5 cells (Figure 2A) and inhibited the proliferation of HepG2 cells (Figure 2B; $P \leq 0.001$), while only the undiluted ADSMC CM was capable of reducing the proliferation of PLC-PRF-5 cells (Figure 2B; $P = 0.001$).

Effect of ADMSCs on HepG2 and PLC-PRF-5 cell apoptosis

Resistance to cell death or apoptosis is a crucial process in malignant cells. It has been shown that bone marrow derived MSCs (BMSCs) induce apoptosis and cell cycle arrest in G0/G1 phase[27]. To elucidate the mechanism of growth suppression by ADMSCs, HepG2 and PLC-PRF-5 cells were cultured alone (control), indirectly co-cultured with ADMSCs or treated with ADMSC CM. Apoptosis was assessed by flow cytometry after removal of ADMSCs in the case of coculture. Our results showed that ADMSCs significantly increased the apoptotic rate of HepG2 (21.54% ± 4.1% *vs* control = 1.94% ± 0.3%, $P < 0.05$) and of PLC-PRF-5 (2.91% ± 0.2% *vs* control = 0.5% ± 0.1%, $P < 0.001$). As shown in Figure 2C-E, HepG2 and PLC-PRF-5 apoptosis was also significantly increased when treated with undiluted ADMSC CM compared to control cells (11% ± 0.5% *vs* control, $P < 0.01$ and 3.8% ± 0.15% *vs* control, $P < 0.01$, respectively).

Effect of ADMSCs on AFP and DCP expression

To monitor the malignant status of HCC cell lines, the levels of biochemical markers AFP and DCP were measured in the supernatant of ADMSCs (negative control), HCC cells (positive control), and HCC cells co-cultured directly with ADMSCs or treated with ADSMC CM. The ADMSCs in the negative control did not express AFP or DCP (data not shown). As illustrated in Figure 3, we found that AFP protein levels dramatically declined upon co-culturing HepG2 and PLC-PRF-5 cells with ADMSCs compared to control cells ($P < 0.001$). Similar results were obtained when HepG2 cells

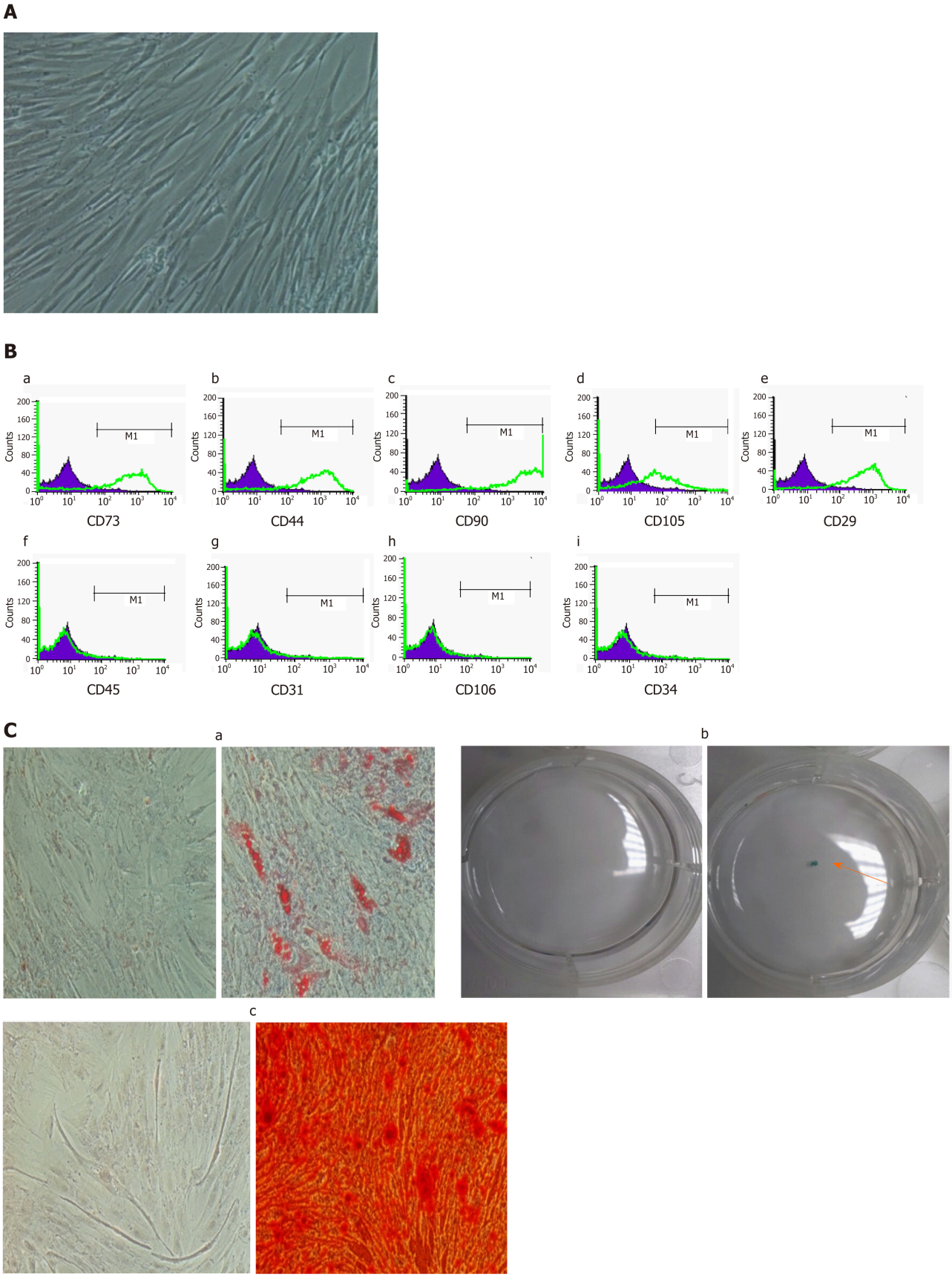

Figure 1 Characterization of adipose-derived mesenchymal stem cells. A: Microscopic observation (magnification 100 ×) showed that isolated ADMSCs have a fibroblast-like morphology; B: Immunophenotyping of ADMSCs using flow cytometry analysis. The flow cytometry histograms of the ADMSCs at passage 1 for a representative donor are displayed. The percentage of positively stained cells is indicated in the middle right section of each histogram. The green line indicates the positively stained cells; whereas the purple line indicates the isotype-matched monoclonal antibody control. Histograms showed that ADMSCs were positive for (B-a) CD73, (B-b) CD44, (B-c) CD90, (B-d) CD105, (B-e) CD29, and were negative for (B-f) CD45, (B-g) CD31, (B-h) CD106, (B-i) CD34; C: Differentiation capacity of ADMSCs incubated for 3 wk in adipogenic, chondrogenic and osteogenic medium. (C-a) Representative images of adipogenic differentiation marked by the formation of intracellular lipid droplets colored using Oil Red O compared to control. (C-b) Solid chondrogenic micromass colored by Alcian blue, indicating the presence of

glycosaminoglycans. (C-c) Representative images of osteogenic differentiation confirmed by the presence of calcium deposits colored by Alizarin Red. All immunophenotyping and differentiation experiments were repeated four times. ADMSCs: Adipose-derived mesenchymal stem cells.

and PLC-PRF-5 cells were treated with different concentrations of ADMSC CM ($P <$ 0.001 and $P < 0.05$, respectively, Figure 3A).

We next examined whether AFP was also repressed at the mRNA level. Our data show that ADMSCs and their CM significantly reduced AFP mRNA expression in HepG2 and PLC-PRF-5 cells ($P \leq 0.001$ and $P < 0.05$ respectively, Figure 3B).

In addition to AFP, we also assessed DCP levels in HCC cells alone (control), co-cultured with ADMSCs or treated with ADMSC CM. We observed that ADMSCs significantly decreased DCP levels in HepG2 and PL-PRF-5 cells ($P < 0.001$). ADMSC CM, undiluted or diluted 1:5, significantly reduced DCP secretion by HepG2 cells ($P <$ 0.001). In contrast, only the undiluted ADMSC CM significantly decreased DCP levels in PLC-PRF-5 cells ($P < 0.001$) (Figure 3C).

ADMSCs and ADMSC CM reduce HCC cell line migration and invasion

Cell migration and invasion are important processes in tumor development and metastasis. Bone marrow-derived MSCs have previously been found to promote microvascular HCC[28]. Conversely, they have also been shown to inhibit tumor invasion[26]. Thus, we tested whether ADMSCs altered HCC cell migration using the wound healing assay. The migratory rate of wounded cells was measured at different times (0 h, 6 h, 12 h, and 24 h). As shown in Figure 4A and B, the migration rate of HepG2 and PLC-PRF-5 cells was dramatically inhibited when directly co-cultured with ADMSCs for 24 h compared to when they were cultured alone (control) ($P <$ 0.001). In addition, the ADMSC CM, undiluted or diluted 1:5, significantly reduced HepG2 cell migration rate ($P < 0.001$). However, the ADMSC CM had no effect on PLC-PRF-5 cell migration rates (Figure 4A-C).

The effect of ADMSCs on cell migration was confirmed using the Transwell migration technique. As shown in Figure 4D, the ADMSCs and their CM significantly decreased HepG2 cell migration rate ($P < 0.01$). ADMSCs also inhibited PLC-PRF-5 cell migration rate ($P < 0.001$, Figure 4D).

Using the Transwell invasion assay, our data show that HepG2 cell invasiveness was significantly reduced when co-cultured with ADMSCs at 1:1 and 1:2 ratios or ADMSC CM compared to control cells ($P < 0.05$). Conversely, ADMSCs had no significant effect on PLC-PRF-5 cell invasion (Figure 4E).

Tissue inhibitor metalloproteinases are overexpressed in HCC cell lines

Migration and invasion are initially controlled by the dysregulated expression of MMPs and tissue inhibitor metalloproteinases (TIMPs). To examine whether TIMPs contribute to the decreased migration and invasion capacity of HepG2 and PLC-PRF-5 cells upon ADMSC or ADMSC CM coculture, we examined TIMP-1, -2 and -3 expression by real time PCR. We observed that ADMSCs and their CM significantly increased TIMP-1, -2 and -3 mRNA levels in HepG2 cells (Figure 5A), but only TIMP-1 and -3 in PLC-PRF-5 cells ($P < 0.05$, Figure 5).

P53, RB, c-Myc and hTERT expression

Excessive proliferation and resistance to cell death are regulated by deactivation of tumor suppressor genes and activation of oncogenes. P53 and RB are two tumor suppressor genes implicated in the regulation of apoptosis and cell cycle[29,30]. C-Myc, a proto-oncogene and growth regulator, is overexpressed in HCC[31]. Human telomerase reverse transcriptase (hTERT), the catalytic unit of telomerase, is highly expressed in HCC[32]. To assess the influence of ADMSCs and their CM on tumor suppressor genes and growth regulators, HepG2 and PLC-PRF-5 cells were cultured alone (control), cocultured with ADMSCs or treated with ADMSC CM, and the mRNA levels of P53, RB, c-Myc and hTERT were measured using RT-PCR. In HepG2 cell lines, we found that ADMSCs and their CM significantly induced RB and P53 expression while significantly decreasing hTERT expression ($P < 0.05$). c-Myc mRNA expression remained unchanged (Figure 6A). However, in PLC-PRF-5 cells, ADMSCs and their CM significantly upregulated of c-Myc and RB mRNA levels and downregulated hTERT mRNA expression ($P < 0.05$), while having no effect on P53 mRNA levels (Figure 6B).

DISCUSSION

HCC is a malignant condition with higher incidence and no effective treatment[33].

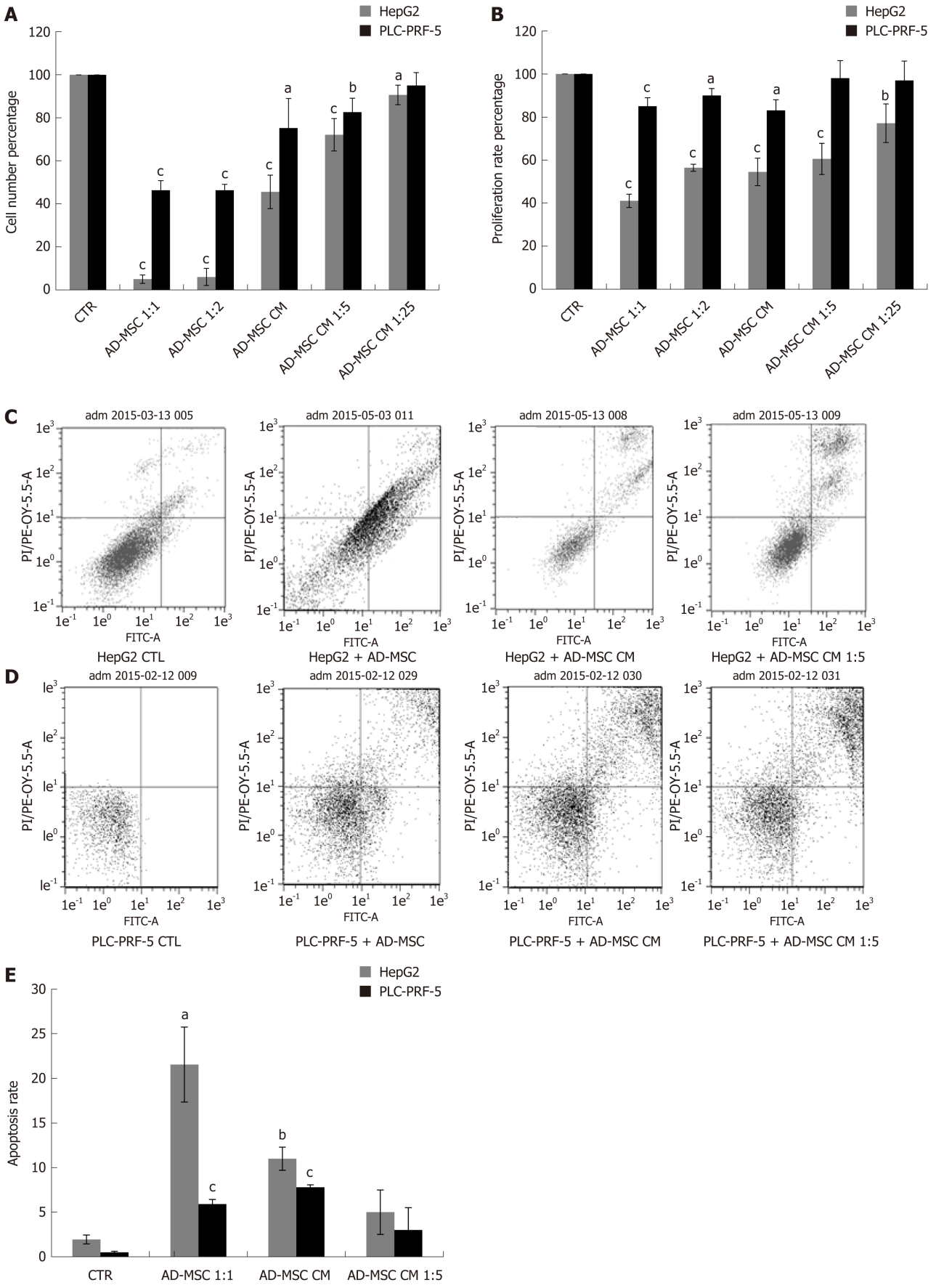

Figure 2 Effect of adipose-derived mesenchymal stem cells and their conditioned media on hepatocellular carcinoma cell proliferation and apoptosis. HCC cells (2×10^5) were seeded in six-well coculture plates in the presence or absence of ADMSCs and ADMSC CM, undiluted or diluted 1:5 or 1:25 for 48 h. The proliferation of HepG2 and PLC-PRF-5 HCC cell lines were measured by (A) cell count assay and (B) WST-8 proliferation tests; The apoptosis of HepG2 (C) and PLC-PRF (D) cells co-cultured as above was measured by flow cytometry using Annexin V/PI test kit; C and D: Two representative experiments of apoptosis in HepG2 and PLC-PRF cells, respectively; E: The average rate of apoptosis in HepG2 and PLC-PRF-5 cells induced by ADMSCs, ADMSC CM. Data are representative of three

independent experiments, each repeated in triplicate. All data are represented as mean ± SD ([a]$P < 0.05$, [b]$P < 0.01$, [c]$P < 0.001$). CTR: Control; ADMSC: Adipose-derived mesenchymal stem cell; CM: Conditioned media; HCC: Hepatocellular carcinoma.

Adipose-derived SCs have been proven to have therapeutic efficacy in many diseases[34-37]. Their CM has been shown to inhibit HCC proliferation and increase apoptosis[38]. Adipose-derived SCs secrete anti-inflammatory cytokines and growth factors and have immunomodulatory effects. However, many controversies have been noted concerning their role in cancer. Therefore, the effect of ADMSCs and their CM on cancer is not clear. In our study, we investigated the role of ADMSCs and their CM in HCC by using two cell lines, HepG2 and PLC-PRF-5. ADMSCs inhibited cell proliferation, decreased the expression of the diagnostic cancer markers AFP and DCP and promoted apoptosis. In addition, ADMSCs decreased cancer cell migration and invasion by increasing TIMP expression. Thus, we suggest that ADMSCs might offer an alternative cell-based therapy for HCC patients.

There are several findings concerning the effect of MSCs on cancer cells, sometimes contradictory within the same type of cancer or between different types of cancer[39]. ADMSCs can be recruited by prostate cancer cells and stimulate tumor growth by increasing tumor vascularity[40]. It has been reported that the interaction of MSCs with tumor cells contributes to gastric carcinoma[41]. In addition, the co-culture or co-injection of MSCs with osteosarcoma cells enhanced tumor growth in mice and promoted osteocarcinoma proliferation. Furthermore, ADMSCs support breast tumor growth and progression[42] but can inhibit proliferation of pancreatic cancer cells *in vitro* and *in vivo*[40]. MSCs can also inhibit tumor growth in Kaposi's sarcoma[43], colon cancer[44,45], hepatoma[46,47], prostate[48,49], pancreatic[50,51], lung cancer[47] and other tumor models[52]. Similar controversies were reported concerning the effect of the SC secretome. For example, bone marrow MSC CM has been shown to have anti-tumor effects on non-small lung cancer cells[53] and stimulatory effects on myeloma cells[54]. In contrast, ADMSC CM had no effect on human glioblastoma cancer SC subpopulations[55]. In addition, it has been demonstrated that human umbilical cord embryonic SC CM has anti-tumor effects on proliferation, apoptosis and tumor cell invasiveness[56]. These findings confirm that in certain types of cancer, MSCs could enhance tumor growth, but in others it can inhibit invasiveness and metastasis[26,57,58]. This might be explained by the complexity of the MSC source, the malignant cell type involved, and the interaction between the MSCs and the tumor cells. The MSC number and microenvironment might also influence tumor cell growth or inhibition. A recent review by Hill *et al*[59] focused primarily on the key mechanisms in which MSCs differentiate into tumor-associated MSCs and cancer-associated fibroblasts to promote pro-metastatic and growth states when in contact with the tumor microenvironment. Despite the described pro-metastatic role of MSCs when in contact with a tumor microenvironment, many other studies have reported that when LEAD MSCs are in contact with cancer cells, it might reduce tumorigenicity[60,61]. The paracrine effects of SCs, their trophic effects when in contact with a stimulatory environment, might provide them the potential to be anti-tumorigenic. We suggest that the type of cancer might dictate the secretion profile of the SCs. Therefore, the microenvironment, cell-cell interactions, and origin of MSCs contribute and direct MSCs to be tumorigenic or anti-tumorigenic.

There have not been any studies involving the direct effect of ADMSCs on HCC proliferation and apoptosis. In our study, ADMSCs inhibited proliferation and induced HepG2 and PLC-PRF-5 cancer cell death when co-cultured. Zhao W *et al*[38] reported that ADMSC CM inhibited HCC cell line proliferation and increased apoptosis. In our data, the inhibition was more significant when ADMSCs were co-cultured rather than using their CM. We hypothesize that this is due to a mechanism underlying cell-cell contact, suggesting that the interactions of receptors and ligands on ADMSCs and cancer cells contribute to the inhibition of proliferation, and thus, trigger cell death. This mechanism of cell-cell interaction should be further investigated. In addition, the upregulation of the tumor suppressor gene P53 and RB and downregulation of c-Myc and hTERT might also contribute to overall tumor suppression. However, tumor suppression is not confined only to inhibition of proliferation by cell-cell interactions and SC CM; it is much more complicated and liver cancer cells are primed for invasiveness and metastasis. Metalloproteinases play major roles in the progression and metastasis of numerous cancers including HCC[62]. This could suggest that inhibiting metalloproteinases is a possible way to decrease HCC invasiveness and metastasis.

In this study, we report the increase in the secretion of the tissue inhibitor metalloproteinases TIMP-1, -2, and -3 by ADMSCs, which may be partially responsible for the decreased HCC cell migration and invasion. The inhibition of

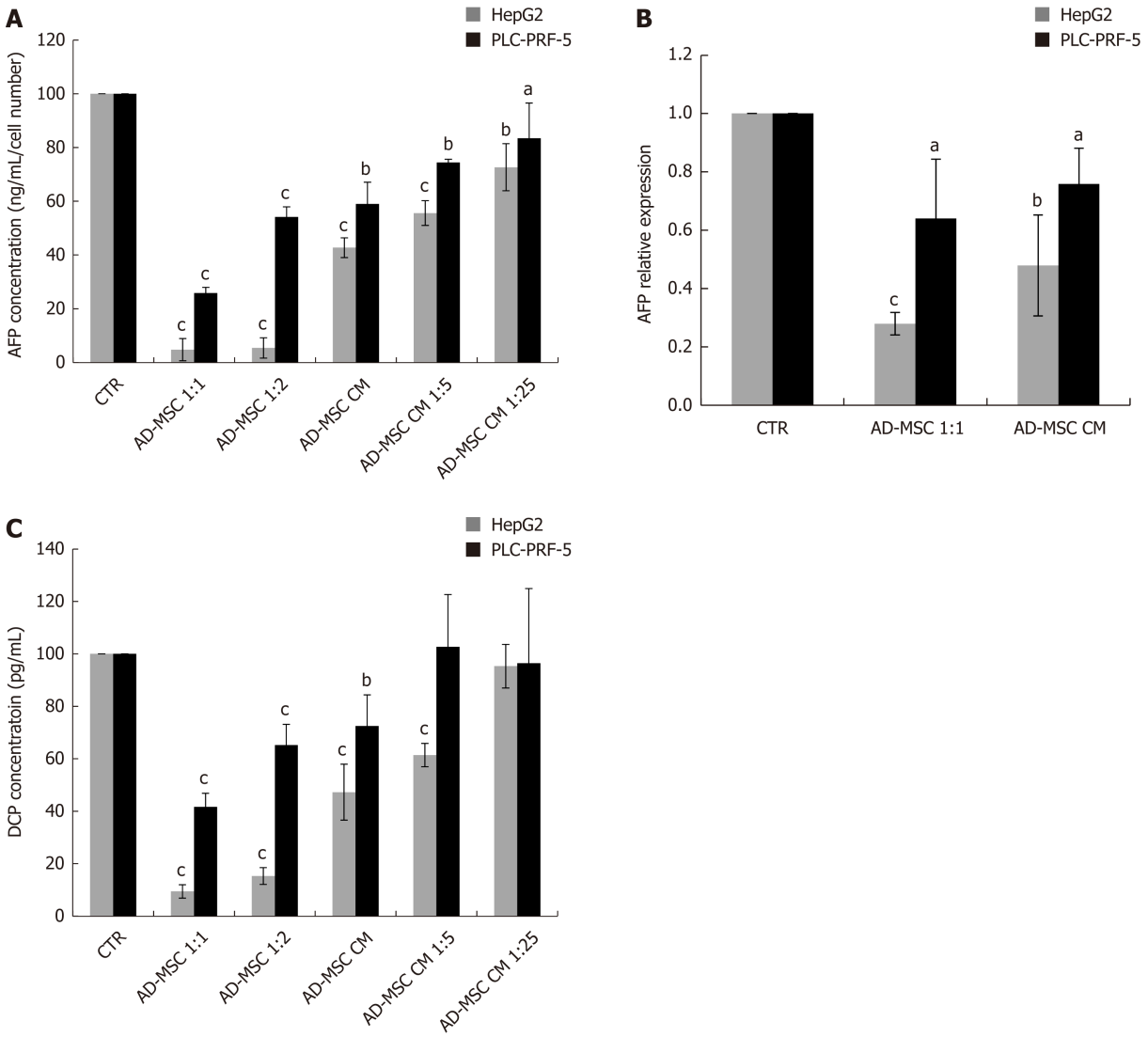

Figure 3 Expression of hepatocellular markers in hepatocellular carcinoma cells. HCC cells (2×10^5) were seeded in six-well coculture plates in the presence or absence of ADMSCs and ADMSC CM, undiluted or diluted 1:5 or 1:25 for 48 h. The protein levels of AFP (A) and DCP (C) was measured in the cell supernatant by ELISA; B: AFP mRNA was assessed by quantitative PCR. Results re displayed as percentage of controls. Data are represented as mean ± SD of five independent experiments, each repeated in triplicate ($^aP < 0.05$, $^bP < 0.01$, $^cP < 0.001$). CTR: Control; ADMSC: Adipose-derived mesenchymal stem cell; CM: Conditioned media; HCC: Hepatocellular carcinoma; AFP: alpha-fetoprotein; DCP: Des-gamma-carboxyprothrombin.

invasion is also explained by the inhibition of TGF-β secretion (data not shown), which is well known to be associated with decreased metastasis and invasiveness[63] through feedback mechanisms. In the future, we aim to demonstrate the exact role of these factors, especially TIMPs, on cancer cell migration and invasion using specific siRNAs.

The increase of TIMP secretion in the presence of ADMSCs or ADMSC CM might be a mechanism that SCs use to restrain tumor invasion. Other important steps in the attempt of ADMSCs to protect against invasion is the decrease in AFP and DCP expression. AFP and DCP have been used as serum markers in HCC patients with and as detectors of tumor progression and malignant proliferation, respectively. When ADMSCs were co-cultured with HCC cell lines, both AFP and DCP were significantly decreased, which might be an indication of SCs attempt to halt proliferation and tumor progression. This is the first report to demonstrate a decrease in DCP in HCC cell lines. Our *in vitro* results will be subsequently confirmed in an *in vivo* study. The primary results of the pilot study confirm the effect of ADMSCs on tumor growth and AFP levels (data not shown).

To conclude our study, we report novel molecules contributing to the effect of adipose-derived SCs on HCC. The increase in TIMPs and in apoptosis, the inhibition of proliferation and invasiveness, and the decrease in AFP and DCP are a coordination attempt from SCs as strategic management to inhibit tumor cell proliferation, progression and invasiveness.

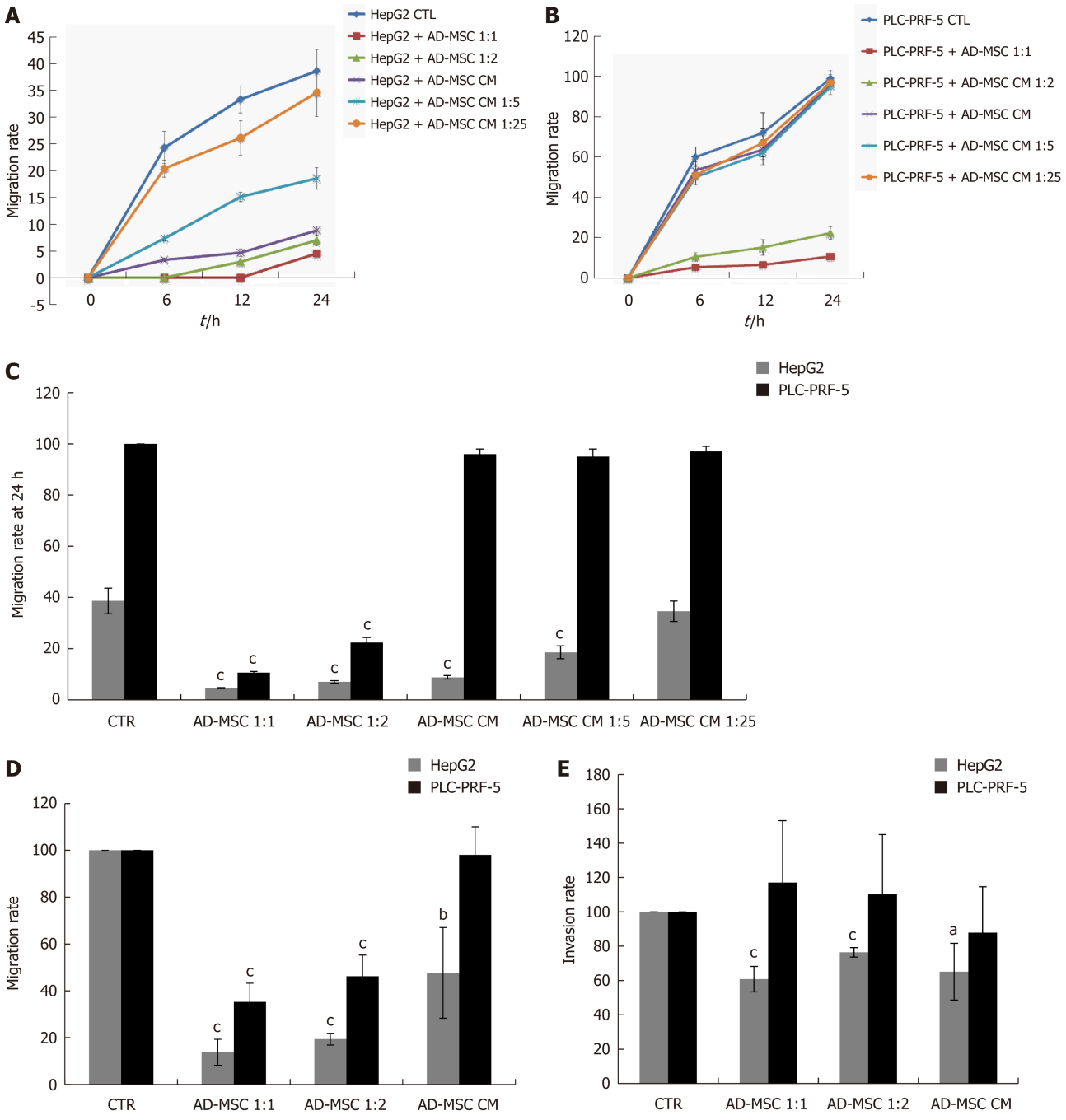

Figure 4 Effect of adipose derived mesenchymal stem cells and their conditioned media on of hepatocellular carcinoma cell migration and invasion. HCC cells (2×10^5) were seeded in six- well co-culture plates in the presence or absence of ADMSCs (at ADSMCs: HCC ratio of 1:1 or 1:2) or ADMSC CM, undiluted or diluted 1:5 or 1:25. The migration of (A) HepG2 and (B) PLC-PRF-5 cells was assessed by wound healing assay. The migration rate at 24 h is represented in (C); D: A Transwell migration assay was performed to confirm the results of the wound healing assay; E: HCC cell invasiveness was measured by Transwell invasion assay. In the Transwell migration and invasion assay, 3×10^5 HCC cells alone, co-cultured with ADMSCs, or treated with ADMSC CM were seeded into the apical chamber of Transwell plates and allowed to migrate or invade through the uncoated polycarbonate membrane or collagen-coated polycarbonate membrane, respectively (8 μm pore size) to the lower chamber for 24 h or 48 h, respectively. The migratory or invasive cells were stained with crystal violet cell stain solution and extracted using an extraction solution provided in the kit. The level of migration and invasion was measured using a plate reader at the absorbance of 560 nm. Values shown are representative of five independent experiments, each performed in triplicate. Data are represented as mean ± SD of five independent experiments, each performed in triplicate ($^aP < 0.05$, $^bP < 0.01$, $^cP < 0.001$). CTR: Control; ADMSC: Adipose derived mesenchymal stem cell; CM: Conditioned media; HCC: Hepatocellular carcinoma.

