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Gastrointestinal neuromuscular apparatus: An underestimated target of gut microbiota

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Abstract

Over the last few years, the importance of the resident intestinal microbiota in the pathogenesis of several gastrointestinal diseases has been largely investigated. Growing evidence suggest that microbiota can influence gastrointestinal motility. The current working hypothesis is that dysbiosis-driven mucosal alterations induce the production of several inflammatory/immune mediators which affect gut neuro-muscular functions. Besides these indirect mucosal-mediated effects, the present review highlights that recent evidence suggests that microbiota can directly affect enteric nerves and smooth muscle cells functions through its metabolic products or bacterial molecular components translocated from the intestinal lumen. Toll-like receptors, the bacterial recognition receptors, are expressed both on enteric nerves and smooth muscle and are emerging as potential mediators between microbiota and the enteric neuromuscular apparatus. Furthermore, the ongoing studies on probiotics support the hypothesis that the neuromuscular apparatus may represent a target of intervention, thus opening new physiopathological and therapeutic scenarios.

Key words: Microbiota; Gastrointestinal motility; Smooth muscle; Enteric nervous system; Probiotics; Irritable bowel syndrome

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Core tip: This article reviews the current evidence of gut microbiota and neuromuscular apparatus connection that results to be both direct and indirect. Besides dysbiosis-driven mucosal inflammatory mediators, recent evidence suggests that gut neuromuscular apparatus can be modulated directly by microbiota metabolic products or circulating bacterial molecular components translocated from the intestinal lumen.

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INTRODUCTION

Microbiota and gut motility are clearly associated, but it's difficult to establish what plays the major role in influencing the other. According to the classical theory, gastrointestinal (GI) motility can affect the microbiota in terms of amount, location and diversity. This concept is mainly supported by the association between different GI motility disorders and small intestinal bacterial overgrowth (SIBO)^[1,2]. GI motility disorders and alterations of migrating motor complex (MMC), that eliminates residual content through the GI tract during periods of fasting, predispose to SIBO because bacteria are not swept from the small bowel into the colon, as reported in experimental models and specific clinical conditions^[3-5]. Neuropathic and myopathic diseases, such as scleroderma and polymyositis, seem to be associated with SIBO^[1,6] as well as conditions associated to long-standing diabetes, such as gastroparesis^[7].

On the other hand, both *in vivo* and *in vitro* evidence highlights that microbiota can affect GI motility^[8,9]. In studies conducted on germ-free animals, impairment of neural and motor functions of the GI tract due to reduced expression of neurotransmitters and contractile proteins, were reversed by gut colonization^[10]. Moreover, probiotics have been shown to affect GI motility *in vivo* and *in vitro*. Prebiotic or probiotic therapies are associated with a significant clinical improvement in irritable bowel syndrome (IBS)^[11,12] and animal studies suggest that the neuromuscular apparatus could represent a target for probiotics^[13-15]. Finally, dysbiosis is associated with significant alterations in intestinal transit time^[16].

By interacting directly with mucosal environment, the microbiota impacts intestinal mucosal functions and permeability, and influences local and systemic inflammatory activity^[12]. In normal conditions neuromuscular apparatus is not in contact with the luminal content and quite inaccessible by the luminal microbes. However, dysbiotic conditions cause an increase in mucosal inflammation and intestinal paracellular permeability^[17,18] (Figure 1) with possible translocation of pathogens, toxins, antigens and bacteria in the circulatory system^[16,19,20]. GI motility might then be affected by microbiota essentially by two mechanisms: an indirect mechanism driven by the inflammatory mediators released by the mucosal immune system and a direct mechanism driven both by the release of end products of bacterial

fermentation and bacterial substances.

INDIRECT EFFECTS

The potential for the microbiota to produce inflammatory alterations in the gut microenvironment deranging gastrointestinal motor function prompts to a unifying hypothesis for the role of the microbiota in the pathogenesis of IBS. To support a role of the microbiota in IBD pathophysiology is the evidence that an acute episode of gastroenteritis precedes the onset of IBS, a specific condition called post-infectious IBS (PI-IBS)^[11,21,22]. PI-IBS is characterized by persistent abdominal discomfort, bloating and diarrhea, despite the elimination of the causative pathogen. In this condition, the imbalance in microbiota composition leads to low-grade inflammation followed by alteration of the sensory and motor bowel functions. An increased amount of immune cells in the colonic, ileal, and jejunal mucosa of IBS patients has been largely reported^[23,24]. The persistent inflammatory state is also characterized by increased mucosal interleukin 1 β levels and mast cells count, as well as activation of entero-endocrine cells (EC), mainly those producing serotonin (5-HT)^[25-28]. The interesting data is that most of these mucosal alterations persist for over a year and thus could contribute to the persistence of a PI-IBS. Therefore, the mucosal inflammation resulting from an acute infection can lead to a dysfunction of intestinal motility and 5-HT could play a pivotal role as its release increases motility and secretion, features which may explain diarrheal symptoms frequent in PI-IBS patients^[29]. With an experimental model of primary infection with *Trichinella spiralis*, that causes hypercontractility of intestinal muscle persisting for over 20 d after the infection was cleared, it was shown that chronic immune response may extend to smooth muscle layers^[30]. In this model, the levels of Th2 cytokines (interleukins 4, 5, and 13) resulted increased during the acute infection but not thereafter, whereas cyclooxygenase-2 (COX-2) and relative enzymatic activity localized to muscle remained significantly increased. These effects did not occur in athymic mice, suggesting a crucial role of T cells in the impairment of intestinal muscle function in post-infective disorders^[30]. The role of COX-2 in muscle impairment during inflammation has been reported both in animal and humans. During severe mucosal inflammatory conditions, it has been shown in colonic muscle cells an altered expression of contractile key-signaling molecules and an increase in nuclear factor NF- κ B DNA binding, which is low or absent in normal colonic muscle cells^[31-33]. In human colonic smooth muscle, NF- κ B activation leads to inflammatory gene expression of COX-2 and to production of prostaglandin E, both widely considered responsible for muscle cell impairment^[34-37].

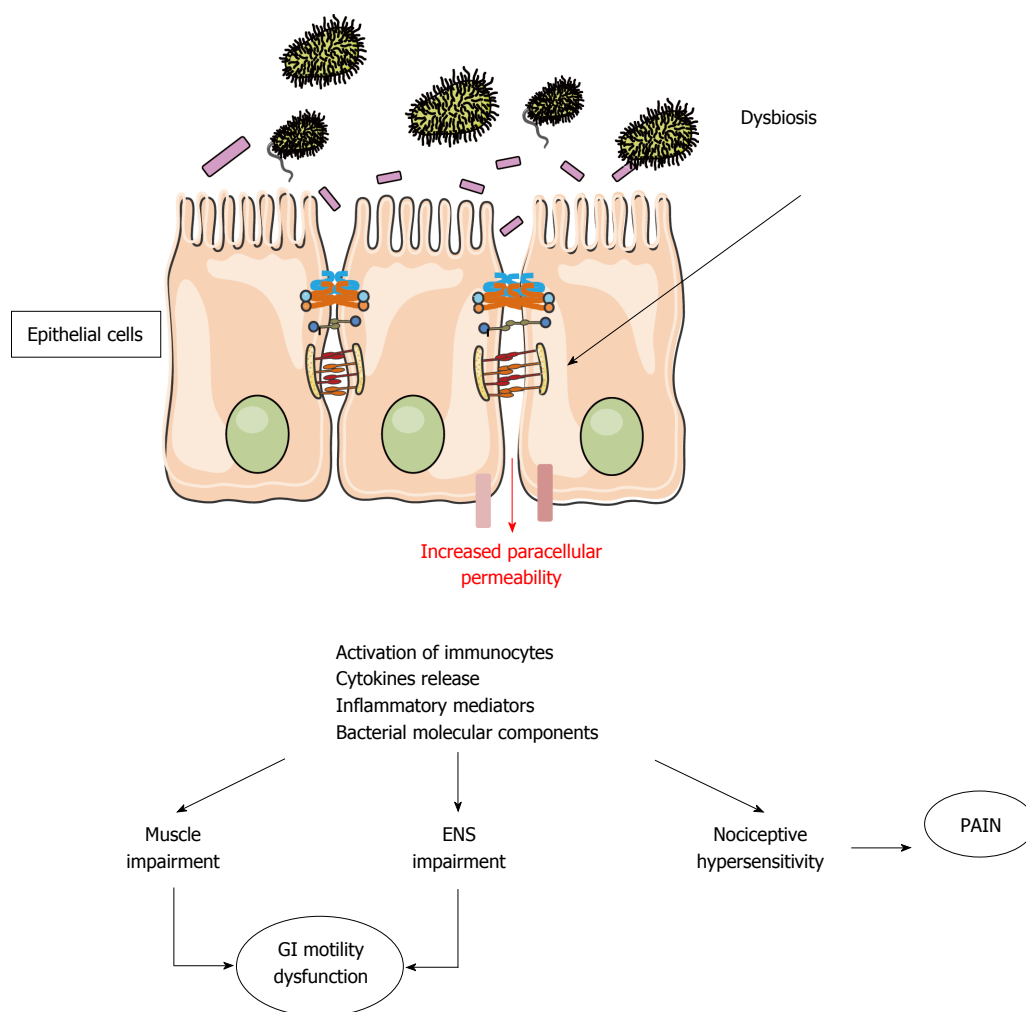


Figure 1 Dysbiosis and intestinal motility disorders. One hypothesis regarding the pathogenesis of functional intestinal disorders suggests that dysbiosis increases paracellular permeability leading to translocation of luminal contents with activation of immunocytes, cytokines and inflammatory mediators release. The activation of this state of inflammation and the presence of bacterial components, such as LPS, lead to nociceptive hypersensitivity, thus explaining the pain, and to enteric nervous system (ENS) or muscle impairment, thus explaining the intestinal motor disorders. LPS: Lipopolysaccharide.

Mediators released by the colonic mucosa of IBS patients are able to activate aberrant responses in the enteric nervous system^[38,39] and to impair contractility of human colonic smooth muscle likely through a receptor-dependent mechanism^[40]. Histamine and proteases, two soluble inflammatory products obtained from IBS biopsy supernatants, are able to excite visceral afferents neurons and to cause hyperalgesia and allodynia when introduced into the colon of mice^[41,42]. Beside increased visceral sensory activation, the soluble products found in supernatants derived from the colon of IBS patients have been shown to evoke excitatory cholinergic longitudinal muscle contractions in the guinea pig ileum^[43]. This effect correlates with the number of mast cells and the activation of the nerve fibers appears to be mediated by the activation of different receptors, including transient receptor potential vanilloid subfamily member 1 (TRPV1), purinergic and prostanoids receptors^[43].

Many studies have been conducted in attempt to identify a specific pattern of intestinal faecal microbiota

in IBS patients and, although heterogeneity of IBS patients, qualitative and quantitative alterations in intestinal microflora have been found. Differently from traditional microbial culture-based techniques, studies using DNA-based techniques showed that specific fecal and mucosal microbiota composition are associated with different subgroups of IBS patients, even if these investigations have produced non univocal results. Some studies reported increased abundance of *Proteobacteria* and *Firmicutes* and reduction in *Actinobacteria* and *Bacteroidetes* in patients with IBS^[11,44] while others reported a decreased amount of *Lactobacilli* and *Bifidobacteria*^[45]. A very recent meta-analysis demonstrated that composition of IBS patients microbiota vary across geographical regions. The study reported a decreased numbers of *Bifidobacteria* and *Lactobacillus* and increased numbers of *Escherichia coli* and *Enterobacterium* in Chinese IBS patients with no significant differences in the abundance of *Bacteroides* and *Enterococcus*. On the other hand, a decreased numbers of *Bifidobacteria* and increased numbers of

Bacteroides were found in IBS patients from other regions of the world^[46]. The strict relationship between dysbiosis and GI motility in IBS need to be further elucidated as one of the major challenges in IBS is the absence of an animal model that fully represent this condition.

DIRECT EFFECTS

New physiopathologic and therapeutic scenarios have arisen by the recent evidence highlighting that microbiota metabolic products or bacterial molecular components can directly affect enteric nerves and smooth muscle cells functions.

Fermentation products

The microbiota is a formidable metabolic “organ”, not only able to capture calories from food but also to elaborate a large amount of compounds such as short-chain fatty acids (SCFAs), neurotransmitters homologs and gases that can act directly with the enteric neuromuscular apparatus^[47].

SCFAs such as acetate, propionate, and butyrate are produced by bacterial fermentation of dietary fibers. SCFAs exert multiple beneficial effects and act both as signal transduction molecules, *via* G-protein coupled free fatty acid receptors (FFAR2, FFAR3, OLFAR78, GPR109A) and regulators of gene expression^[48]. Besides improving the intestinal environment, SCFAs directly affect various host peripheral tissues, generate potent motor responses and have a considerable role in regulating the propulsive activity of the gut, both in animal models and in humans. SCFAs, when administered into the human terminal ileum, have been shown to increase parietal tone and stimulate ileal propulsive contractions^[49,50]. These compounds are suggested to act *via* either extrinsic or intrinsic afferent neurons which can ultimately stimulate myenteric cholinergic neurons^[51]. Most of these responses are not observed in mucosal free preparations, suggesting that SCFAs receptors are located on mucosal EC cells. In particular, propionate acts on receptors in the mucosa causing the release of 5-HT from EC cells that activates, through 5-HT₄ receptors on the endings of intrinsic primary afferent neurons, the enteric peristaltic reflex pathways^[51]. In the rat distal colon, propionate causes also tonic contraction *via* prostaglandin release^[52]. Similarly, butyrate and acetate may also affect GI motility through several mechanisms including direct effects on smooth muscle and myenteric neurons^[53] and production of mucosal 5-HT^[54]. SCFAs receptors have been also localized in mucosal EC cells containing peptide YY that might represent another important messenger in transducing this contractile signal^[55]. However, the effect of these metabolites still remain controversial; a recent human study found no significant differences in global motility index after intracolonic infusion of SCFAs^[56].

Deconjugated bile salts, another bacterial metabolite^[57], have also been reported to affect gastrointestinal motility through activation of transmembrane G-protein coupled receptor (TGR5)^[58]. In animals, TGR5 have been detected in inhibitory intestinal motor neurons and on gallbladder smooth muscle cells^[59]. The direct activation of TGR5 causes relaxation of the smooth muscle cells and inhibition of gallbladder contractility resulting in gallbladder filling. In humans, treating normal gallbladder muscle cells with a hydrophobic bile acid, the tauro-chenodeoxycholic acid, results in impairment of contraction to cholecystokinin due to a significant reduction in receptor binding and an increase in inflammatory mediators and oxidative stress^[60,61]. These latter abnormalities, observed also in gallstone patients, are prevented by treatment with the hydrophilic ursodeoxycholic acid^[61,62].

Among microbiota compounds that might influence GI motility, there is tryptamine, a secondary metabolite resulting from the transformation of the aromatic amino acid tryptophan, that mimics the serotonin stimulatory effects on motility in *ex vivo* preparations of guinea pig ileum^[63]. It is of note that most genes encoding amino-acid-metabolizing enzymes involved in the synthesis of neurotransmitters (catecholamines, serotonin/melatonin, acetylcholine) are present in the microbiota genome^[64]. Commensal bacteria have also been shown to be a significant source of nitric oxide (NO), a key molecule in the control of gut motor functions^[65].

Finally, fermentation by the anaerobic flora of the undigested polysaccharide fraction of certain carbohydrates generates gases, mostly hydrogen (H₂) and methane (CH₄). Even if clinical studies are still controversial, experimental evidence has been provided that methane is not an inert intestinal gas since it can affect the intestinal neuromuscular function^[66]. In animal models, it has been shown that intestinal methane infusion slowed down small intestinal transit time and augmented ileal circular muscle contractile activity^[66,67]. In turn, in an *ex vivo* experiment on guinea pig gut, H₂ by itself has been reported to significantly shorten colonic transit times, this effect being restored by methane^[68]. Finally, the resident sulfate-reducing bacteria produce hydrogen sulfide (H₂S) that inhibits intestinal contractile activity acting on interstitial cells of Cajal and enteric extrinsic neurons^[66]. The effects of fermentation products on GI motility are summarized in Table 1.

Bacterial molecular components

One of the main mechanisms of bacterial recognition are toll-like receptors (TLRs) a family of pattern recognition receptors that are emerging as potential mediators between microbiota and the enteric neuromuscular apparatus. TLR-dependent signaling regulates structural integrity in both the myenteric and submucosal plexus^[69,70]. The mRNA encoding for TLRs

Table 1 Direct effect of bacterial fermentation products on gastrointestinal motility

Fermentation product	Effect on GI motility	Mechanism	Ref.
Short-chain fatty acids	Increase of ileal tone and propulsive contractions Smooth muscle and myenteric neurons activation	Activation of G-protein coupled free fatty acid receptors (FFAR2, FFAR3, OLFAR78, GPR109A) Release of 5-HT from EC cells Release of prostaglandins	[45-55]
Deconjugated bile salts	Relaxation of gallbladder smooth muscle cells Inhibition of gallbladder contractility	Activation of transmembrane G-protein coupled receptor Reduction in cholecystokinin receptor binding Increase of inflammatory mediators and oxidative stress	[58-61]
Tryptamine	Stimulation of ileum motility	Synthesis of neurotransmitters	[63-65]
Gases	Decrease of small intestinal transit time Augmented ileal circular muscle contractile activity Shortening of colonic transit times Inhibition of intestinal contractile activity	Methane (CH ₄) production Hydrogen (H ₂) production Hydrogen sulfide (H ₂ S) production	[66,67] [68] [66]

GI: Gastrointestinal.

have been detected on neurons^[71], glial^[72] and smooth muscle cells^[73]. TLR-2 activation on smooth muscle leads to the production of neurotrophins that enhance the structural and functional integrity of the enteric nervous system^[74].

In acute inflammatory conditions an excessive increase of mucosal permeability leads to luminal bacteria/endotoxins translocation^[75]. Bacteria or bacterial products can migrate from the intestinal lumen to mesenteric lymph nodes, or the circulation, due to the disruption of the normal host/flora equilibrium as reported in cirrhosis^[76], inflammatory bowel diseases^[77] and recently in diarrhea-predominant IBS patients^[78].

Most evidence on the effects of bacterial components on the neuromuscular apparatus derives from studies on lipopolysaccharide (LPS), the major component of the outer membrane of Gram-negative bacteria. Although the exact mechanisms whereby LPS is able to impair muscle contractility are still to be established, various targets have been demonstrated. LPS can directly activate muscular TLR4 inducing a time- and concentration-dependent impairment of contractility associated to cytoskeleton alterations, together with an intracellular oxidative imbalance as shown on human colonic smooth muscle cells^[79] (Figure 2). Many of these effects persisted even after LPS withdrawn suggesting that motility dysfunction might play a pivotal role both during an acute infective process and after its resolution. In an experimental model that enables to stimulate human intestinal mucosa in a polarized fashion with LPS^[37], it has been shown that LPS affects enteric contractility both through translocation from the mucosa and submucosa, with subsequent activation of TLR expressed in muscle, and through mucosal production of oxygen free radicals. LPS effects on human smooth muscle were reversed by the H₂O₂ scavenger catalase, by NFκB transcription inhibitors and by indomethacin, which blocks activation of COX2^[37]. Besides, LPS can directly activate macrophages embedded within

the intestinal muscularis externa that produce inflammatory mediators that indirectly alter smooth muscle contractility^[80,81].

Interestingly, the expression of multiple TLRs receptors subtypes differentially activated by bacterial antigens on the enteric neuromuscular apparatus seems to allow a discrimination between pathogens and probiotics, as reported for both human enteric glial^[72], smooth muscle cells^[73,82]. The crosstalk between TLRs subtypes is emerging as an important regulatory defense mechanism also in neuromuscular apparatus^[83]. On human colonic smooth muscle cells, it has been observed that the activation of TLR2, whose ligands are the components of the outer membrane of Gram-positive bacteria, prevents LPS-induced muscular alterations. By interacting with this receptor, *Lactobacillus rhamnosus* GG (LGG) is able to reduce LPS-induced NFκB activation and inflammatory IL6 secretion cytokine and to restore the levels of secretion of anti-inflammatory cytokine IL10^[82]. These *in vitro* studies support the recent evidence that indicates the neuromuscular apparatus as possible target for probiotics^[13-15]. *Escherichia coli* strain Nissle 1917 specifically modulates contractility of human colonic muscle strips^[84], *Lactobacillus* species regulate jejunal motility^[14], colonic neuron excitability^[15] and attenuate post-infective muscle hypercontractility^[85]. *Bifidobacterium* and *Lactobacillus* also alleviate visceral hypersensitivity and recover intestinal barrier function as well as inflammation^[86]. Also in humans recent evidence further suggests that probiotics might be effective in neuro-motor disorders^[87,88].

CONCLUSION

In summary, the current working hypothesis is that dysbiosis-driven mucosal alterations induce the production of several inflammatory/immune mediators which affect gut neuro-muscular functions suggesting a potential for disturbances in the microbiota to elicit directly intestinal dysmotility or, if sustained,

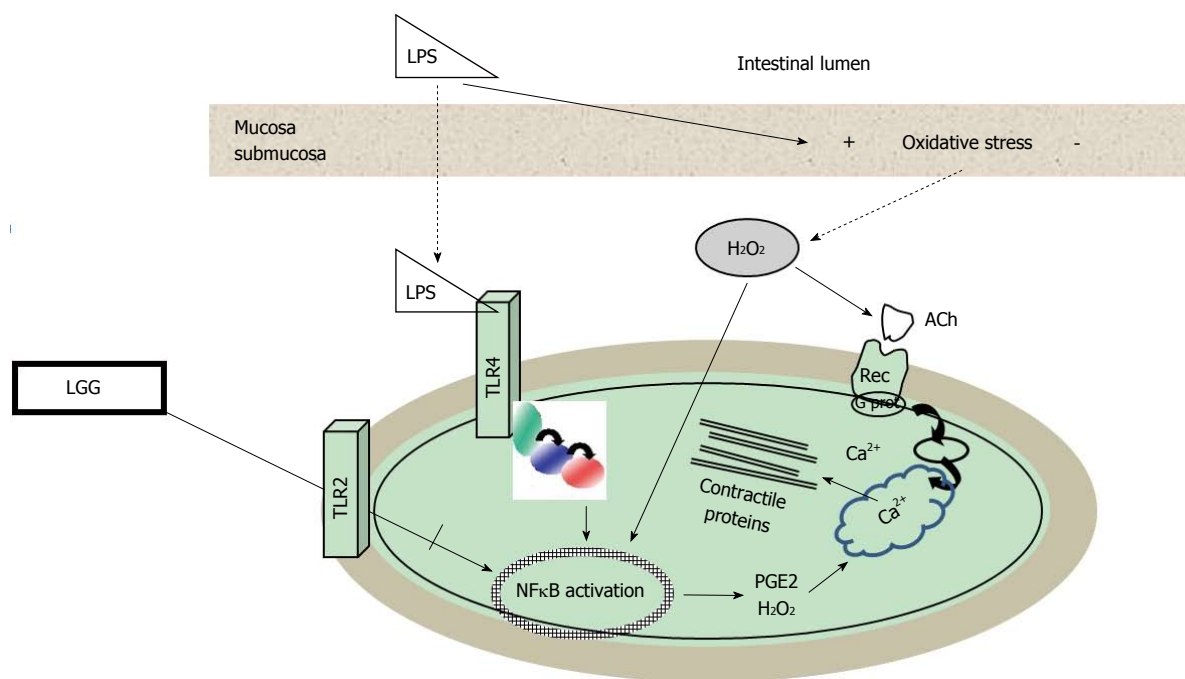


Figure 2 Role of toll-like receptors on human colonic smooth muscle cells. LPS affects intestinal contractility by activating oxidative stress in the mucosa and, once translocated, by activating TLR4 expressed in colonic muscle cells. Activation of muscular TLR4 impairs cell contractility by activation of the nuclear factor κ B transcription with intracellular increase of oxidative stress and by prostaglandin E2 (PGE2) that block intracellular calcium release. The oxygen free radicals, produced in the mucosa, impair cell contractility with a similar mechanism and also by de-regulation of contractile receptors. The activation of TLR2, whose ligands are the components of the outer membrane of Gram-positive bacteria, such *Lactobacillus rhamnosus* GG (LGG), prevents LPS-induced muscular alterations. TLR4: Toll-like receptor 4; LPS: Lipopolysaccharide.

to lead to chronic sensory-motor dysfunction. The understanding in these fields would hopefully open new therapeutic scenarios in GI disease with underlying neuromuscular disorders as manipulation of gut microbiota composition could also correct the mechanisms promoting development and maintenance of symptoms.