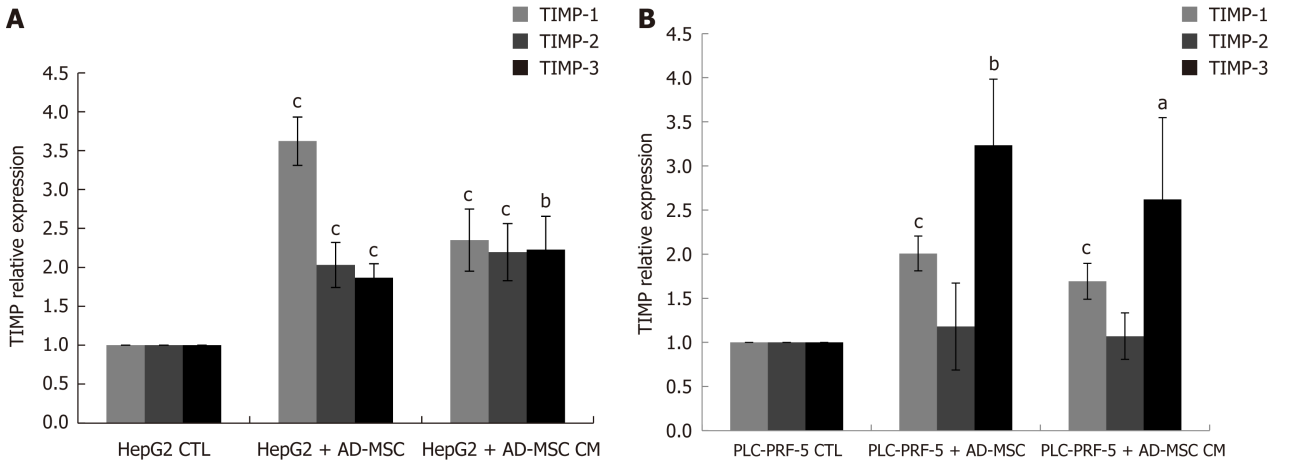

Figure 5 Effect of adipose-derived mesenchymal stem cells and adipose-derived mesenchymal stem cell conditioned media on tissue inhibitor metalloproteinase mRNA levels in hepatocellular carcinoma cells. HCC cells (2×10^5) were seeded in six-well co-culture plates in the presence or absence of ADMSCs or of undiluted ADMSC conditioned media (CM). The mRNA levels of TIMP-1, TIMP-2, and TIMP-3 in HepG2 (A) and PLC-PRF-5 (B) cells after the removal of ADMSCs in the case of coculture was measured by quantitative PCR. Data are represented as mean ± SD of five independent experiments, each performed in triplicate ($^aP < 0.05$, $^bP < 0.01$, $^cP < 0.001$). ADMSC: Adipose-derived mesenchymal stem cell; CM: Conditioned media; HCC: Hepatocellular carcinoma.

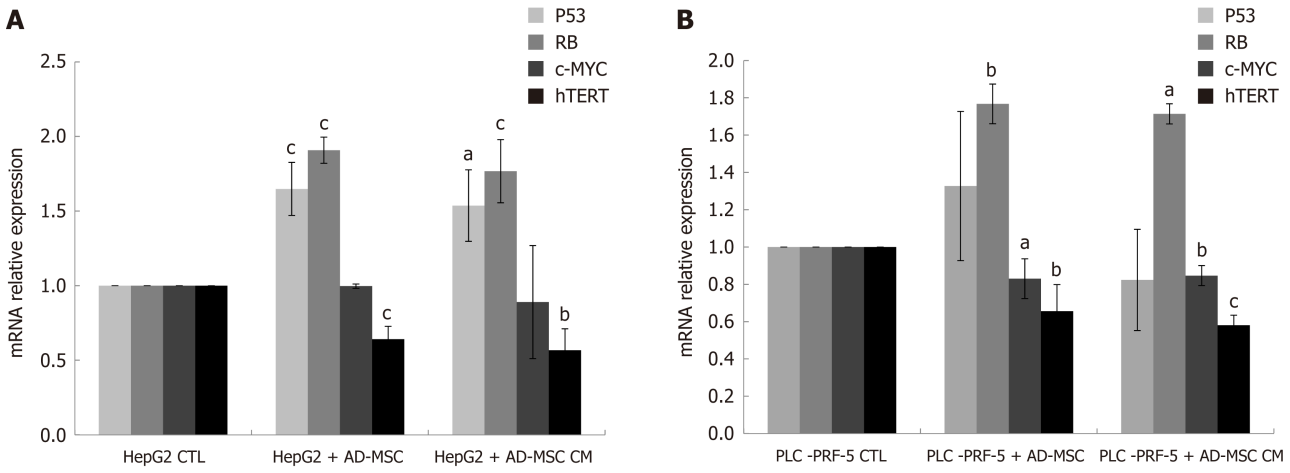

Figure 6 Expression of tumor suppressor genes and oncogenes in hepatocellular carcinoma cells. HCC cells (2×10^5) were seeded in six-well co-culture plates in the presence or absence of ADMSCs or undiluted ADMSC CM for 48 h. The mRNA expression of the tumor suppressor genes P53/RB, oncogene c-Myc and the enzymatic component of telomerase hTERT were assessed by RT-PCR in (A) HepG2 and (B) PLC-PRF-5 cells after the removal of ADMSCs in the case of co-culture. Data are represented as mean ± SD of five independent experiments, each performed in triplicate ($^aP < 0.05$, $^bP < 0.01$, $^cP < 0.001$). ADMSC: Adipose-derived mesenchymal stem cell; CM: Conditioned media; HCC: Hepatocellular carcinoma.

ARTICLE HIGHLIGHTS

Research background

Hepatocellular carcinoma (HCC) is a malignant condition with a high incidence and no effective treatment. Mesenchymal stem cells (MSCs) secrete cytokines and growth factors known to have paracrine, trophic and immunomodulatory effects. Due to their paracrine and differentiation potential, adipose-derived SCs have proven therapeutic efficacy in many diseases. Their conditioned media (CM) has been shown to inhibit proliferation and increase apoptosis in HCC. However, many controversies have been noted concerning their role in cancer.

Research motivation

Many studies have demonstrated the effect of SCs or their CM on cancer, and some reports have shown that they suppress and inhibit tumor growth. Other studies have reported enhanced tumor growth and proliferation. There have not been any studies that reported the effect of adipose-derived MSCs (ADMSCs) on HCC proliferation and apoptosis. Thus, our aim was to investigate the therapeutic effects of adipose-derived SCs and their CM on HCC, specifically their effects on cancer cell marker expression, the proliferation and metastatic potential of cancer cells, and their effect on modulating cancer cell death.

By discovering that adipose-derived SCs and their CM modulate cancer marker expression and liver cancer cell proliferation and metastasis, we have opened a new path for research on the

mechanism of action by which MSCs can affect cancer. If the results were to increase and stimulate cancer cells, then further investigations need to be pursued on two levels: (1) to study the behavior of SCs along with the factors contributing to the stimulatory effects; and (2) inhibition of the pathways leading to this progressive effect. In contrast, if the ADMSCs were to inhibit cancer and induce apoptosis, then ADMSCs could be a potential therapy for HCC, which currently has no cure. To achieve this goal, *in vivo* animal models and clinical studies need to be pursued.

Research objectives

In our study, the main objective was to investigate the role of ADMSCs and their CM in HCC using two cell lines, HepG2 and PLC-PRF-5. In particular, we wanted to study the effect of ADMSCs on alpha-fetoprotein (AFP) and Des-gamma-carboxyprothrombin (DCP) expression and the capacity of ADMSCs to modulate metastasis or proliferation of the above cancer cell lines. We studied TIMPs, P53, and RB. ADMSCs inhibited cell proliferation, decreased AFP and DCP expression and promoted apoptosis. In addition, ADMSCs decreased cancer cell migration and invasion by increasing TIMP expression. Our study has shed light on a novel apoptotic effect of MSCs on cancer. This will direct us and other researchers to further investigate the effect on other cell markers playing roles in cancer and the mechanisms by which ADMSCs exert their anti-cancer effects.

Research methods

HCC cell lines purchased from ATCC were cultured in low glucose DMEM media. Adipose-derived MSCs isolated from lipoaspirates were cultured in DMEM nutrient mix F12. The isolation method of MSCs was modified and improved to obtain a high yield of living ADMSCs using a minimal quantity of fat and collagenases. Isolated ADMSCs were characterized to demonstrate their viability and capacity of multilineage differentiation.

The coculture conditions and treatment with ADMSC CM were extensively studied in order to determine the number of cells that should be used in all experiments. After co-culturing HCC cells with ADMSCs or stimulating with ADMSC CM, AFP and DCP protein and mRNA levels were detected using ELISA kits and real time PCR, respectively.

In addition, the proliferation level and apoptosis rate of HCC cells were measured using a WST-8 proliferation test and annexin V-FITC kit, respectively. Along with these tests, the mRNA levels of P53, RB, hTERT and c-Myc genes involved in the regulation of proliferation and apoptosis were quantified using real time PCR.

Furthermore, using wound healing assays and migration and invasion tests, we studied the effect of ADMSCs and their CM on HCC cell line metastasis. In parallel, TIMP mRNA levels were measured using real time PCR. TIMPs have been reported to play a major role in in inhibiting metastasis.

In all assays, the experiments were repeated at least three times in order to obtain statistically significant results.

Research results

In our study, ADMSCs inhibited cancer cell proliferation and increased cancer cell death when co-cultured with HepG2 and PLC-PRF-5. This effect was more significant in the case of direct co-culture, likely due to cell-cell interactions. The upregulation of the tumor suppressor genes P53 and RB and downregulation of c-Myc and hTERT might be the factors responsible for the mentioned findings. The mechanisms of these results should be further investigated.

We reported increased secretion of TIMP-1, -2, and -3, which may be partially responsible for the decreased HCC cell migration and invasion. Future studies should be performed to confirm this relation. In addition, further investigations are needed to study the involvement of the metalloproteinases MMP-2 and MMP-9 in the inhibition of metastasis. We also found that ADMSCs and ADMSC CM decreased HCC cell line migration and invasion.

We observed decreased AFP and DCP levels after coculturing HCC cells with ADMSCs or stimulating HCC cells with ADMSC CM. This might be an indication of an attempt by SCs to obliterate proliferation and tumor progression. These findings will be confirmed and used subsequently in an *in vivo* animal study.

Research conclusions

This study reported many novel findings about the effects of ADMSCs on HCC. This is the first report to demonstrate a decrease in DCP expression in HCC cell lines. No other study has investigated the direct effect of ADMSCs on HCC proliferation and apoptosis. We reported novel molecules contributing to the effect of adipose-derived SCs on HCC, particularly TIMPs. We reported that coculture of ADMSCs with HepG2 or PLC-PRF-5 cell lines had an anti-cancer effect. This is explained by the inhibition of proliferation and cell death of the cancer cells. We also showed for the first time the effect of direct cell-cell interactions, which is a new mechanism by which ADMSCs might inhibit tumor cell proliferation. The indirect contact of ADMSCs with HCC cell lines inhibited their proliferation and metastasis and increased their apoptosis, though to a lesser extent than the direct coculture, suggesting that the paracrine effects of ADMSCs contribute to their antitumor effects.

These findings confirm that in certain types of cancer, MSCs could enhance tumor growth and in others, it can inhibit invasiveness and metastasis. This might be explained by the complexity of MSC sources, the malignant cell type involved, and the interaction between MSCs and tumor cells. The number of MSCs and the microenvironment might also influence tumor cell growth or inhibition.

Research perspectives

Our findings will lead to many investigations: (1) the mechanism of cell-cell contact by which ADMSCs inhibit cancer cells (autophagy); (2) identification of the factors exerting these inhibitory effects. In particular, two candidates are STC-1 and DKK-1, which are known to have roles in suppressing the expression of genes involved in proliferation, migration and invasion and in the overexpression of apoptotic genes. These results should be demonstrated using WST-8 proliferation assays, apoptosis annexin/PI assays, and migration and invasion tests.

In vivo studies will be pursued to confirm our results, mainly the effect of ADMSCs and their CM on tumor growth, apoptosis and metastasis, as well as the paracrine effects of ADMSCs.

To understand and quantify the changes in hepatic cancer cell morphology when in direct contact with ADMSCs, a study will be conducted in collaboration with the Department of Physics at the American University of Beirut. This will also help us determine the mechanism by which ADMSCs induce HepG2 cell death.

In summary, ADMSCs are cells with complex mechanisms that have the capacity to interact with adjacent cells to exert trophic and paracrine effects, thus altering the microenvironment. Their role in each disease must be vigorously studied to elucidate their therapeutic effects. In this study, we determined the inhibitory effects of ADMSCs on cancer cell markers and on key factors known to play a major role in inflammation, invasion and metastasis. Our study has shed new light on the role of ADMSCs on HCC.

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ORIGINAL ARTICLE

Basic Study

Claudin-7 gene knockout causes destruction of intestinal structure and animal death in mice

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Abstract

BACKGROUND

Claudin-7, one of the important components of cellular tight junctions, is currently considered to be expressed abnormally in colorectal inflammation and colorectal cancer. However, there is currently no effective animal model to study its specific mechanism. Therefore, we constructed three lines of *Claudin-7* knockout mice using the Cre/LoxP system.

AIM

To determine the function of the tumor suppressor gene *Claudin-7* by generating three lines of *Claudin-7* gene knockout mice.

METHODS

We crossed *Claudin-7*-floxed mice with CMV-Cre, vil1-Cre, and villin-CreERT2 transgenic mice, and the offspring were self-crossed to obtain conventional *Claudin-7* knockout mice, conditional (intestinal specific) *Claudin-7* knockout mice, and inducible conditional *Claudin-7* knockout mice. Intraperitoneal injection of tamoxifen into the inducible conditional *Claudin-7* knockout mice can induce the knockout of *Claudin-7*. PCR and agarose gel electrophoresis were used to identify mouse genotypes, and Western blot was used to confirm the knockout of *Claudin-7*. The mental state, body length, and survival time of these mice were observed. The dying mice were sacrificed, and hematoxylin-eosin (HE) staining and immunohistochemical staining were performed to observe changes in intestinal structure and proliferation markers.

RESULTS

We generated *Claudin-7*-floxed mice and three lines of *Claudin-7* gene knockout mice using the Cre/LoxP system successfully. Conventional and intestinal specific *Claudin-7* knockout mice were stunted and died during the perinatal period, and intestinal HE staining in these mice revealed mucosal gland structure

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disappearance and connective tissue hyperplasia with extensive inflammatory cell infiltration. The inducible conditional *Claudin-7* knockout mice had a normal phenotype at birth, but after the induction with tamoxifen, they exhibited a dying state. Intestinal HE staining showed significant inflammatory cell infiltration, and atypical hyperplasia and adenoma were also observed. Intestinal immunohistochemistry analysis showed abnormal expression and distribution of Ki67, and the normal intestinal proliferation balance was disrupted. The intestinal crypt size in inducible conditional *Claudin-7* knockout mice was increased compared with control mice (small intestine: 54.1 ± 2.96 *vs* 38.4 ± 1.63; large intestine: 44.7 ± 1.93 *vs* 27.4 ± 0.60; $P < 0.001$).

CONCLUSION
The knockout of *Claudin-7 in vivo* causes extensive inflammation, atypical hyperplasia, and adenoma in intestinal tissue as well as animal death in mice. *Claudin-7* may act as a tumor suppressor gene in the development of colorectal cancer.

Key words: Claudin-7; Gene knockout; Inflammation; Adenomas; Colorectal carcinoma

Core tip: The intestinal tract of conventional and intestinal specific *Claudin-7* knockout mice was characterized by extensive and severe inflammation. The development of inducible conditional knockout mice can control the knockout of *Claudin-7* in a temporal and compartment specific manner and prolong the survival time of mice, which exhibited atypical hyperplasia and adenoma in the intestine. This study revealed the inhibitory role that Claudin-7 plays in colorectal inflammation and colorectal cancer.

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INTRODUCTION

Members of the Claudin family serve as important components of cellular tight junctions (TJs), and they mainly function to maintain cell polarity, regulate intercellular small molecule flux, and facilitate cell proliferation and differentiation[1-3]. Claudin-7 (Cldn7), one of the 27 members of the Claudin family, is mainly distributed in the stomach, lung, intestine, bladder, and kidney. Cldn7 was originally found in an extracellular Cl^- barrier and Na^+ channel and shown to affect extracellular permeability[4]. However, recent studies have shown that Cldn7 is abnormally expressed in different cancer tissues, especially in colon cancer, suggesting that alterations in its expression may affect the normal structure and function of TJs and be related to the occurrence of intestinal tumors[5-8]. Cldn7 is currently considered to play an inhibitory role in colorectal inflammation and colorectal cancer by most scholars[9-11].

The most effective way to study inhibitors *in vivo* is to knock out the gene in an animal and observe its overall phenotype. In recent years, the Cre/LoxP recombinase system has been widely used in novel gene targeting[12,13]. LoxP was inserted at both ends of the *Cldn7* sequence to obtain heterozygous floxed mice. After crossing with CMV-Cre and vil1-Cre mice, the sequence between the two LoxP sites was excised and inherited by daughter cells. Shimizu was the first to report time-specific gene knockout animal models in which the time of gene knockout could be artificially controlled by injection with an inducer[14]. Therefore, we constructed conventional *Cldn7* gene knockout (CKO) mice and conditional knockout (cKO) mice using the Cre/LoxP system. We also generated inducible conditional *Cldn7* knockout (ICKO) mice and induced Cre expression by injecting tamoxifen. Hematoxylin-eosin (HE) staining showed that the intestinal structures in the CKO and cKO mice were severely damaged, and numerous inflammatory cells infiltrated. By injecting tamoxifen into

the ICKO mice, we successfully established atypical hyperplasia and intestinal adenoma models. Immunohistochemistry analysis indicated that the expression and distribution of Ki67 in the intestinal tissues were dysregulated. The successful construction of mouse intestinal inflammation and intestinal adenoma models could provide a basis for further studying the role of Cldn7 in intestinal tumors.

MATERIALS AND METHODS

Experimental animal species and animal care and use statement

We inserted a LoxP site into the intronic sequence downstream of exon 4 of the *Cldn7* gene and inserted the FRT-neo-FRT-LoxP element into the upstream intronic sequence of exon 2 to obtain *Cldn7*-floxed mice. The CMV-Cre mice were purchased from the National Resource Center for Mutant Mice, the vil1-Cre mice were obtained from the China Pharmaceutical University (from the Jackson Laboratory, USA), and the villin-CreERT2 mice were donated by professor Sylvie Robine. All mice were housed according to specific pathogen-free grade animal feeding standards at an indoor temperature of 20-26 °C and a 12-h day/night cycle. The mice were fed a standard diet after sterilization and had free access to food and water. All animals were euthanized for tissue collection. All animal assay protocols were reviewed and approved by the Medical Ethics Committee of the Capital Medical University Affiliated Beijing Shijitan Hospital Institutional Review Board.

Construction of Cldn7-floxed mice

We constructed a targeting vector as shown in Figure 1A. After the final vector was sequenced for validation, it was transfected into embryonic stem (ES) cells by electroporation. From the 8th-10th day, ES cell clones were picked, and genomic DNA was extracted, amplified, digested with the *Eco*RV enzyme overnight, and slowly electrophoresed for 36 h. The target clones were screened by long range PCR and Southern blot.

Approximately 4-wk-old C57BL/6N female mice were selected and injected with pregnant mare serum gonadotropin and human chorionic gonadotropin to promote ovulation. Embryos were harvested on the 2nd day after cohousing the female mice with the male mice, and 12-15 ES cells were injected into each blastocyst after culturing overnight. After the injection, the blastocysts were cultured for 3 h in an incubator, and those with a normal morphology and intact transparent bands were selected for transplantation. After 8-10 wk of sexual maturation, female C57BL/6N mice were selected for uterine blastocyst transplantation. The mice born after successful transplantation were identified by PCR, and those with the *fln/wt* genotype were deemed to be chimeric mice. Chimeric mice were crossed with Flper mice and then backcrossed with wild-type C57BL/6N mice to obtain *Cldn7*-floxed mice (genotype: *fl/wt*), which were missing the entire *Neo* resistance gene.

Construction of three lines of knockout mice

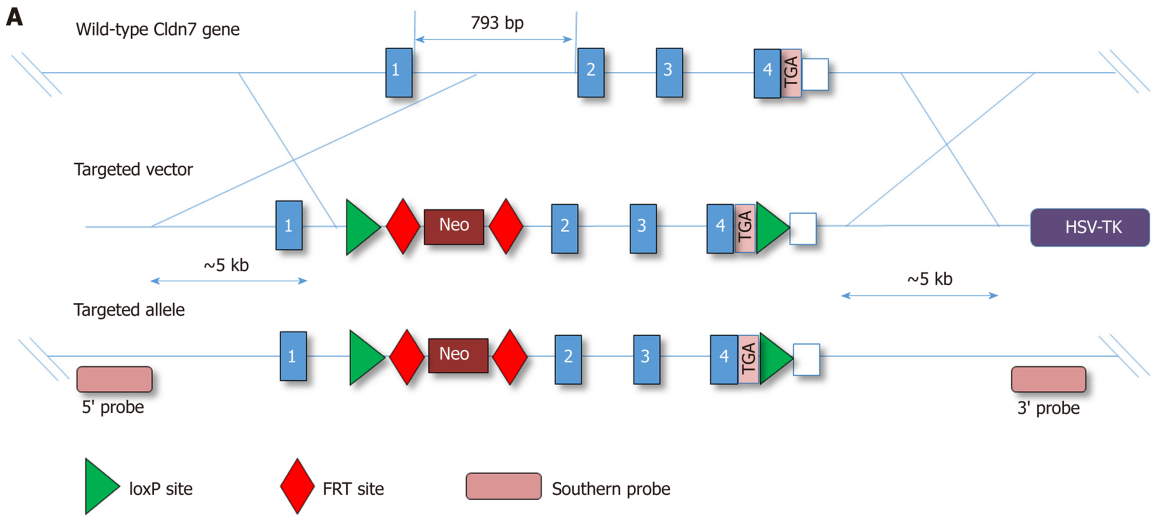
Cldn7-floxed mice were crossed with CMV-Cre mice, vil1-Cre mice, and villin-CreERT2 mice to obtain *Cldn7* CKO mice, *Cldn7* cKO mice, and *Cldn7* ICKO mice, respectively. Next, 50 mg of tamoxifen was dissolved in 5 mL of sterilized sunflower oil and mixed for 30 min to obtain the tamoxifen dilution. Six- to eight-wk-old ICKO mice were intraperitoneally injected with 100 μL of the tamoxifen dilution every 5 d to induce the *Cldn7* knockout.

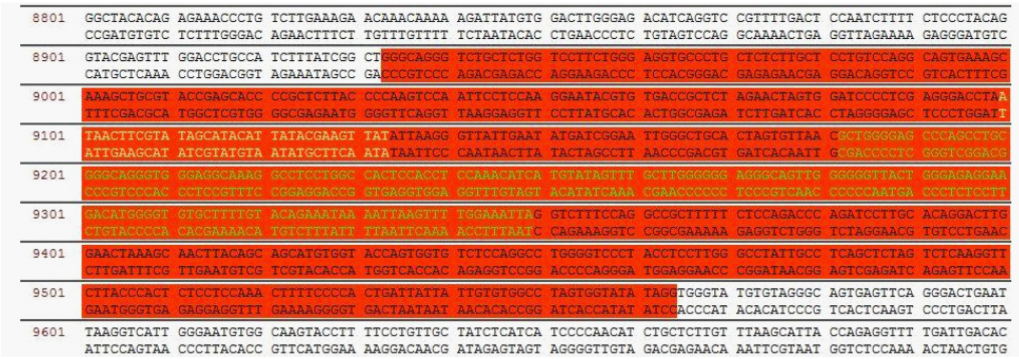
Western blot

Various *Cldn7* knockout mouse tissues were minced on ice and mixed with appropriate grinding beads and total protein extraction reagents containing different protease inhibitors; a tissue homogenizer was then used to extract total protein. Proteins were separated by SDS-PAGE and transferred onto nitrocellulose membranes. The membranes were incubated with a diluted rabbit polyclonal anti-Cldn7 antibody (1:1000, ab27487, Abcam, United States) at 4 °C overnight and then with a donkey anti-rabbit IgG antibody (1:10000, ab175780, Abcam). After blotting, the signals were detected with a Western blot scanner. GAPDH was used as the internal reference.

HE staining

The intestinal tissues of *Cldn7* knockout mice and control mice were washed in PBS and then placed in 10% formalin/PBS at 4 °C. After dehydration and clearing, the tissues were immersed in wax and then cut into 5-8-micron-thick sections. The sections were then dewaxed and stained with HE.





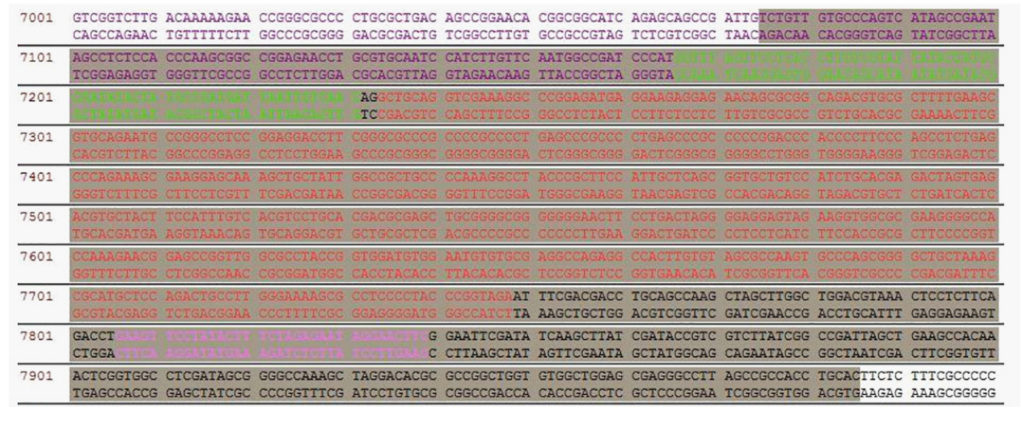


Figure 1 Strategic design and final vector sequencing results. A: Schematic diagram of the *Cldn7* gene knockout targeting vector; B-D: The first and second regions in red show the sequencing results at LoxP and FRT 5', while the grey region shows the FRT 3' sequencing results; B-D indicate that the *Cldn7* targeting vector was correct. Cldn7: Claudin-7.

Immunohistochemical staining

All tissues were embedded in wax blocks and cut into paraffin sections. After dewaxing, hydration, and antigen retrieval, the tissue sections were incubated for 10 min in 3% H_2O_2 and washed with 0.01 mol/L PBS. The sections were then incubated with a diluted rabbit polyclonal anti-Cldn7 antibody (ab27487, 1:200, Abcam, United States) and a rabbit monoclonal anti-Ki67 antibody (ab16667, 1:1000, Abcam, United States), followed by incubation with the corresponding horseradish peroxidase-labelled secondary antibody. The proteins were then developed in 3,3′-diaminobenzidine for coloration and assessment.

Statistical analysis

Statistical analyses were performed using IBM SPSS version 17.0 and GraphPad Prism version 6.0. All data are expressed as the mean ± SD. Differences between two groups were analysed by Student's *t*-test and considered significant at $P < 0.05$.

RESULTS

Cldn7-floxed mice are constructed successfully

We constructed the *Cldn-7* gene knockout targeting vector as shown in Figure 1A, and sequenced the final vector using a unidirectional primer (LoxPtF: GTACGAGTTTGGACCTGCCA) to detect whether the 34 bp LoxP site was inserted correctly (Figure 1B). The LoxP site is shown in yellow, and the 3′-untranslated region is shown in green. FRT sequencing from the 5′ end was performed using a unidirectional primer (Cldn7-FRT-tF: CTGATCTGGGTGTCCCACGT), as the FRT site serves as a screening marker for *Neo*. By removing the *Neo* resistance gene, the FRT site was also removed. The second LoxP site is shown in green, the FRT site is shown in yellow, and *Neo* is shown in purple (Figure 1C). FRT 3′ sequencing was then performed (FRT-tR: CGATGAAACCGTTCCAGGTA), and the presence of another FRT site is shown in pink font (Figure 1D). Therefore, the final gene targeting vector was correct.