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Hepatic steatosis and fibrosis: Non-invasive assessment

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Abstract

Chronic liver disease is a major cause of morbidity and mortality worldwide and usually develops over many years, as a result of chronic inflammation and scarring, resulting in end-stage liver disease and its complications. The progression of disease is characterised by ongoing inflammation and consequent fibrosis, although hepatic steatosis is increasingly being recognised as an important pathological feature of disease, rather than being simply an innocent bystander. However, the current gold standard method of quantifying and staging liver disease, histological analysis by liver biopsy, has several limitations and can have associated morbidity and even mortality. Therefore, there is a clear need for safe and non-invasive assessment modalities to determine hepatic steatosis, inflammation and fibrosis. This review covers key mechanisms and the importance of fibrosis and steatosis in the progression of liver disease. We address non-invasive imaging and blood biomarker assessments that can be used as an alternative to information gained on liver biopsy.

Key words: Hepatic steatosis; Fibrosis; Non-invasive assessment; Blood biomarker; Ultrasound

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Core tip: Ongoing hepatic fibrosis and steatosis are well recognised features of chronic liver disease. Liver biopsy is currently the gold standard for assessing the disease although this has an associated but low morbidity and mortality risk. Therefore, alternative methods of non-

invasive assessment of liver disease are of relevance and importance. We outline the mechanisms of hepatic fibrosis and steatosis and review uses of non-invasive imaging and blood biomarkers as an alternative to liver biopsy.

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CLINICAL PROBLEM

Chronic liver disease is a major cause of morbidity and mortality worldwide. The presence of chronic inflammation and consequent fibrosis leads to the development of cirrhosis and its complications. Whilst the exact prevalence of chronic liver disease is unknown, cirrhosis of the liver was attributed for more than one million deaths worldwide in 2010, although these figures probably reflect heavy under-reporting^[1]. The total worldwide prevalence of cirrhosis has been estimated at around 1% with significant regional variation owing to the presence of viral hepatitis, the metabolic syndrome and alcohol consumption^[2].

Chronic liver disease has a varied aetiology, including viruses, such as hepatitis B (HBV) and hepatitis C (HCV). Worldwide, over half a billion people may be chronically infected with either of these viruses^[3,4]. Metabolic causes include the increasing prevalence of non-alcoholic fatty liver disease (NAFLD). Toxic causes, such as excess alcohol consumption, aflatoxin exposure^[5,6] and autoimmune disorders, such as primary biliary cirrhosis and autoimmune hepatitis, contribute to the disease burden. Over half of the deaths attributable to cirrhosis and nearly 80% of those attributable to primary liver cancers occur in those who have chronic HBV and HCV infection^[7], while in many developed nations excess alcohol consumption is the commonest cause. In the developing world, aflatoxin exposure further complicates the picture, leading to high rates of hepatocellular carcinoma (HCC).

Chronic liver disease usually takes many years to progress from inflammation, associated with hepatocyte injury, to fibrosis and mostly requires long-term exposure to the causative agent. Progressive scarring or fibrosis develops during the period of time between initiation and end-stage disease. The resulting pre-cirrhotic fibrosis is a target for therapies aimed at reducing the rate of progression to cirrhosis, or even reversal of fibrosis^[8].

Effective antiviral therapies and the advent of antifibrotic drugs have led to increasing demand for non-invasive, accurate and reliable biomarkers of

hepatic disease severity. It is well recognised that the current "gold standard", histological analysis of liver biopsy, has limitations and engenders risk to the patient. Sampling variability and the subjective interpretation of scoring systems means that the consistency and representation of the true disease state is questionable. The procedure frequently causes discomfort and if the patient has a malignancy, there is a risk of tumour seeding^[9]. Furthermore, there is frequent associated morbidity and a small, but significant, mortality rate^[10] in all but a few cases. Liver biopsy is rarely performed in lower income countries, often due to a lack of expertise to interpret results^[11]. On the other hand, in the United States and the developed world, magnetic resonance techniques allow a quantitative assessment of different aspects of disease from metabolic markers of inflammation, through to assessment of fibrotic load, markers of portal hypertension and prognostic indicators of the complications of cirrhosis^[12]. There is a clear need for reliable and effective non-invasive markers of hepatic inflammation and fibrosis, both for the management of individual patients and for the development of new anti-fibrotic therapies. Novel imaging modalities and non-invasive biomarkers have the potential to fulfil this role and offer significant benefit in treatment monitoring and have the potential also to be of use in resource-constrained settings.

NATURAL HISTORY OF CHRONIC LIVER DISEASE

Chronic liver injury leads to initiation and perpetuation of inflammatory processes, which, by a cascade of inter-related processes and pathways, leads to deposition of fibrous tissue (Figure 1). By convention, fibrosis has been considered potentially reversible, while the end-stage of the pathological process, cirrhosis, has been considered irreversible. However, with elimination of the cause of liver injury, a number of studies have demonstrated regression of all stages of fibrosis in animal models and in humans^[13-17]. Elucidation of the process of fibrogenesis enables markers of disease severity and potential targets for therapeutic intervention to be developed.

KEY MECHANISMS OF FIBROGENESIS

Fibrosis is a dynamic process of hepatic homeostasis mediated by several cellular mediators in response to an inflammatory process. In particular, hepatic stellate cells (HSC) have a central role in the pathogenesis of liver fibrosis^[8,18]. These cells comprise 15% of liver cell mass^[19]. HSCs are activated following liver injury from a relatively quiescent lipid and vitamin A-storing phenotype to a myofibroblastic phenotype, capable of proliferation, contraction and fibrogenesis. However,

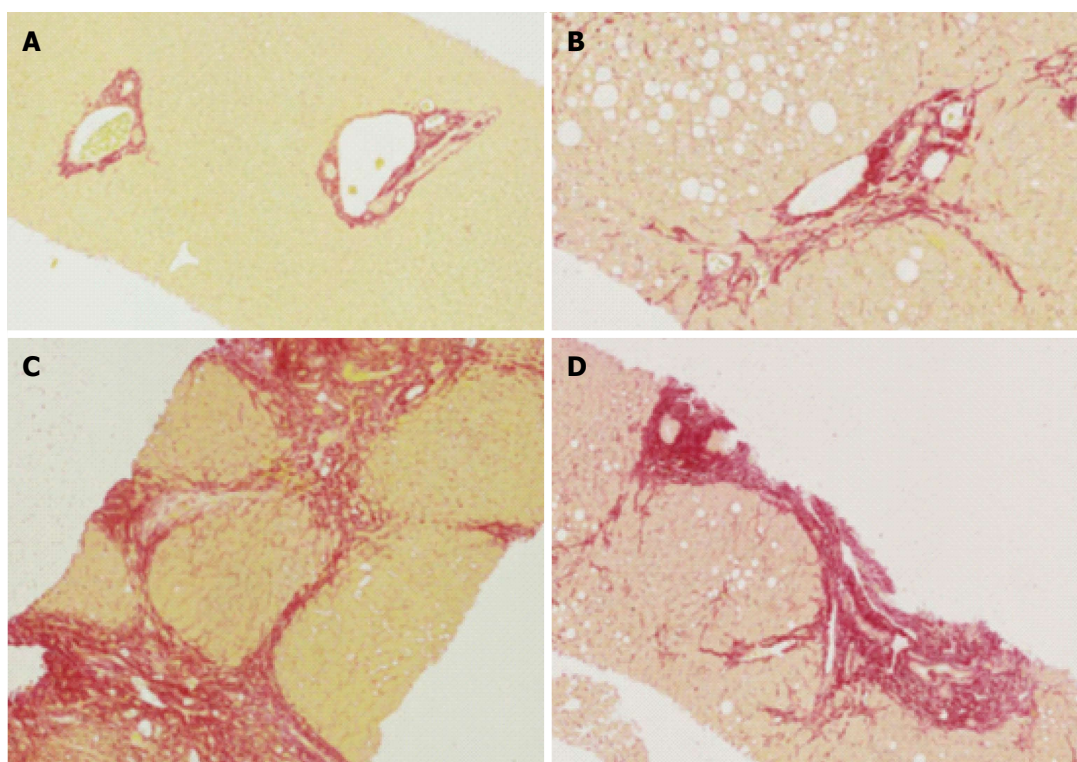


Figure 1 Histology of normal liver, fibrosis and cirrhosis. A: Representative histological images (using Sirius red staining), normal liver; B: Mild to moderate fibrosis with portal tract expansion (METAVIR F = 2, Ishak stage 3); C: Moderate "bridging" fibrosis (METAVIR F = 3, Ishak stage 4); D: Cirrhosis (METAVIR F = 4, Ishak 5 or 6).

other myofibroblastic cell populations have also been shown to be involved in fibrogenesis, including portal fibroblasts^[20,21].

HSC activation occurs in two stages: initiation and perpetuation and each involves characteristic pathways, as described by Friedman^[18]. Initiating events may consist of any chronic perturbation of hepatic homeostasis, which is often, but not always associated with the presence of inflammatory cells on liver biopsy. Such perturbations of hepatic homeostasis may lead to the net over-production of unstable reactive oxygen species (ROS) derived from hepatocytes, macrophages, stellate cells and inflammatory cells. ROS are potent mediators of the initiation and perpetuation of liver injury and cause lipid peroxidation of cellular membranes. Perpetuation of HSC activation is comprised of a number of cellular responses, which lead to increased expression and responsiveness to growth factors. Perpetuation is a dynamic process, occurring along a number of pathways associated with different HSC responses. These responses include release of largely proinflammatory cytokines, proliferation and increased contractility of HSCs, chemotaxis of inflammatory cells and the net deposition of pathological ECM^[18].

Resolution of fibrosis

For resolution of fibrosis to occur, removal of the injurious agent is a prerequisite. Activation of HSCs

has been shown to be crucial for the development of fibrosis, while in recovery and resolution of fibrosis, the number of activated HSCs is reduced. The latter may occur as a result of reversion to the quiescent form or by apoptosis. A return to the quiescent form has been observed *in vitro*^[22], but not *in vivo*. Apoptosis has been postulated as the predominant mechanism for removal of HSC activity. It has been shown that spontaneous apoptosis of HSCs occurs *in vitro*. Conversely, it has also been shown that MMP-2 levels correlate with apoptosis and may be stimulated by apoptosis^[23] and that TIMP-1, by inhibition of MMP, leads to inhibition of activated HSC apoptosis^[24].

HEPATIC STEATOSIS

Liver fibrosis is the key pathological feature of progressive liver disease. However, the accumulation of excessive hepatic triglyceride, hepatic steatosis, is increasingly recognised as an important factor in the pathogenesis of a number of chronic liver diseases, not simply an "innocent bystander".

Definitions

Hepatic steatosis is a pathological lesion, defined as the presence of large and small vesicles of fat, predominantly triglycerides, accumulating within hepatocytes^[25]. Hepatic steatosis is frequently associated with obesity, insulin resistance and dyslipidaemia

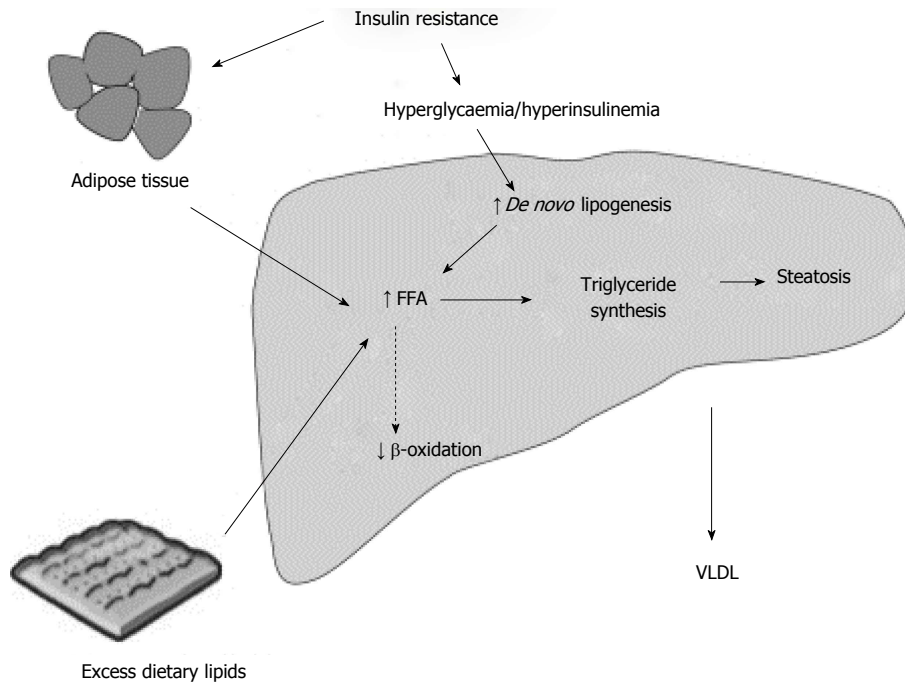


Figure 2 Summary of metabolic mechanisms leading to hepatic steatosis. Reproduced from Dowman *et al.*^[155] with permission from Oxford University Press.

in those who do not consume excessive quantities of alcoholic drinks, where it is termed non-alcoholic fatty liver disease^[26] (NAFLD). Hepatic steatosis may also be a result of secondary causes, which include alcoholism, HCV, severe weight loss, total parenteral nutrition and drugs (such as amiodarone, diltiazem, tamoxifen, steroids and highly active antiretroviral therapy)^[27]. NAFLD may thus be considered a syndrome of various aetiologies, excluding alcohol and, by convention, HCV.

Epidemiology

In a systematic review on the epidemiology of NAFLD, Vernon and colleagues reported the prevalence of NAFLD in the United States ranges from 10%-35% in published studies, depending on the investigative assessment modality used and the study population^[1]. Worldwide, NAFLD prevalence ranges from 6%-35% with a median of 20%^[1].

Key issues in pathophysiology

The development of hepatic steatosis occurs when the rate of synthesis or import of fatty acids by hepatocytes exceeds the rate of export or catabolism. Such an imbalance can occur in a number of ways and is summarised in Figure 2 with increased uptake of fatty acids by hepatocytes in obesity, increased hepatic fatty acid triglyceride synthesis, impaired fatty acid mitochondrial β -oxidation and reduced VLDL and triglyceride synthesis all being components to consider to a greater or lesser extent^[28]. Importantly, with respect to hepatic triglyceride export, HCV core protein has been shown to inhibit this process, providing a

direct route to hepatic steatosis in HCV infection^[29,30].

CURRENT APPROACHES TO THE ASSESSMENT OF CHRONIC LIVER DISEASE

The evaluation of patients with chronic liver disease is largely based on assessment of the clinical history, physical examination and measurement of non-specific liver enzymes. Most pre-cirrhotic chronic liver disease is asymptomatic, where clinical signs are subtle and non-specific. Clinical chemistry, so-called "liver function tests" (aminotransferases, alkaline phosphatase, bilirubin and gamma-glutamyl transferase) and haematological indices (cell-counts and the pro-thrombin time) may be abnormal in cirrhotic and pre-cirrhotic disease, but alone, their sensitivity and specificity for the diagnosis and staging of chronic liver disease is limited^[2].

Liver biopsy - the "gold standard" for disease assessment

Histological assessment of liver biopsy is the mainstay for the diagnosis and staging of chronic liver disease. Epidemiological and pathophysiological data support fibrosis being the hallmark of chronic liver disease and a predictor of outcome^[31]. Most liver biopsies for the assessment of chronic liver disease are performed percutaneously, by passing the biopsy needle between the ribs into the right lobe of the liver. Guidelines for safe and effective liver biopsy have been published by the British Society of Gastroenterology^[32].

Validity of histological scoring systems

Fibrosis and inflammation: The interpretation of the histology of liver tissue in chronic liver diseases is based on categorisation or scoring of inflammatory features (grade) and fibrosis and architectural disruption (stage)^[33]. The first major scoring system was developed in 1981, and based on the identification of periportal necrosis, intralobular necrosis, portal inflammation and fibrosis, assessed separately and assigned a score^[34], although it was later modified to include additional components^[35]. The first three components of the score contribute to the necroinflammatory grade, while the fibrosis score, from 0-6, is based on architectural changes in the pattern and expansion of fibrous bands. From a practical perspective, cirrhosis is represented by both stage 5 (incomplete cirrhosis) and 6 (probable or definite cirrhosis).

The METAVIR scoring system was developed to look specifically at HCV-related liver disease. The histological activity is based on piecemeal and lobular necrosis, while the fibrosis stage is scored from 0-4, with 4 representing cirrhosis^[36]. All scoring systems are designed to place a numeric value to architectural features. They consist of ordered categorical data representing qualitative and semi-quantitative descriptions, so are not quantitative measures of fibrosis. Studies of liver tissue using digital image analysis have shown that the area of fibrous tissue is not linearly related to the fibrosis score. Moreover, inflammation may cause expansion of fibrous tracts^[33]. Nevertheless, histological fibrosis assessment has been validated by successive clinical studies, making the features clinically relevant^[31].

Steatosis: Scoring systems quantify fat on the basis of visible hepatic lipid droplets within hepatocytes. The grade of steatosis is based on the proportion of hepatocytes containing visible lipid and is expressed semi-quantitatively on a scale of 0-3 (0, < 5%; 1, 5%-33%; 2, > 33%-66%; 3, > 66%)^[37,38]. Grading is considered in the context of other histopathological lesions, such as inflammatory infiltration and ballooning of hepatocytes. However, there are considerable limitations to liver biopsy for the quantification of hepatic lipid. Quantification of lipid is based only on visible lipid droplets, so invisible (for example, membrane) lipid is not included. The assessment is only semi-quantitative, being a two-dimensional estimation of the proportion of hepatocytes including lipid, and not considering the volume of droplets. Finally, the composition of the fatty acid components is not assessed, although, using immunohistochemical techniques, the presence of lipid peroxidation products may be determined^[39].

Limitations of liver biopsy

Morbidity and mortality: Percutaneous liver biopsy has a small, but quantifiable, risk of mortality, quoted

as between 1 in 1000 and 1 in 10000 patients^[10,40,41]. Minor complications, such as post-procedural pain or localised haematoma, occur in between 3% and 30% of cases. More severe complications, including intraperitoneal haemorrhage (requiring transfusion) or perforation of a viscus (including pneumothorax) may occur in 0.3% to 0.6% of cases^[32,42]. Tumour seeding following biopsy of suspected carcinomas in cirrhosis may also occur in 2.7% of cases^[43]. Good management of clotting disorders and the appropriate use of the transjugular approach for liver biopsy may improve outcome^[32].

Sampling variability: Percutaneous liver biopsy typically samples less than 1/50000th of the liver, so any heterogeneity of pathological features may lead to sampling variability^[44]. Autopsy studies have demonstrated that cirrhosis may be missed on a single pass liver biopsy in between 10% and 30% of cases^[45,46], while a study using laparoscopic biopsy of both left and right lobes of the liver found a difference of at least one fibrosis stage between lobes in over 30% of patients^[47]. The size of the liver biopsy specimen influences sampling variability. Smaller biopsy sizes (in length and breadth) were shown to lead to a lower probability of observing characteristics of more severe diseases and, consequently, led to underestimation of disease severity^[48].

Subjectivity and inter-observer variation: Histological scoring systems are designed to be objective and reproducible, but interpretation is still a source of error. Inter-observer variability is low for the assessment of fibrosis, but higher for the assessment of activity or inflammation^[49]. In a study of intra- and inter-observer variability, agreement was better for fibrosis than inflammation and also amongst experienced pathologists than more junior pathologists^[50]. These authors concluded that the experience of the pathologist had more influence on agreement than the characteristics of the biopsy itself. Histology of liver biopsy specimens is still the mainstay for the definitive diagnosis of liver diseases and for investigation of co-existing pathology. Nevertheless, there are risks inherent in the technique and scoring systems should be interpreted in the knowledge of the limitations.

NON-INVASIVE TECHNIQUES

Non-invasive assessment techniques are suited to longitudinal studies as the risk to patients is more limited than liver biopsy. However, the measured parameters may be more susceptible to influence by confounding factors and so specificity is important to consider. Non-invasive tests of chronic liver disease may be broadly divided into serum (or blood) markers and imaging-based technologies.

Serum markers

Serum markers have been studied in detail to detect early fibrotic changes as blood tests are quick and acceptable to patients. While the results are objective, there is the possibility of the presence of confounding factors from extrahepatic disease. These have been reviewed extensively^[51-53]. Serum markers may broadly be divided into "indirect" and "direct" markers, single tests and panels. Indirect markers are markers of liver function, which reflect liver fibrosis, while direct markers include serum extracellular matrix components and intermediates of fibrogenesis^[54]. The strengths and limitations of the most widely used models are summarized below.

APRI

APRI (AST to Platelet Ratio Index) was proposed as an alternative to biopsy in patients with chronic HCV infection^[55] and it is calculated as (AST/upper limit of normal range)/platelet count ($10^9/L$) $\times 100$. A recent meta-analysis by Lin and colleagues showed APRI had AUROC scores for the diagnosis of significant fibrosis, severe fibrosis, and cirrhosis of 0.77, 0.80, and 0.83 respectively, demonstrating a potential use for identifying HCV-related fibrosis^[56]. However, APRI fails to identify a significant proportion of people in the earlier stages of fibrosis and therefore is limited in its ability to identify only significant and untreated chronic HCV-related fibrosis^[55,57].

Enhanced liver fibrosis score

The enhanced liver fibrosis score (ELF[®]) score (Siemens, Munich, Germany) combines three direct markers of fibrosis including hyaluronic acid (a component of the extracellular matrix), TIMP-1 (an inhibitor of matrix metalloproteinases, which break down collagen) and PIINP (a marker of collagen synthesis at disease site). Therefore, the premise of this scoring system is that a higher score will indicate a higher rate of fibrogenesis. ELF[®] has been shown to have good performance for the detection of significant fibrosis in chronic HCV (93% sensitivity and 83% specificity)^[58] but also in NAFLD (sensitivity 89% and specificity 96%) and ALD (100% sensitivity and 16.7% specificity)^[59], although results for the latter two have been less rigorously evaluated. Results also need to be adjusted appropriately as scores can be influenced by gender, age, and sex^[60].