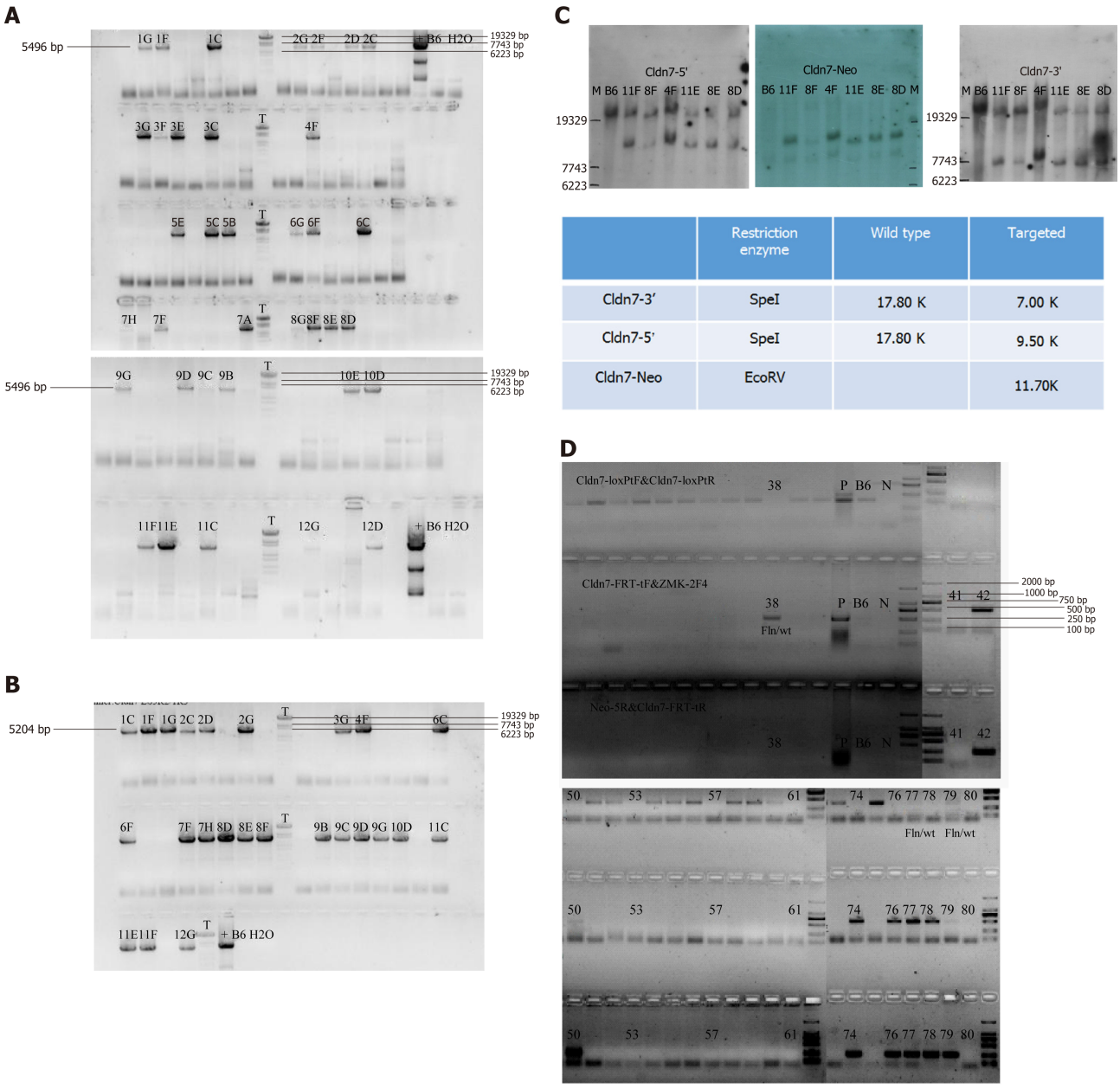
The targeting vector was electroporated into B6/BLU ES cells for targeting, and some drug-resistant ES cell clones were obtained. Two methods were used to prevent false-positive results and detect target clones, long range PCR and Southern blot. First, we tested whether the 5' homologous arm was correct (Figure 2A). The 5496 bp product was a positive clone containing LoxP. The 3′ end was also detected (Figure 2B), and the 5204 bp product was a positive clone. The Southern blot results are shown in Figure 2C. Genomic DNA extracted from the transfected ES cells was digested with *Spe* I restriction endonuclease. Gene fragments of 17.8 kb and 9.5 kb were obtained from the *Cldn7* 5′ end of the wild-type and target clones, respectively, and gene fragments of 17.8 kb and 7 kb were obtained from the *Cldn7* 3′ end. When using the *Eco*RV restriction enzyme (probe on Neo), an 11.7 kb gene fragment was obtained from the target clone. The Southern blot results showed that clones 8D, 8E, 4F, 11E, and 11F were the final positive clones.

The positive clone 8D was selected for blastocyst injection, and the newborn mice after blastocyst transfer were genotyped by PCR. The primer information is shown in Table 1. The mice numbered 50 and 77 were deemed chimeric mice with the genotype *fln/wt* (Figure 2D). The chimeric mice were crossed with Flper mice to remove the *Neo* resistance gene, and the resulting mice were mated with wild-type C57BL/6N mice to completely delete the *Neo* resistance gene, successfully yielding *Cldn7*-floxed mice.

Cldn7 CKO mice die in the perinatal period and have severe intestinal damage

Cldn7-floxed mice were crossed with CMV-Cre mice, and the offspring were then self-crossed. Genomic DNA was isolated from the tails for genotyping. The primer information is shown in Table 2. The mouse with the Null/Null CreW genotype was considered the *Cldn7* CKO mouse. We considered newborn mice from the same litter as an example. We first evaluated whether *Neo* was completely deleted. None of the samples showed an fln band at 515 bp, indicating that *Neo* had been deleted completely. Furthermore, none of the samples showed the Cre band at 481 bp, suggesting that the genotype of all samples was CreW. Next, the banding results showed that mice numbered 1, 5, 6, and 10 had a null band at 640 bp, suggesting the presence of Cre-mediated recombination. The final step was to identify whether the mice were homozygous, and mouse 6 was determined to be homozygous for the Null/Null genotype. Therefore, mouse 6 was deemed the *Cldn7* CKO mouse with the Null/Null CreW genotype (Figure 3A).

Cldn7 CKO mice were born similarly to heterozygous and wild-type mice. However, the lengths of the *Cldn7* CKO mice increased significantly slower than those



	Restriction enzyme	Wild type	Targeted
Cldn7-3′	SpeI	17.80 K	7.00 K
Cldn7-5′	SpeI	17.80 K	9.50 K
Cldn7-Neo	EcoRV		11.70K

Figure 2 Long range PCR and Southern blot for detecting target clones. A: The 5' homologous arm band was first detected, and the product with a length of 5496 bp was a positive clone containing LoxP; B: The 3' end was then detected, and the product with a length of 5204 bp was a positive clone; C: Southern blot results showing that clones 8D, 8E, 4F, 11E, and 11F were the final targeted clones; D: After blastocyst transfer, newborn mice were genotyped by PCR. The mice numbered 50 and 77 were deemed chimeric mice with the genotype *fln*/*wt*.

of the control mice ($P < 0.05$).

Beginning on the third day, CKO mice were thin, lacked energy, showed signs of lethargy, exhibited decreased body temperature, and had reduced activities, all of which suggested a state of dying (Figure 3B).

The dying mice were sacrificed, and their lungs, stomachs, bladders, kidneys, small intestines, and large intestines were collected. Western blot analysis showed that Cldn7 was not expressed in any of the tissues analyzed in CKO mice, while the control mice expressed Cldn7 in all of these tissues (Figure 3C). Intestinal HE staining showed obvious atrophy, thinning or loss of intestinal mucosa, connective tissue hyperplasia with inflammatory cell infiltration, residual intestinal mucosal epithelial vacuolar degeneration, villus shortening, and lymphatic expansion. Intestinal HE staining of the control mice showed no obvious histopathological changes (Figure 3D). Therefore, *Cldn7* CKO mice showed significantly slow growth and appeared to be dying on the third day. HE staining showed severe intestinal destruction, loss of intestinal mucosal structure, and infiltration of numerous inflammatory cells.

Cldn7 cKO mice have longer survival times

Table 1 PCR primers for chimeric mouse genotyping and corresponding band results

Sequence (5'-3')	Results
Cldn7 loxP-tF primer : GTACGAGTTTGGACCT GCCA	Fln/Fln = 493 bp; Fln/wt = 493/375 bp; wt/wt = 375 bp
Cldn7-LoxP-tR primer : TGTGCAA GGATCTGGGTCTG	
Cldn7-FRT-tF primer : CTGATCTGGGTGTCCCACGT	Fln/Fln = 507 bp; Fln/wt = 507/0 bp; wt/wt = 0bp
ZMK-2F4 primer: GCATCGCATTGTCTGAG TAGGTG	
Neo-5R primer : GGCTGG ACGTAAACTCCTC	Fln/Fln = 259 bp; Fln/wt = 259/0bp; wt/wt = 0 bp
Cldn7-FRT-tR primer: CGATGAAACC GTTCCAGGTA	

Cldn7-floxed mice were crossed with vil1-Cre mice, and the offspring were then self-crossed. Genomic DNA was isolated for genotyping analysis. The mouse with the *Cldn7*$^{fl/fl}$; villin-CreT (*fl*/*fl* CreT) genotype was deemed the *Cldn7* cKO mouse. None of the samples showed a fln band at 515 bp or a null band at 640 bp, indicating that *Neo* had been deleted completely and that no Cre recombination occurred. We next detected whether flper recombination occurred by the detection of an *fl* band at 756 bp. The results showed that No. 213 mouse had the *fl*/*wt* genotype, while all other mice had the *fl*/*fl* genotype. Finally, Cre was detected, and a band at 481 bp, which corresponded to CreT, was observed in mice numbered 213, 215, and 217, while all other mice displayed a band corresponding to CreW. Therefore, the mice numbered 215 and 217 were deemed *Cldn7* cKO mice (Figure 4A).

Cldn7 cKO mice were normal at birth compared to control mice. But *Cldn7* cKO mice were obviously thin, their body length increased slowly from the 5th day after birth, and their growth rate was significantly slower than that of control mice ($P < 0.05$). On the 9th day, *Cldn7* cKO mice appeared to be languid, with reduced or even inactive activities, leaving only a slight breath. The body temperature of the mouse was reduced and it was in a state of dying (Figure 4B).

The dying mice were sacrificed, and we found that the expression levels of Cldn7 in the lung, stomach, bladder, and kidney tissues of cKO mice were normal, while Cldn7 expression was absent in the small and large intestines. All control mouse tissues expressed Cldn7 (Figure 4C). Intestinal HE staining showed obvious mucosal atrophy, mucosal gland structure disappearance, and connective tissue hyperplasia with extensive inflammatory cell infiltration. Inflammatory lesions were observed everywhere (Figure 4D), and mucosal epithelial vacuolar degeneration was observable after magnification. No obvious histopathological changes were observed in the mucosal glands, submucosa, or muscular layers of the intestines of *fl*/*fl* CreW mice. *Cldn7* cKO mice excluded the influence of other organs lacking Cldn7 expression on mice and prolonged the survival time. However, because the cKO mice still showed intestinal inflammation, we generated *Cldn7* ICKO mice on this basis to try to obtain an adenoma model.

Cldn7 ICKO mice display atypical hyperplasia and adenoma and dysregulated proliferation in the intestine

Cldn7-floxed mice were crossed with villin-CreERT2 mice, and the offspring were then self-crossed. PCR and agarose gel electrophoresis results showed the mice numbered 135-137, 139-142, and 144 were *Cldn7* ICKO mice with the genotype of Cldn7$^{fl/fl}$; villin-CreERT2 (*fl*/*fl* CreERT2) (Figure 5A).

Cldn7 ICKO mice were normal at birth and developed smoothly, unlike Cldn7$^{fl/fl}$; villin-CreW (*fl*/*fl* CreW) mice (Figure 5B left). A tamoxifen solution (10 mg/mL, 100 μL) was intraperitoneally injected into 6 to 8-wk-old ICKO mice every 5 d. Beginning at the 7th injection, the ICKO mice were lethargic and lack of activity, appeared thin, and exhibited a dying state, while the control mice showed no abnormalities (Figure 5B left). All ICKO mice died within 75 d (15 tamoxifen injections, Figure 5B right).

Both the dying mice and control mice were sacrificed. The expression levels of Cldn7 in the lung, stomach, bladder, and kidney tissues of ICKO mice were normal, but Cldn7 expression was absent in their small and large intestines. All control mouse tissues expressed Cldn7 (Figure 5C). Intestinal HE staining showed obvious inflammatory manifestations (Figure 5D, E, G, and H), numerous infiltrated inflammatory cells, abnormal or absent intestinal villi and intestinal gland structure, mucosal epithelial cell shedding, and disordered residual intestinal villus mucosal epithelial cells that lacked polarity. Atypical hyperplasia (Figure 5J and M) and intestinal adenoma (Figure 5K and N) were also observed. The intestinal structure was normal and intact in the control mice (Figure 5F, I, L, and O), with no obvious pathological changes observed.

Table 2 PCR primers for gene knockout mouse genotyping and corresponding explanations

Primer sequence (5'-3')	Results	Description
Cldn7-FRTtF2: CCTGGGATCTGATCTGGGTG Cldn7-FRTtR2: GGCAGGTAGCCTTAGGATGG	wt = 600 bp, fln = none; fl = 756 bp, Null = none	Check if flper is reorganized
ZMK2F4: GCATCGCATTGTCTGAGTAGGTG Cldn7-FRTtF2: CCTGGGATCTGATCTGGGTG	wt = none, fln = 515 bp; fl = none, Null = none	Check if *Neo* is deleted completely
Cldn7-loxPtF2: CTTGGGAGACATCAGGTCCG Cldn7-loxPtR2: GAGGCAATAGGCCCAAGGAG	wt = 512 bp, fln = 630 bp; fl = 630 bp, Null = none	Homozygous identification
Cldn7-FRTtF2: CCTGGGATCTGATCTGGGTG Cldn7-loxPtR2: GAGGCAATAGGCCCAAGGAG	wt = none, fln = none; fl = none, Null = 640 bp	Check if Cre mediated recombination occurs
Cre-up: GCCTGCATTACCGGTCGATGC Cre-low: CAGGGTGTTATAAGCAATCCC	T: 481 bp; W: none	Detect Cre

Immunohistochemistry analysis was also used to detect the expression of intestinal Cldn7 and the nuclear proliferation marker Ki67 in ICKO and control mice, revealing that Cldn7 was strongly expressed in the intestinal epithelial junction of control mice, but was significantly weakened in the intestines of ICKO mice (Figure 6A-D). In addition, the expression of Ki67 was also altered. In control mice, Ki67 was mainly distributed in portions of the crypt, while Ki67-positive cells occupied the entire intestinal crypt in ICKO mice (Figure 6E-H). The intestinal crypt size in ICKO mice was increased compared with that in control mice (Figure 6I and J). Furthermore, Ki67 was mostly expressed in the crypt area in control mice, while Ki67 in the intestinal tract of the ICKO mice was no longer confined to the crypt area. Cells expressing Ki67 were observed throughout the entire intestinal villi, and this manifestation was more pronounced in the large intestine.

DISCUSSION

Abnormal expression of Cldn7 can lead to the destruction of TJ structure and function as well as cell proliferation and migration abnormalities[15], which are closely related to the occurrence and development of malignant tumors, such as lung cancer, ovarian cancer, and gastric cancer[16-18]. However, the specific mechanisms underlying these phenomena remain elucidated.

Cldn7 is widely considered to be a tumor-suppressor gene[19-21], and one of the best methods for researching tumor-suppressor genes is to knock out the gene *in vivo* and then observe the phenotype of the entire animal[22]. Tamura *et al*[23] generated *Cldn15*$^{-/-}$ mice using the conventional gene targeting strategy. Cldn15 is similar to Cldn7 and strongly expressed in the duodenum, jejunum, ileum, and colon, while other Claudin family proteins, such as Cldn6, 9, 10, 11, and 14, are not expressed in the intestine[20]. *Cldn15*-deficient mice formed mega-intestines, in which the upper small intestine was two times larger than the normal intestine. Moreover, because *Cldn15* deletion is not lethal, researchers can observe intestinal development at different time points, such as at 1 wk, 4 wk, and 10 wk after birth[22]. Similarly, we constructed *Cldn7* CKO mice using the same strategy, and this mouse model showed severe intestinal defects that included mucosal ulcerations, epithelial cell sloughing, and inflammation. However, *Cldn7* CKO mice died beginning on the third day, which was not conducive to long-term observation or subsequent experiments. Additionally, the effects of *Cldn7* deletion in other organs on survival time and morphological changes could not be excluded. Therefore, prolonging the survival time of *Cldn7* knockout mice is necessary for further studying the function of Cldn7.

The Cre/LoxP technology makes it possible to knock out a gene in a site- or time-specific manner[22]. Cre recombinase can be expressed in specific cell types, and the crossing between floxed mice and Cre mice can then be used to remove the sequence between two LoxP sites in specific tissues. When Cre recombinase is fused to a ligand-binding domain of a mutant human estrogen receptor (ER), it becomes a tamoxifen-dependent Cre recombinase (Cre-ERT)[24]. Time-specific gene knockout can be achieved by injecting tamoxifen into transgenic mice at different growth stages. Using this method, we constructed intestinal specific *Cldn7* cKO mice. A Western blot assay confirmed that only intestinal *Cldn7* was ablated. While *Cldn7* cKO mice had a normal phenotype after birth, by the fifth day, the mice grew slowly and lacked energy. On the 9th day, the dying mice were sacrificed; their intestinal tracts showed severe

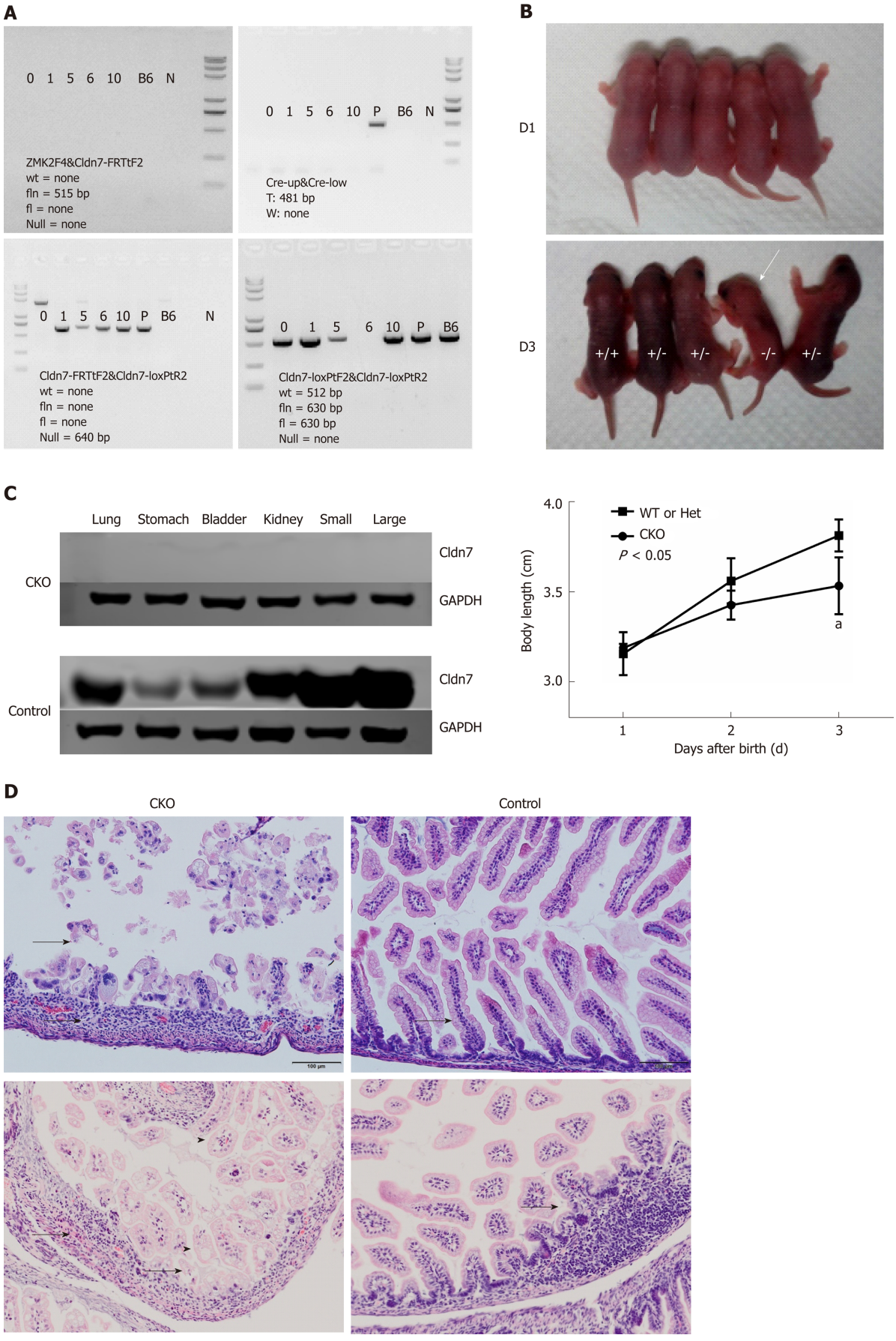


Figure 3 Phenotypic and intestinal pathological changes in *Cldn7* CKO mice. A: Genotype analysis showed that No. 6 mouse was a *Cldn7* CKO mouse with the genotype *Null*/*Null* CreW. Marker: 8000/5000/3000/2000/1000/750/500/250/100 bp; B: *Cldn7* CKO mice were similar to control mice at birth but grew slower than control mice ($P < 0.05$); from the third day, CKO mice appeared in a dying state; C: CKO mice expressed no Cldn7 in any tissues, while all tissues of control mice expressed Cldn7; D: Intestinal HE staining in *Cldn7* CKO mice showed obvious atrophy of intestinal mucosa with inflammatory cell infiltration (as indicated by the

arrow), villus shortening, and lymphatic expansion (as indicated by the arrowhead); the control mice showed no obvious histopathological changes (as indicated by the arrow). Cldn7: Claudin-7; CKO: Conventional knockout.

inflammation, and their mucosal or glands appeared abnormal. Tanaka *et al* also constructed intestinal specific *Cldn7* cKO mice[25], which had a longer survival period of 28 d due to the use of a different Cre enzyme mouse model. They found that intestinal *Cldn7* knockout changed only the paracellular flux of small molecule solutes and did not completely destroy the TJ structure. However, both knockout mice are intestinal inflammation models not yet showing adenomas or tumors. We further constructed *Cldn7* ICKO mice and controlled their survival time and intestinal morphological changes by changing the dose and frequency of tamoxifen. When the mice received 1 mg of tamoxifen every day, they began dying after the 5th injection, and severe inflammation was observed in the intestine (data not shown). When the mice received 1 mg of tamoxifen every 5 d, they began to show signs of dying after the 7th injection, and all mice died within 15 tamoxifen injections. Approximately 71.4% of the mice developed adenomas in different regions, including the duodenum, jejunum, ileum, and colon.

Immunohistochemical staining showed that compared with that in control mice, the intestinal crypt size in ICKO mice was increased, and cells positively expressing Ki67 covered the entire crypt. Ki67 expression was no longer limited to the crypt but rather to the crypt-villus axis. These phenomena suggested that the loss of *Cldn7* led to both expansion of the intestinal crypt and proliferation of cells at the crypt, and the normal proliferation-differentiation balance of intestinal cells along the crypt-villus axis was disrupted.

In the intestine, proliferating epithelial cells are specifically confined to the crypts[26]. Recent studies have shown that the crypt microenvironment is important for the generation and maintenance of proliferating cells[27]. Because TJs play a critical role in maintaining intestinal homeostasis, they may be important for formation of the niche, the microenvironment of the crypt for stem and transit-amplifying cells[24]. Therefore, the construction of *Cldn7* ICKO mice is of great significance for studying the relationship among intestinal barrier destruction, dysregulation of intestinal stem cell proliferation and differentiation along the crypt-villus axis, changes in the intestinal crypt microenvironment, and intestinal tumor formation.

In conclusion, we report the novel findings that *Cldn7* knockout caused extensive inflammation, atypical hyperplasia, and adenoma in intestinal tissue as well as animal death in three lines of knockout mice. Therefore, *Cldn7* may act as a tumor suppressor gene in the development of colorectal cancer, and the specific mechanism of Cldn7 can be elucidated by performing further research on the ICKO mouse model.

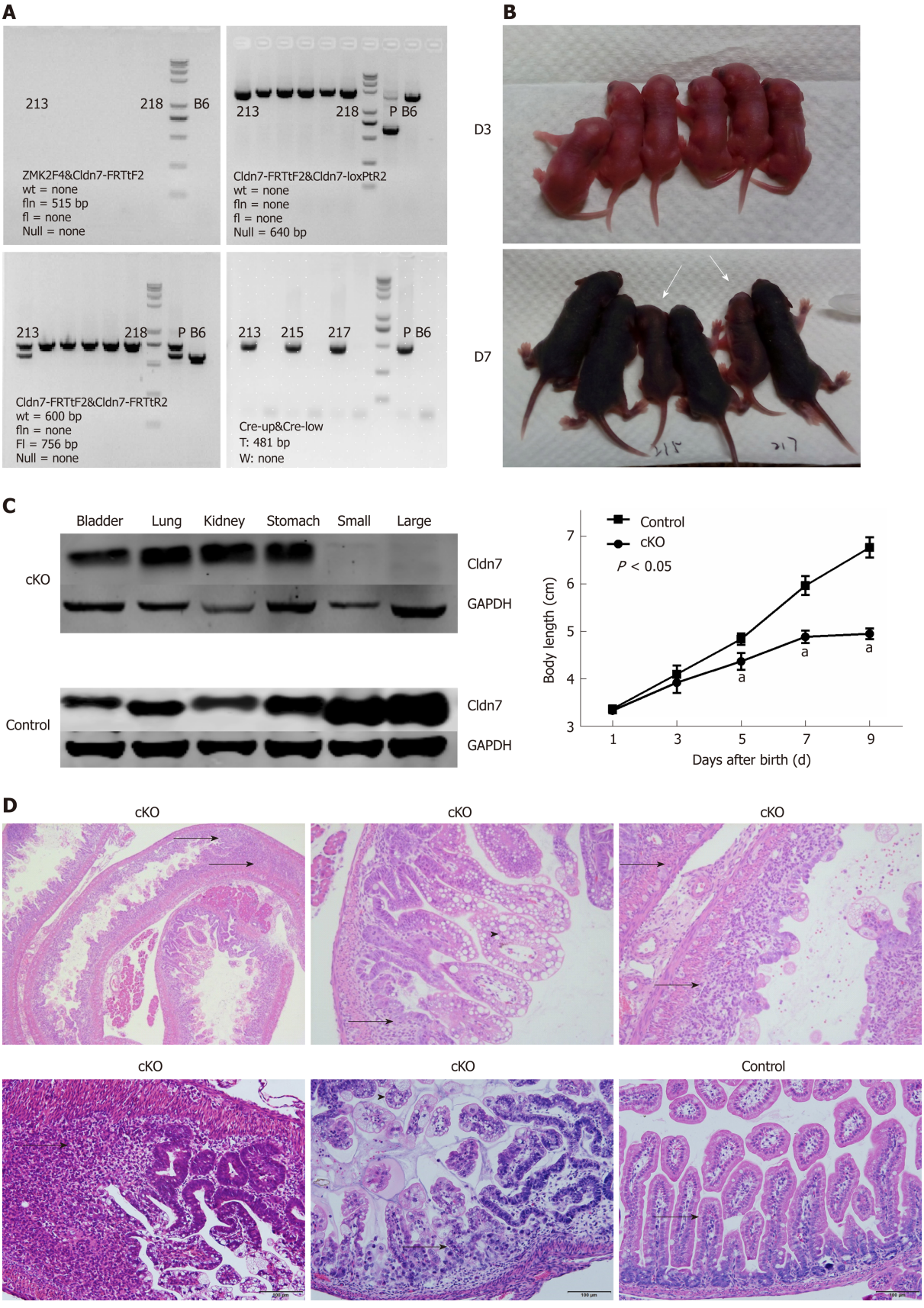


Figure 4 Phenotypic and intestinal pathological changes in *Cldn7* cKO mice. A: Genotype analysis showed the mice numbered 215 and 217 were intestinal specific *Cldn7* cKO mice with the genotype Cldn7fl/fl; villin-CreT; B: Cldn7 cKO mice were normal at birth, but their body lengths increased more slowly from the fifth day after birth than those of control mice ($P < 0.05$); on the 9th day, cKO mice were in poor spirits and died thereafter; C: Cldn7 expression was normal in the lung, stomach, bladder ,and kidney tissues of cKO mice but absent in the small and large intestines; all tissues of the control mice expressed Cldn7; D: Intestinal hematoxylin-eosin staining in *Cldn7* cKO mice showed connective tissue hyperplasia with extensive inflammatory cell infiltration in the submucosa (as indicated by the arrow); mucosal epithelial vacuolar degeneration can be observed after magnification (as indicated by the arrowhead); there were no obvious histopathological changes in *fl/fl* CreW mice. Cldn7: Claudin-7; cKO: Conditional knockout.

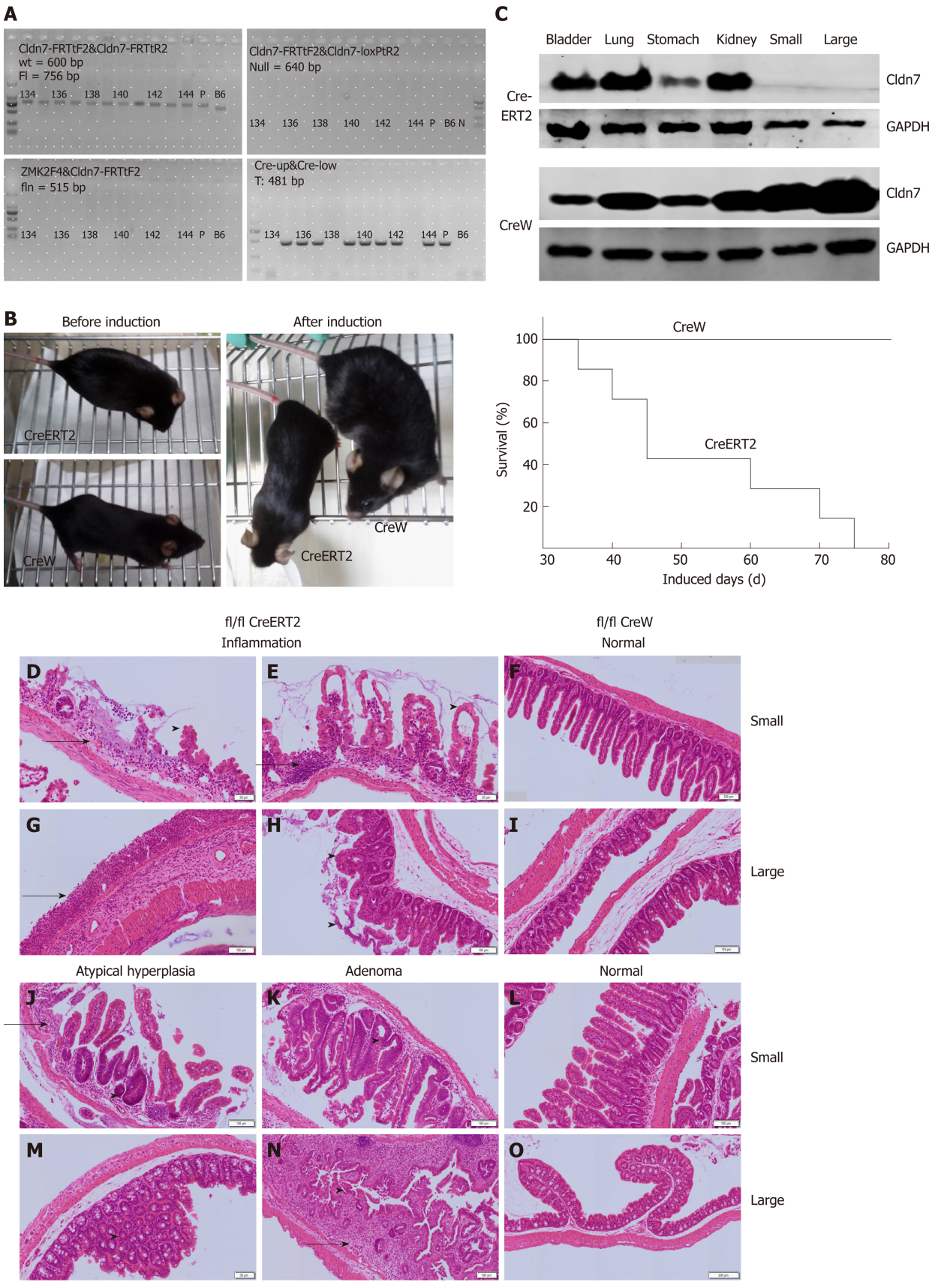


Figure 5 Phenotypic and intestinal pathological changes in *Cldn7* ICKO mice. A: Genotype analysis showed that mice numbered 135-137, 139-142, and 144 were *Cldn7* ICKO mice with the genotype Cldn7fl/fl; villin-CreERT2; B: Cldn7 cKO mice were normal at birth and developed smoothly, but beginning at the 7th injection, ICKO mice were in poor health and appeared to be dying; all ICKO mice died within 15 tamoxifen injections; C: Cldn7 was expressed at normal levels in the lung, stomach, bladder, and kidney tissues of ICKO mice but absent in the small and large intestines; all tissues of the control mice expressed Cldn7; D-O: Intestinal hematoxylin-eosin staining in ICKO mice showed obvious inflammatory manifestations; atypical hyperplasia and intestinal adenoma were also observed; the intestinal structure of the control mice was normal. Tamoxifen was dissolved in sunflower oil at a concentration of 10 mg/mL, and each mouse was intraperitoneally injected at 1 mg every 5 days. Cldn7: Claudin-7; cKO: Conditional knockout; ICKO: Inducible conditional knockout.

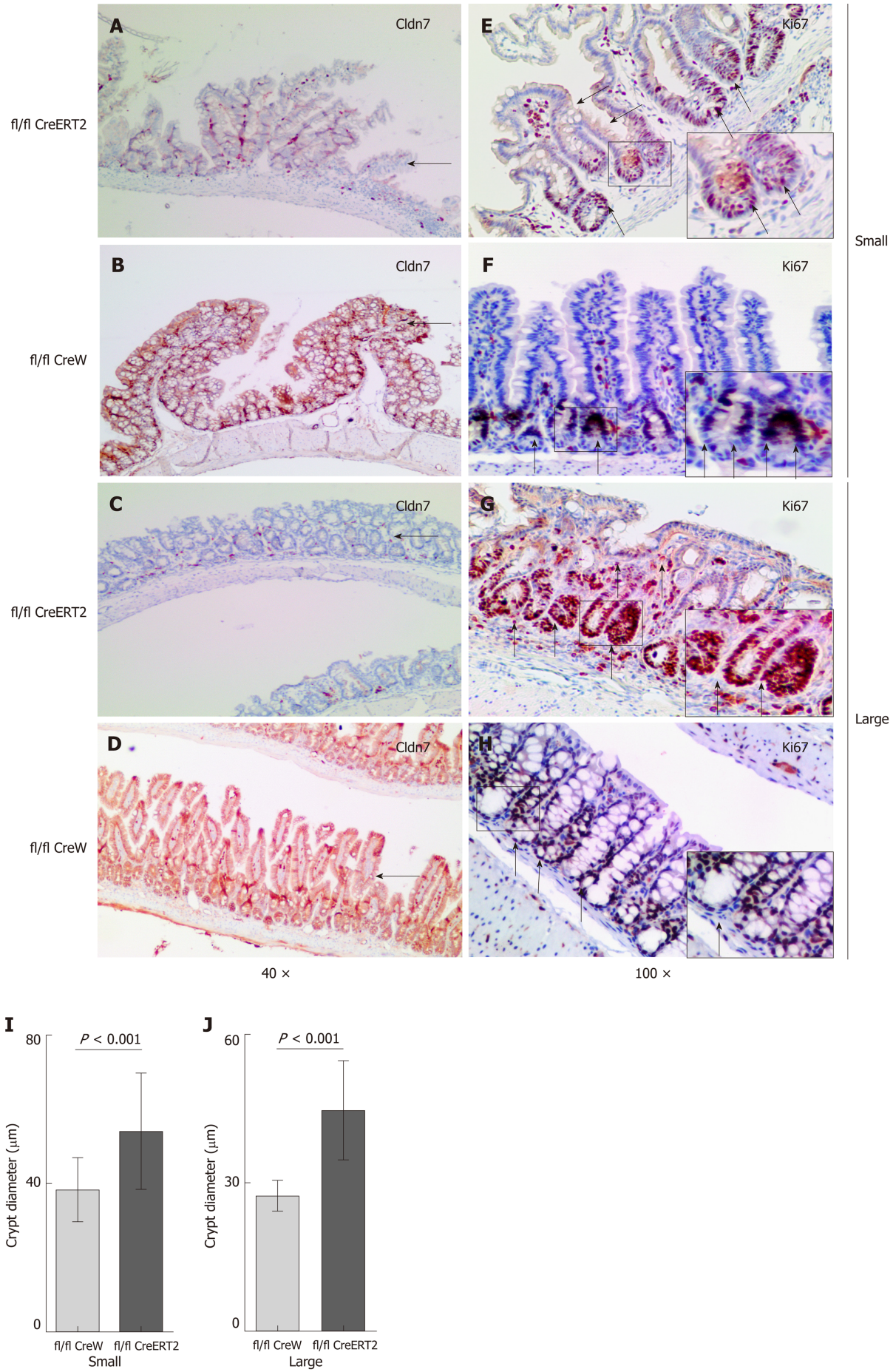


Figure 6 Increased intestinal proliferation in *Cldn7* ICKO mice. A-D: Immunohistochemistry staining showed that Cldn7 was strongly expressed in the intestinal epithelial junctions of control mice but significantly weakened in ICKO mice; E-H: In control mice, Ki67 was mainly distributed in parts of the crypt, while Ki67-positive

cells occupied the entire intestinal crypt and could be observed throughout the intestinal villi in ICKO mice; I-J: The size of the intestinal crypt in ICKO mice was increased compared with that in control mice ($P < 0.001$). Cldn7: Claudin-7; ICKO: Inducible conditional knockout.

ARTICLE HIGHLIGHTS

Research background

Claudin-7, one of the important components of cellular tight junctions, is currently considered to be expressed abnormally in colorectal inflammation and colorectal cancer. However, there is currently no effective animal model to study its specific mechanisms. Therefore, we constructed three lines of *Claudin-7* knockout mice using the Cre/LoxP system to provide a basis for further studying the role of Claudin-7 in intestinal tumors.

Research motivation

Claudin-7 is currently considered to play an inhibitory role in colorectal inflammation and colorectal cancer. The most effective way to study inhibitors *in vivo* is to knock out the gene in an animal and observe its overall phenotype. Therefore, we constructed conventional *Claudin-7* gene knockout (CKO) mice and conditional *Claudin-7* gene knockout (cKO) mice using Cre/LoxP system, and we also generated inducible conditional *Claudin-7* gene knockout (ICKO) mice and induced Cre expression by injecting tamoxifen. The successful construction of these mouse lines as intestinal inflammation and intestinal adenoma models could provide a basis for further studying the role of Claudin-7 in intestinal tumors.

Research objectives

The main objective was to construct three lines of *Claudin-7* gene knockout mice to achieve space- and time-specific knockout of *Claudin-7* and prolong the survival time of mice. Due to the prolonged growth time of mice, the organs matured when *Claudin-7* was knocked out. So this animal model can provide a basis for further study of Claudin-7.

Research methods

We constructed three lines of *Claudin-7* knockout mice using the Cre/LoxP system. First, chimeric mice were constructed by transfecting the target vector into embryonic stem cells, screening the hybrid clones and injecting them into the female mouse blastocysts, and transplanting the blastocysts into the female mouse uterus. Chimeric mice were then purified to obtain *Claudin-7*-floxed mice. Second, *Claudin-7*-floxed mice were crossed with CMV-Cre mice, vil1-Cre mice, and villin-CreERT2 mice ot obtain *Claudin-7* CKO mice, *Claudin-7* cKO mice, and *Claudin-7* ICKO mice, respectively. ICKO mice were induced by intraperitoneal injection of tamoxifen to knockout *Claudin-7* in intestinal tissue. Finally, Western blot was used to verify the knockout efficiency of Claudin-7. Hematoxylin-eosin (HE) staining was used to confirm the structural changes and pathological changes of the intestinal tract in *Claudin-7* knockout mice. Immunohistochemical staining was used to observe the proliferation markers. The construction of cKO mice prolonged the lifespan of CKO mice, and the ICKO mouse was the first animal model to specifically knock out *Claudin-7* in a spatial and temporal manner.

Research results

We generated *Claudin-7*-floxed mice and three lines of *Claudin-7* gene knockout mice successfully. *Claudin-7* CKO mice and *Claudin-7* cKO mice were stunted and died during the perinatal period, and intestinal HE staining revealed mucosal gland structure disappearance and connective tissue hyperplasia with extensive inflammatory cell infiltration. *Claudin-7* ICKO mice had a normal phenotype at birth, but after the induction with tamoxifen, the mice exhibited a dying state. Intestinal HE staining showed significant inflammatory cell infiltration, and atypical hyperplasia and adenoma were also observed. Intestinal immunohistochemistry analysis showed abnormal expression and distribution of Ki67, and the normal intestinal proliferation balance was disrupted.

Research conclusions

This study innovatively constructed three lines of *Claudin-7* gene knockout mice, which clarified that Claudin-7 plays an inhibitory role in colon inflammation and colon adenoma, and initially found that Claudin-7 may promote the development of colon adenomas by affecting proliferation. This study successfully simulated intestinal inflammation and intestinal adenoma, and proposed new animal models. This study clarified the role of Claudin-7 in colonic inflammation and tumors, laying the groundwork for finding early clinical diagnosis and potential therapeutic targets.

Research perspectives

This article describes the construction of *Claudin-7*-floxed mice and the process of crossing with three Cre mice. Based on this experience, we can construct ICKO mice in which *Claudin-7* is ablated in the kidney, skin, and some other organs, and then observe changes in mice before and after *Claudin-7* deletion. In the next step of the study, the dose of tamoxifen should be clarified,

and the tumor should be induced in the intestinal tract of ICKO mice. The direction of the future research is to clarify the specific mechanism of Claudin-7 in inflammatory bowel disease and intestinal tumorigenesis, invasion, and metastasis; and to explore the relationship between Claudin-7 and stem cells as well as its role in intestinal development.

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ORIGINAL ARTICLE

Retrospective Cohort Study

Zinc deficiency in patients with chronic pancreatitis

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Abstract

BACKGROUND

Zinc is a key element in numerous proteins and plays an important role in essential cell functions such as defense against free radicals and DNA damage repair. Chronic pancreatitis (CP) is a chronic inflammation with progressive fibrosis of pancreas ultimately resulting in pancreatic exocrine insufficiency (PEI), which is associated with malnutrition. Studies analyzing zinc levels in patients with CP are sparse and lead to conflicting results.

AIM

To investigate serum zinc levels in patients with CP of various etiologies.

METHODS

Between October 2015 and March 2018, patients with a diagnosis of CP were identified and recruited from the Pancreatic Outpatient Clinic at the Karolinska University Hospital in Stockholm, Sweden. Demographic, clinical and laboratory data were analyzed. Etiology of CP was determined according to the M-ANNHEIM classification system into the following etiological subcategories: alcohol consumption, nicotine consumption, hereditary factors, efferent pancreatic duct factors and immunological factors. Pancreatic exocrine function

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was defined as normal (fecal elastase 1 > 200 μg/g), mildly reduced (100-200 μg/g) and severely reduced (fecal elastase 1 < 100 μg/g).

RESULTS

A total of 150 patients were included in the analysis. Zinc deficiency (< 11 μmol/L) was present in 39 (26.0%) of patients: 22 females and 17 males. In the group of patients with zinc deficiency, 76.7% of patients had an exocrine pancreatic insufficiency (FE-1 < 200 μg/g). Older age was significantly associated with low zinc levels. Following a univariate analysis, patients aged 60-69 and patients ≥ 70 years of age had a significantly higher prevalence of zinc deficiencies compared to patients < 40 years of age [OR: 3.8, 95%CI (1.08-13.4); P = 0.04]; [OR 6.26, 95%CI (1.94-20.2), P > 0.002]. Smoking and number of pack-years were additionally associated with low zinc levels. The risk of zinc deficiency in current smokers and smokers with ≥ 20 pack-years was approximately three times higher compared to those who had never smoked. Gender, body mass index, etiology of CP, presence of diabetes mellitus, levels of glycated hemoglobin (HbA1c), bone mineral density, alcohol intake and presence of PEI were not associated with low zinc levels.

CONCLUSION

Zinc deficiency is common in patients with CP and is significantly associated with age ≥ 60, smoking and the number of pack-years, but not with PEI.

Key words: Zinc; Pancreas; Pancreatic exocrine insufficiency; Chronic pancreatitis; Malnutrition

Core tip: Normal levels of zinc are pivotal to maintain a homeostasis in a wide variety of important cellular systems and immune response. Chronic pancreatitis (CP) is a chronic inflammation of the pancreas resulting in pancreatic exocrine insufficiency, which is associated with malnutrition. There are conflicting published results of zinc levels in patients with CP. Most of the studies were restricted to patients with alcoholic etiology of CP and had limitations due to the small number of studied patients. We are presenting the results of the largest study so far comparing serum zinc levels in relation to different etiological groups of CP.

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INTRODUCTION

Zinc is a key element in numerous proteins and plays an important role in essential cell functions such as defense against free radicals and DNA damage repair[1]. Approximately 10% to 40% of dietary zinc is absorbed in the small bowel and 0.5 to 1.0 mg/day is secreted into the biliary tract followed by passing the small and large bowel[2]. Pathophysiology of zinc deficiency in patients with chronic pancreatitis (CP) is not fully elucidated. It has been proposed that decreased secretion of binding proteins in the pancreatic juice explain compromised absorption of zinc in pancreatic exocrine insufficiency (PEI)[3]. Zinc deficiency may be the effect of reduced absorption and can be a contributory factor in disease progression, *via* the reduction of free radicals[4]. It is known that zinc affects many aspects of the immune system, from the barrier of the skin to gene regulation in lymphocytes, and is crucial for development and function of neutrophils and natural killer cells[5].

There are conflicting published results of zinc levels in patients with CP[6]. Most of the studies included only patients with alcoholic etiology of CP and small number of patients. We are presenting the results of the largest study so far comparing serum zinc levels in patients with CP of various etiologies.

MATERIALS AND METHODS

Patients and methods

Between October 2015 and March 2018, patients with a diagnosis of CP were identified from a database maintained by the Pancreatic Outpatient Clinic at Karolinska University Hospital in Stockholm, Sweden. Demographic, clinical and laboratory data were analyzed. Alcohol intake was recorded, including the number and type of drinks per week and the frequency of consumption.

Smoking status was recorded as current/former/never. For former or current smokers, the number of cigarettes per day over a given number of years was recorded, and pack-years were calculated accordingly. Etiology of CP was determined according to the M-ANNHEIM classification system as: alcohol, nicotine, hereditary, efferent pancreatic duct and immunological factors[7]. Levels of fecal elastase-1 (FE-1) > 200 μg/g, 100-200 μg/g and < 100 μg/g were considered as normal pancreatic exocrine function, mild PEI and severe pancreatic insufficiency, respectively[8]. Zinc was analyzed in serum or plasma on a Cobas C system, Roche Diagnostics, Mannheim, Germany using a colorimetric assay from Sentinel Diagnostics, Milan, Italy. Zinc levels of 11-17 μmol/L were considered as normal.

Statistical analysis

The frequency of zinc deficiency was tabulated according to patients' characteristics. Logistic regression was used to assess the association between patients' characteristics and the presence of zinc deficiency. Factors showing statistical significance at univariable analysis were subsequently included in a multivariable model. Analyses were performed using the SAS software (version 9.4, Cary, NC, United States). All tests were two-sided and *P*-values < 0.05 were considered statistically significant.

Ethics

The study was approved by the Stockholm Ethic Committee (SLL), numbers 2014/1094-31, 2016/491-3172 and 2016/1571-31.

RESULTS

In the observed period, 226 patients with CP were diagnosed. In 76 patients zinc results were not available and these patients were excluded from analysis. A total of 150 patients were included in the analysis. Zinc deficiency (< 11 μmol/L) was present in 39 (26.0%) of patients: 22 females and 17 males. In the group of patients with zinc deficiency, only 23.3% of patients had normal FE-1. Demographic data are presented in Table 1.

Age ≥ 60 years was significantly associated with low zinc levels. Following a univariate analysis, patients aged 60-69 and patients ≥ 70 years of age had a significantly higher prevalence of zinc deficiency compared to patients < 40 years of age [OR: 3.8, 95%CI (1.08-13.4); $P = 0.04$]; [OR 6.26, 95%CI (1.94-20.2), $P > 0.002$]. Smoking and number of pack-years were additionally associated with low zinc levels. The risk of zinc deficiency in current smokers and smokers with ≥ 20 pack-years was approximately three times higher compared to those who had never smoked. Gender, body mass index (BMI), etiology of CP, presence of diabetes mellitus (DM), levels of glycated hemoglobin (HbA1c), bone mineral density, alcohol intake and presence of PEI were not associated with low zinc levels (Table 1). The associations with age and smoking were confirmed to be statistically significant in a multivariable analysis (Table 2).

DISCUSSION

Normal levels of zinc, an essential mineral and trace element, are pivotal to maintain a homeostasis in a wide variety of important cellular systems and immune response[1,5]. In this large study of 150 patients with CP, one in four patients was shown to have a zinc deficiency. CP is a chronic inflammation of the pancreas triggered by various factors including alcohol misuse, smoking, autoimmunity, anatomical variants and genetic factors. Due to progressive fibrosis and destruction of the pancreas, both enzyme and insulin production ultimately become severely impaired, resulting in pancreatic exocrine and endocrine insufficiency. Deficiency of enzymes (exocrine insufficiency) leads to maldigestion and malnutrition which are associated with reduced absorption of fat-soluble vitamins. The essential role of zinc and its deficiency was described in 1963[9]. There is accumulating evidence that different patient groups

Table 1 Factors associated with low zinc levels in patients with chronic pancreatitis (*n* = 150)

	Total, *n*	Zinc		Univariable analysis	
		≥ 11 μmol/L, *n*	< 11 μmol/L, *n* (%)	OR (95%CI)	*P* value
All patients	150	111	39 (26.0)		
Age					
0-39	43	38	5 (11.6)	1.00	
40-49	18	13	5 (27.8)	2.92 (0.73-11.7)	0.13
50-59	34	27	7 (20.6)	1.97 (0.57-6.87)	0.29
60-69	24	16	8 (33.3)	3.80 (1.08-13.4)	0.04
70+	31	17	14 (45.2)	6.26 (1.94-20.2)	0.002
Gender					
Female	86	64	22 (25.6)	1.00	
Male	64	47	17 (26.6)	0.95 (0.46-1.99)	0.89
Body mass index					
Underweight	12	9	3 (25.0)	0.74 (0.18-2.96)	0.67
Normal weight	77	53	24 (31.2)	1.00	
Overweight	38	30	8 (21.1)	0.59 (0.24-1.47)	0.26
Obese	20	16	4 (20.0)	0.55 (0.17-1.83)	0.33
Etiology					
Alcohol	40	27	13 (32.5)	1.00	
Autoimmune	45	32	13 (28.9)	0.84 (0.34-2.13)	0.72
Efferent duct factors	11	8	3 (27.3)	0.78 (0.18-3.43)	0.74
Smoking	15	11	7 (38.9)	1.32 (0.42-4.20)	0.64
Hereditary	18	14	1 (6.7)	0.15 (0.02-1.25)	0.08
Idiopathic	21	19	2 (9.5)	0.22 (0.04-1.08)	0.06
Diabetes					
No	108	82	26 (24.1)	1.00	
Yes	42	29	13 (31.0)	1.41 (0.64-3.11)	0.39
Diabetes treatment					
No diabetes	108	82	26 (24.1)	1.00	
Diet	3	3	0 (0.0)	-	0.99
Per oral	7	5	2 (28.6)	1.26 (0.23-6.89)	0.79
Insulin	32	21	11 (34.4)	1.65 (0.70-3.88)	0.25
HbA1c					
Normal	9	7	2 (22.2)	1.00	
Elevated	30	21	9 (30.0)	1.50 (0.23-6.02)	0.65
Bone mineral density					
Normal	41	30	11 (26.8)	1.00	
Osteopenia	14	10	4 (28.6)	1.09 (0.28-4.21)	0.90
Osteoporosis	5	2	3 (60.0)	4.09 (0.60-27.8)	0.15
Smoking					
Never	76	62	14 (18.4)	1.00	
Former	25	19	6 (24.0)	1.40 (0.47-4.14)	0.54
Current	48	29	19 (39.6)	2.90 (1.28-6.58)	0.01
Smoking pack-years					
Never	76	62	14 (18.4)	1.00	
1-19 pack-years	21	16	5 (23.8)	1.38 (0.43-4.41)	0.58
≥ 20 pack-years	51	31	20 (39.2)	2.86 (1.27-6.41)	0.01
Alcohol					
No	105	81	24 (22.9)	1.00	
< 30g	13	8	5 (38.5)	2.11 (0.63-7.05)	0.23
≥ 30g	31	21	10 (32.3)	1.61 (0.67-3.88)	0.29
Pancreatic exocrine insufficiency					
No	60	46	14 (23.3)	1.00	

Mild/moderate	14	11	3 (21.4)	0.90 (0.22-3.67)	0.88
Severe	66	45	21 (31.8)	1.53 (0.70-3.38)	0.29

OR and 95%CI obtained from univariable logistic regression model; OR: Odds ratios; CI: Confidence intervals; HbA1c: Levels of glycated hemoglobin.

are at risk of developing zinc deficiency[10]. Zinc is not stored in the body, and the level of zinc is determined by the balance of dietary intake, absorption, and losses[11].

Results of studies on zinc levels in patients with CP have been controversial. Van Gossum *et al*[12] reported no difference in serum zinc levels when comparing 35 alcoholic CP patients with healthy controls. Similar results were noted in 32 pediatric patients with cystic fibrosis and PEI determined either at the time of diagnosis or one year later[13]. However, both studies were performed either in a small group of patients or in children with a short duration of PEI. In contrast, Lindkvist *et al*[14] found 7.1% of zinc deficiency among 56 patients, mostly with alcoholic etiology. In a group of 101 patients from India with alcoholic and tropical etiology of CP, erythrocyte zinc levels were significantly reduced in CP compared to controls, and zinc levels were significantly lower in patients with tropical CP compared to alcoholic etiology[4].

Our study is the first to determine zinc levels in patients with CP based on all known key etiologies of CP (alcohol, autoimmune, efferent duct factors, idiopathic and hereditary). Zinc was shown to be deficient in 26% of patients regardless of CP etiology. Interestingly, zinc deficiency was present in patients with and without PEI as reported by others, suggesting that impaired secretion of pancreatic enzymes is not the main factor resulting in a reduced absorption or increased excretion of zinc[14]. Due to the lower sensitivity of the fecal elastase-1 test for diagnosing mild and moderate forms of CP, the true prevalence of PEI may be underestimated suggesting that a number of CP patients are misclassified as not having PEI[15]. However, zinc deficiency was even noted in CP patients in which PEI was excluded by a ^{13}C-mixed triglyceride breath test which has a higher sensitivity for detecting PEI compared to a standard fecal elastase-1 test[14].

Pancreatic endocrine insufficiency with DM has been identified as an independent risk factor for zinc deficiency in a previous study of CP patients[4]. However, in our study this correlation was not confirmed. This difference can be explained by different patient numbers and different etiologies (*e.g.*, tropical CP has a faster progression with an earlier onset of DM)[4].

Smoking and number of pack-years were correlated with low zinc levels in our patient cohort, and the risk of zinc deficiency in current smokers and smokers with ≥ 20 pack-years was approximately three times higher compared to those who had never smoked. In a large study from France with the inclusion of 1821 women and 1307 men aged 45-60 years, zinc serum levels were significantly reduced in women who were current smokers but not men[16]. Others found reduced seminal zinc levels in smokers, which was associated with oxidative stress reduced DNA integrity and diminished sperm vitality[17].

We were also able to demonstrate a correlation between BMI and zinc values. The lack of other anthropometric parameters such as grip strength, triceps skin-fold thickness and mid upper arm circumference is a limitation of the study, as these parameters are established methods for identifying patients with malnutrition. Apart from serum markers of malnutrition, BMI was recorded which represents a further parameter indicating malnutrition (< 18.5 kg/m^2)[6]. Numerous studies indicate that zinc deficiency has a strong effect on key immunological functions, and zinc deficiency has been shown to increase the risk of infectious diseases[5]. Zinc deficiency results not only in decreased lymphocyte concentrations but also in depressed T and B lymphocyte function[5]. It has been shown that zinc deficiency *in vivo* increase oxidative stress increase DNA damage in rat peripheral blood cells[1]. However, the exact mechanisms by which zinc deficiency affects DNA damage is not elucidated. Several studies have demonstrated the benefits of zinc supplementation in patients with infectious diseases[5]. A Cochrane review included six studies with a total of 5193 children (2-59 mo of age) and evaluated the role of zinc in the prevention of pneumonia. Zinc supplementation was associated with a reduced incidence rate of pneumonia[18]. Another meta-analysis including 22 studies demonstrated that zinc supplementation reduces the duration and severity of acute and chronic diarrhea in children in developing countries[11].

Furthermore, epidemiological studies reveal an association between low circulating zinc concentrations and increased risk of cancer[19,20]. The relationship between serum zinc and cancer mortality appeared to be nonlinear with a significantly reduced risk for people with higher zinc values, according to a US national survey including 3000

Table 2 Multivariable analysis of risk factors for low zinc levels in chronic pancreatitis (*n* = 150)

	Multivariable analysis	
	OR (95%CI)	*P* value
Age		
0-39	1.00	
40-49	2.82 (0.68-11.7)	0.15
50-59	1.30 (0.34-4.91)	0.70
60-69	2.64 (0.69-10.1)	0.16
70+	6.30 (1.87-21.3)	0.003
Smoking		
Never	1.00	
Former	0.99 (031-3.17)	0.98
Current	3.14 (1.23-8.02)	0.02

OR and 95%CI obtained from multivariable logistic regression model; OR: Odds ratios; CI: Confidence intervals.

men and 3244 women[19]. It is largely unknown how zinc may modulate the chronic inflammatory process in the pancreas in CP patients. A positive correlation between erythrocyte zinc and erythrocyte superoxide dismutase activity suggests connection between zinc deficiency and oxidative stress in CP and is another possible mechanism by which zinc deficiency may impact on the pathogenesis of CP and its complications[4]. Of note, it is well established that CP harbors the risk of pancreatic cancer. Future studies are needed to establish whether the risk of malignant transformation in CP patients is increased by zinc deficiency by triggering DNA damage and oxidative stress. Both mechanisms are implicated in carcinogenesis.

Assessing zinc levels in the plasma represents a limitation of the present study. Measuring zinc in erythrocytes has the advantage of reflecting the zinc status over a longer period compared to the rapid turnover of the plasma pool of zinc[19,20]. However, the high number of analyzed patients, a well-balanced ratio of male and female patients together with the inclusion of patients with a large array of different CP etiologies represents a strength of this study.

In conclusion, this is the first study to analyze the prevalence and risk factors of zinc deficiency in a large number of patients with different etiologies of CP. According to our data, zinc deficiency is relatively common in patients with CP and is significantly associated with higher age, smoking and the number of pack-years. Further studies are warranted to better define how zinc modulates chronic pancreatic inflammation in patients with CP.