FibroTest[®]

FibroTest[®] (BioPredictive, Paris, France) uses five different serum markers in its model and has been validated in meta-analysis in multiple aetiologies including NAFLD (AUROC 0.84; 95%CI: 0.76-0.92), alcohol-related liver disease (AUROC 0.86; 95%CI: 0.80-0.92) and both chronic HBV infection (AUROC 0.80; 95%CI: 0.77-0.84) and HCV infection (AUROC 0.85; 95%CI: 0.82-0.87)^[61]. However, results are

limited by false-positive results, attributed to increases in bilirubin or decreases in haptoglobin; in particular HCV patients on ribavirin. Results can also be affected by acute inflammation, Gilbert's syndrome and cholestasis^[52].

FIB-4 index: The FIB-4 index combines several markers of liver function into the following formula: age (years) \times AST [U/L]/(platelets [$10^9/L$] \times (ALT [U/L])). The FIB-4 index was specifically developed as an alternative to biopsy in patients with chronic HCV infection, although it has shown use in other causes of liver disease. In a study of 529 HCV-infected patients, the FIB-4 index enabled the correct identification of patients with severe fibrosis (F3-F4) and cirrhosis with an Area under Receiver Operated Curve (AUROC) of 0.85 (95%CI: 0.82-0.89) and 0.91 (95%CI: 0.86-0.93), respectively^[62]. However, a lack of universal agreement amongst studies for positive and negative cut-off values has proved problematic.

Forns index: The Forns index is another formula that assesses liver function by combining age, cholesterol, gamma-glutamyltranspeptidase and platelet count. Forns and colleagues showed using a best cut-off score of < 4.2 , the presence of significant fibrosis (F2-F4) could be excluded with high accuracy (negative predictive value of 96%) in 125 (36%) of 351 patients with chronic HCV infection^[63]. The Forns index has also been shown to be more accurate than other serum markers including FIB-4 and aspartate APRI (AUROC 0.795, 0.764 and 0.774 respectively) in the prediction of significant fibrosis in patients with chronic HCV^[64].

IMAGING-BASED MODALITIES AS ALTERNATIVES TO BIOPSY

Various imaging modalities have been proposed as alternatives to liver biopsy. Given that there is strong evidence that liver stiffness measurements (LSM) increases with the degree of fibrosis^[65,66], the most successful imaging modalities have used techniques to measure liver stiffness and correlate this with the degree of fibrosis, most notably ultrasound-based transient elastography (TE) and acoustic radiation force impulse imaging (ARFI[®], Siemens, Munich, Germany). Their potential use in the measurement of hepatic steatosis and differentiation of hepatic steatosis grade has also been investigated. The use of other modalities, including MR techniques, to assess morphological and biochemical changes in chronic liver disease (CLD) will also be discussed.

TE

TE was first developed as Fibroscan[®] (Echosens, Paris, France), where a vibrator generates low frequency shear waves through the liver which are then transmitted to an ultrasound receiver. The velocity of the waves is

dependent on the tissue elasticity and therefore, the rate of propagation through the liver can be used as a measure of liver stiffness and converted into a numerical value (kPa). Meta-analyses have shown TE has a very high sensitivity and specificity for detecting cirrhosis, but its accuracy was much reduced in the earlier stages of fibrosis^[67,68]. This is partly due to the lack of validation for stiffness cut-off values in earlier stages of disease, but also possibly due to the multiple processes that contribute to liver stiffness other than fibrosis^[67,69]. Fibroscan[®] machines now have been calibrated to measure hepatic steatosis levels by using a novel Controlled Attenuation Parameter (CAP[®]; Echosens, Paris, France), with results showing a sensitivity and specificity between 78% and 100% and an excellent correlation between different steatosis grades, determined by the percentage of hepatocytes with fatty infiltration (Spearman Rank $\rho = 0.81$, $P < 10^{-16}$)^[70]. Its use has been validated in patients with chronic hepatitis C infection (AUROC 0.80; 95%CI: (0.75-0.84) for $S \geq 1$, 0.86; 95%CI: 0.81-0.92 for $S \geq 2$ and 0.88; 95%CI: 0.73-1.00 for $S = 3$) respectively and has shown correlation in patients with NAFLD (0.49, $P = 0.00069$) although this accuracy reduces with increasing steatosis grade in patients with NAFLD^[71,72].

Acoustic radiation force impulse imaging[®]

Acoustic radiation force impulse imaging (ARFI[®]) (Siemens, Munich, Germany) is another form of ultrasound elastography that measures soft tissue displacement following the exposure of high-energy acoustic pulses. The measurement of displacement can then be quantified and interpreted as a measurement of liver stiffness. Meta-analysis by Friedrich-Rust and colleagues used AUROC to show ARFI[®] is effective with scores of 0.87 for discriminating significant fibrosis, 0.91 for severe fibrosis and 0.93 for cirrhosis^[73]. Additionally, ARFI[®] has also shown comparable results with TE for detection of significant fibrosis and cirrhosis and was significantly more likely to obtain reliable measurements^[74]. However, its uses for detecting earlier stages of fibrosis though remain limited.

ElastPQ[®]

ElastPQ[®] (Philips, Best, Netherlands) is a newer ultrasound method of non-invasive assessment of liver stiffness and uses two-dimensional shear wave elastography to generate an absolute measurement of liver stiffness. ElastPQ[®] showed comparable accuracy to ARFI[®] when differentiating 176 patients with and without chronic liver disease (83.7% vs 83.1%), although measurements of liver stiffness for ElastPQ[®] were significantly lower, compared to ARFI[®] and thus required different cut-off values^[75]. Additionally, in a study of 291 patients with chronic HBV, who underwent biopsy or partial hepatectomy, ElastPQ[®] values showed good correlation between the stage of liver fibrosis and grade of necroinflammatory activity while

being unaffected by levels of steatosis^[76]. However, whilst ElastPQ[®] remains an exciting prospect, further comparisons with other non-invasive imaging modalities and liver biopsy are needed.

Standard ultrasound

Ultrasound is widely used clinically to detect hepatic steatosis, but it can also detect the vascular changes of chronic liver disease with contrast enhancement^[77]. Ultrasound is also able to detect hepatic steatosis, based on the premise that steatosis causes increased echogenicity of the hepatic parenchyma, leading to a brighter image when compared to the cortex of the ipsilateral kidney^[78]. Other conditions, such as fibrosis may also lead to increased echogenicity, resulting in a potential for confusion. A review of the non-invasive measurement of fat content found the sensitivity of ultrasound for the detection of steatosis to range from 60% to 94%, while the specificity ranged from 84% to 95%^[79]. However, histologically-assessed mild steatosis resulted in a sensitivity of just 55%^[80]. In addition, obesity reduces the accuracy of ultrasound due to technical considerations and increased attenuation of signal caused by subcutaneous fat. Ultrasound performs more poorly for the *quantification* of hepatic lipid, although subjective grading systems, categorizing steatosis into mild, moderate and severe groups have been proposed^[81]. Dynamic microbubble contrast-enhanced studies are thought to exploit the intra- and extrahepatic vascular changes that generate shortening of hepatic vein transit times with increasing disease severity^[82,83] (Figure 3).

Computed tomography

CT enables the assessment of hepatic steatosis on the basis of radiographic density^[84], although the ability to quantify hepatic lipid is not clear^[85]. Ionising radiation associated with CT-scanning confers a small excess risk to subjects, making it less suited for repeated measurements.

Magnetic resonance imaging

T₁ and T₂ mapping are MR techniques that can be used for *in vivo* tissue characterisation either individually or in combination. T₁ relaxation times correlate with increased levels of extracellular fluid associated with inflammation and fibrosis, whereas T₂ mapping primarily reflects the amount of iron deposition^[86]. For example, T₁ mapping of the liver has used a modified Look-Locker inversion recovery sequence with motion recovery^[87] and T₂ on multi-gradient-echo acquisition^[88]. Since elevated iron levels interfere with T₁ measurements, correction algorithms are applied to provide more accurate readings^[86]. T₁ mapping has shown good correlation with the histological degree of fibrosis in a cohort of 79 patients with chronic liver disease of multiple aetiologies with a ROC of 0.94 for any degree of fibrosis^[88].

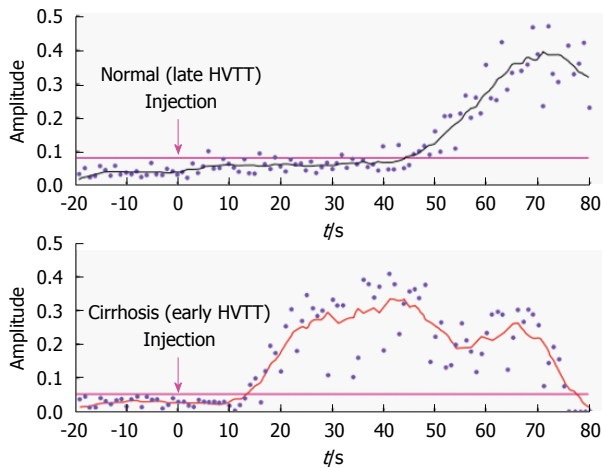


Figure 3 Hepatic vascular transit times in normal patients and patients with cirrhosis. Time intensity curves from the hepatic vein show earlier arrival of contrast in the cirrhotic liver^[83].

Additionally, Pavlides and colleagues have used T_1 mapping to show a correlation with the degree of liver disease and the risk for developing clinical events in 112 patients. Optimal T_1 cut-off points were created by comparing multiparametric MR data with histological staging of fibrosis from previous studies. This was used to create a “liver inflammation and fibrosis” (LIF) staging score. Pavlides subsequently found that patients with severe liver disease ($LIF > 3$) were at higher risk for developing clinical events, compared to patients with $LIF < 1$ ($P = 0.02$) and $LIF 1-1.99$ ($P = 0.03$)^[86]. T_1 mapping has also been shown to differentiate Child-Pugh A patients from Child-Pugh B/C patients effectively ($P < 0.00001$)^[89]. T_1 mapping is a promising diagnostic tool that has shown to be effective in differentiating different stages of fibrosis and also has shown potential for predicting clinical events. However, further research is needed to validate effective scoring systems and the influences of other compounding factors for it to become a valid alternative to liver biopsy in clinical practice.

Magnetic resonance elastography

While ultrasound-based transient elastography techniques such as Fibroscan® measure liver stiffness in a defined region (about 5 cm³, right lobe of the liver), Magnetic Resonance Elastography (MRE) can be used to predict fibrosis stage effectively in patients with chronic liver disease, while producing a wider and more representative map of liver stiffness in both 2D and 3D planes. This is performed using similar principles to TE, whereby propagating shear waves are generated and imaged using phase contrast MRI, which includes oscillating motion sensitising gradients (MSGs). The subsequent cyclic spin displacement of protons, which in the presence of synchronised MSGs, are encoded as phase shifts within the MRI signal^[90].

MRE has been validated in multiple studies and

meta-analyses^[91-94]. One meta-analysis by Singh and colleagues using 12 retrospective studies, comprising 697 patients with CLD of varying aetiology, showed MRE had high diagnostic capability for detecting significant fibrosis ($F \geq 2$), advanced fibrosis ($F \geq 3$), as well as cirrhosis with ROC values of 0.88 (0.84-0.91), 0.93 (0.90-0.95), and 0.92 (0.90-0.94) respectively, as determined by liver biopsy^[91]. Another meta-analysis by Su and colleagues, comprising 13 studies and 989 patients, also demonstrated good sensitivity and specificity of MRE for the staging of fibrosis. The pooled sensitivity and specificity for $F \geq 1$, $F \geq 2$, $F \geq 3$ and $F = 4$ were 0.87 (95%CI: 0.84-0.89) and 0.92 (95%CI: 0.87-0.96), 0.87 (95%CI: 0.84-0.90) and 0.92 (95%CI: 0.89-0.95), 0.88 (95%CI: 0.85-0.91) and 0.91 (95%CI: 0.88-0.93), 0.91 (95%CI: 0.87-0.94) and 0.92 (95%CI: 0.89-0.94), respectively. The pooled ROC values for $F \geq 1$, $F \geq 2$, $F \geq 3$ and $F = 4$ were 0.9502, 0.9663, 0.9644, and 0.9768, respectively^[92]. MRE can also be used to stratify risk of cirrhosis progression in patients with chronic HCV infection^[95] and may have greater potential than TE for assessment of NAFLD in patients with high risk of NASH or cirrhosis, due to the multiparametric nature of MRI which allows for a comprehensive assessment of the liver^[96].

Recent studies have shown that three-dimensional spin-echo echoplanar imaging (3D-SE-EPI) could have better diagnostic accuracy than conventional two-dimensional gradient-recalled echo (2D-GRE). In a study of 179 patients with either chronic HBV or HCV infection, Shi and colleagues showed AUCs for the characterisation of $F \geq 1$, $F \geq 2$, $F \geq 3$, and $F = 4$ were 0.957 (95%CI: 0.913-0.983), 0.971 (0.932-0.991), 0.991 (0.961-0.999), and 0.979 (0.942-0.995) for 3D-SE-EPI compared with the AUCs for 2D-GRE at each fibrosis stage which were 0.948 (0.901-0.977), 0.959 (0.915-0.981), 0.979 (0.943-0.995), and 0.976 (0.938-0.994) respectively^[97]. A higher diagnostic accuracy for 3D-SE-EPI compared with 2D-GRE has also been shown for patients with NAFLD advanced fibrosis^[98].

Proton magnetic resonance spectroscopy

Since a Magnetic Resonance Spectroscopy (¹H MRS) spectrum of the liver is dominated by lipid and water resonances, ¹H MRS has been used for the assessment of hepatic fat. The percentage liver fat has been estimated from the number of protons in the lipid and water resonances by calibration with hepatic lipid extracts^[99]. Such measures have been used to assess racial differences in the prevalence of hepatic steatosis and the technique has also been applied in a population of over 2000 participants^[100,101]. Simpler lipid-to-water resonance ratios have been used to compare hepatic steatosis between obese and lean individuals and have demonstrated a change in intrahepatic lipid in response to dietary intervention^[102-104]. ¹H MRS also has also been applied to the assessment of steatosis in living-

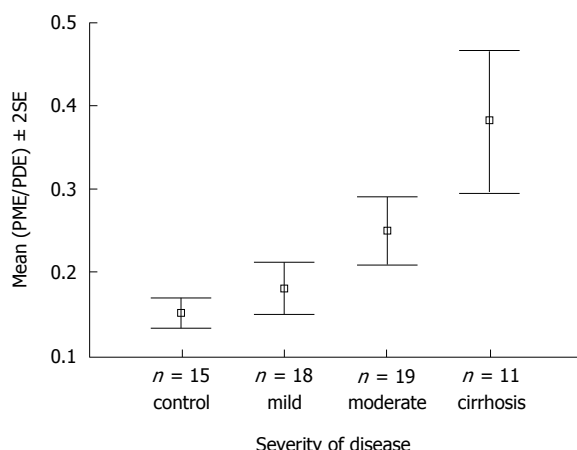


Figure 4 ^{31}P magnetic resonance spectroscopy of patients with increasing severity of liver disease vs controls. PME/PDE ratios obtained from *in vivo* hepatic ^{31}P MRS correlating with severity of liver disease in patients with hepatitis C^[116]. MRS: Magnetic resonance spectroscopy; PME: Phosphomonoester; PDE: Phosphodiester.

donor liver transplantation^[105] and for quantification of steatosis in HIV mono-infected individuals who are at greater risk of liver fibrosis and cirrhosis^[106].

Proton density fat fraction

MRI techniques can also be used to calculate the proton density fat fraction (PDFF), a marker of hepatic steatosis, using MRS as a reference. PDFF is defined as the ratio of density of mobile protons from triglycerides and the total density of protons from mobile triglycerides and mobile water which then reflects the concentration of fat within that tissue in the absence of confounding factors^[107]. PDFF has shown at least equivalence in accuracy for quantifying hepatic steatosis with both ^1H MRS and with histological grade, across several studies with various aetiologies of chronic liver disease^[108-112]. Additionally, in a retrospective study of data from 506 adults, PDFF estimation accuracy was not affected by age, sex and BMI with the authors concluding these confounders have a clinically negligible effect^[113]. Further validation of these techniques would be of benefit as ^1H MRS techniques are not widely available.

Phosphorus MRS

Phosphorus (^{31}P) MRS currently remains a research tool and allows observation of metabolites associated with energy metabolism as well as membrane phospholipid turnover. The latter include phosphomonoesters (PME), thought broadly to represent membrane precursors including phosphoethanolamine, phosphocholine and phosphodiesters (PDE), which are thought to represent membrane degradation products including glycerophosphocholine and glycerophosphoethanolamine. Studies in chronic liver disease both *in vitro*^[114,115] and *in vivo*^[116,117] have demonstrated a correlation between the PME/PDE and disease state or severity (Figure 4). A study in

patients with acute hepatitis A demonstrated an acute rise in PME/PDE, which decreased with resolution of disease^[118]. Moreover, in patients with hepatitis C, the PME/PDE decreased significantly in those responding to antiviral treatment but did not change in non-responders^[119].

Combination techniques

Combinations of non-invasive techniques can be used to improve accuracy. Serum biomarker models can be combined to create more accurate algorithms, such as the Fibropaca algorithm, Leroy algorithm and SAFE biopsy, with some studies suggesting their use could reduce the number of liver biopsies by 79%^[120]. However, the use of serum biomarkers in combination with imaging techniques has proved highly useful. One study of 183 patients with chronic HCV by Castera and colleagues demonstrated that a combination of serum FibroTest[®] and ultrasound-based Fibroscan[®] (TE) (the Bordeaux algorithm) showed an increased AUROC score for F2 (0.88 vs 0.83) and F3 (0.95 vs 0.90), compared to Fibroscan[®] alone, thus avoiding the need for biopsy in a large proportion of patients^[121]. The Anger's algorithm combines the serum biomarker model, Fibrometer[®] (Echosens, Paris, France) and ultrasound-based Fibroscan[®]. TE has also demonstrated good diagnostic accuracy and required significantly fewer biopsies than the Bordeaux algorithm for significant fibrosis (20.2% vs 28.6%, $P = 0.02$) and cirrhosis (9.3% vs 25.3%, $P = 0.001$)^[122]. Most recently, the role of combination techniques as a cost-effective screening tool has been validated by Harman and colleagues in a community setting in Nottingham, United Kingdom (practice population 10479). High-risk patients were identified using risk factors for chronic liver disease and subsequently investigated them using a serial biomarker algorithm and liver stiffness measurement (Figure 5). Of the 504 identified as being high risk, 62 patients (12.3%) had normal biomarkers and were not further investigated. 378 patients then agreed to undergo TE which found 98 patients (26.8% of valid scans) had clinically significant fibrosis (defined as LSM < 8kPa). Most interestingly, 71/98 patients (72.4%) of these patients had normal liver enzymes and would have been otherwise missed by conventional algorithm models. This techniques also managed to identify 140% more patients with definite cirrhosis ($n = 11$)^[123].

IMAGING MODALITIES TO DETECT FEATURES OF CLD

Imaging modalities can be used to detect a number of signs or physical parameters, which are of relevance to chronic liver disease.

Morphological changes

The later stages of chronic liver disease are cha-

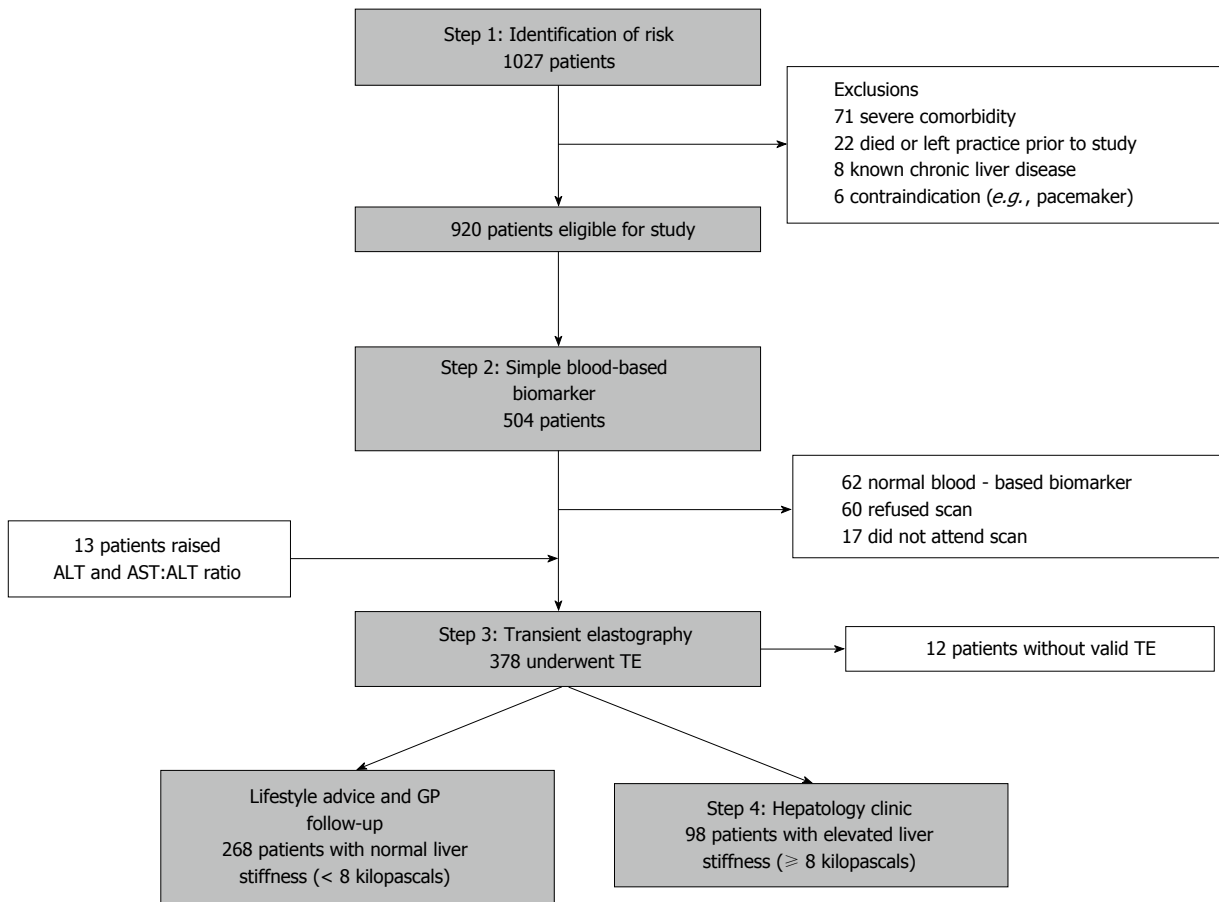


Figure 5 Diagnostic algorithm and patient flow chart of the non-invasive biomarker and transient elastography pathway^[123]. ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; GP: General practitioner; TE: Transient elastography.

racterised by a number of intra- and extrahepatic structural changes. A number of these structural changes are manifestations or consequences of other pathological processes. The cirrhotic liver is typically small with an uneven border or perimeter. These features are caused by the contraction of thick bands of fibrous tissue interspersed by regenerative nodules. Ultrasound-visible features, such as liver surface nodularity, caudate lobe hypertrophy and the presence of detectable hepatic venous blood flow have been shown to identify those with severe fibrosis or cirrhosis from a cohort of patients with chronic liver disease, although it is doubtful that the severe fibrosis group alone would be identifiable by such a technique^[124]. Routine MRI, computed tomography (CT) and ultrasound indices can detect gross morphological changes associated with cirrhosis, but none is sensitive or specific. Moreover, pre-cirrhotic disease is not adequately discriminated^[125-127]. However, dynamic superparamagnetic iron oxide-enhanced and gadolinium-enhanced MRI demonstrates reticular-nodular patterns, thought to represent septal hepatic fibrosis, the presence of which, taken with an overall (subjective) qualitative assessment, allows the discrimination of moderate and severe from mild fibrosis^[128] (Figure 6). Digital image processing

of unenhanced CT scans has enabled assessment of the distribution of high-attenuation patterning, again presumed to represent fibrosis and distinction between moderate and severe fibrosis^[129]. However, these techniques do not provide a quantitative measure of disease severity, and a number of the techniques remain unvalidated by other centres. Water molecules are tightly bound in the fibrotic extracellular matrix, providing the rationale behind the application of diffusion-weighted MR imaging to chronic liver disease. An apparent diffusion coefficient (ADC) is derived, representing proton, and hence water, mobility. A reduced ADC is observed in cirrhosis and with increasing fibrosis stage, and has been interpreted as being due to restriction of water diffusion in fibrotic tissue^[130,131], or possibly by reduced capillary perfusion^[132]. However, the precise relationship between the ADC and fibrosis is currently unclear.