ARTICLE HIGHLIGHTS

Research background

Malnutrition with deficiencies of fat-soluble vitamins, minerals and trace elements are well-known consequences of maldigestion and poor absorption of nutrients. Malnutrition has been frequently identified in patients with chronic pancreatitis (CP) and pancreatic exocrine insufficiency (PEI). Zinc is important for normal functioning of immune system and its deficiency was recognized more than 50 years ago. Studies analyzing zinc levels in patients with CP are sparse and lead to conflicting results. However, studies analyzing zinc levels in patients with CP are sparse and most of them included only patients with alcoholic etiology. In this study, prevalence and risk factors of zinc deficiency in a large number of patients with different etiologies of CP were determined.

Research motivation

The main topic was zinc insufficiency and its relation to patients' demographic and clinical parameters: Gender, age, smoking, alcohol intake, body mass index (BMI), diabetes mellitus (DM), bone mineral density, PEI and etiology of CP.

Research objectives

In this large study of 150 patients with CP, one in four patients was shown to have a zinc deficiency, which was the main objective. Zinc deficiency was not related to gender, BMI, etiology of CP, presence of DM, bone mineral density and alcohol intake. It is of significance that zinc deficiency was present in patients with and without PEI, suggesting that impaired secretion of pancreatic enzymes is not the main factor resulting in a reduced absorption or increased

excretion of zinc. These objectives should be better analyzed in future research in this field.

Research methods

We performed retrospective analysis of demographic, clinical and laboratory data, with detailed information on alcohol intake and smoking status. For former or current smokers, the number of cigarettes per day over a given number of years was recorded, and pack-years were calculated accordingly. Results showed that smoking status was very important in this study: Number of pack-years was correlated with low zinc levels and the risk of zinc deficiency in current smokers and smokers with ≥ 20 pack-years was approximately three times higher compared to those who had never smoked.

Research results

We showed that zinc deficiency is significantly associated with higher age, smoking and the number of pack-years. On the other hand, zinc deficiency was not related to gender, BMI, etiology of CP, presence of DM, bone mineral density, alcohol intake and presence of PEI. The fact that zinc deficiency was present in patients with and without PEI is very important. Future studies on this topic are necessary for better understanding of mechanisms included in zinc insufficiency.

Research conclusions

This is the first study to analyze the prevalence and risk factors of zinc deficiency in a large number of patients with different etiologies of CP. One in four patients was shown to have a zinc deficiency. There is significant association of zinc deficiency with higher age, smoking and the number of pack-years. We showed no correlation of zinc deficiency with gender, BMI, etiology of CP, presence of DM, bone mineral density, alcohol intake and presence of PEI. Due to the lower sensitivity of the fecal elastase-1 test for diagnosing mild and moderate forms of CP, the true prevalence of PEI may be underestimated. Future studies with ^{13}C-mixed triglyceride breath test, which has a higher sensitivity for detecting PEI compared to a standard fecal elastase-1 test, can help us to solve this problem.

Research perspectives

Assessing zinc levels in the plasma represents a limitation of the present study. Future studies with measuring of zinc in erythrocytes can be of interest due to the advantage of reflecting the zinc status over a longer period compared to the rapid turnover of the plasma pool of zinc. Impaired secretion of pancreatic enzymes is probably not the main factor resulting in a reduced absorption or increased excretion of zinc. Future studies on this topic are necessary. The results from the present study suggest that zinc deficiency is relatively common in patients with CP. Clinicians dealing with CP, regardless of etiology, should be aware of the clinical importance of zinc malnutrition, especially in older patients and smokers.

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ORIGINAL ARTICLE

Retrospective Study

Analysis of intrahepatic sarcomatoid cholangiocarcinoma: Experience from 11 cases within 17 years

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Author contributions: Kim DK is the first author, designed the study, collected and analyzed the data, and drafted the manuscript; Kim BR helped in analyzing the findings of the imaging studies; Jeong JS helped in analyzing the histopathologic findings of the biopsies; Baek YH designed and supervised the study and revised the manuscript for important intellectual content; All authors have read and approved the final version of the manuscript to be published.

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Abstract

BACKGROUND

Intrahepatic sarcomatoid chonalgiocarcinoma (s-CCC) is an extremely rare disease, accounting for less than 1% of hepatobiliary system malignancies, and its pathophysiology is not well known. On the hypothesis that its clinical, serologic, or radiologic diagnosis are not fully understood and its prognosis is poor, we investigated the distinguishing features of s-CCC compared with those of intrahepatic bile duct adenocarcinoma [cholangiocellular carcinoma (CCC)] in patients from a single center.

AIM

To analyze the clinical, serologic, imaging, and histopathologic characteristics of intrahepatic s-CCC patients diagnosed in a single center.

METHODS

The clinical, serologic, imaging, and histopathologic features of 227 patients diagnosed with intrahepatic cholangiocarcinoma (IHCC) in a single medical center during the last 17 years were analyzed. The characteristics of 11 patients with s-CCC were compared with those of 216 patients with CCC.

RESULTS

The number of patients with s-CCC who presented fever and abdominal pain and past history of chronic viral hepatitis or liver cirrhosis (LC) was higher than that of patients with CCC. In imaging studies, patients with s-CCC showed relatively aggressive features. However, no clear distinction was observed between s-CCC and CCC based on other clinical, serologic or radiologic

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examination results. An accurate diagnosis could be made only *via* a histopathologic examination through immunohistochemical staining. The clinical course of s-CCC was generally aggressive, and patients had a relatively poor prognosis.

CONCLUSION
In patients with s-CCC, early diagnosis through biopsy and aggressive treatment, including surgical resection, are important.

Key words: Intrahepatic sarcomatoid cholangiocarcinoma; Immunohistochemical staining; Survival; Prognosis; Surgical resection

Core tip: Intrahepatic sarcomatoid cholangiocarcinoma is rare condition. Patients usually present with an advanced stage of the disease, and they have a poor prognosis. Diagnosis based on histopathologic examination is important because serologic and radiologic examinations cannot help in distinguishing such condition from intrahepatic bile duct adenocarcinoma or other intrahepatic masses. Thus, patients must be diagnosed as early as possible and should receive aggressive treatment, including surgical resection.

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INTRODUCTION

Epithelial tumors with a sarcomatoid feature can develop in several organs, such as the skin, kidney, esophagus, stomach, gallbladder, thyroid, urinary bladder, uterus, and lungs. However, its pathologic mechanism has not been clearly elucidated[1]. Most malignant tumors in the intrahepatic bile duct are adenocarcinomas which are commonly referred to as cholangiocellular carcinoma (CCC). In contrast, intrahepatic sarcomatoid cholangiocarcinoma (s-CCC) is an extremely rare condition, accounting for less than 1% of hepatobiliary system malignancies, and its pathophysiology is not well known[2,3]. The most common primary hepatic malignancy with a sarcomatoid feature is hepatocellular carcinoma (HCC), and generally, it occurs due to secondary changes in tumor cells after embolization or chemotherapy. However, the relationship between the occurrence of s-CCC and previous treatment has not been clearly understood, and it is often considered as a change according to the natural history of CCC[4]. In some previous studies, the prognosis of s-CCC was reported worse than that of CCC, which was due to a more frequent metastasis to other organs or invasion to adjacent vasculatures, and only few established data about its clinical features, diagnosis, and treatment methods are available[5,6]. In this study, we hypothesized the pathogenesis of s-CCC, investigated the clinical, serologic, imaging, and histopathologic features of s-CCC, and compared with those of CCC in patients from a single center.

MATERIALS AND METHODS

Patients selection

From January 2001 to June 30, 2018, a total of 228 patients with intrahepatic cholangiocarcinoma (IHCC) diagnosed *via* surgery or ultrasonography (US)-guided liver biopsy at Dong-A University Hospital were screened. Among them, 12 were diagnosed with s-CCC. One patient who was diagnosed with s-CCC *via* surgery in August 2001 was excluded from the analysis due to the lack of data about the clinical course of the disease and imaging study results. Two patients presented with mixed histopathologic features of HCC and IHCC were included in the study.

Patients assessment

In 11 patients with s-CCC who were enrolled, the admission history, accompanying symptoms or signs, past history of the hepatobiliary system, and findings of serologic examinations, such as liver function tests and tumor marker analysis were assessed. In terms of imaging studies, such as US, computed tomography (CT) scan, and magnetic resonance imaging (MRI), the characteristic features, the size and number of primary masses, distant and lymph node (LN) metastases, and the TNM stage were investigated and compared with those of HCC, IHCC, and other hepatic tumors. Histopathologic examination and immunohistochemical staining of hepatic mass were also conducted. Finally, the types of treatment, consequent clinical course, follow-up results, and survival time were investigated.

Statistical analysis

Data were reported as means ± standard deviation and median value. Data analysis was performed using the Statistical Package for the Social Sciences (SPSS) software version 20.0. Continuous variables were compared using the unpaired student *t*-test, Mann-Whitney *U* test, and Kruskal-Wallis test. Categorical variables were compared with the chi-square test and Fisher's exact test, and they were reported as frequencies and percentages. $P < 0.05$ were considered statistically significant. Survival curves were obtained using the Kaplan-Meier method and compared using the log-rank test.

RESULTS

Clinical findings

Among the 11 patients diagnosed with s-CCC, 9 (81.8%) were men and 2 (18.2%) were women. The median age of the patients was 61 (range: 45-68) years. During their first visit at the hospital, the patients mainly presented abdominal pain ($n = 10$, 90.9%), and fever ($n = 4$, 36.4%), which were more frequent in s-CCC patients compared with CCC patients. Past history of chronic viral hepatitis and liver cirrhosis (LC) were frequent in s-CCC patients: chronic hepatitis B (CHB), $n = 3$ (27.3%); chronic hepatitis C (CHC), $n = 1$ (9.1%); LC, $n = 5$ (45.5%). One of the patients was diagnosed with HCC at another hospital 1 mo before admission and was taking sorafenib. In addition, 1 (9.1%) patient had a previous history of *Clonorchis sinensis* infection and another (9.1%) patient presented with gallstones at the time of diagnosis. Meanwhile, 3 (27.3%) patients had a history of cholecystectomy. All 11 patients presented with stage IV disease by the TNM stage, of whom 4 (36.4%) had stage IVA and 7 (63.6%) had stage IVB (Tables 1 and 2).

Laboratory findings

The laboratory findings were as follows: serum aspartate aminotransferase (AST) level, 23-80 (median: 34, normal: < 40) U/L; alanine aminotransferase (ALT) level, 10-96 (median: 31, normal: <40) U/L; serum total bilirubin (TB) level, 0.3-1.1 (median: 0.7, normal: < 1.2) mg/dL; and direct bilirubin (DB) level, 0.1-0.8 (median: 0.4, normal: < 0.4) mg/dL. In all patients, the serum alkaline phosphatase (ALP) level was elevated at 252-1520 (median: 757, normal: < 120) U/L. The gamma-glutamyl transferase (GGT) level was measured in only 9 patients, ranging from 32 to 323 (median: 137, normal: < 64) U/L, and it was elevated above the normal limit in 7 patients.

Serum carcinoembryonic antigen (CEA) levels were elevated in only 1 (9.1%) patient, ranging from 0.1 to 12.7 (median: 1.8, normal: < 5) ng/mL. The carbohydrate antigen 19-9 (CA19-9) level ranged from 2.0 to 1809.57 (normal: < 37) U/mL, and it was elevated in 5 patients (45.5%). Alpha-fetoprotein (AFP) level was high in 2 (18.2%) patients (cases 1 and 2), of which 1 patient presented with CHB, LC, and HCC and treated with sorafenib, and the other patient had CHC and LC (Table 3).

Imaging findings

The imaging findings were newly reviewed by a radiologist. The initial radiologic impression obtained *via* abdominal US and CT scan was very variable as IHCC, HCC, lymphoma, and hepatic abscess. (Table 4, Figure 1). The size of the main tumor lesion on US, CT scan and MRI ranged from 2.5 to 10 (median: 7.5) cm, and the number of intrahepatic tumors was 1 in 6 (54.5%) patients, and multiple tumors were observed in the other 5 (45.5%) patients. All patients had stage IV disease by TNM stage. Multiple LN metastases were observed in all patients, and distant metastases to other organs were observed in 7 (63.6%) patients (Table 1).

All patients had lobulated heterogeneous mass lesions on abdominal US. Hypoechoic features were observed in 3 (27.3%) patients, hyperechoic features in 3

Table 1 Clinical findings of the patients with intrahepatic sarcomatoid cholangiocarcinoma

Case	Sex	Age	Chief complaint	Stone	CS	Chronic hepatitis	LC	HCC	Tumor size (cm)	Number of mass	Metastasis	Stage (TNM)
1	M	45	RUQ pain	-	-	CHB	+	+	7.5	7	LN, IH	IVB
2	M	67	Lt. flank pain	-	-	CHC	+	-	2.5	1	Bone, LN	IVB
3	M	55	RUQ pain, fever	CST	+	-	-	-	6.5	2	LN	IVA
4	M	66	RUQ pain, fever	GB stone	-	-	-	-	10.0	1	Lung, LN	IVB
5	M	56	RUQ pain, Fatigue	-	-	CHB	+	-	8.0	1	Thymus, LN	IVB
6	F	66	RUQ pain	CST	-	-	-	-	7.5	1	LN	IVB
7	M	68	Wt. loss, Fatigue	CST	-	-	+	-	6.0	1	Lung, Bone, LN	IVB
8	F	55	RUQ pain, fever	-	-	-	-	-	8.5	3	LN	IVA
9	M	49	LUQ pain, fever	-	-	CHB	+	-	9.5	3	LN	IVA
10	M	65	RUQ pain	-	-		-	-	9.5	15	LN, IH	IVA
11	M	61	RUQ pain	-	-		-	-	5.0	1	Diaphragm, LN	IVB

CHB: Chronic hepatitis B; CHC: Chronic hepatitis C; CS: Clonorchis sinensis; CST: Cholecystectomy; GB: Gallbladder; HCC: Hepatocellular carcinoma; IH: Intrahepatic; LC: Liver cirrhosis; LN: Lymph node; LUQ: Left upper quadrant; RUQ: Right upper quadrant.

(27.3%) patients, and mixed echoic features in 5 (45.5%) patients. A hypodense mass with heterogeneous, ill-defined, or lobulated features was observed in the CT non-enhanced phase which was performed in only 5 patients. Enhanced CT scans were performed in all 11 patients. In the arterial phase, a hepatic mass presented with irregular rim enhancement (n = 5), peripheral enhancement (n = 5), or diffuse heterogeneous enhancement (n = 1). The CT portal phase showed gradual centripetal enhancement (n = 6), irregular rim enhancement (n = 3), and irregular peripheral enhancement (n = 1). In 1 patient with heterogeneous enhancement of the mass in the arterial phase, the enhancement was more prominent in the portal phase. CT scan in the delayed phase was performed in 4 patients and its findings were not significantly different from those in the portal phase (Table 4). MRI was performed in 3 patients with heterogeneous hyperintensity (n = 3) on the T2-weighted image, gradual centripetal (n = 2) or rim (n = 1) enhancement on the dynamic enhancement scan, and diffusion restriction (n = 3) on diffusion weighted image.

Histopathologic and immunohistochemical findings

Figure 2 shows histopathological findings in case 9. In low-magnified microscopic findings, liver biopsy showed relatively well-demarcated tumor, characterized by poorly differentiated cancer cells without organoid structures, interspersed by stromal tissue, which is associated with necrosis (Figure 2A and B). Individual or clustered cancer cells consisted of enlarged epithelioid cells with pleomorphic and hyperchromatic nuclei and prominent nucleoli, and spindle cells with hyperchromatic and enlarged nuclei (Figure 2A). Occasionally, mucin-secreting cancer cells were observed. Inflammatory cell infiltration was present in the abundant stroma.

Immunohistochemical study had shown that cancer cells expressed cytokeratin 9 (CK8), cytokeratin 19 (CK19) (Figure 2C), and vimentin (Figure 2D), but not hepatocyte-specific antigen (HSA) (Figure 2E) and AFP. Based on the microscopic findings and immunohistochemical results, this liver tumor was diagnosed as s-CCC. In the remaining 10 patients, cells showing polymorphic and polygonal spindle features were observed on cytopathologic examination. Hepatocellular features were found in 2 patients. Immunochemical staining revealed positive vimentin expression in all patients and CK19 expression in 10 (90.9%) patients. HSA staining was performed in 9 patients, and all patients tested negative. Immunohistochemical findings are summarized in Table 5.

Treatment and outcome

In this study, all s-CCC patients presented with stage IV disease at the time of diagnosis. Among them, 9 (81.8%) patients died due to disease progression, 1 (9.1%) patient was lost follow-up, and another (9.1%) patient survived. The mean follow-up period of all patients was 93.4 ± 106.3 (median: 47, 14-379) d. The mean survival time of 9 patients who died, from diagnosis to death, was 67.2 ± 53.4 (median: 47, 14-148) d (Table 6).

Table 2 Baseline clinical characteristics of the patients of intrahepatic bile duct adenocarcinoma and sarcomatoid cholangiocarcinoma *n* (%)

		Adenocarcinoma	Sarcomatoid	*P* value
Gender	Male	155 (71.8)	9 (81.8)	0.732
	Female	61 (28.2)	2 (18.2)	
Age	< 60	73 (33.8)	5 (45.5)	0.518
	≥ 60	143(66.2)	6 (54.5)	
Abdominal pain	No	106 (49.1)	1 (9.1)	0.011
	Pain	110 (50.9)	10 (90.9)	
Fever	No	195 (90.3)	7 (63.6)	0.022
	Fever	21 (9.7)	4 (36.4)	
Jaundice	No	199 (92.1)	11 (100.0)	1.000
	Jaundice	17 (7.9)	0 (0.0)	
Weight loss	No	201 (93.1)	10 (90.9)	0.561
	Weight loss	15 (6.9)	1 (9.1)	
GB stone	No	164 (75.9)	8 (72.7)	0.730
	GB stone	52 (24.1)	3 (27.3)	
Hepatitis	No	190 (88.0)	7 (63.6)	0.063
	CHB	21 (9.7)	3 (27.3)	
	CHC	5 (2.3)	1 (9.1)	
LC	No	183 (84.7)	6 (54.5)	0.022
	LC	33 (15.3)	5 (45.5)	
Serum CEA	< 5 ng/mL	123 (56.9)	10 (90.9)	0.029
	≥ 5 ng/mL	93 (43.1)	1 (9.1)	
Serum CA19-9	< 37 U/mL	64 (29.6)	6 (54.5)	0.098
	≥ 37 U/mL	152 (70.4)	5 (45.5)	
Main mass	Single	145 (67.1)	6 (54.5)	0.513
	Multiple	71 (32.9)	5 (45.5)	
Mass size	< 5 cm	90 (41.7)	1 (9.1)	0.054
	≥ 5 cm	126 (58.3)	10 (90.9)	
LN metastasis	No	84 (38.9)	1 (9.1)	0.057
	LN meta.	132 (61.1)	10 (90.9)	
Distant metastasis	No	140 (64.8)	4 (36.4)	0.104
	Distant meta.	76 (35.2)	7 (63.6)	
TNM stage	I or II	76 (35.2)	0 (0.0)	0.018
	III or IV	140 (64.8)	11 (100.0)	

CA19-9: Carbohydrate antigen 19-9; CEA: Carcinoembryonic antigen; CHB: Chronic hepatitis B; CHC: Chronic hepatitis C; LC: Liver cirrhosis; LN: Lymph node.

None of the s-CCC patients could undergo surgical treatment. Seven (63.6%) of them underwent chemotherapy, all of whom were not treated with anticancer drugs for more than 4 cycles due to rapid disease progression, deterioration of systemic condition, or presence of adverse events. Among the 7 patients with chemotherapy, 1 patient was lost follow-up and the other 6 patients died within 6 months (mean survival time: 92 ± 48.5 d, range: 31-148 d). Three (27.3%) of 11 s-CCC patients who underwent conservative treatment died within 3 wk (mean survival time: 17 ± 3.2 d, 14-20 d) (Table 6). The survival of patients who received chemotherapy was significantly longer than that of patients who only received conservative treatment (P = 0.013). In addition, 1 patient (case 11) with a 5-cm sized single s-CCC with LN and diaphragm metastasis refused chemotherapy and was treated with ABNOBA VISCUM M® (viscum album) *via* subcutaneous injection as the second option. Despite the advanced stage, the treatment was well tolerated by the patient, and slow progression was observed. He went to regular follow-up for 379 d.

Comparison of intrahepatic s-CCC and intrahepatic bile duct adenocarcinoma

Compared with 216 patients with CCC, abdominal pain (P = 0.011), fever (P = 0.022), past history of LC (P = 0.022), advanced TNM stage (P = 0.018) were more common in

Table 3 Laboratory findings of the patients with intrahepatic sarcomatoid cholangiocarcinoma

Case	CEA (ng/mL)	CA19-9 (U/mL)	AFP (ng/mL)	PIVKA-II (mAU/mL)	AST (U/L)	ALT (U/L)	TB (mg/dL)	DB (mg/dL)	ALP (U/L)	GGT (U/L)
1	0.74	> 1200.00	131.67	69	25	19	1.0	0.4	387	115
2	1.45	3.38	66.45	16	31	10	0.7	0.2	252	32
3	0.10	3.00	2.54	35	54	96	0.4	0.2	892	137
4	2.35	1809.57	1.73	N/A	42	30	0.9	0.6	1385	N/A
5	1.81	2.33	2.31	20	43	57	1.0	0.6	1520	203
6	12.70	710.38	3.92	N/A	23	39	0.7	0.3	1133	253
7	1.18	12.59	2.70	20	23	16	0.6	0.4	638	224
8	3.15	> 1200.00	1.71	N/A	30	31	1.1	0.6	757	323
9	1.08	< 2.00	1.52	N/A	80	30	0.7	0.5	476	98
10	3.56	599.14	1.02	N/A	37	47	1.1	0.8	804	N/A
11	1.81	5.77	3.02	14	34	36	0.3	0.1	369	35

AFP: Alpha-fetoprotein; ALP: Alkaline phosphatase; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; CA19-9: Carbohydrate antigen 19-9; CEA: Carcinoembryonic antigen; DB: Direct bilirubin; GGT: Gamma-glutamyl transferase; N/A: Not applicable; PIVKA-II: Protein-induced by vitamin K antagonist-II; TB: Total bilirubin.

patients with s-CCC. The incidence of chronic viral hepatitis, larger mass size, LN metastasis, and distant metastasis tended to be more common in s-CCC patients, although there was no statistical significance. Increased serum CEA (P = 0.029) and CA19-9 (P = 0.098) levels were more common in patients with CCC than in those with s-CCC (Table 2). The initial radiologic impressions were variable, however, there was no patient diagnosed with s-CCC by imaging studies (Table 4).

Thirty-three (15.3%) of the patients with CCC underwent surgical treatment, whereas all of the patients with s-CCC could not undergo surgery and received chemotherapy or conservative treatment. The mean survival time of patients with s-CCC was significantly shorter than that of patients with CCC (P = 0.001) (Figure 3).

DISCUSSION

Sarcomatoid tumor of the epithelial origin is commonly found in the bladder or lungs, whereas it is rarely observed in the liver or biliary tract and is referred to as "carcinosarcoma", "sarcomatoid carcinoma", "carcinoma with sarcomatoid change", "pseudosarcoma", "malignant mixed tumor" and "spindle cell carcinoma"[3,7,8]. HCC with sarcomatoid feature accounts for 1.8% of surgically resected HCC and 3.9%-9.4% of autopsy cases. According to some previous reports, it is known to occur due to a secondary change in tumor cells after previous treatment[4,8]. By contrast, s-CCC accounts for 4.5% of bile duct cancer, and has no definite relationship with previous anticancer therapy. Rather, sarcomatoid change is believed due to the natural progression of bile duct cancer[4,9,10].

The pathogenesis of sarcomatoid changes in cancers including cholangiocarcinoma, has not been elucidated yet, and the following possibilities have been suggested: First, sarcomatoid trans-differentiation or de-differentiation of primary carcinoma cells of epithelial origin, which is also explained as epithelial-mesenchymal transition (EMT) or metaplastic transformation. The EMT is an important mechanism in sarcomatoid changes, and this is a biologic process in which epithelial phenotypes are converted into mesenchymal phenotypes by biochemical changes, including enhanced migratory capacity, invasiveness, elevated resistance to apoptosis, and increased production of extracellular matrix components[11]; Second, biphasic differentiation from pluripotent stem cells to carcinoma or sarcoma in various directions, which is divided into a combination and a collision depending on the type of each tumor growth; Finally, a sarcomatoid re-differentiation of cancer cells with multi-potent differentiation potency derived from carcinoma cells[6]. In this study, underlying CHB, CHC, and LC more common in patient with s-CCC, suggesting that these underlying diseases could have been the causative factors for de-differentiation or biphasic differentiation. This hypothesis about the mechanism of the sarcomatoid change is also an evidence supporting the poor prognosis of s-CCC.

Sarcomatoid combined hepatocellular-cholangiocarcinoma is primary hepatic tumor in which hepatocellular carcinomas and cholangiocarcinomas coexist within

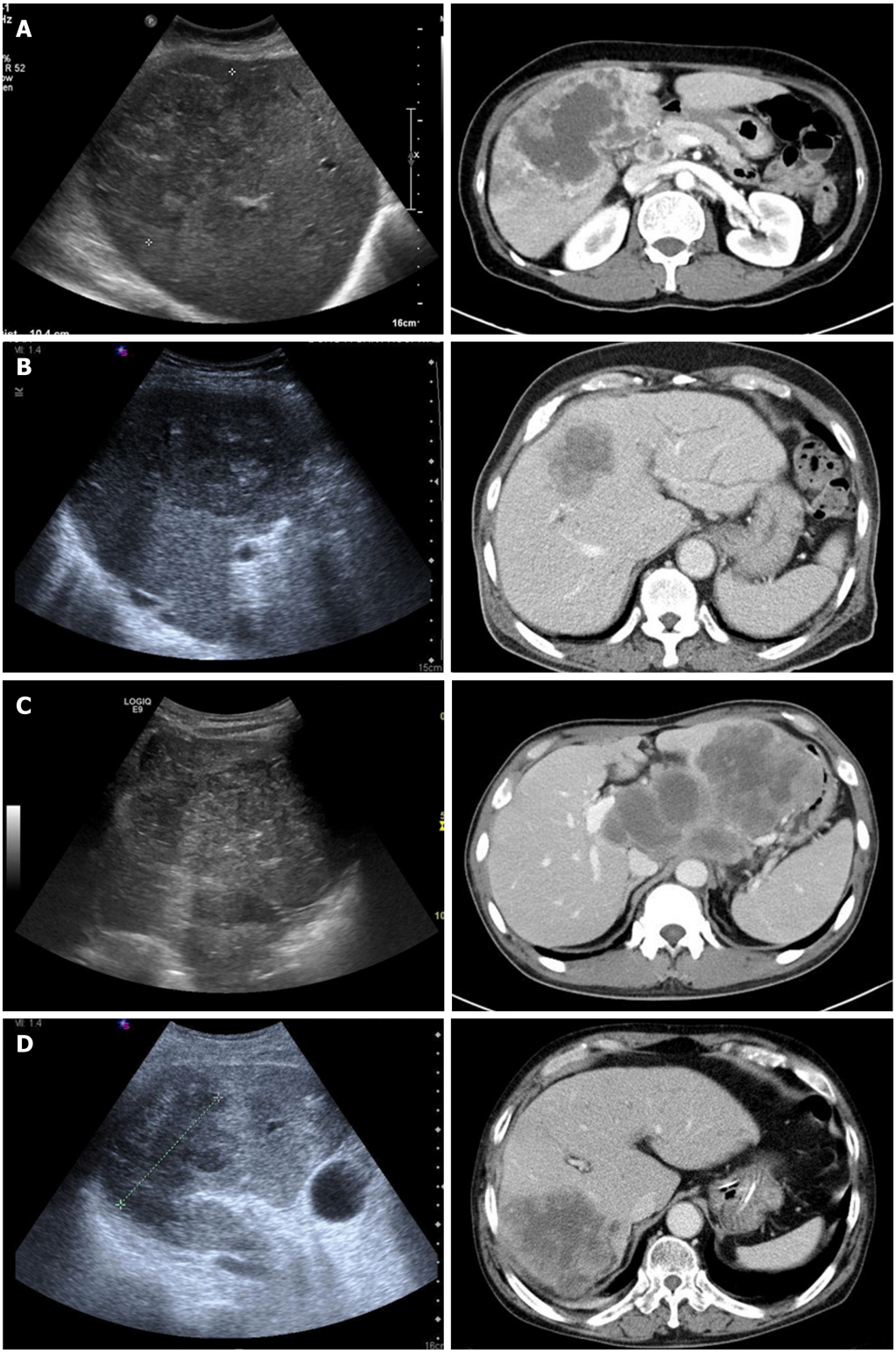

Figure 1 Imaging findings of the patients with various initial diagnoses based on radiologic findings. A: The initial radiologic impressions were intrahepatic cholangiocarcinoma; B: Hepatocellular carcinoma; C: Lymphoma; D: Hepatic abscess, respectively.

the same hepatic tumor components showing distinct tumor pathologic features, and is observed in less than 1% of all patients with liver malignancies[12-14]. In this study, 2 (0.9%) patients presented with mixed histopathologic findings similar to sarcomatoid combined hepatocellular-cholangiocarcinoma (cases 1 and 2). These 2 patients had LC due to CHB and CHC, respectively, and they presented with increased serum AFP levels. At time of diagnoses of these 2 patients, it was difficult to distinguish HCC from IHCC, and the imaging diagnoses were even HCC (Figure 4). These 2 cases can be inferred due to the "sarcomatoid regeneration" of multipotent cancer cells derived from carcinoma cells, which is one of the hypotheses for the mechanism of

Table 4 Imaging findings of the patients with intrahepatic sarcomatoid cholangiocarcinoma

Case	US	CT Non-E	CT arterial	CT portal	CT delayed	Initial radiologic impression
1	Lobulated, heterogeneously hyperechoic	N/A	Thin rim enhancement	Gradual centripetal enhancement	Gradual centripetal enhancement	HCC
2	Lobulated hypoechoic	Ill-defined hypodense	Minimal to mild heterogeneous enhancement	Progressive heterogeneous enhancement	Progressive heterogeneous enhancement	HCC
3	Lobulated, heterogeneously hypoechoic internal hyperechoic	N/A	Non to minimal rim enhancement	Mild irregular rim enhancement	N/A	IHCC
4	Ill-defined hypoechoic	heterogeneously hypodense	Irregular rim enhancement	Gradual centripetal enhancement	N/A	Hepatic abscess
5	Lobulated, heterogenoeusly hypo- and hyper-echoic	N/A	Peripheral enhancement	Gradual centripetal enhancement	Gradual centripetal enhancement	HCC
6	Lobulated hyperechoic	N/A	Irregular rim enhancement	Irregular rim enhancement	N/A	IHCC
7	Lobulated, heterogeneously hypoechoic internal hyperechoic	Ill-defined hypodense	Irregular peripheral enhancement	Gradual centripetal enhancement	Gradual centripetal enhancement	HCC
8	Ill-defined heterogenoeusly hypo- and hyper-echoic	Ill-defined hypodense	Irregular peripheral enhancement	Gradual centripetal enhancement	N/A	IHCC
9	Lobulated, heterogenoeusly hypo- and hyper-echoic	N/A	Irregular peripheral enhancement	Gradual centripetal enhancement	N/A	Lymphoma
10	Lobulated, heterogeneously hyperecmoic	N/A	Minimal rim enhancement	Rim enhancement	N/A	IHCC
11	Well-defined, heterogeneously hypoechoic	Lobulated hypodense	Irregular peripheral enhancement	Irregular peripheral enhancement	N/A	IHCC

CT: Computed tomography; HCC: Hepatocellular carcinoma; IHCC: Intrahepatic cholangiocarcinoma; N/A: Not applicable; Non-E: non-enhanced; US: Ultrasonography.

sarcomatoid cholangiocarcinoma.

s-CCC could be asymptomatic, as is the case with other hepatic tumors, and it may be accompanied by non-specific symptoms or signs such as abdominal pain, nausea, fatigue, fever, and weight loss, of which abdominal pain and fever are more specific. In this study, 10 (90.9%) patients had abdominal pain and 4 (36.4%) patients had fever, which is similar to those reported in the previous articles[3,15]. It can be thought that these clinical features are more common in s-CCC patients because of the mass effect or tumor necrosis caused by relatively large mass size or rapid progression.

According to previously reported cases about s-CCC, serologic tumor markers, such as AFP, CEA, and CA19-9 levels, were negative or low[3,8,11,16-22]. Watanabe *et al*[23] investigated the clinical characteristics of operated sarcomatous intrahepatic cholangiocarcinoma and ordinary intrahepatic cholangiocarcinoma, and there was no significant difference in serum CEA and CA19-9 levels between the two groups. By comparison, in this study, CA19-9 level showed an increase in only 45.5% of s-CCC patients and CEA level increased more frequently in CCC patients with statistical significance. AFP levels were with normal range in most cases of s-CCC. Therefore, tumor markers were not helpful in the diagnosis of s-CCC. Additionally, as previously mentioned, two patients with elevated AFP levels were all suspected to be sarcomatoid combined hepatocellular-cholangiocarcinoma *via* histopathological examination.

A low echogenic liver mass on US, which shows hypo-attenuation and peripheral region enhancement after contrast injection on CT scan, is a commonly known imaging characteristic of s-CCC. In the present study, the hepatic masses showed

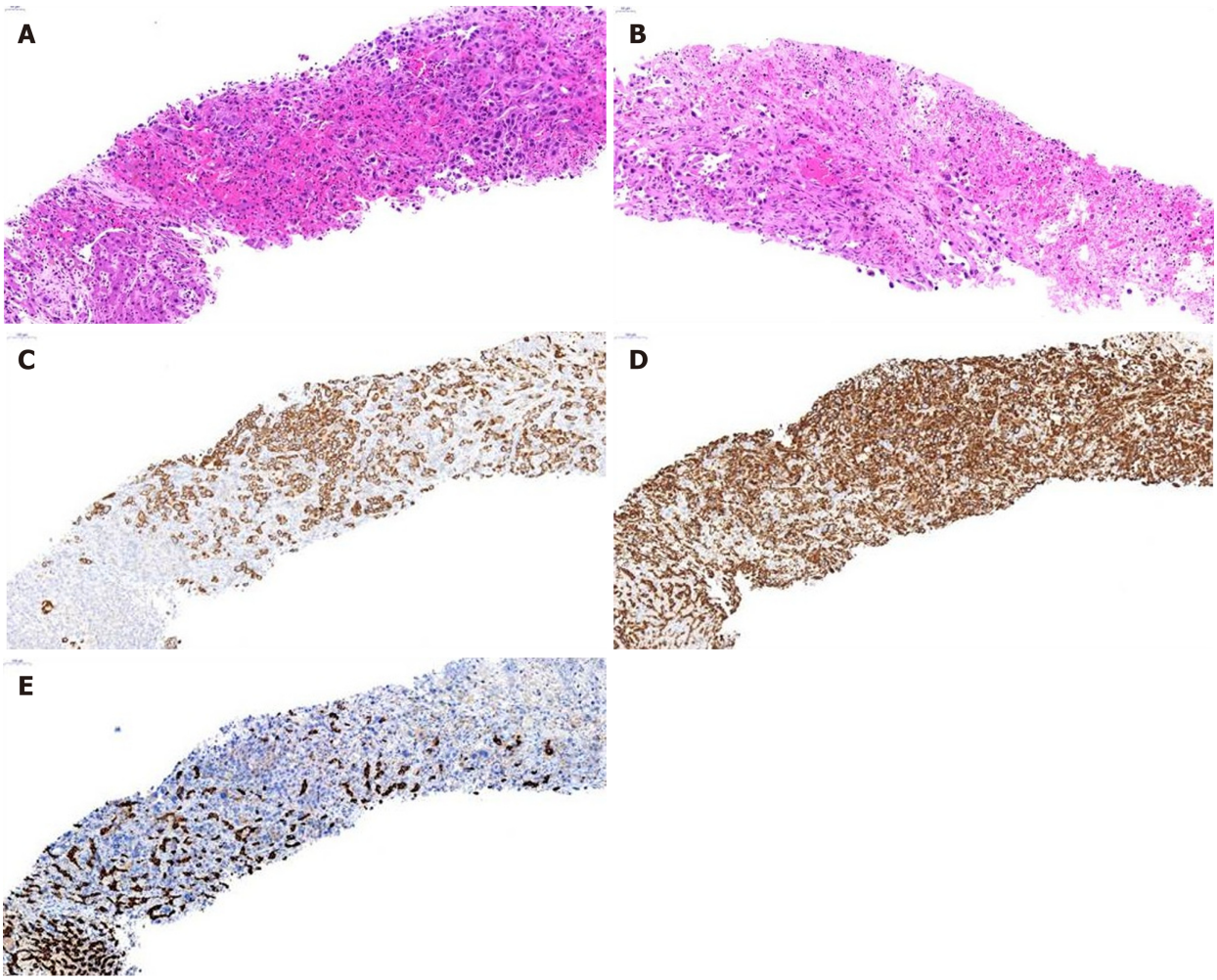

Figure 2 Histologic findings of liver biopsy of case 9. A and B: Hematoxylin and eosin stain, × 100; C-E: Immunohistochemistry, × 50; C: Cytokeratin 19; D: Vimentin; E: Hepatocyte specific antigen. The liver shows a mass composed for proliferating anaplastic tumor cells with ovoid or rather spindle shape and no organoid structures (A), and necrosis (B). The tumor cells express CK19 (C) and vimentin (D), not HSA (E).

various echoic, but mainly hypoechoic features on US. CT scan showed heterogeneous, lobulated hypodense hepatic masses with irregular rim or peripheral enhancement. These are similar to those reported in previous studies[3,24-26]. The imaging findings of 11 patients tended to show relatively aggressive features, which included large mass size (mean: 7.3 ± 2.2 cm, median: 7.5 cm), frequent LN and distant metastases, and extensive metastatic lymphadenopathy accompanied by invasion of portal vein or hepatic parenchyma (n = 2). In addition, portal vein thrombosis (n = 1), multiple intrahepatic metastasis (n = 2), and seeding metastasis (n = 1) were aslo observed. However, these findings are also observed in other mass-forming IHCC. Distal intrahepatic ductal dilatation (n = 5), capsular retraction (n = 8), satellite nodule (n = 3), peripheral enhancement (n = 10), and gradual centripetal enhancement on dynamic enhancement study are also well-known imaging findings of common mass-forming IHCC. Thus, even though the imaging findings of s-CCC show relatively aggressive features, these are not distinguishing from those of other hepatic tumors, especially IHCC. Indeed, none of the 11 patients were not clearly diagnosed with s-CCC during the first imaging study. Moreover, there were cases of underlying LC (n = 5), mimicking HCC (n = 4), and requirement for differentiation from lymphoma (n = 1), or hepatic abscess (n = 1) (Figure 1). In previous study, there was no meaningful difference in tumor size, number of tumors, and LN metastasis between sarcomatous intrahepatic cholangiocarcinoma and ordinary intrahepatic cholangiocarcinoma[23]. Therefore, it is nearly impossible to identify s-CCC only *via* imaging studies.

Biopsy is an indispensable test for the final diagnosis. In this study, polymorphic and polygonal cells exhibiting spindle features were commonly observed. In immunohistochemistry, all patients were positive for vimentin, and 10 (90.9%) patients were positive for CK19, and 9 patients who underwent HSA tested negative, which was considerably helpful for the final diagnosis of s-CCC. These results were similar to those of previous studies[9,27,28].

Table 5 Immunohistochemistry of the patients with intrahepatic sarcomatoid cholangiocarcinoma

Case	Specimen	Positive IHC results	Negative IHC results
1	Needle biopsy	CK19, vimentin	HSA, CD10
2	Needle biopsy	CK, vimentin, CEA, AFP	CK7, CK19, HSA, c-kit, CD117
3	Needle biopsy	CK, CK19, vimentin	CK8, Desmin, EMA, CEA, c-kit, S-100
4	Needle biopsy	CK, CK8, CK19, vimentin, CEA, EMA	HSA, AFP, TTF-1
5	Needle biopsy	CK, CK8, CK19, vimentin, SMA	HSA, CD5, CD68, HMW-CK
6	Needle biopsy	CK7, CK8, CK19, vimentin, CEA	HSA
7	Needle biopsy	CK7, CK8, CK19, vimentin, CD34	HSA, CEA, HMW-CK
8	Needle biopsy	CK19, vimentin, CEA, p53	CD31, CD34
9	Needle biopsy	CK19, vimentin, CEA	CK7, Desmin, HSA, SMA, c-kit, S-100
10	Needle biopsy	CK, CK19, vimentin, CEA	HSA, CD31
11	Needle biopsy	CK7, CK19, vimentin, MUC1	HSA, CD10

AFP: Alpha-fetoprotein; CD: Cluster of differentiation; CEA: Carcinoembryonic antigen; CK: Cytokeratin; EMA: Epithelial membrane antigen; HMW-CK: High molecular weight cytokeratin; HSA: Hepatocyte specific antigen; IHC: Immunohistochemistry; MUC1: Human mucin-1; SMA: Smooth muscle actin; TTF-1: Thyroid transcription factor-1.

Although no definitive treatment for s-CCC is available, surgical resection is generally recommended first, and in patients undergoing surgical resection, the survival rate was significantly higher than that of patients who have not undergone surgery[3,23,29].

Adjuvant chemotherapy along with the combination of gemcitabine and cisplatin has been proposed as a treatment to improve survival[10,30]. Nevertheless, the prognosis of s-CCC is extremely poor compared with that of CCC, and there is no established treatment that can significantly prolong survival after surgery. This is due to the fact that LN or distant metastasis is common at time of diagnosis, and the rate of tumor growth is relatively fast. The tumor is also frequently accompanied by tumor thrombi, and the recurrence rate after treatment is high[3,6,10,30,31]. In addition, several studies have shown that survival prolongation was obtained with iniparib, cisplatin, ifosfamide, dacarbazine, doxorubicin, cyclophosphamide, taxol, thalidomide and paclitaxel treatment as postoperative adjuvant chemotherapy for carcinosarcoma in obstetrics and gynecology patients[2,32-34], however, there are also some reports on unsuccessful outcomes[35,36]. Among the 11 s-CCC patients of this study, no one could undergo surgical treatment and only seven patients underwent chemotherapy. In the remaining 3 patients with conservative treatment, shortened survival time may be due to the lack of the chemotherapy effect, but it is also thought to be due to the higher incidence of deteriorated general condition, old age, or poor performance status, which made the patients die earlier, regardless of cancer progression.

ABNOBA VISCUM M® (viscum album), also called mistletoe, is an extract of a plant that is hemiparasitic to various host plants. This is an immunomodulator and somewhat different from the cytotoxic drugs, target agents, or immune checkpoint inhibitors, and it is mainly, along with chemotherapy or radiation therapy, used to increase the therapeutic effect, decrease side effects, and enhance immunity. In some papers, various positive results regarding the use of viscum album as a palliative therapy in patients with terminal cancer who do not respond to conventional chemotherapy or for those with lung cancer or accompanying malignant pleural effusion and hematologic diseases[37-39]. However, there has been no report about the therapeutic effect of this drug alone for IHCC including s-CCC. Although the patient (case 11) of this study is an unusual case, whether the reason for the good prognosis in this patient is due to the effect of the viscum album or the slow progression of the tumor still remains unclear.

This study has some limitations. First, because only 11 patients with s-CCC were analyzed, some aspects of the comparison between s-CCC and CCC were not statistically significant. Second, a relatively large number of follow-up loss was observed; about 12% of the patients with CCC lost follow-up. This is believed that this study was conducted in a single center, and a large number of patients did not continue with aggressive treatment and wanted conservative treatment only, or to go to another hospital.

In conclusion, intrahepatic s-CCC of the liver is an extremely rare disease, and it is frequently characterized by abdominal pain, fever, and commonly diagnosed at an

Table 6 Treatment and outcome of the patients with intrahepatic sarcomatoid cholangiocarcinoma

Case	Sex	Age	Tumor size (cm)	Number of mass	Stage (TNM)	Treatment	Outcome	F/U duration (d)
1	M	45	7.5	7	IVB	Chemotherapy	Died	47
2	M	67	2.5	1	IVB	Chemotherapy	Died	148
3	M	55	6.5	2	IVA	Chemotherapy	Died	129
4	M	66	10.0	1	IVB	Supportive	Died	20
5	M	56	8.0	1	IVB	Chemotherapy	Died	72
6	F	66	7.5	1	IVB	Chemotherapy	Died	125
7	M	68	6.0	1	IVB	Supportive	Died	19
8	F	55	8.5	3	IVA	Chemotherapy	Died	31
9	M	49	9.5	3	IVA	Chemotherapy	F/U loss	43
10	M	65	9.5	15	IVA	Supportive	Died	14
11	M	61	5.0	1	IVB	viscum album	Survived	379

F/U: Follow-up.

advanced stage. Its prognosis is extremely poor due to the rapid progression of the disease. Early diagnosis and appropriate treatment are important. Because clinical, serologic, and imaging findings are not helpful in distinguishing s-CCC from CCC, HCC, or other hepatic masses, biopsy should be performed to accurately diagnose and predict prognosis. Although there has been no fully established treatment thus far, surgical resection is usually prioritized, and in some cases, the survival rate can be improved through adjuvant chemotherapy. Further prospective, multicenter studies about the characteristic features, complementary treatment effect of various therapeutic options, and the effect of surgery on the survival rate must be conducted based on the pathogenesis of this disease.

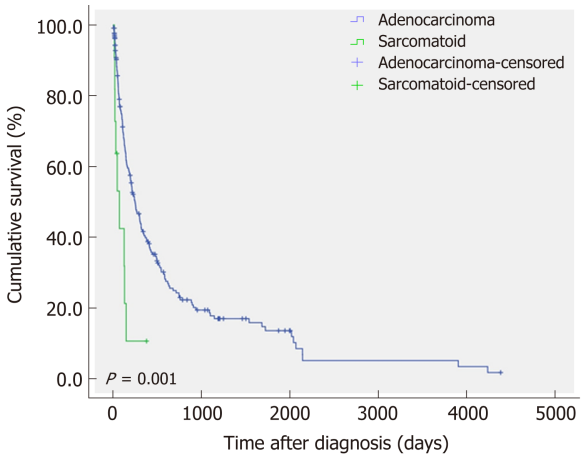

Figure 3 Comparison of cumulative survival rates. Survival rate of intrahepatic bile duct adenocarcinoma group was better than that of sarcomatoid cholangiocarcinoma group.

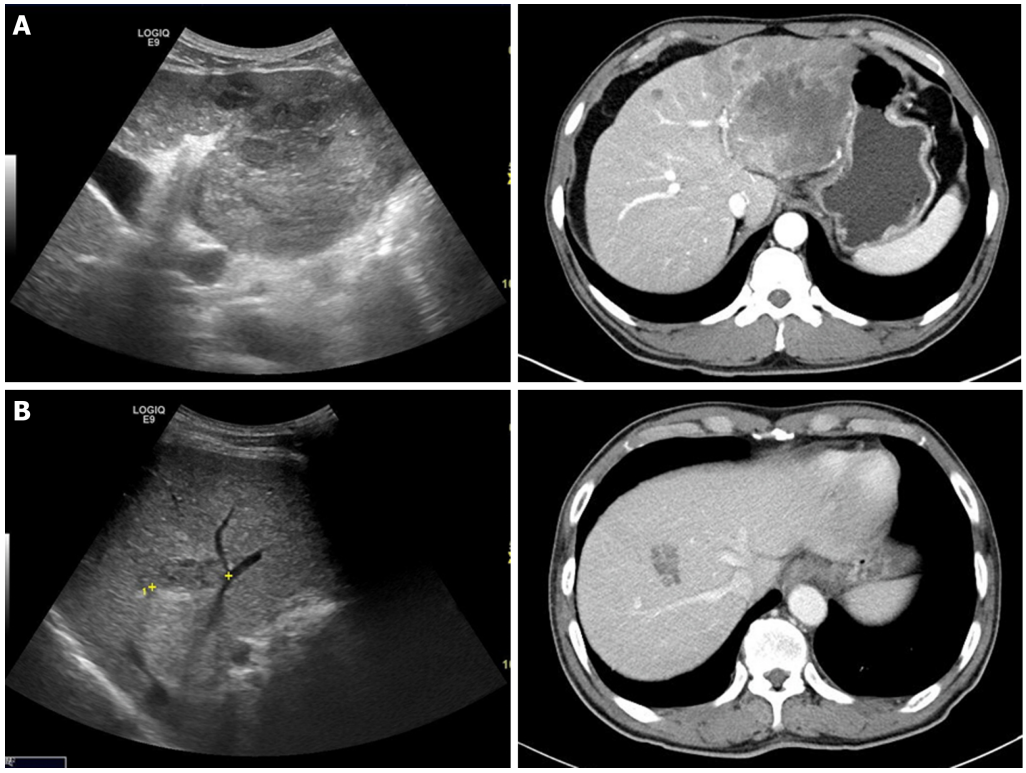

Figure 4 Imaging finding of the patient with mixed pathological findings of hepatocellular carcinoma and intrahepatic cholangiocarcinoma. A: Case 1; B: Case 2.

ARTICLE HIGHLIGHTS

Research background

Intrahepatic sarcomatoid chonalgiocarcinoma (s-CCC) is extremely rare, and its pathophysiology is not well known. Because of the poor prognosis, early diagnosis and aggressive treatment is important.

Research motivation

Clinical, serologic, and imaging findings are known to be not helpful to distinguish s-CCC from intrahepatic bile duct adenocarcinoma (CCC) or other hepatic tumors. There is no established treatment option to prolong survival, except surgery.

Research objectives

This study aimed to analyze the distinct characteristics of s-CCC patients for early diagnosis and

appropriate treatment.

Research methods
This retrospective study was conducted in a single center of South Korea for assessment of 11 patients with s-CCC diagnosed for 17 years. We analyzed the clinical, serologic, imaging, and histopathologic features of s-CCC patients and compared with those of CCC patients.

Research results
The patients with s-CCC tended to present abdominal pain or fever as the chief complaint and have past history of liver cirrhosis (LC) or chronic viral hepatitis more frequently, compared with the patients with CCC. In addition, s-CCC showed relatively aggressive features on imaging studies. However, no clear distinction in other clinical and serologic, or radiologic examination results between s-CCC and CCC patients. Only a histopathologic examination with immunohistochemical staining was helpful and essential for an accurate differential diagnosis of s-CCC. The clinical course of s-CCC was relatively aggressive, and patients had poor prognoses. Surgery is generally recommended first, however in this study, we could not obtain meaningful results of the surgical treatment or chemotherapy for s-CCC.

Research conclusions
s-CCC is extremely rare disease which presents aggressive clinical course and poor prognosis. Clinical, serologic, and imaging studies are not helpful in diagnosis of s-CCC. In patients with s-CCC, early diagnosis through biopsy and aggressive treatment, including surgical resection, are important.

Research perspectives
Although s-CCC has a poor prognosis, its pathogenesis and the effects of non-surgical treatment are not well established. In addition, there is no definite strategy for differential diagnosis except a histopathological examination. A large-scale, prospective, and multicenter study involving larger number of patients should be conducted in the future.

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ORIGINAL ARTICLE

Retrospective Study

Hepatocellular carcinoma: Can LI-RADS v2017 with gadoxetic-acid enhancement magnetic resonance and diffusion-weighted imaging improve diagnostic accuracy?

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Institutional review board statement: This study was approved by the Ethics Committee of West China Hospital.

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

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Abstract

BACKGROUND

The Liver Imaging Reporting and Data System (LI-RADS), supported by the American College of Radiology (ACR), has been developed for standardizing the acquisition, interpretation, reporting, and data collection of liver imaging examinations in patients at risk for hepatocellular carcinoma (HCC). Diffusion-weighted imaging (DWI), which is described as an ancillary imaging feature of LI-RADS, can improve the diagnostic efficiency of LI-RADS v2017 with gadoxetic acid-enhanced magnetic resonance imaging (MRI) for HCC.

AIM

To determine whether the use of DWI can improve the diagnostic efficiency of LI-RADS v2017 with gadoxetic acid-enhanced magnetic resonance MRI for HCC.

METHODS

In this institutional review board-approved study, 245 observations of high risk of HCC were retrospectively acquired from 203 patients who underwent gadoxetic acid-enhanced MRI from October 2013 to April 2018. Two readers independently measured the maximum diameter and recorded the presence of each lesion and assigned scores according to LI-RADS v2017. The test was used to determine the agreement between the two readers with or without DWI. In addition, the sensitivity (SE), specificity (SP), accuracy (AC), positive predictive value (PPV), and negative predictive value (NPV) of LI-RADS were calculated. Youden index values were used to compare the diagnostic performance of LI-RADS with or without DWI.

RESULTS

Conflict-of-interest statement: All authors declare no conflicts of interest related to this article.

Data sharing statement: No additional data are available.

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Almost perfect interobserver agreement was obtained for the categorization of observations with LI-RADS (*kappa* value: 0.813 without DWI and 0.882 with DWI). For LR-5, the diagnostic SE, SP, and AC values were 61.2%, 92.5%, and 71.4%, respectively, with or without DWI; for LR-4/5, they were 73.9%, 80%, and 75.9% without DWI and 87.9%, 80%, and 85.3% with DWI; for LR-4/5/M, they were 75.8%, 58.8%, and 70.2% without DWI and 87.9%, 58.8%, and 78.4% with DWI; for LR- 4/5/TIV, they were 75.8%, 75%, and 75.5% without DWI and 89.7%, 75%, and 84.9% with DWI. The Youden index values of the LI-RADS classification without or with DWI were as follows: LR-4/5: 0.539 *vs* 0.679; LR-4/5/M: 0.346 *vs* 0.467; and LR-4/5/TIV: 0.508 *vs* 0.647.

CONCLUSION
LI-RADS v2017 has been successfully applied with gadoxetate-enhanced MRI for patients at high risk for HCC. The addition of DWI significantly increases the diagnostic efficiency for HCC.

Key words: Hepatocellular carcinoma; Liver Imaging Reporting and Data System; Magnetic resonance imaging; Diffusion-weighted imaging; Diagnosis

Core tip: The aim of this study was to determine whether the use of diffusion-weighted imaging (DWI) improves the diagnostic efficiency of the Liver Imaging Reporting and Data System (LI-RADS) v2017 with gadoxetic acid-enhanced magnetic resonance (MR) for hepatocellular carcinoma (HCC). A total of 245 observations in 203 patients were analyzed. The Youden index values of the LI-RADS classification without or with DWI were as follows: LR-4/5: 0.539 *vs* 0.679; LR-4/5/M: 0.346 *vs* 0.467; and LR-4/5/TIV: 0.508 *vs* 0.647. Using LI-RADS v2017 with gadoxetic acid-enhanced MR combined with DWI may result in a more accurate diagnosis of HCC.

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the fifth most common cancer and the third leading cause of cancer-related deaths[1]. In high-risk patients, HCC can be diagnosed noninvasively by computed tomography (CT) and magnetic resonance imaging (MRI) without the need for further histopathological confirmation when the imaging features are characteristic[2-4].

The Liver Imaging Reporting and Data System (LI-RADS), supported by the American College of Radiology (ACR), has been developed for standardizing the acquisition, interpretation, reporting, and data collection of liver imaging examinations in patients at risk for HCC. Initially released in 2011, the system has been updated in 2013, 2014, 2017, and 2018 based on the evolution of published evidence, integration of new technology, and incorporation of user feedback[5-7]. Each liver observation is categorized according to its probability of HCC, from LR-1 to LR-5 (definitely benign, probably benign, intermediate, probably HCC, and definitely HCC)[5]. In LI-RADS v2017[8], if an observation is probably or definitely malignant but is not specific for HCC, LR-M is allocated. Moreover, a new diagnostic category, LR-NC and LR-TIV (previously LR-5V), has been added. Ancillary features can be applied to upgrade or downgrade the initially assigned LI-RADS category based on major features only[9].

MRI can be used for categorization of liver observations and diagnosis of HCC based on the major and ancillary features of LI-RADS[10-12]. Gadoxetic-acid disodium (Gd-EOB-DTPA), a hepatobiliary contrast agent, could provide information on tumor vasculature and hepatocyte function[13-15]. Diffusion-weighted imaging (DWI) can

further quantitatively measure tissue proton diffusion and reflect tumor cellularity[16-18]. Thus, the combination of Gd-EOB-DTPA-enhanced MRI and DWI has the potential to improve the sensitivity (SE) and overall accuracy (AC) for diagnosing HCC.

The aim of this retrospective study was to evaluate the interrater reliability and diagnostic AC of LI-RADS v2017 with Gd-EOB-DTPA-enhanced MRI for HCC and to determine the incremental value of the ancillary feature "restricted diffusion" on DWI images.

MATERIALS AND METHODS

Patients

This retrospective cohort study was approved by our institutional review board, and the requirement for patient consent was waived. Between October 2013 and April 2018, a total of 414 consecutive patients who were at high risk [hepatitis B virus (HBV) infection, hepatitis C virus (HCV) infection, or hepatic cirrhosis] for HCC were enrolled. All included patients were confirmed by surgical pathology, needle biopsy, or more than two years of follow-up. Among these patients, 211 were excluded because of the exclusion criteria (Figure 1). The exclusion criteria were as follows: (1) previously treated for HCC (n =105); (2) pathologically proven HCC or benign lesions before gadoxetic acid-enhanced MRI (n = 52); (3) less than 18 years old (n = 17); and (4) underwent nonsurgical treatment without obtaining histopathological results (n = 22) or lesions that could not be conclusively diagnosed based on 2-year follow-up imaging (n = 15).

Imaging techniques

For all examinations, studies were carried out by using a 3.0 T MR system (MAGNETOM Skyra, Siemens Healthcare, Erlangen, Germany). An 18-channel phased-array torso coil was used for all measurements. Routine MRI sequences included in the standardized scanning protocol were a respiratory-triggered axial T2-weighted turbo spin echo (TSE) sequence with fat suppression; in and out of phase T1-weighted imaging acquired with a gradient recalled (GRE) dual echo sequence; and pre- and postcontrast T1-weighted three-dimensional VIBE sequences acquired with a GRE sequence in the arterial phase (20 s), portal venous phase (60 s), and delayed phase (180 s) after the injection of Gd-EOB-DTPA (Primovist, Bayer Pharma AG, Berlin, Germany) at a rate of 2 mL/s. The delay time for the hepatobiliary phase was 20 min. The detailed parameters of each acquisition sequence are shown in Table 1.

Image analysis

Two radiologists (with more than ten years of experience in abdominal radiology) who were blinded to the clinical, laboratory, and pathology results reinterpreted the MR images. Each reader measured the maximum diameter and recorded the presence of each lesion and assigned scores according to LI-RADS v2017[11]. The scoring categories were as follows: LR-1 was definitely benign, LR-2 was probably benign, LR-3 was an intermediate probability of malignancy, LR-4 was probably HCC, and LR-5 was definitely HCC. Findings that were probably or definitely malignant but not HCC specific were categorized as LR-M and those with definite tumor in vein as LR-TIV. The final category results were compared with the pathology to assess diagnostic AC.

Statistical analysis

Categorical variables are reported as the number of cases and percentages. The *kappa* test was first used to determine the agreement between the two independent radiologists in each item. A *kappa* value of 0 indicates no agreement, *kappa* values of 0.01-0.20 represent slight agreement, 0.21-0.40 fair agreement, 0.41-0.60 moderate agreement, 0.61-0.80 good agreement, 0.81-0.99 almost perfect agreement, and 1 perfect agreement[19]. In addition, the SE, specificity (SP), AC, positive predictive value (PPV), and negative predictive value (NPV) were calculated for the diagnostic performance of LI-RADS. Youden index values were used to compare the diagnostic performance of LI-RADS with or without DWI. All statistical analyses were performed using SPSS 23.0 (SPSS Inc, Chicago, IL, United States).