Portal hypertension

Portal hypertension is the cause of much morbidity and mortality associated with chronic liver disease, through development of varices of porto-systemic anastomoses and through activation of vasodilatory pathways and development of ascites and the hepatorenal

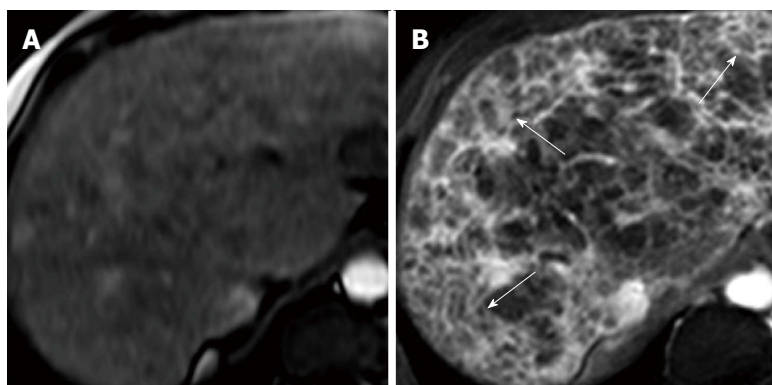


Figure 6 Transverse MR images of cirrhotic liver *in vivo*^[128]. A: SPIO-enhanced two-dimensional spoiled gradient echo (SPGR) image with echotime of 2.65 ms; B: Double-enhanced SPGR image at the same level, showing hyperintense reticulations and hypointense nodules (arrows), thought to represent fibrous septal bands surrounding regenerative nodules.

syndrome. Increased portal pressure is caused by increased intrahepatic resistance to flow which results from both vascular factors and fibrosis^[133]. The structural results of portal hypertension, such as splenomegaly, ascites and the presence of venous collaterals are also readily assessed by conventional imaging techniques, but these features tend to be associated with decompensated cirrhosis and not pre-cirrhotic disease stages. A number of ultrasound-based studies have aimed to assess portal pressure indirectly as a surrogate for disease severity^[134]. However, ultrasound Doppler indices of portal flow were found not to correlate reliably with increasing severity of disease^[135,136]. A number of studies have pointed to a relationship between portal hypertension and liver stiffness measurement (LSM) measured by Fibroscan[®]. Foucher and colleagues demonstrated a correlation between liver stiffness measurement and splenomegaly, the presence of oesophageal varices and a history of bleeding varices^[137]. A relationship between LSM, measured by Fibroscan[®] and the presence of varices has also been described, although evidence for the relationship between LSM and size of varices is mixed^[138,139]. Vizzutti and colleagues went on to demonstrate correlation between LSM and the hepatic venous pressure gradient (HVPG), particularly at lower HVPG values (< 10–12 mmHg). This represents a complex relationship, which was less apparent at higher HVPG values^[139]. It has been stated that the “progressive rise in portal pressure...[is]...due mainly to an increase in intrahepatic vascular resistance from the accumulation of fibrillar extracellular matrix”^[140]. However, increased arterial and portal inflow may contribute directly to the liver stiffness, while haemodynamic changes characteristic of advanced portal hypertension, including extrahepatic haemodynamic changes, may not be detected by changes in liver stiffness.

Intrahepatic vascular changes

Vascular remodeling is increasingly seen as a patho-

logical feature of chronic liver disease. In the development of fibrosis, obliteration of the small hepatic and portal veins may lead to a congestive hepatopathy, which is exacerbated by a co-existent hyperdynamic circulation^[141]. This results in inflammation and oxidative stress, both triggers for fibrogenesis. Intrahepatic vascular remodeling within the fibrotic liver is performed by the contractile HSCs, mediated by changes in levels of nitric oxide (NO), consequent to derangement of endothelial NO synthase. This contributes to high resistance and constricted sinusoidal vessels^[142]. Such mechanisms may also contribute to the development of intrahepatic vascular shunts. Imaging techniques assess changes in physical properties consequent to vascular alteration. While vascular changes occur with increasing fibrosis, imaging techniques do not assess fibrosis directly, so may be considered surrogate markers in this context.

Inflammation and cell turnover

Hepatic inflammation is associated with cellular inflammatory infiltrate, tissue oedema and hepatocyte swelling. Each of these is likely to affect the physical properties of liver tissue and, as such, can be measured by imaging modalities. These properties include: nuclear relaxation (T_2), assessed by MR techniques; water perfusion and diffusion, as assessed by DWI; liver stiffness; changes in attenuation, assessed by CT and echogenicity, assessed by B-mode ultrasound.

Liver stiffness

The association between liver stiffness on Fibroscan[®] and disease activity, or necroinflammatory score on histology has been shown by a step-wise increase of liver stiffness measurements (LSM) with necroinflammatory activity in a cohort of patients with disease of varied aetiology^[143]. The relationship between LSM and biochemical activity in patients with chronic viral hepatitis has also been studied using Fibroscan[®]. The LSM was lower, stage-for-stage, in those with biochemical

remission (assessed by ALT) than those with a higher ALT^[144]. Studies have specifically addressed the effect of hepatic inflammation on LSM. In one, 18 patients without a past history of liver disease, but with acute viral hepatitis, were studied. The LSM on Fibroscan® at the peak aminotransferase level exceeded 12kPa (the cut-off for prediction of cirrhosis) and furthermore, in all but one subject, the LSM returned to within normal range (below 7kPa). In addition, the LSM correlated with the aminotransferases at onset and with the AST at follow-up^[145]. In another Fibroscan® paper, 20 patients with acute hepatitis of varying aetiology were studied. In those followed up longitudinally, the aminotransferases returned to a level commensurate with the fibrosis stage at biopsy^[146]. While it is known that acute hepatitis is associated with an inflammatory infiltrate, tissue oedema and hepatocyte swelling, (all of which are likely to affect LSM), there was no histological confirmation of these features in these studies, as liver biopsy was not clinically indicated^[147].

The development of ultrasound-based TE has enabled the rapid acquisition of objective liver stiffness measurements *in vivo*^[65]. Multiple regression analysis in early studies demonstrated a relationship between elasticity measurements and fibrosis stage, but not to the histologically-measured disease activity, necroinflammatory score or the degree of steatosis^[65,66]. A number of studies have confirmed the correlation between liver stiffness and hepatic fibrosis in chronic HCV infection^[66,148], and other chronic hepatic conditions^[149-151] with meta-analyses and systematic reviews also being recently published^[68,152]. However, studies have also indicated the presence of co-existing factors that may contribute to liver stiffness, including inflammation, portal hypertension and possibly steatosis^[137,147,150,151]. It has also been stressed that "biological tissue is a composite material and it is difficult to separate the influence of each component of the tissue on the total in modulus estimates"^[153]. Substantial differences in cut-off values for cirrhosis have been observed on Fibroscan® between those with chronic hepatitis and those with alcohol-related chronic liver disease and NAFLD, perhaps representing modification of liver stiffness by coexisting fat^[150,151,154]. Thus, there is circumstantial evidence that steatosis affects liver stiffness although the magnitude of the effect is likely to be smaller than that of other contributing factors, such as fibrosis, inflammation and portal hypertension.

Summary

The development of chronic liver disease is a major cause of morbidity and mortality worldwide and usually occurs over many years from progressive fibrosis and associated hepatocellular injury, including steatosis. Non-invasive assessments using imaging modalities and serum markers have now been shown to be effective at detecting significant fibrosis and

cirrhosis to at least some degree. However, recent evidence suggests that various combinations of these techniques may be helpful both as an alternative to liver biopsy, which has significant associated morbidity and mortality, and as a cost-effective tool to identify sub-clinical disease.

SUMMARY OF KEY POINTS

Chronic liver disease develops over many years following ongoing injury and is characterised by progressive scarring caused by fibrosis. Hepatic steatosis is increasingly being recognised as an important factor in a number of chronic liver diseases and occurs when the rate of synthesis or import of fatty acids by hepatocytes exceeds the rate of export or catabolism. Serum markers measure both direct and indirect markers of liver fibrosis although these have had limited success as individual markers of fibrosis. Imaging modalities to assess liver disease can be used to quantify morphological changes, portal hypertension, vascular remodelling and liver stiffness associated with increasing fibrosis. The most widely used and successful of these are transient elastography and acoustic radiation force impulse imaging although MRI techniques are very promising. Combination techniques involving transient elastography and various serum markers can provide good diagnostic accuracy and a reduced need for liver biopsy in patients with significant fibrosis. This has also proved successful as a screening tool in for patients in a community setting.

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Contraindications for video capsule endoscopy

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Abstract

Video capsule endoscopy (VCE) has been applied in the last 15 years in an increasing field of applications. Although many contraindications have been put into perspective, some precautions still have to be considered. Known stenosis of the gastrointestinal tract is a clear contraindication for VCE unless surgery is already scheduled or at least has been considered as an optional treatment modality. In patients with a higher incidence of stenosis, as in an established diagnosis of Crohn's disease, clinical signs of obstruction, prior radiation or surgical small bowel resection, a preceding test with the self-dissolving patency capsule can override this contraindication. Endoscopic placement of the capsule should be considered in patients with swallowing disorders to avoid aspiration. Esophageal or gastric motility disorders may require endoscopic capsule transport or application of prokinetics if the real-time viewer proves delayed transit. In pregnant women, VCE should be restricted to urgent cases where diagnosis cannot be postponed after delivery, as data on safety are missing. There is theoretical and clinical evidence that patients with implanted cardiac devices such as a pacemaker, cardioverters or left heart assist devices, can safely undergo VCE in spite of still existing contraindication by manufacturers. Children from the age of 2 years have safely undergone VCE. Although video capsules are not proven safe with magnetic resonance imaging (MRI), first single cases of patients incidentally undergoing MRI with an incorporated capsule have been reported, showing susceptibility artifacts but no signs of clinical harm.

Key words: Video capsule endoscopy; Contraindications; Stenosis; Pacemaker; Aspiration; Pregnancy; Magnetic

resonance imaging

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Core tip: Video capsule endoscopy has emerged as a first line diagnostic tool for small bowel visualization. The few existing contraindications are discussed in this review and put into perspective. Special situations are to be considered for patients with gastrointestinal stenosis, swallowing and motility disorders, or implanted electromagnetic cardiac devices, pregnant women, young children, and magnetic resonance imaging for patients with a retained capsule. Appropriate precautions are discussed in this paper.

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INTRODUCTION

Video capsule endoscopy (VCE) was introduced in 2001 as a well-tolerated, non-invasive, radiation free, disruptive method to visualize the gastrointestinal (GI) tract, in particular the small bowel. The wireless video capsule consists of one or more cameras with a corresponding lens and light source, batteries, a video chip, and an electronic circuit to either store or transmit the captured images. Depending on the manufacturer, the capsule measures 24-32 mm in length and 11-13 mm in diameter. The capsule is swallowed by the patient and then progresses through the gastrointestinal tract by peristalsis until it is excreted naturally. Only the colon capsule endoscopy needs an additional booster-solution during the procedure. The most commonly used VCE systems transmit the captured images in real-time to an external sensor array and recorder. The transmission technique is based on radiofrequency (Pillcam, Medtronic plc, Dublin, Ireland; EndoCapsule, Olympus Medical Systems Corp., Tokyo, Japan; OMOM capsule, Jinshan Science and Technology Co. Ltd., Chongqing, China) or electrical current *via* Human Body Communication (MiRoCam, IntroMedic Co. Ltd., Seoul, South Korea). Images captured by the CapsoCam capsule (CapsoVision Inc., Saratoga, CA, United States) are stored on-board in an integrated flash-drive, thus obviating the need for an external recorder, but requiring retrieval of the capsule to download the data^[1].

Based on these properties of VCE systems and the modality of the procedure, contraindications were established by the manufacturers. Up to today, millions

of VCE studies have been performed worldwide. For example, Covidien/Medtronic announced that more than 1.5 million PillCam capsules were used by the end of 2014. With this vast clinical experience, many of the initially pronounced contraindications can now be put into perspective.

This review summarizes the contraindications to VCE provided by the manufacturers and critically analyzes the theoretical reasons, the existing clinical evidence in the literature and technical data, as well as statements and guidelines of national or international societies.

CONTRAINDICATIONS FOR VCE BASED ON MANUFACTURERS' RECOMMENDATIONS

Listed below (Table 1) are the contraindications and relative contraindications for VCE as stated by the manufacturers. Detailed contraindications are summarized based on the underlying pathophysiology (*i.e.*, radiation enteritis, large small bowel tumor, extensive abdominal surgery, extensive small bowel or colon diverticulosis, GI perforation and fistulas are summarized under GI obstruction/obstacles).

VCE IN PATIENTS AT RISK FOR GASTROINTESTINAL STENOSIS

Known or suspected obstruction of the gastrointestinal tract bears the risk of capsule retention and consecutive complications. Intestinal obstacles like extensive diverticulosis or fistulas can have a similar effect. Capsule retention is defined by consensus as having a capsule endoscope remaining in the GI tract for a minimum of 14 d or if a directed medical, endoscopic or surgical intervention has to be implemented to retrieve the capsule^[2]. The consequences of capsule retention can be a total or subtotal obstruction^[3], gastrointestinal perforation^[4,5], or capsule disintegration^[6]. As these rare complications may occur late in previously asymptomatic patients, retrieval of a retained capsule should be considered. A case report has documented asymptomatic retention for up to 12 years. This 43-year-old patient underwent procto-colectomy for familial adenomatous polyposis (FAP) in 2000 and capsule endoscopy in a pilot study in 2004. The patient was lost at follow-up. In 2016 an abdominal computed tomography (CT) detected the capsule proximal of an anastomotic stricture. After failed endoscopic retrieval, the capsule was recovered surgically^[7]. This very rare necessity of surgery for retrieval is the reason that some manufacturers include the inability to undergo surgery as a contraindication for VCE.

In a systematic review of 22840 VCE procedures, the overall retention rate was as low as 1.4% (CI:

Table 1 Contraindications by manufacturer

Product Condition	Medtronic Patency capsule	Medtronic PillCam	Olympus EndoCapsule	IntroMedic Mirocam	Capsovision CapsoCam	Jinshan Science OMOM capsule
Known or suspected GI obstruction/obstacles, Fistulae, relevant (small bowel) diverticulosis		C	C	C	C	RC
Motility disorder incl. indigestion or slow gastric emptying			C	C	C	
Cardiac pacemakers or other implanted electromedical devices		C	C	C		RC
Swallowing disorder (dysphagia)	C	C	C	C	C	RC
Pregnancy		RC	C	C	C	RC
Children under the age of (yr)	2	2 (SB3)		2		
		18 (Colon, Eso)				
Strong electromagnetic fields <i>i.e.</i> , MRI			C	C	C	
Inability to endure capsule retrieval surgery			C			C
Inability to communicate sufficiently				C		
Concomitant heart disease or epilepsy (due to electromagnetic radiation)				C		

PillCam Rapid 8 User Manual (DOC-2051-02) <http://www.medtronic.com/content/dam/covidien/library/us/en/product/diagnostic-testing/rapid-v83-user-manual.pdf>; Olympus EC 10 System User Manual (DE-8602257); IntroMedic Mirocam User Manual v3.9 (MM1100-U-1511); Capsovision CapsoCam SV1 Manual (Doc. No. 1151, Rev. G, ECO 11-0098; OMOM User's Manual Version 1 (ZSSM-OM00-002). C: Contraindication; GI: Gastrointestinal; MRI: Magnetic resonance imaging; RC: Relative contraindication.

1.2-1.6). Categorized by indication, retention rates in obscure gastro-intestinal bleeding (OGIB) were 1.2% (CI: 0.9-1.6), in Crohn's disease (definite or suspected) 2.6% (CI: 1.6-3.9) and in the neoplastic lesions subgroup 2.1% (CI: 0.7-4.3). Out of 104 reported capsule retentions, 88 were asymptomatic (85%) and 16 had signs of partial or total intestinal obstruction. Of the retained capsules 58.7% were removed surgically, 12.5% endoscopically, or passed either spontaneously or after medical treatment in 15.8%, others were not reported in detail. In 136 cases a cause for retention was reported: Crohn's disease 35.3%, neoplastic lesions 22.1%, NSAID-induced enteropathy 18.4%, postsurgical stenosis 7.4%, ulceration 3.7%, intestinal adhesion 2.9%, tuberculosis or radiation enteritis each 2.2%, ischemia-induced stenosis, Meckel's diverticulum or pouch each 1.5%, peptic ulcer scar with stricture or cryptogenic multifocal ulcerous stenosing enteritis each 0.7%^[8]. In addition, case reports documented capsule retention in a Zenker's diverticulum^[9], a duodenal diverticulum^[10], in an ileo-rectal fistula^[11], in an epiphrenic diverticulum^[12], and the appendix orifice^[13].

An analysis of 5428 VCE procedures in Spain came to a similar conclusion. The overall retention rate was 1.9%, and 1.5% in the OGIB subgroup and 3.3% in the inflammatory bowel disease subgroup. Retention rate raised to 5.7%-30% if at least two of these clinical symptoms were present prior to the VCE study: abdominal pain, distension and nausea/vomiting^[14].

In patients with suspected GI obstruction a patency test capsule can be administered prior to the actual VCE study. If the capsule is excreted intact within 30 h, GI patency is presumed. After 30 h the lactose body of the patency capsule dissolves, leaving only a slim cellophane coating and a small tag^[15]. Passage of an intact patency capsule predicts uneventful VCE^[16].

Established Crohn's disease is an indication for capsule endoscopy with an increased rate of capsule retention. A Swedish analysis found an odds ratio of 9.39 (95%CI: 3.32-26.54, $P < 0.001$) for capsule retention in patients with known Crohn's disease compared to bleeding indication^[17]. Even if current studies could not confirm retention rates of 13% as reported in the era before the advent of the patency capsule^[18], a retention rate of 2%-3% seems to be realistic^[19]. Clinical assessment, MR-enteroclysis and the use of a patency capsule can help to identify high-risk patients.

In a retrospective study 134 patients with known Crohn's disease underwent VCE. Patients with obstructive symptoms, a history of bowel obstruction and NSAID/aspirin medication were previously excluded and 1/3 had prior small bowel follow through. Although no patency capsule test was performed on this selected group of patients, no cases of a capsule retention were observed^[20]. This is in accordance with a recent retrospective multicenter study including 406 patients with known Crohn's disease. A patency capsule test in every patient with Crohn's disease did not show a reduction in the capsule retention rate compared to a selective use of the patency capsule in high risk patients with clinical signs of obstruction, or prior abdominal surgery^[19]. In a prospective study, 57 patients with known Crohn's disease and mild symptoms or in remission, who underwent MR-enteroclysis evaluated by two radiologists, had a good sensitivity (92.3% and 100%, respectively) and a negative predictive value (96.3% and 100%, respectively) for retention of the patency capsule as a predictor for functional stenosis test^[21].

In 2009 the joint consensus of the Organisation Mondiale d'Endoscopie Digestive and the European Crohn's and Colitis Organization recommended using

imaging techniques before VCE in suspected Crohn's disease^[22]. However, in 2015 based on broader evidence, the European Society for Gastrointestinal Endoscopy (ESGE) recommended not using cross sectional imaging or patency capsule before VCE in patients with suspected Crohn's disease in the absence of obstructive symptoms. In contrast, in established Crohn's disease, imaging techniques and patency capsule are recommended to precede VCE^[23].

Patients with a small bowel (SB) tumor seem to have a slightly higher risk of retention. A suspected tumor as an indication for VCE was associated with an odds ratio of 3.9 (95%CI: 1.2-12.8, $P = 0.026$)^[17]. However, clinical symptoms of such tumors are typically bleeding or iron deficiency anemia. As tumors only present in a small subgroup of patients presenting with bleeding/anemia, retention even in this subgroup is rare, mostly asymptomatic, and diagnostic rather than a complication, ESGE recommends against routine precautions tests before VCE in bleeding patients. However, if a tumor is suspected by imaging techniques, device assisted enteroscopy with the option of obtaining histology is preferred over VCE^[23].

In sum, suspected or known GI stenosis is a contraindication unless intestinal patency is proven, best by the passage of an intact patency capsule. The risk for capsule retention should be assumed in patients with known Crohn's disease, clinical or radiologic signs of obstruction, a history of abdomino-pelvic radiation, and after small bowel resection. Patients undergoing VCE for mid-GI bleeding without the above risks do not require preceding radiology or a patency capsule.

VCE IN PATIENTS WITH MOTILITY DISORDERS

VCE is not indicated for the diagnosis of GI motility disorders. For this purpose, a specifically designed, non-imaging wireless motility capsule (SmartPill, Medtronic plc, Dublin, Ireland) has been developed. Data from sensors measuring pH, pressure, and temperature are transmitted wirelessly for up to 5 d allowing diagnosis of gastroparesis, and prolonged transit times in the small bowel, colon or combined disorders^[24-27].

Nevertheless, standard video capsule was applied in 18 patients with chronic intestinal dysmotility in the search for associated mucosal lesions. Three capsules were retained in the stomach for > 2 h, one of them during the entire recording time. However, no permanent retention, symptoms, or need for interventional treatment occurred^[28]. Another study included 36 patients with severe symptomatic intestinal motor disorders for analysis of VCE image patterns compared with controls. No adverse events were mentioned in this report^[29].

Although indication of VCE for diagnosis of GI motility disorders has yet to be considered as

experimental, known or yet undiagnosed motility disorders may jeopardize routine VCE performed for other indications. Prolonged esophageal or gastric passage may lead to incomplete visualization of the small bowel, *i.e.*, the cecum is not reached during working capacity of the batteries. Moderate prolongation seems to be compensated by longer battery life span in newer capsule generation^[30].

VCE systems using an external recorder have the ability to display transmitted images in real-time during the procedure^[31-33]. Significantly prolonged gastric transit time can be identified by this real-time viewer and a prokinetic agent can be administered^[34]. A single center study reported a higher completion rate and diagnostic yield when a real time viewer was used and the capsule was placed endoscopically into the duodenum in the case of prolonged gastric transit time (> 60 min)^[35]. The unselected primary endoscopic placement of the capsule into the duodenum to circumvent possible gastroparesis had no effect on complete small bowel visualization in a single center analysis of 687 hospitalized or out-patients compared to swallowing the capsule^[36]. In a prospective single-center study including 100 VCE studies, a pathologic Gastroparesis Cardinal Symptoms Index questionnaire could not predict a prolonged gastric transit time nor did a delayed gastric passage have any clinical significance^[37].