RESULTS

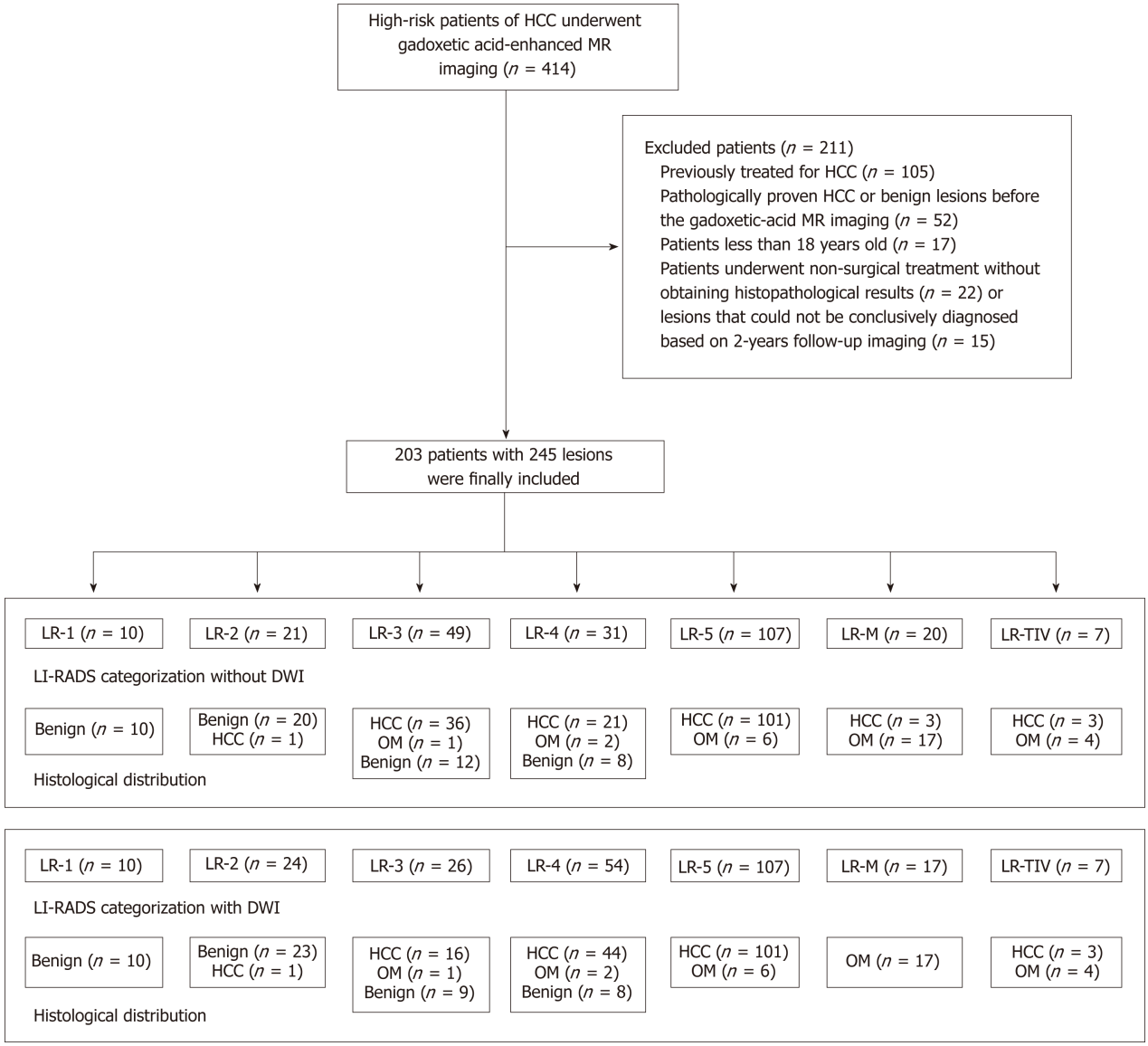

Figure 1 Flow diagram of the study population. HCC: Hepatocellular carcinoma; OM: Other non-hepatocellular carcinoma malignancy.

Clinicopathologic characteristics

During the study period, 414 consecutive patients were selected for potential inclusion. Of these patients, 203 (mean age: 50.31 ± 10.87 years; range: 26-77 years) with 245 hepatic lesions, including 157 (77.34%) men (50.06 ± 10.05 years old; range: 26-77 years old) and 46 (22.66%) women (51.17 ± 11.67 years old; range: 30-77 years old) who met the inclusion criteria, were ultimately included. In the study cohort, 19 patients had multiple HCCs. Of these patients, 195 had Child-Pugh A, and 8 had Child-Pugh B. In addition, 194 (95.57%) patients had HBV infection, 8 (3.94%) had HCV infection, and 1 (0.4%) had both HBV and HCV infections. The baseline characteristics of all patients are summarized in Table 2.

Histologic results

Of all 245 hepatic lesions, 195 (79.59%) were confirmed as malignant by histologic analysis, including 165 (67.35%) lesions diagnosed as HCC (Figure 2), 22 (8.98%) as intrahepatic cholangiocarcinoma (ICC) (Figure 3), 5 (2.04%) as combined HCC (cHCC), and 3 (1.22%) as sarcomatoid HCC (SHC). In addition, 50 (20.41%) lesions were diagnosed as benign by liver biopsy (n = 5) or two-year follow-up using CT or MRI (n = 45). The median diameter for hepatic lesions was 5.3 cm (range: 1.1-12.8 cm).

Interobserver agreement

The agreement of the LI-RADS classification was almost perfect between the two observers [*kappa* = 0.813; 95% confidence interval (CI): 0.748-0.871]. When DWI images were jointly viewed for LI-RADS classification, the agreement between the two

Table 1 Parameters of diffusion-weighted imaging, T1-weighted imaging, T2-weighted imaging, and VIBE sequence

Parameter	DWI	In/out of phase T1-weighted imaging	T2-weighted imaging	VIBE
Repetition time (ms)	5600	81	2160	3.95
Echo time (ms)	68	1.4	100	1.92
Field of view (mm^2)	380 × 289	400 × 325	433 × 433	400 × 296
Scan matrix	100 × 76	352 × 286	320 × 20288	352 × 256
Slice thickness (mm)	6	6	6	2
Slice gap (mm)	1	2.7	2.7	0
Number of excitation	…	1	2	1

DWI: Diffusion-weighted imaging.

observers was markedly increased (*kappa* = 0.882; 95%CI: 0.834-0.928).

LI-RADS lesion categories and diagnostic efficiency

When MR images were reviewed without DWI, HCCs were diagnosed in zero of 10 (0%) LR-1 lesions, one (5%) of 21 LR-2, 36 (73.5%) of 49 LR-3, 21 (67.7%) of 31 LR-4, 101 (94.4%) of 107 LR-5, 3 (15%) of 20 LR-M, and 3 (42.9%) of 7 LR-TIV. However, when the MR and DWI images were jointly viewed for the LI-RADS classification, HCCs were diagnosed in zero of 10 (0%) LR-1 lesions, one (4%) of 24 LR-2, 16 (61.5%) of 26 LR-3, 44 (81.5%) of 54 LR-4, 101 (94.4%) of 107 LR-5, zero (0%) of 17 LR-M, and 3 (42.9%) of 7 LR-TIV. Regarding the diagnostic efficiency, when considering only lesions classified as LR-5, the diagnostic SE, SP, and AC values were 61.2%, 92.5%, and 71.4% without DWI and 61.2%, 92.5%, and 71.4% with DWI, respectively. For LR-4/5, the values were 73.9%, 80%, and 75.9% without DWI and 87.9%, 80%, and 85.3% with DWI, respectively. The Youden index value of this LI-RADS classification with DWI (0.679) was higher than that without DWI (0.539). For LR-4/5/M, the values were 75.8%, 58.8%, and 70.2% without DWI and 87.9%, 58.8%, and 78.4% with DWI, respectively. The Youden index value of this LI-RADS classification with DWI (0.467) was higher than that without DWI (0.346). For LR- 4/5/TIV, the values were 75.8%, 75%, and 75.5% without DWI and 89.7%, 75%, and 84.9% with DWI, respectively. The Youden index value of this LI-RADS classification with DWI (0.647) was higher than that without DWI (0.508). Detailed information about the diagnostic efficiency is summarized in Table 3.

DISCUSSION

The results of this study demonstrated that the use of LI-RADS v2017 on gadoxetic acid-enhanced MR can provide high diagnostic efficacy for HCC. Furthermore, when DWI and MR images were jointly viewed for LI-RADS classification, the diagnostic AC was significantly increased. Thus, using LI-RADS v2017 with gadoxetic acid-enhanced MR combined with DWI may result in a more accurate diagnosis of HCC.

Several studies have compared the interobserver agreement of liver nodule classification based on LI-RADS. Liu *et al*[20] showed that the interobserver agreement was 0.44, and another study showed that the observer consistency was 0.748[21]. The interobserver agreement in our study was higher than that in previous studies, which might be explained by the application of the hepatobiliary phase (HBP) on gadoxetic acid-enhanced MRI. As Gd-EOB-DTPA is a liver-specific contrast agent with a unique EOB group, it can be specifically taken up by normal hepatocytes (approximately 50% uptake rate), thereby producing an enhancing effect in liver cells after Gd-EOB-DTPA administration[22]. However, dysfunctional liver cells cannot take up special liver contrast agents. Therefore, this imaging modality could provide useful information to distinguish abnormal hepatocytes (including HCC) from normal hepatocytes. In addition, the interobserver agreement of LI-RADS categorization was increased when DWI and MR were jointly viewed for the classification. The combination of Gd-EOB-DTPA-enhanced MRI and the DWI sequence can significantly improve both the diagnostic AC and SP for chronic liver disease-associated HCC[23]. This finding might be explained by the ability of DWI to reflect the cellularity of tissue. Compared with normal tissue, tumor tissue with high cellularity could result in decreased

Table 2 Patients' baseline characteristics

Characteristic	Value
Clinical information	
Age (yr)	50.31 ± 10.87 (range: 26-77)
Male/female	157/46
Etiology of liver disease	
Hepatitis B virus	194 (95.57%)
Hepatitis C virus	8 (3.94%)
Both hepatitis B and C virus	1 (0.49%)
Tumor size (cm)	Median 5.3 (range: 1.1-12.8)
AFP level (ng/mL)	102.3 ng/mL (range 1.2-15926.0)
Serum AST (≥ 35 IU/L)	107 (52.7%)
Serum ALT (≥ 40 IU/L)	82 (40.4%)

AFP: Alpha-fetoprotein; ALT: Alanine aminotransferase; AST: Aspartate aminotransaminase.

extracellular space and limited water diffusion, represented by high signal intensity. In our study, a small number of lesions were classified as LR-3 without DWI; once DWI was added, these lesions were downgraded to LR-2 due to unrestricted diffusion. In addition, some lesions previously classified as LR-3 were upgraded to LR-4 due to restricted diffusion. Compared with the final pathological results, lesions degraded to LR-2 included atypical hemangioma and hepatic angiomyolipoma, and lesions upgraded to LR-4 were HCCs.

Our study shows that LI-RADS v2017 on gadoxetic acid-enhanced MR and DWI can improve the diagnostic efficiency for the evaluation of patients at risk for HCC. When considering only lesions classified as LR-5, the diagnostic efficiency of HCC did not change with or without DWI. In LI-RADS v2017, DWI is an ancillary feature that can be applied for category adjustment but cannot be used to upgrade to LR-5. In addition, observations classified as LR-5 in our study strongly suggested HCCs based on major features; accordingly, no observations were downgraded to LR-4. For LR-4/5, LR-4/5/TIV, and LR-4/5/M, all diagnostic efficiencies were improved when DWI was added (LR-4/5: 0.539 *vs* 0.679; LR-4/5/M: 0.346 *vs* 0.467; and LR-4/5/TIV: 0.508 *vs* 0.647). Although DWI is an ancillary feature, we could upgrade some LR-3 observations to LR-4, and many observations were confirmed as HCCs. Therefore, the diagnostic efficiency was improved. Our results were consistent with those of Kim *et al*[23] who reported similar diagnostic efficacy of LI-RADS on gadoxetate-enhanced MRI. However, compared with previous studies, the advantage of this study was the application of LI-RADS v2017, which offered more consummate categorization.

In our study, one of 21/24 (without and with DWI, respectively) category 2 lesions was ultimately diagnosed as HCC (well-differentiated). Some previous studies have also confirmed a few LR-2 lesions[20,24] as HCCs. For LR-3, 36 out of 49 lesions were HCCs without DWI; however, 23 out of the 49 LR-3 lesions mentioned above were reclassified as LR-2 (n = 3) with hypointensity and LR-4 (n = 20) with hyperintensity on DWI. Moreover, the final pathological outcomes confirmed the 20 reclassified LR-4 lesions as HCCs and the remaining 3 LR-2 lesions as benign. In addition, 3 LR-M lesions were reclassified as LR-4 due to DWI characteristics, and all of these lesions were confirmed as HCCs. Therefore, the application of DWI readjusted the categorization and enhanced the diagnostic efficiency for HCC. For LR-1, LR-5, and LR-TIV, the categorization was the same with or without DWI because the imaging signs for these LI-RADS classifications were sufficient to make accurate diagnoses. Some non-HCC malignancies in our study were categorized as LR-3, LR-4, or LR-5, demonstrating the difficulty of obtaining a perfectly specific diagnosis of HCC using LI-RADS v2017.

However, our study had several limitations. First, a selection bias may have been present due to the single-center, retrospective design. Thus, validating these results with studies in other centers with a prospective design is necessary. Second, a major feature, the growth threshold, was not investigated because of patients with doubtful liver malignancy who underwent liver surgery without a long-term follow-up. Thus, we lacked patients to meet the criterion. Third, definitely or probably benign observations were reported at the observers' discretion; hence, the numbers of LR-1 and LR-2 observations were lower than the actual numbers of benign lesions, which may decrease the diagnostic AC for true negative patients. Fourth, the latest version

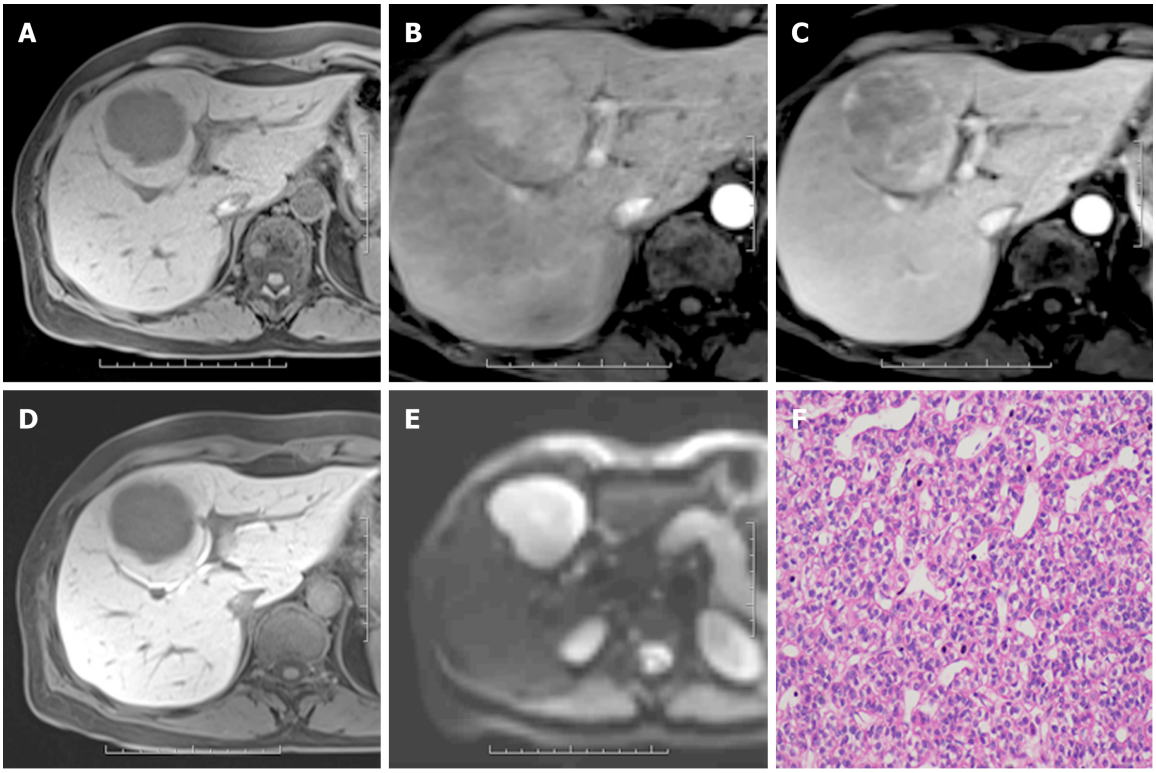

Figure 2 Pathologically confirmed hepatocellular carcinoma in a 59-year-old woman. A: A 55.8-mm hypointense mass is seen on the precontrast image; B: The image shows hyperenhancement (not rim) in the arterial phase; C: Nonperipheral "washout" and enhancing capsule in the portal venous phase; D: Hypointensity in the hepatobiliary phase; E: Restricted diffusion in diffusion-weighted imaging; F: The mass was confirmed as hepatocellular carcinoma at 200 × magnification after hematoxylin-eosin staining.

of LI-RADS (v2018) was not applied to these patients. However, we believe that our data, a pool of categorization results by several readers during actual MRI interpretation, can better reflect clinical practice and may be broadly applied.

In conclusion, LI-RADS v2017 has been successfully applied with gadoxetate-enhanced MRI for patients at high risk for HCC. The addition of DWI significantly increases the diagnostic efficiency for HCC. However, these results need to be validated with studies in other centers in a prospective form.

Table 3 Sensitivity, specificity, and accuracy of the liver imaging reporting and data system category for diagnosing hepatocellular carcinoma with gadoxetic-acid enhanced magnetic resonance imaging

Group	DWI	Sensitivity (100%)	Specificity (100%)	Accuracy (100%)	PPV (100%)	NPV (100%)	Youden index
LR-5	A-DWI	61.2 (101/165)	92.5 (74/80)	71.4 (175/245)	94.4 (101/107)	53.6 (74/138)	0.537
	P-DWI	61.2 (101/165)	92.5 (74/80)	71.4 (175/245)	94.4 (101/107)	53.6 (74/138)	0.537
LR-4/5	A-DWI	73.9 (122/165)	80 (64/80)	75.9 (186/245)	88.4(122/138)	59.8 (64/107)	0.539
	P-DWI	87.9 (145/165)	80 (64/80)	85.3 (209/245)	90.1 (145/161)	76.2 (64/84)	0.679
LR-4/5/M	A-DWI	75.8 (125/165)	58.8 (47/80)	70.2 (172/245)	79.1 (125/158)	54 (47/87)	0.346
	P-DWI	87.9 (145/165)	58.8 (47/80)	78.4 (192/245)	81.5 (145/178)	70.1 (47/67)	0.467
LR-4/5/TIV	A-DWI	75.8 (125/165)	75 (60/80)	75.5 (185/245)	86.2 (125/145)	60 (60/100)	0.508
	P-DWI	89.7 (148/165)	75 (60/80)	84.9 (208/245)	88.1 (148/168)	77.9 (60/77)	0.647

A-DWI: Absence of diffusion-weighted imaging; P-DWI: Presence of diffusion-weighted imaging; PPV: Positive predictive value; NPV: Negative predictive value.

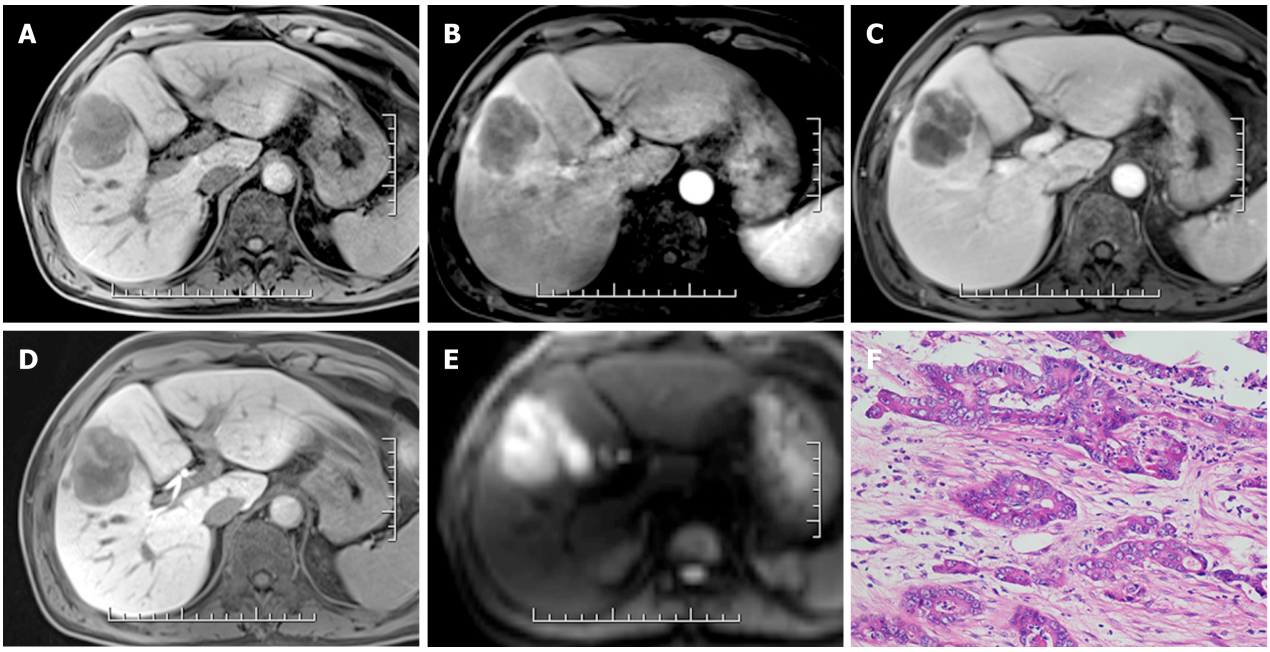

Figure 3 Pathologically confirmed intrahepatic cholangiocarcinoma in a 47-year-old man. A: A 53.2-mm hypointense mass is seen on the precontrast image; B: The image shows rim-like hyperenhancement in the arterial phase; C: Peripheral "washout" in the portal venous phase; D: Targetoid appearance in the hepatobiliary phase; and E: Target sign in diffusion-weighted imaging; F: The mass was confirmed as intrahepatic cholangiocarcinoma at 200 × magnification after hematoxylin-eosin staining.

ARTICLE HIGHLIGHTS

Research background

Hepatocellular carcinoma (HCC) is the fifth most common cancer and the third leading cause of cancer-related deaths. The Liver Imaging Reporting and Data System (LI-RADS), supported by the American College of Radiology (ACR), has been developed for standardizing the acquisition, interpretation, reporting, and data collection of liver imaging examinations in patients at risk for HCC. Ancillary features can be applied to upgrade or downgrade the initially assigned LI-RADS category based on major features only.

Research motivation

Magnetic resonance imaging (MRI) can be used for categorization of liver observations and diagnosis of HCC based on the major and ancillary features of LI-RADS. Gadoxetic-acid disodium (Gd-EOB-DTPA), a hepatobiliary contrast agent, could provide information of hepatocyte function. Diffusion-weighted imaging (DWI) can further quantitatively measure tissue diffusion and further reflect tumor cellularity. Thus, the combination of Gd-EOB-DTPA-enhanced MRI and DWI has the potential to improve the diagnostic accuracy (AC) for HCC.

Research objectives

In this study, we aimed to determine the usefulness of DWI in improving the diagnostic AC of LI-RADS v2017 classification. In addition, future research should focus on the comparison of LI-RADS v2017 and v2018.

Research methods

In this institutional review board-approved study, a total of 414 consecutive patients at high risk for HCC were enrolled. Two radiologists who were blinded to the clinical, laboratory, and pathology results reinterpreted the MR images. Each reader measured the maximum diameter and recorded the presence of each lesion and assigned scores according to LI-RADS v2017. The ancillary feature "restricted diffusion" on DWI images could be used at radiologist discretion for category adjustment (upgrade or downgrade). The kappa test was used to determine the agreement between the two independent radiologists in each item. In addition, the sensitivity (SE), specificity (SP), AC, positive predictive value (PPV), and negative predictive value (NPV) were calculated for assessing the diagnostic performance of LI-RADS. Youden index values were used to compare the diagnostic performance of LI-RADS with or without DWI.

Research results

For LR-5, the diagnostic SE, SP, and AC values were 61.2%, 92.5%, and 71.4%, respectively, with or without DWI; for LR-4/5, they were 73.9%, 80%, and 75.9% without DWI and 87.9%, 80%, and 85.3% with DWI; for LR-4/5/M, they were 75.8%, 58.8%, and 70.2% without DWI and 87.9%, 58.8%, and 78.4% with DWI; for LR- 4/5/TIV, they were 75.8%, 75%, and 75.5% without DWI and 89.7%, 75%, and 84.9% with DWI. The Youden index values of the LI-RADS classification without or with DWI were as follows: LR-4/5: 0.539 *vs* 0.679; LR-4/5/M: 0.346 *vs* 0.467; and LR-4/5/TIV: 0.508 *vs* 0.647. The remaining problems that exist should be solved by using prospective, multi-center study to verify our results.

Research conclusions

The ancillary feature "restricted diffusion" on DWI images could be used at radiologist discretion for category adjustment (upgrade or downgrade). The ability of DWI is to reflect the cellularity of tissue. Compared with normal tissue, tumor tissue with high cellularity could result in decreased extracellular space and limited water diffusion, represented by high signal intensity. In our study, a small number of lesions were classified as LR-3 without DWI; however, when DWI was added, these lesions were downgraded to LR-2 due to unrestricted diffusion. In addition, some lesions previously classified as LR-3 were upgraded to LR-4 due to restricted diffusion. Thus, our study also shows that the use of DWI can improve the diagnostic efficiency of LI-RADS v2017 with gadoxetic acid-enhanced MRI for HCC. We believe that our data, a pool of categorization results by several readers during actual MRI interpretation, can better be explained by clinical practice and may be broadly applied.

Research perspectives

Our study shows that LI-RADS v2017 has been successfully applied with gadoxetate-enhanced MRI for patients at high risk for HCC. The addition of DWI significantly increased the diagnostic efficiency for HCC. For the future research, we intend to investigate interobserver or intraobserver variability through a multi-center study and apply the latest 2018 version of LI-RADS.

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SYSTEMATIC REVIEWS

Fatigue in children and adolescents with inflammatory bowel disease

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Abstract

AIM

To identify factors other than active disease and anemia that contribute to fatigue in pediatric inflammatory bowel disease (IBD).

METHODS

We performed an electronic search in Medline and EMBASE from their inception to May 2017 using the search term "fatigue" or the related keywords "physical impairment" and "inflammatory bowel disease" with the filter "child" (age 0-18 years). Cross-sectional and case-control studies were included. We restricted our search to studies published in English. We used the PRISMA checklist and flow diagram. Duplicate articles were manually deleted in End Note. To identify further relevant studies, we checked the reference lists of the selected articles.

RESULTS

We identified 149 papers, of which 19 were retrieved for full text review. Eleven studies were subsequently excluded because fatigue was not evaluated as an outcome measure. Eight papers focused on the desired topic and were discussed in the final analysis. A lack of uniformity of outcome measures made the pooling of data impossible. In all but one study, questionnaires were used to evaluate fatigue. In the remaining study, an accelerometer was used to measure daily activities, sleeping time and their relationships with fatigue in a more

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quantifiable manner. Adolescents with IBD are significantly more fatigued than healthy controls. In addition to active disease, increased anxiety or depression and disturbed family relationships were frequently reported predictors of fatigue. Quantitative measurement of physical activity in patients with Crohn's disease showed a reduction in the number of steps per day, and patients with ulcerative colitis had a shorter duration of physical activity during the day.

CONCLUSION
Fatigue in pediatric IBD is related to a combination of biological, functional and behavioral factors, which should all be taken into account when managing fatigue.

Key words: Adolescents; Children; Fatigue; Inflammatory bowel disease; Physical impairment; Sleep

Core tip: Children and adolescents with inflammatory bowel disease (IBD) often report fatigue as their most severe and distressing symptom. Fatigue is often attributed to active disease and anemia. We systematically reviewed the literature to identify additional factors that contribute to fatigue in pediatric IBD. After a strict selection process, eight studies were suitable for detailed data extraction. Increased anxiety or depression and disturbed family relations were frequently reported predictors of fatigue. This systematic review demonstrates the importance of evaluating biological, functional, and psychobehavioral factors to facilitate the optimal management of fatigue.

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INTRODUCTION

Inflammatory bowel disease (IBD) is a chronic disease of the gastrointestinal tract. The disease is characterized by relapsing periods of inflammation and remission and usually presents with abdominal pain, diarrhea, rectal bleeding and weight loss[1]. The ultimate goal in IBD treatment is to reach clinical remission as quickly as possible. Fatigue and decreased physical fitness may continue to affect a patient's daily life despite disease remission. Ten percent of patients with IBD are diagnosed before the age of 19 years[2].

Fatigue refers to a subjectively overwhelming sense of tiredness, lack of energy, and feeling of exhaustion that decreases one's capacity for physical and mental activity[3]. It is a common, independent, and nonspecific symptom identified in numerous chronic health conditions in childhood[4]. In adults with chronic disease, fatigue can be a major source of disablement and is often reported as being among the most severe and distressing symptoms[5]. It affects physical, emotional, cognitive, and social functioning, impacting quality of life. Nevertheless, fatigue has typically been ignored in the assessment of symptom severity or outcome in many diseases in which it is observed[5].

The quantification of fatigue is challenging due to the lack of a consensus framework, vague terminology, and the multidimensional nature of symptoms. Subjective methods, such as self-reported or parent-reported surveys[6,7], are commonly used but can be distorted by response and recall bias. More objective methods, such as polysomnography and performance tests[8-10], are expensive and time-consuming. Furthermore, the prevalence of fatigue varies among healthy pediatric age groups; it is common in infancy, early childhood, and late adolescence and less frequently observed during mid-childhood; it is more common in girls than in boys[9].

We aimed to systematically review the literature to identify factors that contribute to fatigue in children and adolescents with IBD.