GI motility disorders are no contraindication for VCE. The routine use of a real time viewer directly after swallowing the capsule and after an hour enables detection of aspiration (see below) and esophageal or gastric retention and consecutive intervention.

VCE IN PATIENTS WITH IMPLANTABLE CARDIAC DEVICES

The radio transmitters of the first capsule endoscopes work with a carrier frequency of 434.1 MHz in PillCam and 433.8 MHz in EndoCapsule, similar to the C-Net mobile cellular system (450 MHz). The frequency in the newly available OMOM Capsule is 2.4 GHz. Two studies revealed electromagnetic interference (EMI) between cardiac pacemakers (PM) and the C-Net mobile cellular system in 22.4%-30.7% of the tested pacemakers^[38,39]. However, the radiated power of C-Net mobile phones with 2 W is several factors higher than that of VCE with max. 100 nW. EMI with implantable cardiac devices at 2.4 GHz was also investigated in two studies^[40,41] showing no risk of interference. Nevertheless, users of VCE estimated EMI between capsules and cardiac devices possibly being life-threatening for patients. Since the introduction of VCE, several *in vitro* and *in vivo* studies analyzed EMI between VCE (PillCam and EndoCapsule) and PMs (*in vitro*:^[42-44], *in vivo*:^[44-54]), implantable cardioverter defibrillators (ICD) (*in vitro*:^[55,56], *in vivo*:^[45-48,54,56-59]) and left ventricular assist devices (LVAD) (*in vitro*:

none, *in vivo*:^[52,60-68]).

In order to simulate electrical interactions under physiological conditions in patients, the authors of *in vitro* studies positioned PMs^[43,44] or ICDs in a saline solution with a resistivity corresponding to that of muscle tissue. No interference with any of the PMs was observed. In Dubner's study in one ICD (Belos DR, Biotronik), interference occurred reproducible when placing a test cap (technical data corresponding to first generation PillCam SB1 video capsule) over the ring and the shock coil electrode, but not over the pulse generator itself. This could still be verified even at 30 cm distance from the ICD system^[56]. However, the reason for EMI remained unclear, and *in vivo* validation was missing. This observation is in contrast to our results. We tested five Belos ICDs and found no interference by the capsules at all, even though the devices were investigated in the most sensitive setting^[54]. Furthermore, there are several *in vivo* studies investigating interference between VCE and PMs and ICDs. Interrogation of the devices (in all or some patients) either before and/or after VCE was performed in some studies (PM: ^[44,46-48,52-54], ICD: ^[48,52,54,57-59]) whereas (all or some) patients in other studies were monitored with ECG monitor, telemetry or clinically (PM: ^[44-54] ICD: ^[44-49,52,54,57-59]). No interference with any of the PMs or ICDs in *in vivo* studies was observed. Relevant interference of wireless telemetry has been observed. In some cases, VCE videos had been corrupted^[46,47,51]. If cardiac monitoring is necessary during VCE, wired systems should be used.

With regard to different capsule types, PillCam SB1, SB2, PillCam Colon1, and Olympus EndoCapsule have been studied. For the new PillCam SB3 and PillCam Colon2 with additional remote signals from the DR3 recorder to the capsule in order to adapt frame rates^[69], studies are still warranted.

Only one study investigated EMI between the MiroCam endoscope that uses human body communication to transmit data and PMs ($n = 3$) and ICDs ($n = 3$)^[70]. VCE was safely performed in patients with PMs and ICDs, and images from capsule endoscopy were not affected by cardiac devices. Studies relating to EMI between OMOM-Capsule and cardiac devices are lacking. For CapsoCam with on board storage of images without transmission, interference with cardiac devices is not possible.

EMI between VCE and LVAD was investigated in 10 *in-vivo* studies^[52,60-68]. No interference was observed in any of the studies.

The United States Food and Drug Administration (FDA) and the manufacturers of transmitting capsules (Medtronic GI solutions, Olympus, IntroMedic, and Jinshan) recommend not using VCE in patients with cardiac devices. For CapsoCam without transmission technology there is no such formal contraindication.

Guidelines of the ESGE state that VCE is not contraindicated in patients with PM or ICD^[71], whereas

the American Society of Gastrointestinal Endoscopy guidelines consider cardiac devices as a relative contraindication for VCE^[72]. The German Society of Gastroenterology, Digestive and Metabolic diseases recommends not withholding VCE in patients with a proper indication regardless of implanted cardiac devices^[73].

In accordance with the recommendations of the Biotronik and Medtronic Cardio vascular group, VCE can be used in patients with cardiac devices^[74,75], whereas statements from other manufacturers are not available. Technical data (maximum effective radiated power or output current and transmitter frequency) of VCE (Medtronic, Olympus, Jinshan, IntroMedic) and of the remote transmitting PillCam recorder DR3 were made available to two of the authors (Bandorski D, Stunder D). Based on this data, the maximum electromagnetic radiation in close proximity (5 mm) was calculated for VCE of Medtronic, Olympus, Jinshan as well as for Medtronic recorder DR3. Likewise, for VCE of IntroMedic the maximum obtainable interference voltage at the input of cardiac devices due to the human body communication was evaluated. The determined values are below the safety objectives set by the international product standard for cardiac devices (ISO 14117)^[76] by a factor of 8 to 85.

In conclusion, VCE is safe in patients with PMs/ICDs based on technical data and *in vitro/in vivo* studies. The automatic frame rate control by transmitting a reverse signal from the recorder (DR3) to the capsule also remains without interference. Technical data of manual remote switching between different image acquisition rates in OMOM capsules are lacking. Wireless telemetry can impair recording of VCE images. Regarding patients with LVAD VCE seems to be safe according to *in vivo* results.

VCE IN PATIENTS WITH SWALLOWING DISORDERS

Capsule aspiration is a rare complication of VCE with a presumed incidence of 1 in 600-700^[77,78]. Oral ingestion of the capsule is therefore contraindicated in patients with known swallowing disorder. Yet it is difficult to predict the patient's ability to swallow the capsule safely. Aspiration was reported even if a patency capsule had been administered successfully prior to the procedure^[79] or a barium swallow was uneventful^[78]. In a series of 15 well-documented cases of capsule aspiration, only three patients had a history of dysphagia. The leading symptom during the aspiration was coughing (12/15)^[80], which can stop even if the capsule is still within the trachea^[81]. The aspiration resolved spontaneously by coughing (9/15) or *via* endoscopic retrieval (6/15)^[80].

In one case, asymptomatic retention of a capsule for 6 d within a bronchus and consecutive spontaneous passage through the GI tract was reported^[82]. However,

one patient with capsule aspiration experienced fatal extensive intracerebral hemorrhage, either provoked by initial coughing or during consecutive endoscopy for retrieval^[83].

In case of an increased risk of aspiration, the capsule should be placed endoscopically directly into the duodenum^[73]. This can be achieved *via* an overtube^[84] or a special endoscopic delivery device (AdvanCE, US Endoscopy, Mentor, OH, United States)^[85]. Endoscopic placement with a Roth net is another alternative, but is more frequently associated with mucosal trauma in children than application with the dedicated delivery device^[86].

In conclusion, swallowing disorders with the inability to safely swallow the capsule are a contraindication for standard procedure. However, if endoscopic placement is applied, VCE can be safely performed. The clinical challenge is the identification of patients at risk. Older patients, a history of cerebral stroke, bleeding or trauma, require a thorough history, and test for swallowing function. Children may have a test with swallowing a marshmallow.

VCE IN PREGNANCY

During pregnancy the growing uterus compresses the GI tract. Additionally, gastrointestinal transit is prolonged in the second and third trimester^[87], which theoretically may jeopardize VCE procedure. There are only two published cases of VCE studies about pregnant women. Both reported no adverse events including no retention. The first case was a 30-year-old woman with extensive GI bleeding. A conventional upper endoscopy was uneventful. Lower endoscopy showed fresh blood coming out of the ileocecal valve. VCE revealed an ulcerated jejunal neuroendocrine tumor. Emergency surgery was successful and mother and child were alive and well^[88]. The second case was a 20-year-old woman with a history of cavernous transformation of the portal vein with secondary thrombosis after omphalitis at the age of two. Esophageal varices were treated with sclerotherapy and banding at age 13 and 15. Due to the high risk of upper GI bleeding during pregnancy, the esophagus was examined through the PillCam ESO capsule. No esophageal or gastric varices were detected. The VCE study was uneventful with mother and child alive and well^[89]. The theoretical short-term risk of retention due to altered GI motility in advanced pregnancy was not observed in either of these two cases.

However, there is no data on whether the electromagnetic field of the capsule-recorder-system could harm the unborn child. For comparison, mobile phones seem to have no negative effect^[90]. In contrast, pregnancies of mothers reporting microwave use 6 mo prior to the pregnancy or during the first trimester were more likely to result in miscarriage (OR = 1.28, 95%CI: 1.02-1.59). The odds ratio was raised with an increasing level of exposure with an odds ratio of 1.59

for the highest exposure group (20 or more exposures/month)^[91]. Although microwaves have a higher frequency - from 300 to 3000 MHz - than radio waves, the radio waves used by endoscopic capsules (*e.g.*, 434 MHz for PillCam and EndoCapsule) are within the lower range of microwaves. Another comparator are effects caused by mobile phones with a much higher power than video capsules but not reaching proximity to the unborn as an intra-abdominal source of radio waves. This risk is not relevant for CapsoCam without electro-magnetic emission.

In conclusion, elective capsule endoscopy should be postponed after delivery due to missing data. Nevertheless, VCE may be considered in indications related to maternal symptoms not allowing delay of diagnosis as in relevant small bowel bleeding. Accordingly, the FDA assesses pregnancy only as a relative contraindication to VCE^[92].

VCE IN CHILDREN

There has been an increased use of VCE in the pediatric population due to the possibility of avoiding ionizing radiation, deep sedation and general anesthesia^[93]. The main issue of VCE in children seems to be the ability to voluntarily swallow the capsule and the fear of the capsule not being able to pass the narrow GI tract^[94].

Since it was introduced, the minimum age of VCE has been lowered by the manufacturers and the FDA. In 2009 the FDA approved VCE for children of 2 years or older. The youngest age of a child undergoing a VCE study was 8 mo^[95], and the lowest weight was 7.9 kg^[96]. Voluntary ingestion seems feasible at an age older than 6-8 years^[94], and has already been reported in a child of 4 years^[86]. However, the manufacturer of PillCam recommends not letting children under the age of 8 years swallow the capsule. If endoscopic delivery is necessary, the AdvanCE delivery device was superior to the Roth-net, which caused significant mucosal trauma in 50% in a multicenter trial^[86].

There have been no reports of a capsule aspiration, perforation or complete small bowel obstruction in the studies and meta-analyses of more than 1000 VCE studies with children^[86,93,95-98]. In the largest meta-analysis, the retention rate was 2.3%. The risk for retention was higher in known inflammatory bowel disease (IBD 5.2%), a small bowel follow through suggestive of Crohn's disease (CD 35.7%), and the combination of a body-mass-index below the 5th percentile and known IBD (43%). Retention rates by indication were 1.2% for OGIB, 2.6% for CD, and 2.1% for neoplastic lesions^[93]. In patients with an increased risk of small bowel obstruction, a patency capsule test may reduce the risk of retention^[97,98]. Guidelines of the Spanish Societies for Pediatric Gastroenterology, Hepatology, and Nutrition (SEGHPN) and for Digestive Diseases (SEPD) recommend that in suspected or established Crohn's disease, magnetic resonance

enterography or patency capsule should precede VCE in cases of obstruction symptoms^[94].

MAGNETIC RESONANCE IMAGING IN PATIENTS WITH INCORPORATED CAPSULE

As no testing on magnetic resonance (MR) compatibility of VCEs has been conducted, the FDA requested a warning that a patient should not undergo magnetic resonance imaging (MRI) until excretion of the capsule has been verified^[99]. The feared theoretical complication of performing an MRI scan while a capsule is still within the GI tract is migration of the capsule and the potential for bowel injury or perforation due to heat or high forces^[100]. There are only few reported cases of MRI scans in patients with retained video capsules. In one case, an emergency MRI of the lumbar spine was ordered due to acute lumbar radiculopathy. The localizing sequence showed a focal susceptibility and the MRI was terminated, the capsule was excreted two days later^[100]. In another case, an MRI was performed in a patient with a recurring Crohn's disease. The MRI revealed a capsule that had been retained for two years due to a stenosis. The capsule was retrieved endoscopically with prior dilatation of the stenosis^[101]. The third case was also a patient with symptoms of recurring Crohn's disease. An MRI was performed shortly after VCE with the capsule still lying in the colon^[102]. None of the three cases reported adverse events. Due to the interference of the MRI scan, VCE had no diagnostic value. Unpublished personal experience with three other patients incidentally undergoing abdominal MRI with an incorporated VCE confirms these initial reports.

COLON CAPSULE ENDOSCOPY IN PATIENTS WITH CONTRAINDICATION FOR SODIUM PHOSPHATE

The standard colon preparation prior to a colon capsule endoscopy consists of a PEG solution. In addition, sodium phosphate is used as the standard booster solution during the procedure to ensure that the capsule passes through the entire colon within the lifespan of the capsule's battery. The ESGE guidelines for colon capsule endoscopy recommend the use of sodium phosphate as a booster for all patients with no contraindication^[103]. However, sodium phosphate can cause severe complications like phosphate nephropathy, acute renal failure, hypertension, or mineral imbalance.

In the search for an alternate procedure, a pilot study showed feasibility of a low volume cleansing procedure for colon capsule endoscopy using PEG with ascorbic acid for bowel cleansing and as a boost after swallowing the capsule. CCE could be completed

in 37/49 patients (76%)^[104]. Another pilot trial from Japan, where sodium phosphate is contraindicated in hypertensive patients older than 63 years, proposed a diluted Gastrografin solution as an alternative booster based on a capsule excretion rate during recording of 97% (28/29 patients)^[105].

CONCLUSION

Non-invasive VCE is safe, and formal contraindications can be put into perspective when observing some precautions. Based on uneventful clinical application in children, the minimum age has been lowered to 2 years. There is positive *in vitro* and *in vivo* evidence that cardiac pacemakers and defibrillators are no contraindication to VCE. Due to missing data, VCE in pregnancy should only be performed in very limited indications in cases where a delay of diagnosis until after delivery may put the mother or the unborn at risk. MRI with retained video capsule should be avoided, although the first reports describe only artifacts prohibiting proper image analysis but no harm to the patient. Suspected, known, or likely GI stenosis is a contraindication to VCE unless patency has been proven, or surgery is scheduled and preceding VCE might provide additional relevant information.

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Therapeutic approaches for portal biliopathy: A systematic review

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Abstract

Portal biliopathy (PB) is defined as the presence of biliary abnormalities in patients with non-cirrhotic/non-neoplastic extrahepatic portal vein obstruction (EHPVO) and portal cavernoma (PC). The pathogenesis of PB is due to *ab extrinseco* compression of bile ducts by PC and/or to ischemic damage secondary to an altered biliary vascularization in EHPVO and PC. Although asymptomatic biliary abnormalities can be frequently seen by magnetic resonance cholangiopancreatography in patients with PC (77%-100%), only a part of these (5%-38%) are symptomatic. Clinical presentation includes jaundice, cholangitis, cholecystitis, abdominal pain, and cholelithiasis. In this subset of patients is required a specific treatment. Different therapeutic approaches aimed to diminish portal hypertension and treat biliary strictures are available. In order to decompress PC, surgical porto-systemic shunt or transjugular intrahepatic porto-systemic shunt can be performed, and treatment on the biliary stenosis includes endoscopic (Endoscopic retrograde cholangiopancreatography with endoscopic sphincterotomy, balloon dilation, stone extraction, stent placement) and surgical (bilioenteric anastomosis, cholecystectomy) approaches. Definitive treatment of PB often requires multiple and combined interventions both on vascular and biliary system. Liver transplantation can be considered in patients with secondary biliary cirrhosis, recurrent cholangitis or unsuccessful control of portal hypertension.

Key words: Portal biliopathy; Portal cavernoma; Magnetic resonance cholangiopancreatography; Endoscopic retrograde cholangiopancreatography; Porto-systemic shunt

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Core tip: Portal biliopathy in patients with portal vein thrombosis and portal cavernoma can be symptomatic in about 5%-38% of patients. Therapy includes endoscopic and surgical approaches aimed to improve both portal hypertension and biliary alterations and clinical manifestation. Usually, multiple and combined treatments are required to resolve portal biliopathy.

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INTRODUCTION

Portal biliopathy (PB) is a clinical condition defined as the presence of abnormalities in the biliary tree (including biliary tree and gallbladder) in patients with non-cirrhotic/non-neoplastic extrahepatic portal vein obstruction (EHPVO) and portal cavernoma (PC)^[1]. In literature, this disease has been named as "portal hypertensive biliopathy"^[2], "portal cavernoma-associated cholangiopathy"^[3], "portal cavernomacholangiopathy"^[4], "cholangiopathy associated with portal hypertension"^[5], "pseudosclerosing cholangitis"^[6] and "pseudocholangiocarcinoma"^[7] due to the fact that PB biliary alterations can mimic sclerosing cholangitis or cholangiocarcinoma, respectively.

The spectrum of biliary abnormalities shown at magnetic resonance cholangiopancreatography (MRCP) includes intra- and extra-hepatic biliary stenosis (single or multiple), with or without consensual above dilation; bile duct wall irregularity or thickening; bile duct angulation, varicose veins located at the ductular walls and gallbladder^[3,8].

The aim of this paper is to analyse PB clinical approaches based on classification and characteristics of portal biliopathy through a systematic review of the literature.

For this purpose, a systematic search on MEDLINE was conducted spanning April 1990 to April 2016. Studies were identified using the following terms: "portal biliopathy" OR "portal cholangiopathy" OR "pseudosclerosing cholangitis" OR "pseudocholangiocarcinoma" AND "treatment". In addition, all study references were consulted to identify any other relevant studies. Only studies on humans were considered, and only papers wrote in English were used for the analysis. Inclusion criteria were as follows: patients with PB secondary to non-neoplastic non-cirrhotic portal vein thrombosis (PVT) reporting endoscopic and surgical treatment of PB, both case series and case reports. Exclusion criteria were: review articles, guidelines or comment to other papers; iatrogenic PVT articles; papers about treatment of PB therapy complications or PB medical

therapy; papers about PVT treatment.

A total of 118 articles were initially retrieved. Of these, 69 were excluded according to inclusion/exclusion criteria and the remaining 49 papers were included in this review (Figure 1).

PATHOGENESIS

The development of PB in PC is due to two main mechanisms: a mechanical compression *ab extrinseco* of bile ducts (mainly hepatic biliary duct and common bile duct) by both the PC and the numerous compensatory collateral vein circles that arise after EHPVO formation, and an ischemic damage secondary to the altered vascularization due to EHPVO and PC.

Normally, venous drain of biliary tree is serviced by epicholedochal venous plexus of Saint and the paracholedochal plexus of Petren, whom normally diameter does not exceed 1 mm. In chronic portal vein obstruction these plexus are dilated in response to portal hypertension leading to thickening of biliary duct walls and compression, often with a characteristic radiological/endoscopic image of virtual lumen^[9-11]. In particular, dilation of plexus of Saint causes fine irregularities in biliary walls while dilation of plexus of Petren causes extrinsic compression.

The ischemic damage seems to be related to deficient portal blood supply of the biliary tree secondary to EHPVO and PC and to thrombosis of small bile duct venules, resulting in strictures formation and fibrous^[9]. The prevalence of biliary strictures due to a mechanical compression is about 55% among patients with PB, on the contrary in 45% of cases there was no relationship between stenosis localization and severity and cavernoma/collateral vein compression, suggesting the preponderance of ischemic damage in these cases^[12]. However, both mechanisms can contribute at the same time in PB pathogenesis.

The bile stasis secondary to biliary strictures and hypothetic changes in bile compositions (increased pigment load due to hypersplenism, abnormal enterohepatic circulation of bile acids due to portal hypertension) can contribute to stones formation^[13].

DIAGNOSIS AND CLASSIFICATION

The diagnosis of PB is based on radiological imaging, in particular on cholangiography. MRCP is a non-invasive imaging test that can give a definite outline of biliary ductal abnormalities, and for these reasons it represents the modality of choice for PB diagnosis and evaluation. As previously mentioned, the main alterations evidenced at MRCP are bile duct stenosis, angulations and dilations, both intra and extra-hepatic, parietal irregularities, bile duct angulations, choledochal varices, lithiasis^[3,9]. Condat *et al*^[3] purposed the use of MRCP coupled with magnetic resonance (MR) portography in the initial evaluation of PB, to assess both biliary abnormalities and portal anatomy, which

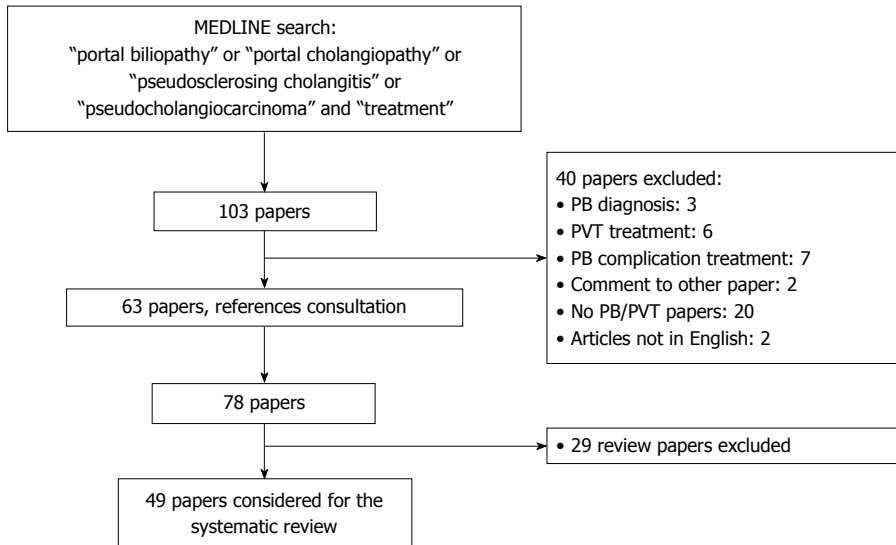


Figure 1 Flow-chart of literature search. PB: Portal biliopathy; PVT: Portal vein thrombosis.

helps in choice of the best therapeutic approach.

Initially, endoscopic retrograde cholangiopancreatography (ERCP) has been for many years the modality of choice to obtain cholangiographic images. Nowadays it has been replaced by MRCP in biliary diagnostic: ERCP is an invasive tool with possible complications, and it has only a therapeutic rather than diagnostic role. ERCP abnormalities are the same demonstrated at MRCP, however some old studies that used ERCP for PB diagnosis described a more frequent and severe left hepatic duct involvement (40%-100%) respect right hepatic duct (40%-57%)^[6,14].

Endoscopic ultrasound (EUS) has a complementary role and can evaluate biliary stenosis and dilations, stones, and in particular it is able demonstrate dilated peri-choledochal venous collateral vessels^[15].

Abdominal ultrasound (US) has not a primary role in PB diagnosis but can be complementary to other techniques to evaluate presence and characteristics of PC, presence of gallbladder varices, signs of portal hypertension, gallstones, biliary tree dilation.

In the literature many classification have been published. Firstly, Chandra and Sarin^[10] identified four PB types according with the biliary abnormalities localization at ERCP: type I, involvement of extrahepatic bile duct only; type II, involvement of intrahepatic bile duct only; type IIIa, involvement of extrahepatic bile duct and unilateral intrahepatic bile duct (left or right); type IIIb, involvement of extrahepatic bile duct and bilateral intrahepatic ducts. Llop *et al.*^[1] classified PB into different degrees of severity: grade I, biliary tree irregularities or angulations; grade II, indentations or strictures without dilation; grade III, strictures with dilation (defined as intrahepatic duct ≥ 4 mm or extrahepatic duct ≥ 7 mm).

At least, Shin *et al.*^[16] distinguished three type of PB base on pathogenetic mechanism: they described

a varicoid type in which biliary irregularities are mainly caused by extrinsic compression, a fibrotic type in which strictures are due to fibrosis and wall thickening that results from ischemic injury, and a mixed type (both kind of alterations and pathogenetic mechanisms are involved).

CLINICAL MANIFESTATIONS AND NATURAL HISTORY

Biliary changes are present in about 77%-100% of patients with PC^[1,3,6]; however, only 5%-38% of patients developed biliary symptoms^[1,3,14]. Symptoms and clinical manifestations of PB can be related to chronic cholestasis and/or biliary stones formations, and they include jaundice, cholangitis, cholecystitis, abdominal pain, cholelithiasis^[17].

Risk factors for symptoms occurrence in PB are older age, longer duration of disease, common bile duct and gallbladder stones, and abnormal liver function tests (LFTs)^[18].

The natural history of PB is still undefined. Dhiman *et al.*^[11] identified four stages in PB progression, shown in Table 1. The progression from stage I to IV is due to worsening of biliary changes, symptoms onset, alterations in liver function tests and complications occurrence. Only one study by Llop *et al.*^[1] investigated the evolution of biliary changes and symptoms in patients with acute and chronic non-cirrhotic non-tumoral PVT: 67 patients were followed with MR angiography (MRA) and MRCP after PVT diagnosis. Among 22 patients with acute PVT, 73% developed biliary alterations at MRA/MR cholangiography (MRC) within a median follow-up of 33 mo (range 1-102): 4 patients had grade I PB, 4 grade II and 8 grade III, while 6 patient didn't had PB. In 14 patients without

Table 1 Characteristics of four stages in portal biliopathy natural history

Stage	Portal cavernoma	Biliopathy	LFTs	Symptoms	Complications
Preclinic	Yes	No	Normal	No	No
Asymptomatic	Yes	Early changes	Normal or abnormal	No	No
Symptomatic	Yes	Advanced changes	Abnormal	Yes	No
Complicated	Yes	Advanced changes	Abnormal	Yes	Yes

LFTs: Liver function tests.

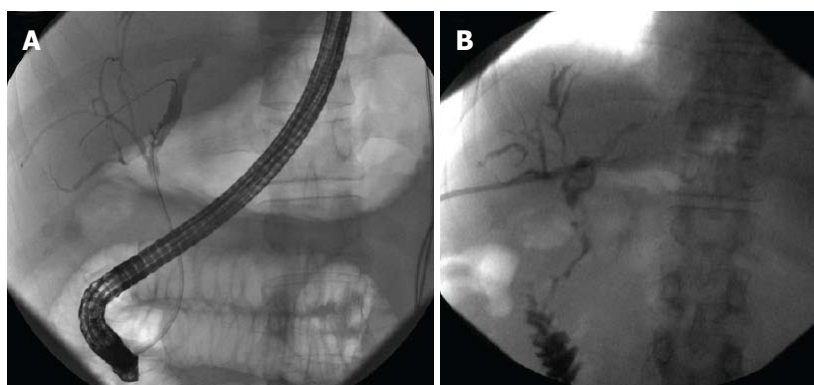


Figure 2 Cholangiographic findings in a patient with symptomatic (jaundice and cholangitis) portal biliopathy secondary to chronic extrahepatic portal vein obstruction and portal cavernoma. Ischemic stenosis with dilatation of the left intrahepatic biliary tree is shown by ERCP (A): patient underwent unsuccessful ERCP with stent insertion and then PTBD placement; PTBD was changed for 3 times in one year because of cholangitis and liver abscess. After clinical and biochemical improvement of BA, patient was treated with surgical splenorenal shunt and after 5 mo PTBD was definitively removed. Last cholangiography obtained before PTBD removal shows significant improvement in biliary dilation (B). Patient is actually asymptomatic for BA and PB management. BA: Bilioenteric anastomosis; ERCP: Endoscopic retrograde cholangiopancreatography; PB: Portal biliopathy; PTBD: Percutaneous transhepatic biliary drainage.

grade III PB, 11 performed another MR during a 43 mo follow-up and no progression to grade III PB was observed. Chronic PVT was present in 45 patients: within a median follow-up of 67 mo (range 0-749), 80% developed PB (3 patients grade I, 7 grade II and 26 grade III). Also in this group, no evolution from grade I - II PB to grade III PB was observed after a mean follow-up of 37 mo. Biliary symptoms occurred in 41% of grade III PB patients, while patients with grade I - II PB remained asymptomatic during follow-up [Positive predictive value (PPV) 41%, Negative predictive value (NPV) 100%]. In conclusion, when PB is established due to the formation of PC in EHPVO, the clinical picture will not change much over time and clinical manifestations will be mainly correlate with site and type of stenosis.

THERAPEUTIC APPROACHES

Treatment of PB is recommended only for symptomatic patients, whereas the presence of biliary abnormalities associated with mild biochemical alterations without clinical manifestations is not an indication for therapeutic intervention but requires a biochemical and clinical follow-up^[11].

Therapy of portal biliopathy should be addressed to firstly reduce portal hypertension and secondly to resolve biliary obstruction. Both surgical and radiological porto-systemic shunt (PSS) can be considered

the treatment of choice to reduce portal hypertension in patients with EHPVO and PC; when PB is related only to a mechanical compression, this approach can resolve both PC and PB at the same time^[19]. However, when the damage of the biliary tree is also ischemic, portal decompression alone is not sufficient to relief biliary obstruction, and in many cases patients need multiple endoscopic and surgical interventions on biliary tree during their life. An example of cholangiographic findings at ERCP before and after endoscopic plastic stent placement is shown in Figure 2.

Medical treatment with ursodeoxycholic acid (UDCA) has a limited role, even if some authors reported resolution or improvement of mild PB symptoms (mainly abdominal pain and biochemical cholestasis without evidence of stones) and prevention in recurrence of cholangitis after UDCA therapy alone^[1,17,20].

At the moment, there is not a standardized flow-chart for PB therapy, and data about different and combined approaches and their outcomes are reported from small series and case reports.

Considering all papers included in this review, a total of 284 PB patients were described, among these, the most frequent described symptoms were jaundice (52.8%), cholangitis (27.1%) and abdominal pain (19.4%). The mean age at PB presentation was 33.5 ± 13.5.

Hereafter we analyze the different types of treatment reported in the included articles.

Endoscopic treatment

Among the total of symptomatic PB patients included in case series and case reports, 114 patients were treated endoscopically at first. Only in 34 patients (29.8%) a single endoscopic treatment resolved PB alterations and symptoms; among these, 13 patients had common bile duct (CBD) stones as PB manifestation resolved after endoscopic sphincterotomy (ES) + stone extraction (SE). In most cases, repeated treatment were necessary to have a complete resolution of biliary abnormalities, often combined with surgical intervention for portal hypertension: after the first treatment, 49 patients (43%) underwent multiple plastic stent exchange, 4 (3.5%) metallic stent placement, 35 (30.7%) surgical treatment with PSS or splenectomy (with or without esophageal devascularisation), 7 (6%) surgical biliary anastomosis, 2 (1.7%) transjugular intrahepatic porto-systemic shunt (TIPS) and 1 (0.9%) liver transplantation (LT). The duration of follow-up, when reported, range between 2 d and 18 years. In these cases the endoscopic approach can be considered safe, even if some complications have been reported. The most frequent is haemobilia, occurred in 24 procedures (in one case after intracholedocal biopsy). The risk of haemobilia during endoscopic treatment for PB is related to the presence of numerous pericholedocal compensatory collateral veins and congestion of biliary duct walls vessels. In particular, the transient pressure elevation in the distal portion of biliary varices during balloon sweeping can increase the risk of bleeding^[21]. For these reasons, a decompressive shunting procedure performed before endoscopic treatment could reduce the risk of haemobilia^[21,22].

Cholangitis were also frequent (reported after 53 procedures), but although they can be seen after ERCP in up to 15.4% of cases^[23], they are often not directly related to the endoscopic treatment but rather to recurrence of sludge or stones inside the biliary stent and can be treated with stent exchange. Sepsis was observed as well in 3 cases. Mortality directly related to endoscopic treatment was 0%, but one patient died for secondary biliary cirrhosis and one for cholangitis, both developed despite multiple stent placements during the treatment period.

The choice of the endoscopic treatment of PB depends on the type of biliary alterations and includes ERCP with ES, SE (with previous mechanical lithotripsy if necessary) for patients with choledocholithiasis, stricture dilation ± biliary stent or nasobiliary drain placement.

The largest series of PB patients endoscopically treated is described by Saraswat *et al.*^[24], who published data about 130 ERCP performed for biliary strictures in 20 symptomatic patients. ES with SE was performed in 8 patients with choledochal stones and 9 patients were treated with plastic stents placement. Eleven patients (for a total of 101 procedures) were treated on the line of postoperative benign biliary strictures, with balloon dilation and plastic stent insertion,

that were exchanged every 3-4 mo until LFTs normalizations; in 8/11 case a cholangiogram normalization was achieved as well. ERCP complications included haemobilia in 9/130 procedures and cholangitis in 40/130 (mainly already present at the moment of ERCP and due to delay in stent exchanged).

Khare *et al.*^[25] published a case series including 13 patients with EHPVO and PC complicated by symptomatic PB divided in 3 groups according to radiological biliary findings: group A included 5 patients with biliary strictures without choledocholithiasis; group B included 3 patients with choledocholithiasis alone; group C included 5 patients with both biliary strictures and choledocholithiasis. In group A, 4 patients were treated endoscopically with biliary stenting alone (n. 3) or with previous dilation (n. 1), followed by surgical PSS for portal hypertension. In 3/4 patients ERCP resolved biliary strictures, while 1 patient showed persistent stenosis at 12 mo and underwent multiple endoscopic procedures with dilation and stenting. In group B, 2/3 patients were treated with endoscopic SE that was successful in one patient; the other patient underwent stent placement and then surgical intervention for portal hypertension (splenectomy with esophagogastric devascularisation). During the follow-up, this patient needed multiple ERCP for choledochus clearance. In group C, four patients were first treated with endoscopic approach but SE failed in all of these patients; after surgical approaches (PSS or splenectomy with esophagogastric devascularisation), 2/4 patients were successfully managed with repeated ERCP with dilation and SE. No complications were observed after ERCP.

In 2009 the group from Birmingham published his experience with 13 patients with symptomatic portal biliopathy^[17]: at the cholangiography (PB was diagnosed with MRC in 12 patients and with ERCP in 1), 12 patients showed biliary strictures, 10 bile duct stones/sludge, 11 gallbladder stones/sludge. In one patients symptoms (right hypocondrial pain and dark urine) resolved spontaneously, while 12 patients underwent therapeutic ERCP that was successful in 8: in one patients with associated Crohn's disease plastic stent insertion did not resolved jaundice and the patient, that was not eligible for surgical portal decompression for PVT extension, was listened for liver and small bowel transplantation; one patient had an excellent response to ES and SE. Among 7 patients with jaundice resolution after plastic stent insertion, 2 patients underwent portal decompression for recurrent cholangitis despite multiple ERCP (one with PSS and one with TIPS), 1 needed repeated plastic stent changes and in 3 patients a metallic biliary stent was placed after several repeat plastic stent changes. Among 4 cases in which ERCP was unsuccessful, 2 had spontaneous resolution. The observed complications during a total of 49 ERCP were haemobilia (4%) and sepsis (6%, two cholangitis and one enterococcal sepsis), and no death directly related to biliary complications was observed during the follow-up.

In particular, the last two articles evidence how complex is to choose the appropriate treatment for PB, and the great variability on patients response to the same therapy, even between patients with the same type of biliary manifestation. This is also due to the difficulty to classify the etiology of PB (varicoid type or fibrotic type) based on cholangiographic findings.

Combined treatment with biliary procedures and percutaneous transhepatic portal vein recanalization (balloon dilation + self-expandable stent placement) have also been reported^[26].

The role of UDCA administration associated with endoscopic procedures is still uncertain: even if some authors reported the absence of biliary symptoms recurrence after endoscopic treatment associated with UDCA therapy^[1,3], equally good results with the only endoscopic management are reported.

All published PB case series and case reports about endoscopic treatment of PB are summarized in Tables 2 and 3^[1,3,15,17,20,22-47], respectively.

Surgical treatment

Indication to surgery in PB patients is given by the need to decompress the portal system through PSS and to resolve the biliary obstruction. In cases in which PB is due to biliary compression by PC, the detension of collateral vessels obtained with the reduction of portal pressure by PSS can resolve in the same time biliary obstruction^[30]. The most common PSS performed are proximal spleno-renal shunt or mesocaval shunt, but other types of surgical shunts include meso-gonadal vein shunt, meso-renal shunt, right-portal ovarian shunt, shunt between a portal varix and cava^[41,48-51].

However, in patients without resolution of biliary abnormalities and symptoms after PSS, a second stage procedure can be required: biliary stenosis can be managed endoscopically, as explain above, or with surgical construction of a bilioenteric anastomosis. In patients without a suitable patent vein, splenectomy associated with esophagogastric devascularisation could reduce pressure in pericholedochal collateral veins and improve biliary symptoms^[52]. A surgical approach with intrahepatic segment 3 bypass has been described to provide definitive treatment for biliary decompression and stone removal in a single time procedure in appropriately selected patients^[47].

The largest PB series^[52] retrospectively included 56 PB patients who underwent surgery from 1996 to 2010; 32/56 (57.1%) were asymptomatic for PB. To reduce portal hypertension, PSS was performed in 40 patients and splenectomy with devascularisation in 16. After first-line surgery, 7 patients required endoscopic treatment for cholangitis or CBD stones, that was successful in 2 patients, while 5 of them required multiple procedures; 2 patients previously treated with PSS at least needed biliary surgery for dominant CBD stricture that required frequent stent exchanges. In addition,

the authors reported a significant reduction in serum biliary levels after first-line surgery (both shunt and no-shunt surgery) and alkaline phosphatases (shunt surgery), confirming that the resolution or improvement in portal hypertension can be effective in relieving biliary obstruction.

Vibert *et al*^[30] published a case series including 19 symptomatic PB patients and propose an interesting 3 steps approach: (1) biliary drainage and antibiotic therapy if cholangitis is present; (2) surgical PSS; and (3) biliodigestive anastomosis with hepatico-jejunal anastomosis with Roux-en-Y. Patients were divided in two groups according to feasibility of PSS: 10 patients were included in the PSS group, 9 patients in the no PSS (NPSS) group. In the first group, one patient with severe sepsis from cholangitis underwent percutaneous transhepatic biliary drainage (PTBD) with extraction of intrahepatic stones. Then, a splenorenal shunt was performed in all 10 patients. Mortality was nil, and complications rate was 27%, including one chylus fistula and 2 early thrombosis, successfully treated with anticoagulant therapy or angiographic-guided pneumatic dilation. Initially, PSS was successful in biliary symptoms resolution in 70% of cases, but within 30 mo after PSS 5/10 patients required a bilioenteric anastomosis because of persistent jaundice or recurrent cholangitis. In the NPSS group, 3 patients were initially treated with endoscopic approach (1 ES and 2 plastic stent placement); PTBD was positioned in 6 patients (in 3 cases because of recurrence of biliary symptoms after endoscopic treatment); biliodigestive anastomosis was performed in 4 patients. Among these, one patient initially treated with endoscopic stent placement, 2 patients initially treated with PTBD and 4 patients treated with biliodigestive anastomosis needed repeated transhepatic cholangioscopies to remove intrahepatic stones and to improve biliary drainage; except for 2 patients lost in follow-up, at long-term follow-up one patient died for severe cholangitis and haemobilia while other patients were asymptomatic.

Overall, 173 patients underwent surgery intervention as first or second step for PB treatment: PSS was performed in 131 patients, PSS with splenectomy in 7, splenectomy with devascularisation in 18, devascularisation in 1 and 16 patients underwent biliary surgery (biliodigestive anastomosis, cholecystectomy, choledochotomy). The reported follow-up after surgical intervention ranged between 4 mo and 15 years. Considering patients underwent PSS alone as first treatment for PB, biliary symptoms relieve in 64.1% of cases, while in patients firstly treated with surgical biliary anastomosis, only in 30% of cases no other treatment was required.

A total of 6 death was reported: 1 patients died after 10 years from splenectomy for recurrence of gastrointestinal bleeding, 1 patient died for decompensated alcoholic cirrhosis, one for perforated duodenal

Table 2 Endoscopic management of portal biliopathy: summary of case series

Ref.	No. of patients	Biliary abnormalities	First treatment	Follow-up	Further treatments	Complications/ outcome
Bhatia <i>et al</i> ^[27] , 1995	4 symptomatic	Stenosis + stones 3 CBD stones 4	ES + SE + NBD 4	3-8 mo	Multiple ERCP 4	None
Perlemuter <i>et al</i> ^[20] , 1996	8 symptomatic	Stenosis 8 CBD stones 2 Cholangitis 1	ES+NBD 3 BD 1 PSS 1 UDCA 2	6-60 mo	Multiple ES 1	Death 2 (cholangitis 1; stroke 1)
Condat <i>et al</i> ^[3] , 2003	7 symptomatic	Cholecystitis/right hypochondrial pain 4 Cholangitis 1 Stenosis 2	Cholecystostomy + SE 1 Stent 1 BA + PTBD 1 UDCA 4	4-25 mo	-	Haemobilia 1
Sezgin <i>et al</i> ^[28] , 2003	10 symptomatic	Stenosis 9 IE stones 1	ES + stent 10 NBD 4 BD 4	3.3 yr (range 1-7)	Multiple ERCP 5	Haemobilia 1 Cholangitis 5 Death 1
Dumortier <i>et al</i> ^[22] , 2003	6 symptomatic	Stenosis 5 CBD stones 2	ES 5 BD 5 SE 2 Stent 1	10 mo (range 2 d-18 mo)	Multiple ERCP + PSS 4	Cholangitis 1 Cholecystitis 4
Khare <i>et al</i> ^[25] , 2005	13 symptomatic	Stenosis 10 CBD stones 8	Stent 4 BD 6 SE 4	-	PSS 8 BA 1 Multiple ERCP 2 Splenectomy + devasc 2	Death 1
Dhiman <i>et al</i> ^[29] , 2007	12 symptomatic	Stenosis 7 CBD stones 5 CBD varices 1 Mirizzi's sdr 1	PSS 5 ES 3 ES + BD 2 Stent 4	19 mo (6-132 mo)	Multiple ERCP in pts initially treated with stent	Cholangitis in 2 pts treated with stent
Vibert <i>et al</i> ^[30] , 2007	19 symptomatic	IE biliary dilation 9 IE stones 7 CBD stones 4	PSS group: PTBD 1 SRS 10 NPSS group: ES + stent 2 ES + SE 1 PTBD 6 BA 4	19 pts 8.3 yr	PSS group: BA 5 NPSS group PTBD 1 after ERCP and 4 after BA	Resolution 17 Death 3
Oo <i>et al</i> ^[17] , 2009	13 symptomatic	Stenosis 13 CBD stones 10 GB stones 11	UDCA 1 Stent 7 ES + SE 1 Failed ERCP 4	2 yr (1-18 aa)	Metallic stent 3 Stent exchange 2 PSS 3 (2 TIPS, 1 surgical) LT 1	Haemobilia 2 Sepsis 3
Llop <i>et al</i> ^[1] , 2011	14 symptomatic	Stenosis 14 CBD stones 6 GB stones 2	ES + SE 6 ES + UDCA 2 Cholecystectomy 2	-	Multiple ERCP 1 BA 1	-
Saraswat <i>et al</i> ^[24] , 2013	20 symptomatic	Stenosis 20 CBD stones 8 GB stones 6	ES + SE 8 Stent 9 BD + stent 11	18 mesi (range 3-188)	Multiple ERCP 11	In 130 procedures: Cholangitis 40 Haemobilia 9 - Resolution 5
Ramchandani <i>et al</i> ^[31] , 2013	5 symptomatic	CBD stenosis 2 CHD stenosis 1 CBD stones 2	Metallic stent 1 Plastic stent 2 BD + stent 1 Intracholedocal lithotripsy 1	6-7 mo	SRS 2 Stent Exchange 1	-
Cellich <i>et al</i> ^[23] , 2015	8 symptomatic 1 asymptomatic	Stenosis 7 CBD stones 3 GB stones 1	ES 7 BD 4 SE 2 Stent 7	-	PSS 1 Stent exchange 3 BA 3	Cholangitis 3 Haemobilia 1

CBD: Common bile duct; GB: Gallbladder; IE: Intrahepatic; ES: Endoscopic sphincterotomy; SE: Stone extraction; NBD: Nasobiliary drainage; BD: Balloon dilation; PSS: Porto-systemic shunt; NPSS: No porto-systemic shunt; UDCA: Ursodeoxycholic acid; ERCP: Endoscopic retrograde cholangio-pancreatography; BA: Bilioenteric anastomosis; SRS: Splenorenal shunt; LT: Liver transplantation; TIPS: Transjugular intrahepatic porto-systemic shunt.

ulcer and one for cholangitis and haemobilia after 8.6 years from biliodigestive anastomosis, 2 patients died for intraoperative bleeding during surgery on bile duct (choledocholithotomy and hepaticojejunostomy), suggesting a higher risk of bleeding from biliary tree due to the presence of numerous compensatory collateral veins and the congestion of biliary and splanchnic venous system. Table 4^[2,3,19,25,30,51-58] summarized

papers about surgical intervention in PB patients.