MATERIALS AND METHODS

Identification and selection of studies

We searched for studies published in Medline and EMBASE up to May 2017. The search strategy for Medline was as follows: ("fatigue" [MeSH Terms] OR "fatigue" [All Fields]) AND ("inflammatory bowel diseases"[MeSH Terms] OR ("inflammatory" [All Fields] AND "bowel" [All Fields] AND "disease" [All Fields] OR "inflammatory bowel disease" [All Fields]); ("physical examination" [MeSH Terms] OR ("physical" [All Fields] AND "examination"[All Fields]) OR "physical examination" [All Fields] OR "physical" [All Fields]) AND impairment [All Fields]) AND ("inflammatory bowel diseases"[MeSH Terms] OR ("inflammatory"[All Fields] AND "bowel"[All Fields] AND "diseases"[All Fields]) OR "inflammatory bowel diseases"[All Fields] OR ("inflammatory"[All Fields] AND "bowel"[All Fields] AND "disease"[All Fields]) OR "inflammatory bowel disease"[All Fields]) , with the filter "child" (age 0-18 years). For EMBASE, the search strategy was as follows: ("fatigue"/exp OR fatigue) AND Inflammatory AND ("bowel"/exp OR bowel) AND ("disease"/exp OR disease). We restricted our search to studies published in English. Duplicate articles identified in both Medline and EMBASE were manually deleted in End Note. To identify additional relevant studies, we checked the reference lists of the selected articles.

We selected cross-sectional or case-control studies reporting on fatigue (or its synonyms) in patients under the age of 19 years with IBD. Two reviewers (Van de Vijver E and Van Gils A) independently screened the abstracts of all identified articles to determine their eligibility. Any disagreements regarding the inclusion of articles were solved by discussion until consensus was reached.

Quality assessment and data extraction

Study quality was assessed using the online criteria for case-control and cross-sectional studies[11]. Each item was scored as "yes", "no", or "not reported".

The guidelines of the PRISMA 2009 Statement were adopted.

RESULTS

Study selection

This study includes paper retrieved by electronic searches up to May 2017. In total, 149 papers were identified, of which 19 were retrieved for full-text review. Eleven were subsequently excluded because fatigue was not evaluated as an outcome measure. Eight focused on the desired topic and were discussed in the final analysis (Figure 1).

The selected studies varied considerably with regard to the fatigue assessment method, which made pooling of data impossible (see Table 1).

Six papers reported fatigue or physical activity related to IBD as their primary outcome. The remaining two studies reported quality-of-life as the primary outcome; one used a quality-of-life questionnaire and evaluated the domain "motor functioning" separately, while the other conducted a semi structured interview with questions about the functional impact of the disease. The methodological quality of the studies is summarized in Table 2.

Assessment of fatigue

Seven of eight papers used subjective methods, such as questionnaires, to evaluate fatigue[6,7,12-16]. Most research teams used self-reported surveys [IMPACT-III, semistructured interviews, Youth Self Report (YSR), Sleep Self Report (SSR), RCMAS, KINDL, KIDSCREEN and TACQOL][7,8,12,13,15,16], while others used a combination of parent proxy-reported and self-reported surveys (PEDSQL multidimensional fatigue scale and PedsQL 4.0 generic care scale)[6]. Only one paper used a parent proxy-reported questionnaire [Child Behavior Checklist (CBCL)][16]. Table 3 describes the myriad of fatigue-related diagnostic tests that were used in the included studies.

Scientists from Chicago and Texas performed a cross-sectional study among 70 children with IBD and 157 healthy controls and their parents[6]. They categorized fatigue as general fatigue (*e.g.*, "feeling tired"), sleep/rest fatigue (*e.g.*, "feeling tired when waking up") or cognitive fatigue (*e.g.*, "attention problem")[6] based on the PedsQL Multidimensional Fatigue Scale[11]. General fatigue and sleep/rest fatigue were more frequently observed in pediatric IBD patients than in healthy control subjects, even when their disease was in remission. Differences in cognitive fatigue were not observed[6]. A Canadian team from Toronto conducted in-depth interviews among 80 children and adolescents who were purposively selected for their variation

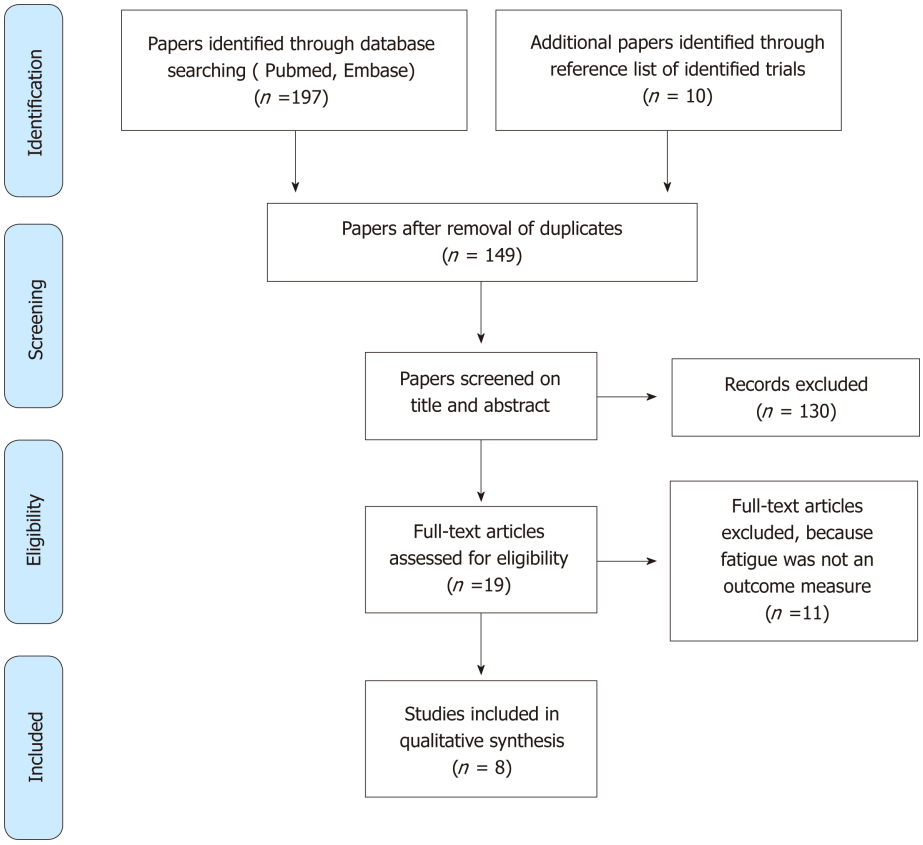

Figure 1 Study selection.

in age and condition and found that children and teenagers with IBD commonly mentioned that "exhaustion" and "malaise" ("having no energy and being tired") had large impacts on their lives[13]. A Finnish research team evaluated sleep problems and daytime tiredness in 160 adolescents by both a parent proxy-reported survey (CBCL) and a self-reported questionnaire (YSR)[17]. Twenty-five percent of parents reported that their adolescent child had trouble sleeping. This was a significantly greater percentage than was found among the parents of healthy controls. Overall, parents of adolescents with IBD more commonly reported sleeping during the day and night and overtiredness than did parents of healthy controls. The self-reported questionnaire did not confirm the high prevalence of sleep-related problems among IBD patients when compared to healthy subjects (11% *vs* 16%)[16].

A Swiss research team evaluated 110 adolescents with IBD who were included in the national IBD Cohort Study[7]. They assessed fatigue as physical activity using the KIDSCREEN 27. Physical well-being (*e.g.*, "feeling fit, being physical active, able to run") was only moderately disturbed in IBD patients compared to healthy controls[7].

A German research group conducted the only study that evaluated fatigue in an objective manner with a wearable device. They assessed physical activity using the SenseWear Pro2 armband (a portable motion sensor) and reported a trend towards a shorter duration of physical activity and significantly prolonged sleep duration in patients with mild IBD compared to controls, but there were no statistically significant differences[8].

Biological factors related to fatigue

Disease activity: All included studies observed a positive correlation between disease activity and fatigue, but the scoring systems used to discriminate active disease from disease remission differed among the papers. The team from Chicago and Texas used the Pediatric Crohn's Disease Activity Index (PCDAI) and defined disease remission as a score < 10. They reported that children with active Crohn's disease (CD) had significantly more symptoms of general fatigue ("feeling tired") and sleep/rest fatigue ("feeling tired when waking up") than children and teenagers in remission[6]. The Finnish study among 160 children and teenagers used a visual analogue scale (VAS) to measure disease activity. Children with severe IBD (VAS scores above 3) had significantly more trouble falling asleep (41% *vs* 22%), felt significantly more overtired (80% *vs* 44%) and had significantly longer sleep duration than adolescents with less

Table 1 General characteristics of the included studies

First author (year of publication)	Study objectives	Age (yr)	Patient population	Percentage of patients with active disease	Main findings related to fatigue
Marcus *et al* (2009)[6]	To evaluate the degree of fatigue and health-related quality-of-life in children with IBD	10-17	52 CD; 13 UC; 5 IBD-U; 157 healthy controls	Remission 56%; Mild 22%; Moderate 17%; Severe 5%	Adolescents with IBD have significantly more fatigue than healthy controls; PedsQL total fatigue, general fatigue, and sleep/rest fatigue were all impaired in patients with IBD; Adolescents with IBD are fatigued even when clinical remission is reached
Nicholas *et al* (2007)[13]	To understand the lived experience and elements of quality-of-life in adolescents and adolescents with IBD	7-19	61 CD; 19 UC	Not reported	Young patients with IBD commonly feel "sick and tired" and have "no energy"
Pirinen *et al* (2010)[16]	To evaluate the effect of disease severity on (the frequency of) sleep problems and daytime-tiredness among adolescents with IBD	10-18	53 CD; 83 UC; 24 IBDU; 236 healthy controls	Not reported	Adolescents with IBD do not report more sleeping problems or overtiredness than their healthy peers Adolescents with active disease have significantly more trouble sleeping, more daytime sleepiness and are overtired compared to adolescents with mild IBD symptoms; Adolescents with severe IBD symptoms have worse quality of sleep and more sleep disturbances than those with less severe IBD
Werkstetter*et al* (2012)[8]	To evaluate whether physical activity is reduced in patients with IBD compared to control subjects	6-20	27 CD; 12 UC; 39 healthy controls	Remission 66%; Mild 34%	Patients with IBD show a trend toward less physical activity, especially among girls and those with mild disease activity; There is no relation between inflammatory markers (CRP) and physical activity
Rogler *et al* (2013)[7]	To examine the determinants of health-related quality-of-life in adolescents and adolescents with IBD	11-15	64 CD; 46 UC	PCDAI > 15 36%; PUCAI ≥ 10 28%	Patients with IBD (in particular boys) have moderate impairments in physical well-being; Impairment in physical well-being is associated with active inflammation; And its symptoms
Loonen *et al* (2002)[12]	To evaluate the impact of IBD on health- related quality of life	8-18	41 CD; 40 UC; 2 IBD-U	Mild 60%; Moderate 23%; Severe 15%; Missing 2%	Adolescents with IBD have impairments in motor functioning (running, walking, playing) and complain more of tiredness, especially those with Crohn's disease.

Tojek *et al* (2002)[14]	To examine family dysfunction, maternal physical symptoms and maternal positive affect as correlates of health status in adolescents with IBD	11-18	36 CD; 26 UC	Not reported	Family dysfunction is related to an increased frequency of fatigue in adolescents; Maternal positive affect is inversely related to fatigue (not significant); Fatigue is independent of maternal negative affect
Ondersma *et al* (1996)[15]	To examine how psychological factors relate to disease severity among adolescents with IBD	11-17	34 CD; 22 UC	Not reported	There is a relationship between negative affect and physical symptoms of fatigue

CD: Crohn's disease; IBD: Inflammatory bowel disease; IBD-U: Inflammatory bowel disease-unclassified; PCDAI: Pediatric Crohn's Disease Activity; PedsQL: Pediatric Quality of Life; PUCAI: Pediatric Ulcerative Colitis Activity Index; UC: Ulcerative colitis.

active disease (VAS score below 3). In that study, the results of the self-reported questionnaires and the parent reports were very similar when the adolescents had higher VAS scores, but this was less true in the parent-adolescent pairs with mild IBD symptoms[10,16].

Medication: The research team from Chicago and Texas evaluated the association between fatigue and medication and concluded that the use of mesalamine, thiopurine or anti-tumor necrosis factor (TNF) were not predictors of fatigue as measured with the PedsQL Fatigue Scale[6].

Psychobehavioral factors related to IBD

Family support: A group from Detroit found a significant association between fatigue and dysfunction in the family[14]. The researchers used the McMaster Family Assessment Device[18]. They also evaluated two additional items created by the authors themselves, which assessed the frequency of IBD-related pain and IBD-related fatigue over the past 3 mo. They found that maternal positive affect, including being attentive, active, and interested, was inversely related to fatigue but the association was not significant. Fathers were not included in the study because they almost never accompanied their children to the clinic, and a considerable proportion of the adolescents did not have fathers living with them[14].

Psychological variables: In another paper, the Detroit team assessed 56 adolescents with IBD (aged between 11 and 17 years) with the Revised Children's Manifest Anxiety Scale and found that adolescents with a negative affect (*i.e.*, those who reported anxiety and depression) also experienced more pain and fatigue[15]. The group from Chicago and Texas used the Children's Depression Inventory and found that adolescents with primarily inactive IBD did not report more depressive symptoms than healthy controls (1.4% *vs* 1.3%)[6].

Functional factors related to IBD

Disease type: The studies that used questionnaires to assess fatigue did not observe differences between CD and ulcerative colitis (UC) patients[8,12,16]. The German research group that evaluated physical activity with a wearable device found that patients with CD tended towards taking fewer steps per day[8], and UC patients had a shorter duration of physical activity compared with healthy controls[8].

DISCUSSION

Eight studies were included in this systematic review. These studies were selected for their focus on fatigue in adolescents with IBD.

Key findings

This review demonstrates that fatigue, exhaustion, diminished physical activity and trouble sleeping are more common in children and adolescents with IBD than in their healthy peers. Fatigue is likely to be a multifactorial phenomenon and includes biological factors (such as disease activity), psychobehavioral factors (such as anxiety, depression and family support) and functional factors (such as decreased functional capacity). The model depicted in Figure 2 addresses the various etiological factors and the connection with the fatigue-related diagnostic tests mentioned in this paper. The

Table 2 Methodology and quality assessment

First author (year of publication) and study type	Patient selection	Disease activity score	Fatigue score	Study quality
Marcus *et al* (2009)[6] Case-control study	Patients: recruited during scheduled clinical appointments at University Hospital, United States; Healthy controls: adolescent children of hospital employees	CD: PCDAI; CU and IBDU: PGA	PedsQL Multidimensional Fatigue Scale, IMPACT-III, PedsQL 4.0 Generic Core Scales Children's Depression Inventory: Short Form	Good: no sample size justification
Nicholas *et al* (2007)[13] Cross-sectional study	Patients: recruited from the database of Reference Children's Hospital, Canada	No distinction made	Semi structured interview designed by author	Poor: Patients purposively selected, questionnaires not validated, participation rate not reported
Pirinen *et al* (2010)[16] Case-control study	Patients: recruited from the database of the Population Register Center, Finland; Healthy controls: matched	VAS disease severity	Youth self-reported questionnaire, Sleep Self Report, child behavior checklist	Medium: Subjective score to assess disease severity, exact sleep duration unknown
Werkstetter *et al* (2012)[8] Case-control study	Patients: recruited from University Hospital, Germany; Healthy controls: matched	CD: PCDAI; UC: PUCAI	SenseWear Pro2 accelerometer, German KINDL, IMPACT III	Good: no sample size justification
Rogler *et al* (2013)[7] Cross-sectional study	Patients: recruited from Swiss IBD cohort study, Switzerland	CD: PCDAI; UC: PUCAI	KIDSCREEN-27	Medium: numbers in text and table do not match
Loonen *et al* (2002)[12] Cross-sectional study	Patients: recruited from a database of two large tertiary referral centers, Netherlands	5-item symptom card (completed by patients)	TACQOL, IMPACT-II	Good: validated questionnaires, the results compared with healthy controls
Tojek *et al* (2002)[14] Cross-sectional study	Patients: recruited from routine outpatient visit in 2 urban pediatric gastroenterology hospitals, United States	No distinction made	Questions designed by author	Medium: parental factors can influence adolescent's health, the converse remains possible, only mothers investigated, questionnaires not validated
Ondersma *et al* (1996)[15] Cross-sectional study	Patients: recruited from 2 pediatric gastroenterology hospitals, United States	No distinction made	10-item Subjective Illness Questionnaire (parts or RCMAS and CDI)	Medium: no sample size justification, parts of validated questionnaires

CDI: Children's Depression Inventory; CD: Crohn's disease; IBD: Inflammatory bowel disease; PCDAI: Pediatric Crohn's Disease Activity Index; PedsQL: Pediatric Quality of Life; PGA: Physician Global Assessment; PUCAI: Pediatric Ulcerative Colitis Activity Index; RCMAS: Revised Children's Manifest Anxiety Scale; UC: ulcerative colitis.

model highlights the importance of the multifaceted nature of fatigue, and this fatigue model could act as a guide on which to base treatment interventions.

Biological factors: Fatigue is a common finding[6] in children and adolescents with IBD, and several studies have shown a positive relationship between the degree of disease activity and fatigue. Adolescents with active IBD experience more fatigue than their peers in disease remission, who, in turn, experience more fatigue than healthy controls. It is plausible that active disease impairs sleep quality due to nocturnal abdominal pain and diarrhea. Inflammation and immune activation, together with the subsequent activation of glial cells and mitochondrial damage, likely account for the severe levels of intractable fatigue and disability seen in patients with autoimmune diseases[19].

Adolescents in clinical remission are fatigued, but patients in deep remission were not assessed: deep remission could have an impact on less fatigue.

Reduced muscle mass[20] and anemia[21-23], both of which are frequently observed in patients with IBD, even when their disease is in remission, may also have affect fatigue, but so far, these factors have not been investigated in the adolescent IBD population.

Psychobehavioral factors: The papers that sought correlations between psychobehavioral factors and fatigue showed conflicting results. One paper[14] linked anxiety, depression and lack of family support with IBD-related fatigue, while another paper failed to show that depression occurs more often in adolescents with IBD than in their healthy peers[6]. Sleep disorders can affect the feeling of being tired,

Table 3 Description of fatigue-related diagnostic tests

Abbreviation	Full name	Details
CBCL	Child Behavior Checklist	Caregiver report form that categorizes problem behaviors in preschool and school-aged children in the following 8 syndromes: aggressive, anxious-depressed, attention, rule-breaking, somatic complaints, social, thought, withdrawn-depressed.
CDI	Children's Depression Inventory	Adolescent self-reported assessment. For each of 26 items, respondents endorsed one of three sentences indicating varying levels of depression.
IMPACT-III	Not applicable	IBD disease-specific health-related quality-of-life questionnaire for pediatric patients. It is composed of 35 items in the following 6 domains: IBD-related symptoms (7 items), systemic symptoms (3), emotional functioning (7), social functioning (12), body image (3) and treatment/intervention-related concerns (3). Each item is scored on a 5-point Likert scale, coded from 0 to 4 points. Higher scores indicate better quality of life.
KIDSCREEN 27	Not applicable	Self-reported survey is a quality of life questionnaire consisting of 27 items measuring physical well-being, psychological well-being, autonomy and parent relations, peers and social support, and school environment.
KINDL	Not applicable	Adolescent self-reported survey consists of 24 Likert-scaled items, which are subdivided into the following six dimensions (subscales) of quality of life: physical well-being, emotional well- being; self-worth, well-being in the family, well-being regarding friendships and well-being at school.
McMaster Family Assessment Device	Not applicable	Adolescent self-reported 60-item instrument that assesses six domains, namely, problem solving, communication, roles, affective responsiveness, affective involvement, behavior control and general functioning of family functioning as well as general family dysfunction.
PedsQL generic scale	Pediatric Quality of Life Inventory	Parent reported and self-reported assessment. A modular approach to measuring health-related quality of life (HRQOL) in healthy children and adolescents and those with acute and chronic health conditions. It contains the following four multidimensional scales: physical functioning, emotional functioning, social functioning, school functioning.
PedsQL Multidimensional Fatigue Scale	Pediatric Quality of Life Inventory Multidimensional Fatigue Scale	Age-appropriate versions and parallel forms for children and parents. It measures the perceptions of fatigue by children and their parents and has been validated in a variety of pediatric chronic diseases.
RCMAS	Revised Children's Manifest Anxiety scale	Adolescent self-reported assessment that is a true/false anxiety measure containing 28 items. The measured key areas are physiological anxiety, worry, social anxiety and defensiveness. The scale differentiates between anxiety-disordered and normal Children.
SSR	Sleep Self Report	Adolescent self-reported assessment to discern sleep patterns and possible difficulties with sleep.
TACQOL	TNO-AZL Children's Quality of life Questionnaire	Generic health-related quality of life questionnaire enabling comparisons between groups of children with varying chronic diseases. It includes 7 scales, involving general physical function, motor function, daily function, cognitive function, social contact, and positive and negative moods.

YSR	Youth Self Report	Adolescent self-reported assessment with the following eight empirically-based syndrome scales: anxious/depressed, withdrawn/depressed and somatic complaints composing the internalizing (*i.e.*, emotional) broad-band scale; rule-breaking behavior and aggressive behavior composing the externalizing (*i.e.*, behavioral) broad-band scale; and these two scales, together with the syndrome scales of social, thought and attention problems, compose the total problems scale.

as shown in 2 of the included papers. Sleep deprivation leads to more anxiety and depression and to an increase in somatic complaints and aggressive behavior[13,16,17]. Sleep itself was not often a research objective; only one study had sleeping problems as an outcome measurement[16], while a German study only reported a trend towards prolonged sleep duration in patients with mild IBD compared to healthy controls[8].

Functional factors: Only one paper focused on functional capacity in relation to fatigue and used a wearable device to plot activity over time; this study did not find a significant difference between patients with IBD and healthy controls. It is rather surprising that only one paper looked at functional capacity in adolescents with IBD because it is a frequently used outcome measurement in other chronic diseases[24-27].

Comparison with adult-oriented publications

In 2010, a systematic review[3] identified 10 papers about fatigue in patients with IBD and mentioned that the topic deserved more attention, as the prevalence of fatigue approached 50% in patients with IBD in remission and up to 86% in patients with active IBD. Cuzber-Dochan and colleagues published a systematic review in 2013 that included 28 papers on adults, and they concluded that the use of terminology regarding fatigue is inconsistent and that knowledge of the causes, severity and ways of measuring IBD fatigue is incomplete[28]. Three years later, the same research grouped repeated the literature search and identified a number of psychosocial and physical factors that could potentially be modified through targeted health interventions to improve fatigue in IBD. As in this study, they concluded that fatigue is multifactorial and is associated with active disease, poor sleep quality, anxiety and depression, but the complex interplay between these factors has yet to be deciphered[29].

In studies among adolescents, disease activity and sleep quality are also related to fatigue, but the relationship with anxiety and depression is unclear. Approximately one-quarter of adolescents with IBD have somatic or cognitive symptoms of depression[30], and this is comparable with the prevalence observed in their healthy peers.

Depression among adult patients with IBD, on the other hand, is more common compared to among control subjects[12,31-33].

Previous studies described a poor to low degree of parent-adolescent agreement on psychosocial symptoms[17,30]. Moreover, adolescents and parents report different symptoms. Therefore, to gain a comprehensive picture of the complaints in adolescents with IBD, both the adolescents and their parents need to be questioned[34].

Methodological limitations of the review

The cross-sectional design of most included studies precludes the ability to draw conclusions concerning the causal relations between variables. Prospective observational cohort studies are needed to gain more insight into the direction and mechanism of the identified associations. If prospective cohort studies are conducted in ethnically and socioeconomically diverse groups of children and adolescents, causative factors of fatigue can be identified, and these could potentially lead to more efficacious ways of treating fatigue in adolescents with IBD.

Implications for clinical practice

Future research opportunities: The mechanism underlying fatigue in children and adolescents with IBD remains poorly understood. Fatigue is a subjective sensation and presents with a multitude of symptoms, which makes it difficult to describe, measure and quantify. Past studies have mainly focused on one aspect of fatigue. Future studies should explore fatigue manifestations at several levels simultaneously, including illness-related aspects (such as ongoing inflammation, disease activity, medication use and pain), physical functioning (health-related quality of life, sleep quality and disability), and psychobehavioral factors.

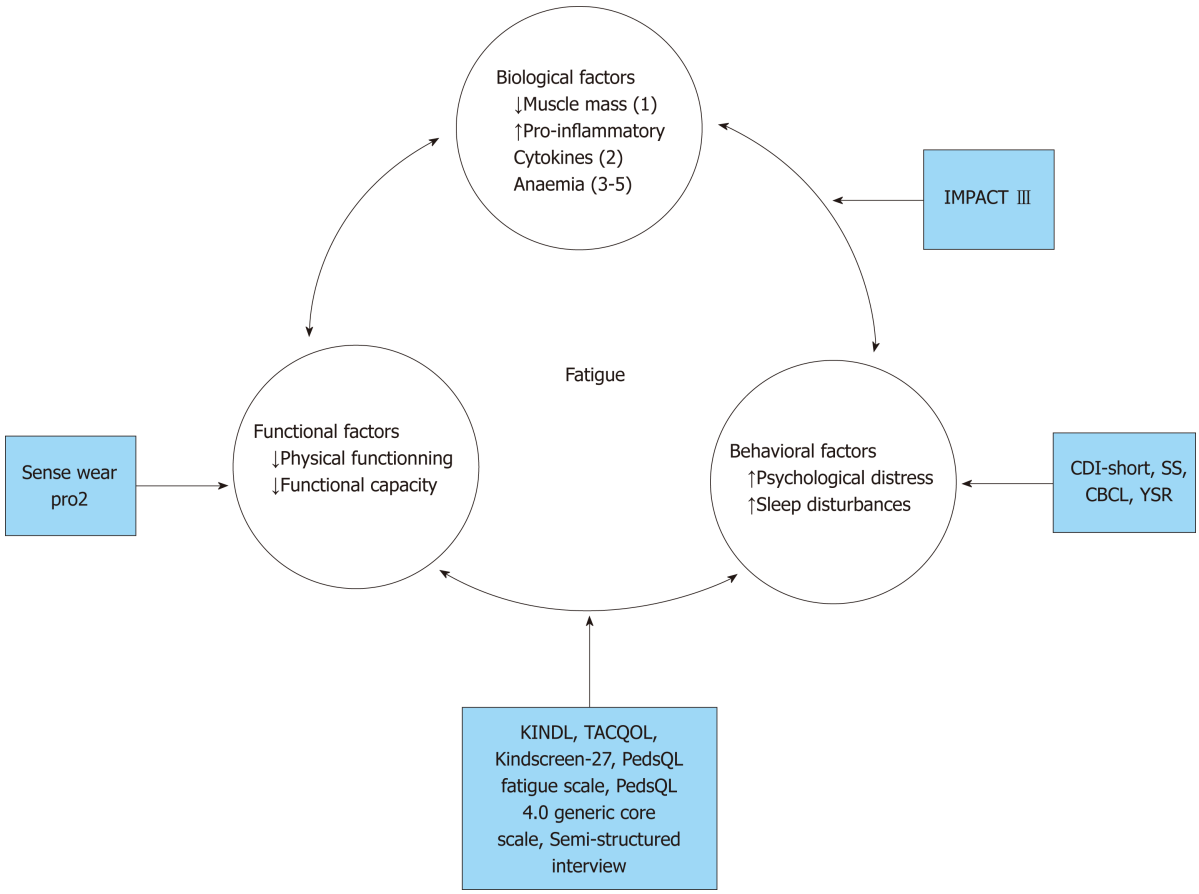

Figure 2 Multidimensional fatigue model depicting the biological, psychobehavioral and functional factors that play roles in the etiology of fatigue. The fatigue-related diagnostic tests mentioned in the rectangles are also mentioned in this paper. CDI: Children's Depression Inventory; CBCL: Child Behavior Checklist; SSR: Sleep Self Report; YSR: Youth Self Report.

Conclusion

Fatigue is a common problem in children and teenagers with IBD, and it is significantly more prevalent among young patients with IBD than in the healthy control population. It is multidimensional and caused by both physical and psychosocial factors. The most predictive factor seems to be disease activity. Health care providers need to pay attention to this problem because it is associated with reduced quality of life, increased sleeping problems and increased anxiety. The multifactorial nature of fatigue necessitates multilevel testing.

ARTICLE HIGHLIGHTS

Research background

Children and adolescents with inflammatory bowel disease (IBD) regularly report fatigue as their most severe and distressing symptom. Fatigue is often attributed to active disease and anemia, but also in quiescent IBD, fatigue can trouble daily life.

Research motivation

The ultimate goal in IBD management is not only to reach disease remission, but also to counteract fatigue and decreased physical fitness

Research objectives

We aimed to systematically review the literature to identify factors that contribute to fatigue in children and adolescents with IBD.

Research methods

We performed an electronic search in Medline and EMBASE from their inception to May 2017 using the search term "fatigue" or the related keyword "physical impairment" and "inflammatory bowel disease" with the filter "child" (age 0-18 years). Cross-sectional and case-control studies were included. We restricted our search to studies published in English. To identify further relevant studies, we checked the reference lists of the selected articles.

Research results

We ultimately identified eight papers that matched the search criteria. A lack of uniformity of outcome measures made the pooling of data impossible. In all but one study, questionnaires were used to evaluate fatigue. In the remaining study, an accelerometer was used to measure daily activities, sleeping time and their relationships with fatigue in a more quantifiable manner. Adolescents with IBD are significantly more fatigued than healthy controls. In addition to active disease, increased anxiety or depression and disturbed family relationships were frequently reported predictors of fatigue. Quantitative measurement of physical activity in patients with Crohn's disease showed a reduction in the number of steps per day, and patients with ulcerative colitis had a shorter duration of physical activity during the day.

Research conclusions

Fatigue is a common problem in children and teenagers with IBD, and it is significantly more prevalent among young patients with IBD than in the healthy control population. It is multidimensional and caused by both physical and psychosocial factors. The most predictive factor seems to be disease activity. Health care providers need to pay attention to this problem because it is associated with reduced quality of life, increased sleeping problems and increased anxiety.

Research perspectives

The multifactorial nature of fatigue necessitates multilevel testing. Fatigue in pediatric IBD is related to a combination of biological, functional and behavioral factors, which should all be taken into account when managing fatigue.

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