TIPS

In addition to surgical PSS, a TIPS placement can be a valid alternative to improve portal hypertension. Since 1996, Görgül *et al*^[59] observed the resolution of "pseudocholangiocarcinoma" sign after TIPS in 3 patients. In a case report, a 45-years-old woman

Table 3 Endoscopic management of portal biliopathy: summary of case reports

Ref.	Patients	Biliary abnormalities	First treatment	Follow-up	Further treatment	Complications
Mörk <i>et al</i> ^[32] , 1998	2	CBD stenosis 2	Stent 2	-	Multiple Stent exchange + PSS 1	Cholangitis 1
Solmi <i>et al</i> ^[33] , 1998	1	Stenosis	Stent	-	-	-
Mutignani <i>et al</i> ^[34] , 2002	3	CBD stenosis 3	Stent 3	-	PSS 3	Haemobilia 3
Perego <i>et al</i> ^[35] , 2003	1	Stenosis + CBD stones	- Stent	3 yr	Multiple stent exchange→PTBD + dilation and SE	-
Umphress <i>et al</i> ^[15] , 2004	M, 62 yr	IE and CBD stones	ES + SE + stent	1 yr	Stent exchange and cholecistectomy	-
Guerrero Hernández <i>et al</i> ^[36] , 2005	M, 29 yrs	CBD stenosis	ERBD + Sugiura	-	-	-
Rosenthal <i>et al</i> ^[37] , 2008	F, 44 yr	CBD stenosis and stones	Stent + SE	-	PSS	-
Ajayi <i>et al</i> ^[38] , 2009	F, 13 yr	Stenosis	ES + BD + stent	6 mo	Multiple stent exchange	-
Layec <i>et al</i> ^[39] , 2009	F, 74 yr	Stenosis	Metallic stent	18 mo	Metallic stent	Haemobilia
Sharma <i>et al</i> ^[40] , 2009	M, 35 yr	CBD stenosis 3	ES+SE 3	-	-	Haemobilia 3
	F, 30 yr					
	M, 25 yr					
Vasiliadis <i>et al</i> ^[41] , 2009	F, 39 yr	CBD stenosis	ES	19 mo	Multiple stent exchange→PSS + cholecistectomy	-
Cantù <i>et al</i> ^[42] , 2010	M, 31 yr	CBD stenosis	ES + stent	4 yr	-	-
Martinez <i>et al</i> ^[43] , 2011	M, 34 yr	IE dilataions	Stent	-	-	-
Mistry <i>et al</i> ^[44] , 2012	M, 28 yr	CBD stenosis	BD + BA + PTBD	-	Percutaneous transhepatic-gastrostomy	Haemobilia
					Cholecistectomy	-
Alam <i>et al</i> ^[45] , 2012	M, 30 yr	CBD stenosis 2	PTBD	-		
	F, 19 yr	GB stones 1	Stent			
		CBD stones 1				
D'Souza <i>et al</i> ^[46] , 2013	M, 49 yr	CBD stenosis	Stent + BD (after stent removal)	-	PSS + splenectomy	Haemobilia (post-biopsy)
Bernon <i>et al</i> ^[47] , 2014	M, 36 yr	CBD stenosis and stones	SE + stent	1 yr	Stent exchange→PTBD→cholecistectomy, intrahepatic 3 semgment bypass	Cholangitis
Hyun <i>et al</i> ^[26] , 2015	M, 33 yr	CBD stenosis	ERBD→PTBD + portal stent	3 mo	-	Haemobilia

CBD: Common bile duct; ES: Endoscopic sphincterotomy; SE: Stone extraction; BD: Balloon dilation; PSS: Porto-systemic shunt; IE: Intrahepatic; BA: Bilioenteric anastomosis; ERBD: Endoscopic retrograde biliary drainage; PTBD: Percutaneous transhepatic biliary drainage.

affected by cholestatic jaundice due to compression on CBD by extrahepatic portal and splenic vein thrombosis with collateral, was treated with TIPS: in order to decompress the biliary tree before the procedure, a biliary stent was placed and the day after TIPS was successfully performed. The biliary stent was removed after 1 wk and, at 14 mo follow-up, the patient was asymptomatic and MR/MRCP showed a significant improvement of biliary alterations and of cavernoma size^[60]. Another case report described resolution of cholangiographic CBD abnormalities and normalization of LFTs after TIPS performed in a patient with PB and portal hypertension secondary to PVT^[61]. In the case series published by Cellich *et al*^[23] including 13 PB patients, one of these was initially treated with TIPS that was unsuccessful; patient underwent PSS and multiple endoscopic treatment and, at least, bilioenteric anastomosis. Oo *et al*^[17] reported 2 patients successfully treated with TIPS: one after repeated ERCP with plastic stent exchanges obtaining resolution of biliary symptoms and reduction in portal pressure; the second was successfully treated with temporary PTBD placement followed by TIPS. Even if the use of TIPS in PB patients is anecdotal, this technique seems effective and safe in treatment of portal hypertension and PB secondary to PC. However, due to the vascular

alterations secondary to PC, TIPS is not always technically feasible.

LT

There are few data about LT for PB patients. In literature only 4 cases are reported (1 regarding a paediatric patient), 2 living donor LT and 2 deceased donor LT^[62-65]. Indication for LT was secondary biliary cirrhosis, recurrent cholangitis (even with suspicion of cholangiocarcinoma) despite multiple endoscopic treatments, often associated with gastrointestinal bleeding^[62-64]; Zhang *et al*^[65] reported 3 successful living donor LT in 3 paediatric patients with PC, one of these with jaundice and evidence of dilated biliary duct due to PC compression, consistent with PB; however, in all 3 cases the major indication for LT was deteriorating liver function ad recurrent gastrointestinal bleeding. In all the 4 cases of LT for PB the outcome was favourable after a follow up of 12-26 mo.

CONCLUSION

PB is a frequent complication in patients with chronic PVT and PC, however symptoms are present in a minority of cases and only symptomatic patients require therapy.

Table 4 Surgical management of portal biliopathy: summary of case series and case reports

Ref.	No. of patients	Biliary abnormalities	First treatment	Follow-up	Further treatments	Complications/outcome
Chaudhary <i>et al</i> ^[53] , 1998	9 symptomatic	Stenosis: 2 CBD stones: 2	BA 2 SRS 7	9 pts -	BA 2 Stent 1 ES + SE 2	Death 1 Resolution 7
Condat <i>et al</i> ^[53] , 2003	7 symptomatic	Cholecystitis/right hypocondrial pain 4 Cholangitis 1 Stenosis 2	Cholecystostomy + ERCP 1 Stent 1 BA+PTBD 1	4-25 mo	-	Haemobilia 1
Gauthier-Villars <i>et al</i> ^[19] , 2005	8 symptomatic (pediatric)	Stenosis 6 Biliary dilation 8	- PSS 8	8 pts 4.5-15 yr	-	Complete resolution 7 Partial resolution 1
Khare <i>et al</i> ^[25] , 2005	13 symptomatic	A: Stenosis 5 B: CBD stones 3 C: Stenosis + CBD stones 5	A: PSS 4; BA 1 B: ERCP 2; PSS + BA 1 C: Unsuccessful ERCP 4; PSS 3; Splenectomy + devasc 2	-	A: Multiple ERCP: 1 B: Splenectomy 1 (post-ERCP) C: Multiple ERCP 2; BA 1; Splenectomy + BA 1	Death 1
Vibert <i>et al</i> ^[30] , 2007	19 symptomatic	IE biliary dilation 9 IE stones 7 CBD stones 4	PSS group: PTBD 1 SRS 10 NPSS group: ES + stent 2 ES + SE 1 PTBD 6 BA 4	19 pts 4-30 mo	PSS group: BA 5 NPSS group PTBD 1 after ERCP and 4 after BA	Resolution 17 Death 3
Dhiman <i>et al</i> ^[2] , 2007	12 symptomatic	Stenosis: 7 CBD stones: 5 Choledochal varices 2 Mirizzi's syndrome 1	PSS 5 ES 3 ES + dilation 2 Stent 4	19 mo (range 6-132)	Multiple stent exchange in pts initially treated with stent	Cholangitis in 2 pts treated with stent
D'Souza <i>et al</i> ^[54] , 2009	1 symptomatic	CBD stenosis + GB stones	Pre-surgery stent→PSS + BA (single stage)	18 mo	-	Resolution
Camerlo <i>et al</i> ^[51] , 2010	3 symptomatic	Stenosis 3	PSS 3 Stent 1 (pre-PSS)	3 pts 2-13 yr	-	Resolution 3
Agarwal <i>et al</i> ^[55] , 2011	39 symptomatic	Stenosis 15 CBD stones 7 GB stones 12 IE dilation 39	SRS 37 BA 2	37 pts 32 mo	ES ± SE 10 BA 12 ES + cholecystectomy 1	Resolution 35
Chattopadhyay <i>et al</i> ^[52] , 2012	24 symptomatic 32 asymptomatic	CBD stenosis 3 Multiple stenosis 5 IE dilation 14 CBD stones 7 GB stones 11 Stenosis 3	ERCP pre-surgery 12 PSS 40 Splenectomy + devasc. 16	43 pts 48 mo (range 14-120)	ES + SE 2 Multiple ES + stent 5 BA 2	Resolution 38 Death 1
Suárez <i>et al</i> ^[56] , 2013	3 symptomatic	Stenosis 3	UDCA 1 BA 1	-	-	-
Bhatia <i>et al</i> ^[57] , 2014	2 symptomatic	GB stones	Cholecistectomy	-	-	Resolution
Liu <i>et al</i> ^[58] , 2015	18	Stenosis 6 Dilations 6 Stenosis + dilations 6	PSS 18	-	-	Resolution 15

CBD: Common bile duct; GB: Gallbladder; IE: Intrahepatic; ES: Endoscopic sphincterotomy; SE: Stone extraction; BD: Balloon dilation; PSS: Porto-systemic shunt; NPSS: No porto-systemic shunt; UDCA: Ursodeoxycholic acid; ERCP: Endoscopic retrograde cholangio-pancreatography; BA: Bilioenteric anastomosis; SRS: Splenorenal shunt; FU: Follow-up.

The majority of patients with PC and PB need multiple treatments during their life, aimed both to decompress portal circle and to resolve biliary abnormalities and symptoms; only few patients have a complete resolution of biliary and vascular problem after the first intervention, and it can be seen more often in PSS performed for PC compressing the bile duct. In most cases a combined approach is required (endoscopy and surgery), but a consensus on the timing and priority of treatments is not still available.

In Figure 3 we propose a possible algorithm for the management of PB. For asymptomatic patients, no specific therapy is required except the eventual

treatment of portal hypertension complications; during follow-up LFTs monitoring is suggested. For symptomatic patients, cholecistectomy is recommended in case of gallbladder stones or cholecystitis alone. In case of PB due to PC compression (varicoid type), surgical PSS or TIPS (when feasible) should resolve both PC and biliary stenosis. However, if cholangitis or choledocholithiasis are present, they should be treated at first. In case of mixed type, an ischemic damage coexist with the compressive one and the only PSS will not solve the problem; in these case, further interventions on the biliary system are needed: consider ES with/without stent placement and, in case of persistence of bili-

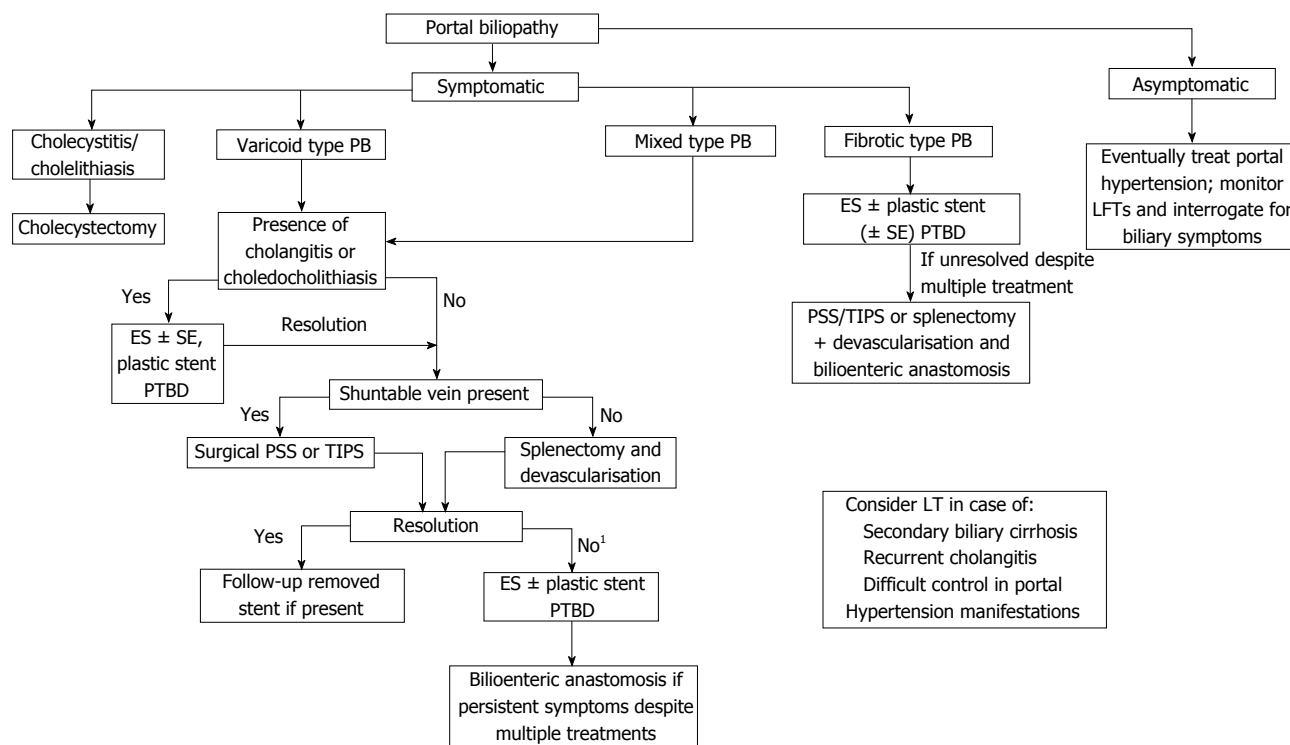


Figure 3 Proposed algorithm for portal biliopathy management. ¹Unsuccessful vascular surgery is frequent in mixed type because of co-presence of ischemic and compressive damage. ES: Endoscopic sphincterotomy; SE: Stone extraction; LFTs: Liver function tests; LT: Liver transplantation; PTBD: Percutaneous transhepatic biliary drainage; PB: Portal biliopathy; PSS: Porto-systemic shunt; TIPS: Transjugular intrahepatic porto-systemic shunt.

ary symptoms despite multiple endoscopic treatments, surgical intervention of bilioenteric anastomosis. When a fibrotic type of PB is diagnosed, PSS will not resolve the biliary alterations. In these cases, PB resolution is difficult to achieve and multiple endoscopic/surgical biliary treatments are required. Patients can be evaluated for LT in case of secondary biliary cirrhosis, recurrent cholangitis despite multiple endoscopic treatments or difficult control in portal hypertension manifestations.

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Understanding the role of PIN1 in hepatocellular carcinoma

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Abstract

PIN1 is a peptidyl-prolyl *cis/trans* isomerase that binds and catalyses isomerization of the specific motif comprising a phosphorylated serine or threonine residue preceding a proline (pSer/Thr-Pro) in proteins. PIN1 can therefore induce conformational and functional changes of its interacting proteins that are regulated

by proline-directed serine/threonine phosphorylation. Through this phosphorylation-dependent prolyl isomerization, PIN1 fine-tunes the functions of key phosphoproteins (*e.g.*, cyclin D1, survivin, β -catenin and x-protein of hepatitis B virus) that are involved in the regulation of cell cycle progression, apoptosis, proliferation and oncogenic transformation. PIN1 has been found to be over-expressed in many cancers, including human hepatocellular carcinoma (HCC). It has been shown previously that overexpression of PIN1 contributes to the development of HCC *in-vitro* and in xenograft mouse model. In this review, we first discussed the aberrant transcription factor expression, miRNAs dysregulation, *PIN1* gene promoter polymorphisms and phosphorylation of PIN1 as potential mechanisms underlying PIN1 overexpression in cancers. Furthermore, we also examined the role of PIN1 in HCC tumourigenesis by reviewing the interactions between PIN1 and various cellular and viral proteins that are involved in β -catenin, NOTCH, and PI3K/Akt/mTOR pathways, apoptosis, angiogenesis and epithelial-mesenchymal transition. Finally, the potential of PIN1 inhibitors as an anti-cancer therapy was explored and discussed.

Key words: Phosphorylation; Hepatocellular carcinoma; PIN1; Isomerization; Hepatocarcinogenesis

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Core tip: PIN1 specifically binds and catalyses isomerization of the pSer/Thr-Pro motif of target proteins, thereby modulating their functions. Many PIN1-interacting proteins are involved in cellular transformation and maintenance of malignant phenotype, and overexpression of PIN1 is frequently found in various cancer types, including hepatocellular carcinoma (HCC). Through interacting with regulatory proteins of key signalling pathways, such as β -catenin, cyclin D1, and HBx, PIN1 drives and amplifies the oncogenic signals essential for the development of HCC. Given its diverse oncogenic functions in hepatocarcinogenesis, PIN1

represents a potential therapeutic target in the treatment of HCC.

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the fifth leading cancer in men and the ninth for women worldwide, with estimated 782000 new cases in 2012. It is one of the most common causes of cancer death, leading to 746000 deaths annually^[1]. Chronic infection with hepatitis B virus (HBV) or hepatitis C virus (HCV) is the major risk factor^[2], accounting for 92% and 43% of HCC cases in developing and developed countries, respectively. HCC is an aggressive malignant tumour associated with a poor prognosis, and only a small proportion of HCCs is detected at an early stage. Early-stage HCCs are amenable to potentially curative treatments, such as surgical resection, liver transplantation, transcatheter arterial chemoembolization (TACE) and radiofrequency ablation (RFA)^[3]. Nonetheless, HCCs are frequently diagnosed at advanced-stage and patients with advanced HCCs are not candidates for curative therapies. Conventional chemotherapy is ineffective for advanced HCCs because of the development of chemo-resistance and early occurrence of metastasis^[4-6]. A thorough understanding of the pathogenesis and biology of HCC provides the mechanistic basis for designing effective HCC therapies.

Protein phosphorylation is a post-translational modification that plays an important role in the regulation of signalling pathways. Through activation of multiple protein kinases, such as cyclin dependent kinases (CDKs) and mitogen activated protein kinases (MAPKs), phosphorylation of proteins in serine or threonine residues preceding proline (pSer/ Thr-Pro) motif has been linked to dysregulated cell proliferation and malignant transformation^[7,8]. The identification of peptidyl-prolyl *cis/trans* isomerase PIN1 provides a new post-phosphorylation regulatory mechanism in cell signalling^[9-12]. PIN1 is a small and highly evolutionarily conserved 18-kDa protein, and is mainly localized in the nucleus^[11,13,14]. It binds specific pSer/Thr-Pro motif in certain proteins through its amino-terminal WW domain, and isomerizes the pSer/Thr-Pro peptide bonds with its carboxyl-terminal prolyl isomerase (PPIase) domain^[7,11,15] (Figure 1). PIN1-catalysed isomerization induces conformational changes of its target proteins, resulting in alterations of their enzymatic activities, phosphorylation status, protein-

protein interaction patterns, subcellular localization, and protein stability. Conceivably, PIN1 plays an important role in diverse cellular processes, including cell cycle progression, differentiation, apoptosis and proliferation, as well as transformation^[11,16-19]. Indeed, many PIN1-interacting partners, such as β -catenin, c-Jun, cyclin D1, cyclin E, Myc, nuclear factor-kappa B (NF- κ B)-p65, p53 and p73, are important in regulating cell cycle progression and cell proliferation, and are often dysregulated in cancer^[17,18,20-25]. Thus, the role of PIN1 in enhancing the oncogenic potential of these proteins *via* phosphorylation-dependent prolyl isomerization is important during cancer development.

In this article, we discuss the possible mechanisms underlying dysregulated PIN1 expression in cancer, the oncogenic roles of PIN1 in hepatocarcinogenesis, and the potential of PIN1 inhibitors as anti-cancer agents.

REGULATION OF PIN1 EXPRESSION AND ACTIVITY

In normal cells, PIN1 expression is usually very low and is tightly regulated by the retinoblastoma protein (Rb)-E2F pathway^[26,27]. The binding between Rb and E2F proteins is controlled by the phosphorylation of Rb. Hypophosphorylated Rb binds E2F transcription factors and inhibits its transcriptional activity towards the *PIN1* gene. In response to cell proliferative stimuli, CDK-cyclin complexes phosphorylate and inactivate Rb to release E2F. In turn, E2F binds to the E2F-binding sites of the *PIN1* promoter and directly activates transcription of the *PIN1* gene. Interestingly, PIN1 has also been found to interact with Rb and enhance its hyperphosphorylation^[28,29]. Therefore, PIN1 inactivates Rb and promotes E2F target gene activation. Since dysregulation of the Rb-E2F pathway is frequently found in various cancers^[30], it is speculated that abnormalities of this pathway may contribute to PIN1 overexpression in cancers. Furthermore, PIN1 interacts with phosphorylated NOTCH1 to enhance NOTCH1 transcriptional activity, which in turn, increases the transcription of *PIN1*, resulting in a positive feedback loop for PIN1 overexpression in cancers^[31]. In addition to E2F and NOTCH1, forkhead box transcription factor FOXO1 also enhances *PIN1* promoter activity, resulting in increased mRNA and protein expression of PIN1 in breast cancer cells^[32].

MicroRNAs (miRNAs) are small non-coding RNA that functions as a negative regulator of gene expression by binding to the 3'UTR of target mRNA to inhibit gene expression at the post-transcriptional level^[33]. Dysregulation of miRNAs expression is frequently observed in cancers^[34]. In HCC, a global reduction of miRNAs expression is associated with HCC progression^[35], suggesting that most of the expressed miRNAs in normal hepatocytes function as tumour suppressors. Some of these miRNAs may target the expression of PIN1 and their reduced expression may

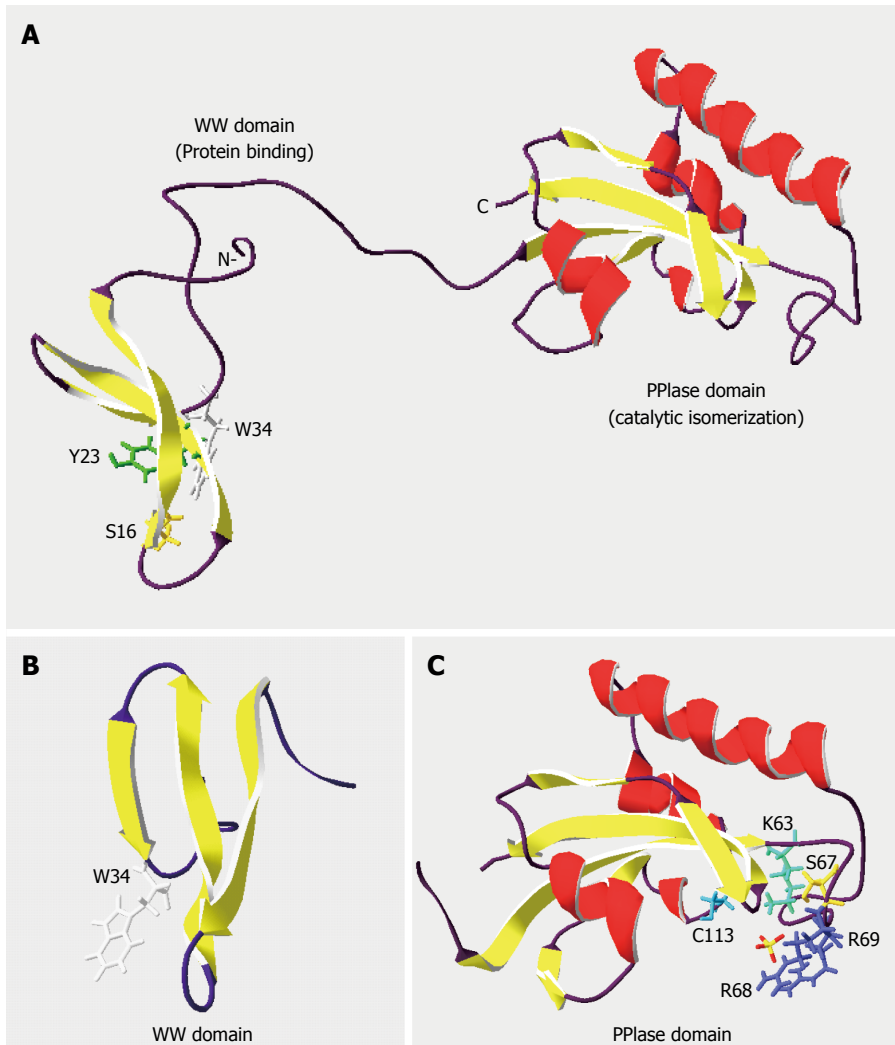


Figure 1 Structure of peptidyl-prolyl-isomerase PIN1 protein. Ribbon diagrams of (A) PIN1 (NCBI Structure No. 1NMV), (B) WW binding domain (NCBI Structure No. 1I8H), and (C) PPIase catalytic domain (NCBI Structure No. 1NMW) were drawn with the Swiss-Pdb Viewer^[11,15,115,116]. α -helices and β -strands are denoted by coils and arrows, respectively. Residues Ser(S)¹⁶, Tyr(Y)²³ and Trp(W)³⁴ in the WW domain are critical for phospho-protein binding, while residues Lys(K)⁶³, Ser(S)⁶⁷, Arg(R)^{68/69} and Cys(C)¹¹³ contribute to the PPIase activity. Adapted from thesis: Identification and characterization of PIN1 binding partners, HKU 2010.

therefore result in the PIN1 overexpression observed in HCC. Currently, three miRNAs have been shown to negatively regulate PIN1 expression in cancers. miR-200b/c and miR-296-5p directly target and suppress PIN1 expression in breast cancer and prostate cancer cells, respectively^[36-38]. However, no specific miRNA has been reported to target PIN1 expression in HCC.

Single nucleotide polymorphism (SNP) of the *PIN1* gene promoter may also contribute to the regulation of PIN1 expression. The promoter region of *PIN1* gene contains two SNPs [rs2233678 (-842G/C) and rs2233679 (-667C/T)] and one synonymous SNP [rs2233682 (Gln33GlnG>A)] in exon 2. Genotype -842CC is associated with lower PIN1 protein expression in peripheral mononuclear cells, whereas -677C/T genotype does not have significant effect on PIN1 expression^[39]. Similar result was also found in squamous cell carcinoma of head and neck (SCCHN), with -842C genotype but not -677C/T associated with a lower *PIN1* promoter activity and a lower risk of

SCCHN^[40].

In addition to the transcriptional regulation, PIN1 level is also regulated through post-translational modification. Phosphorylation at Ser⁶⁵ by Polo-like kinase I (PLK1) inhibits ubiquitination of PIN1, resulting in decreased proteasomal degradation and increased protein level^[41]. Moreover, multiple regulators have also been found to modulate the activity of PIN1 by post-translational modifications. Sumoylation of Lys⁶ on the WW domain and Lys⁶³ on the PPIase domain suppress PIN1 protein-binding and catalytic abilities, respectively. De-sumoylation on those sites by SUMO1/sentrin specific peptidase 1 (SEN1) reverses the inhibitory function on PIN1 and increases PIN1 stability^[42]. In addition, mixed-lineage kinase 3-induced phosphorylation of PIN1 at Ser¹³⁸ enhances PIN1 catalytic activity^[43], whereas death-associated protein kinase 1-induced phosphorylation of PIN1 at Ser⁷¹ abolishes PIN1 catalytic activity^[14]. Furthermore, protein kinase A, ribosomal S6 kinase 2 and Aurora

have also been shown to inhibit PIN1 function by phosphorylation at Ser¹⁶[13,44,45].

PIN1 OVEREXPRESSION IN HCC

As PIN1 regulates cellular signalling, PIN1 overexpression typically results in uncontrolled cell proliferation and tumour formation. A relationship between PIN1 and cancer was first demonstrated in breast cancer, in which PIN1 level positively correlated with tumour grade^[20]. Moreover, PIN1 overexpression has been found in different cancers, including brain, breast, cervical, colon, lung and prostate^[26,46].

Consistently, several studies have confirmed that PIN1 mRNA and protein are over-expressed in HCC tumours, as compared with those of the adjacent non-tumourous liver tissues^[47-50]. In one of the earliest studies, PIN1 overexpression was found in more than 50% of HCC samples^[47]. In addition, a study from our group has also demonstrated that PIN1 overexpression is more frequently observed in HBV-related HCCs^[51]. Shinoda *et al.*^[52] has studied the association between PIN1 expression and clinicopathological characteristics in HCC. HCC tumours with higher PIN1 expression show significantly larger tumour size and higher frequency of portal vein invasion. Moreover, higher PIN1 expression is also significantly associated with poorer prognosis, lower overall survival and higher early recurrence rate (within 3 years) in patients with HCC. Thus, dysregulation of PIN1 expression is closely associated with HCC progression.

ROLES OF PIN1 IN HEPATOCARCINOGENESIS

Through catalysing isomerization of signalling molecules, PIN1 functions as a critical catalyst in many signalling pathways in cancer. The oncogenic property of PIN1 was first demonstrated in breast cancer, as PIN1 can transform breast epithelial cells through up-regulation of cyclin D1^[27]. Several studies have demonstrated that PIN1 expression is positively correlated with cyclin D1 expression in various types of cancer^[20,47,53-56]. A positive correlation has also been shown between PIN1 expression and centrosome amplification in breast cancer. Both *in vitro* and *in vivo* studies have demonstrated that PIN1 induces centrosome duplication, resulting in chromosomes mis-segregation and genomic instability, which in turn promote cell transformation and tumour growth in transgenic mice^[57].

In hepatocytes, overexpression of PIN1 has been shown to promote malignant transformation. PIN1 overexpression in the immortalized but non-tumorigenic liver cell line MIHA leads to anchorage-independent colony formation in soft agar and tumour formation in nude mice *in vivo*^[58]. The number of colonies in soft agar and the size of tumours in nude

mice are positively correlated with PIN1 expression levels. Similarly, PIN1 depletion in human HCC cells results in the suppression of cell proliferation, reduction of colony formation in soft agar and abrogation of tumour development in nude mice. Thus, PIN1 plays a critical role in hepatocarcinogenesis and the pathways involved are further discussed in the following sections (Figure 2).

PIN1 AND β -CATENIN/CYCLIN D1 SIGNALLING PATHWAY

Dysregulation of the β -catenin signalling pathway resulting from β -catenin gene mutations contributes to HCC development. Activation of the β -catenin signalling pathway results in nuclear translocation of β -catenin protein that in turn activates the transcription of its target genes, including cyclin D1 and c-Myc^[59-62]. Up-regulation of cyclin D1 and c-Myc leads to uncontrolled cell proliferation, malignant transformation and tumour development. In addition to β -catenin gene mutations, PIN1 overexpression has been shown to increase the β -catenin transcriptional activity towards its target genes, such as cyclin D1, c-Myc, PPAR- δ and fibronectin^[18]. The mechanism of PIN1 regulating β -catenin transcriptional activity is dependent on the direct interaction between PIN1 and the phosphorylated Thr²⁴⁶-Pro motif of β -catenin. Such interaction stabilizes β -catenin by inhibiting its binding with the adenomatous polyposis coli protein for nuclear export and subsequent glycogen synthase kinase-3 β -mediated degradation of β -catenin. This leads to increased nuclear accumulation and transcriptional activity of β -catenin. We have previously demonstrated that overexpression of PIN1 in HCC is associated with β -catenin accumulation^[47]. More importantly, PIN1 overexpression and β -catenin gene mutations are found to be mutually exclusive events, further underscoring the role of PIN1 overexpression in causing β -catenin accumulation in HCC. Therefore, in addition to the somatic mutations of β -catenin that occur only in 20% of HCCs^[63,64], PIN1 overexpression is the major mechanism leading to β -catenin accumulation in HCC.

In addition to β -catenin, PIN1 also binds other transcription factors to increase their transactivation activity towards the *cyclin D1* gene. In response to activated c-Jun N-terminal kinases or oncogenic Ras, PIN1 interacts with c-Jun *via* its phosphorylated Ser⁶³/⁷³-Pro motif and increases the transcriptional activity of c-Jun on its target genes, such as cyclin D1^[20]. PIN1 binds the phosphorylated Thr²⁵⁴-Pro motif in the p65/RelA subunit of NF- κ B^[23]. This interaction stabilizes NF- κ B by inhibiting its interaction with its inhibitor I κ B to prevent the nuclear export and subsequent ubiquitin-mediated degradation of NF- κ B. Moreover, PIN1 increases NF- κ B transactivation activity by promoting phosphorylation of NF- κ B at Ser²⁷⁶ residue^[52]. The

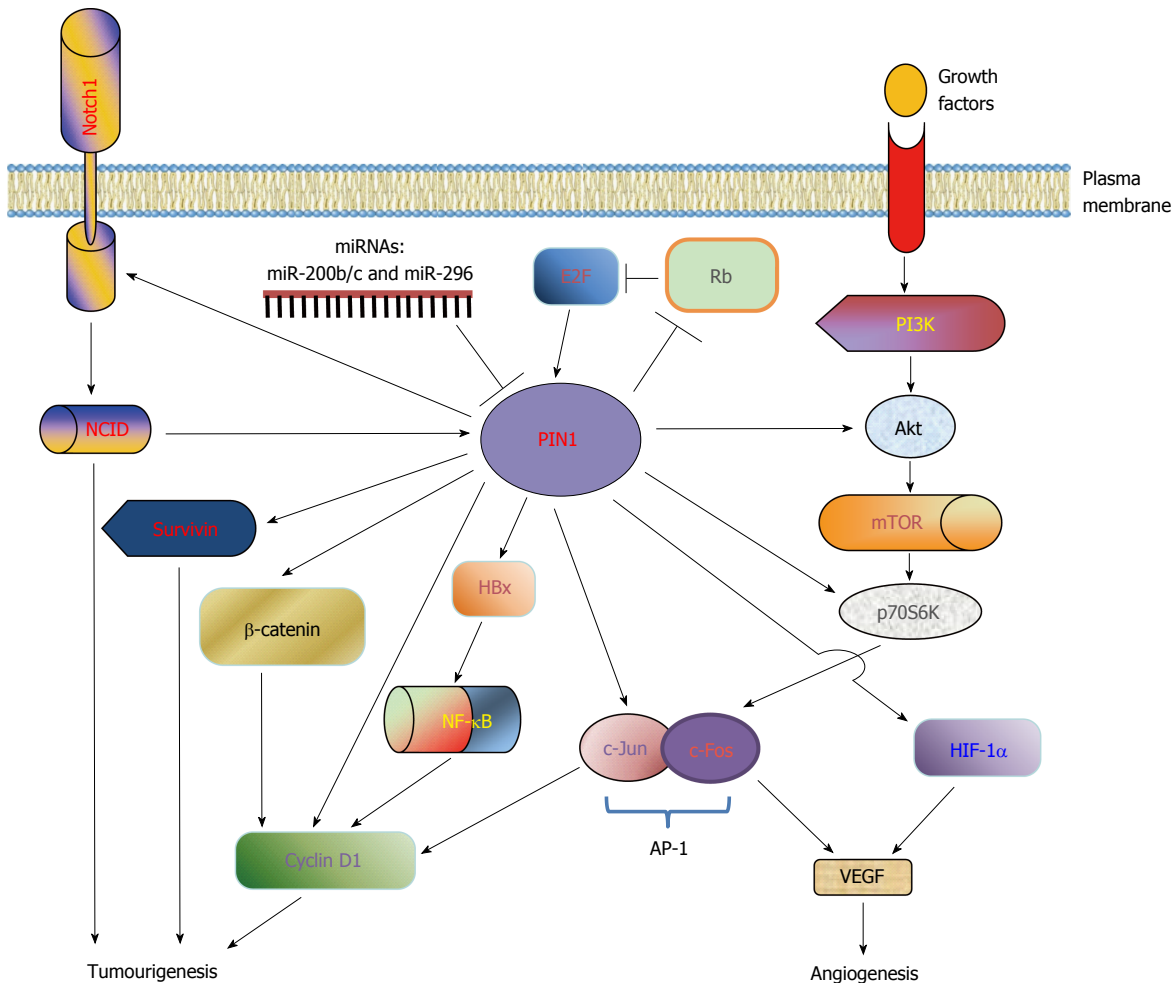


Figure 2 PIN1 dysregulation and targets in hepatocellular carcinoma. PIN1 functions as an amplifier to augment the oncogenic activities of key phosphoproteins involved in HCC tumorigenesis. PIN1 gene expression is up-regulated by various transcription factors, including E2F family and activated NOTCH1 intracellular domain (NCID), but is down-regulated by miRNAs (miR-200b/c and miR-296). In addition, PIN1 inactivates retinoblastoma protein (Rb), resulting in the release of E2F for activation of PIN1 expression. Through phosphorylation-dependent prolyl isomerization, PIN1 activates the β -catenin/cyclin D1 signalling pathway by induction of β -catenin transcriptional activity and stabilization of cyclin D1 protein. In parallel, PIN1 increases the transcriptional activities of c-Jun and NF- κ B, leading to an increase in cyclin D1 transcription. Furthermore, PIN1 stabilizes hepatitis B virus X protein (HBx) and enhances its transactivating activity on downstream target nuclear factor-kappa B (NF- κ B), which in turn increases cyclin D1 transcription. Up-regulation of cyclin D1 leads to uncontrolled cell proliferation and tumourigenesis. In addition, PIN1 increases the antiapoptotic function of survivin to inhibit apoptosis and contribute to tumourigenesis. Through interaction with Akt and ribosomal S6 kinase (p70S6K), PIN1 also activates the PI3K/Akt/mTOR pathway to promote tumourigenesis. PIN1 enhances the transcriptional activities of hypoxia-inducible factor (HIF)-1 α and activator protein (AP)-1, resulting in up-regulation of angiogenic factor vascular endothelial growth factor (VEGF) and promotion of angiogenesis.

PIN1-induced nuclear accumulation and activation of NF- κ B result in increased cyclin D1 expression. Interestingly, PIN1 has also been shown to interact directly with cyclin D1 itself *via* its phosphorylated Thr²⁸⁶-Pro motif, resulting in protein stabilization and nuclear accumulation of cyclin D1^[17].

PIN1 and HBx

Chronic infection with HBV is a major cause of HCC^[65,66]. HBV is a DNA virus that facilitates malignant transformation by integration into the host genome to induce chromosome instability^[67,68] and to alter the expression of cancer-related genes by insertional mutagenesis^[69]. In addition, HBV modulates cell proliferation through the expression of viral proteins, in particular, the hepatitis B virus X protein (HBx)^[70].

HBx is a gene transactivator that contributes to hepatocarcinogenesis through up-regulation of the proto-oncogenes such as c-myc, c-jun and NF- κ B^[71-73]. Studies have also shown that HBx interacts with p53 to inhibit the translocation of p53 into nucleus, resulting in the inhibition of p53-mediated cellular apoptosis and the development of liver tumour in transgenic mouse^[74].

PIN1 overexpression is more frequently observed in HBV-related HCCs. Moreover, PIN1 directly binds to the phosphorylated Ser⁴¹-Pro motif in HBx^[51]. This interaction results in the stabilization of HBx protein, leading to augmentation of its transactivating activity on the downstream target genes Bcl-XL, c-myc, and NF- κ B^[51]. Overexpression of both PIN1 and HBx leads to synergistic increase in cell proliferation *in vitro*

and tumour growth *in vivo*, as compared with cells overexpressing PIN1 or HBx alone. These synergistic effects were totally dependent on the interaction between PIN1 and HBx. Neither the expression of the non-PIN1-binding HBx mutants nor PIN1 mutants that are defective for protein binding or isomerase activity cause any synergistic increase in cell proliferation and tumour growth.

PIN1 and survivin

In addition to the dysregulation of cell proliferation, the tightly regulated programmed cell death (cellular apoptosis) is frequently impaired in cancers. PIN1 has also been found to affect cellular apoptosis in breast, prostate and cervical cancer cells^[56,75,76]. A recent study by our group has demonstrated an interaction between PIN1 and phosphorylated Thr³⁴-Pro motif of survivin, an inhibitor of apoptosis protein (IAP), in HCC cells^[48]. The function of survivin is to inhibit apoptosis by facilitating its interaction with hepatitis B X-interacting protein (HBXIP) and pro-caspase-9, thereby blocking caspase-9 activation^[77,78]. The antiapoptotic function of survivin is critical in a number of cancers, including HCC^[79]. In our study, we showed that PIN1 overexpression increases the binding between survivin and pro-caspase-9 *via* HBXIP, leading to suppression of caspase-9 and caspase-3-dependent apoptosis in HCC cells. Moreover, PIN1 promotes HCC tumour growth through inhibition of apoptosis and PIN1 expression is positively correlated with survivin expression in HCC tumours. Therefore, PIN1 enhances the antiapoptotic function of survivin and plays an important role in hepatocarcinogenesis through inhibition of apoptosis.

PIN1 and phosphoinositide 3-kinase/serine/threonine-specific protein kinase B (Akt)/mammalian target of rapamycin pathway

In addition to the β -catenin signalling pathway, the phosphoinositide 3-kinase (PI3K)/Akt/mammalian target of rapamycin (mTOR) pathway is another important oncogenic pathway involved in HCC. Activation of this pathway is triggered by binding of multiple growth factors, including insulin and cytokines, to their respective cell surface receptors. This leads to the activation of PI3K and its subsequent downstream targets, including Akt, mTOR, translation initiation factor 4E-binding protein (4E-BP1) and ribosomal S6 kinase (p70S6K). Both 4E-BP1 and p70S6K regulate the translation of cell cycle regulatory proteins, and therefore, aberrant activation of the PI3K/Akt/mTOR pathway results in dysregulated cell cycle progression and tumourigenesis. It has been reported that mTOR phosphorylation is found in 15% of HCCs and total p70S6K expression is up-regulated in 45% of HCCs^[80]. The occurrence of multiple serine and threonine phosphorylation events in this pathway suggests that PIN1 may be involved in and contribute to the development of HCC. Recently, PIN1 has been

found to interact with the phosphorylated Thr^{92/450}-Pro motifs of Akt, resulting in the stabilization of both total and activated Akt (phosphorylated Ser⁴⁷³)^[81]. Moreover, PIN1 expression is positively correlated with Akt activation in various types of human tumours including breast, nasopharynx and salivary gland^[81]. Therefore, the PIN1-mediated Akt stabilization may be closely associated with tumourigenesis.

In addition, Lee *et al.*^[44] revealed that PIN1 interacts with p70S6K and facilitates the insulin-induced phosphorylation of p70S6K as well as of its downstream target extracellular signal-regulated protein kinase (ERK)1/2 in human HCC cells. Through p70S6K-ERK signalling, co-expression of PIN1 and p70S6K leads to a synergistic increase in transcriptional activity of activator protein (AP)-1, as compared with expression of PIN1 or p70S6K alone^[44]. The transcription factor AP-1 regulates gene expression through the formation of heterodimeric protein with other DNA binding proteins, such as c-Fos and c-Jun^[82]. Activation of AP-1 regulates various cellular processes, such as cell proliferation, apoptosis and transformation, through activation of genes that encode the cell cycle regulatory proteins including cyclin D1, p53 and p21. Therefore, enforced expression of PIN1 enhances the insulin-induced neoplastic cell transformation, whereas the mTOR inhibitor rapamycin that blocks the activation of p70S6K suppresses PIN1-induced neoplastic cell transformation^[44]. Hence, the PIN1-p70S6K signalling pathway enhances AP-1 activity and mediates the insulin-induced neoplastic cell transformation. Thus, PIN1 promotes hepatocarcinogenesis partly through the activation of the PI3K/Akt/mTOR pathway.

PIN1 and NOTCH1

NOTCH1 signalling pathway plays a critical role in oncogenesis through the regulation of cell proliferation, differentiation and apoptosis. Ligand (γ -secretase)-induced cleavage of the membrane-bound NOTCH1 receptor releases its intracellular domain. This intracellular domain of NOTCH1 translocates into the nucleus and then activates the expression of target genes, including oncogenes c-Myc and HES1. Hence, activation of NOTCH1 signalling has been implicated in tumourigenesis. With immunohistochemistry and immunoblotting, NOTCH1 overexpression was found in 60% of HCCs^[83]. In addition, enforced expression of the activated NOTCH1 intracellular domain completely rescues the inhibitory effect of γ -secretase inhibitor (GSI) on the cell proliferation of HCC cells^[84]. As mentioned above, interaction between PIN1 and NOTCH1 stimulates the NOTCH1 signalling by releasing the activated NOTCH1 intracellular domain^[31]. Therefore, PIN1 overexpression increases the transcriptional activity of activated NOTCH1 intracellular domain and enhances the colony formation *in vitro*. Likewise, down-regulation of PIN1 decreases NOTCH1 transcriptional activity, which in turn abrogates tumour

growth *in vivo*^[31]. Taken together, both *in vitro* and *in vivo* studies demonstrate that activation of NOTCH1 signalling by PIN1 may potentially contribute to hepatocarcinogenesis.

PIN1 and angiogenesis

Angiogenesis is the formation of new blood vessels that enhances the development and progression of tumours. HCC is a highly aggressive vascular tumour that relies on active angiogenesis. Tumour angiogenesis is mainly regulated by the expression of an angiogenic factor, vascular endothelial growth factor (VEGF). VEGF functions to stimulate vascular permeability and subsequently promotes tumour angiogenesis^[85,86]. Its expression is positively correlated with the angiogenic activity or tumour progression in HCCs^[87-91]. Recently, PIN1 has been found to interact with hypoxia-inducible factor (HIF)-1 α under hypoxic conditions^[92,93]. HIF-1 α is an important transcription factor for VEGF gene expression. Down-regulation of PIN1 or suppression of PIN1 activity reduces the protein stability of HIF-1 α under hypoxic conditions, resulting in decreased transcriptional activity of HIF-1 α , down-regulation of VEGF expression and inhibition of angiogenesis *in vivo*^[92]. In addition, PIN1 overexpression has been found to increase the transcriptional activity of AP-1 and the protein level of VEGF in breast cancer cells^[94]. The transcriptional activity of AP-1 is regulated through the formation of heterodimer with c-Fos and c-Jun^[82]. Since PIN1 binds c-Fos and c-Jun, and increases their transcription activity^[20,95], it also enhances the transactivation activity of AP-1 (c-Jun/c-Fos dimer), leading to increased VEGF gene transcription. Therefore, PIN1 may facilitate VEGF-mediated angiogenesis of HCCs through the regulation of AP-1 activity.

ROLES OF PIN1 IN TUMOUR INVASIVENESS

Several studies have reported that PIN1 is involved in cell motility and contributes to cancer cell invasiveness. PIN1 overexpression promotes migration of immortalized human breast epithelial cells and induces epithelial-mesenchymal transition (EMT) with up-regulation of the mesenchymal markers including N-cadherin, Zeb1 and vimentin, and down-regulation of the epithelial marker E-cadherin^[37]. Moreover, the protein binding WW domain and catalytic isomerization PPIase domain of PIN1 are essential for the induction of EMT in breast cancer cells. Consistently, PIN1 silencing suppresses protein expression and promoter activity of EMT regulator SNAIL, leading to increased expression of its downstream effector E-cadherin in tamoxifen-resistant breast cancer cells^[96]. In epidermal growth factor receptor (EGFR) Thr⁷⁹⁰Met mutant lung cancer tissues, PIN1 expression is also positively correlated with the mesenchymal markers vimentin and Zeb1^[97]. PIN1 expression enhances the survival of

EGFR-mutant lung adenocarcinoma cells with an EMT phenotype. In prostate cancer, PIN1 induces cellular migration and invasion by interacting with Smad2/3^[98], while PIN1 depletion by siRNA inhibited migration, invasion and wound healing ability^[75]. In addition to EMT, PIN1 also regulates focal adhesion kinase (FAK), which is a critical focal adhesion component for cell-cell interaction and migration. By binding to protein tyrosine phosphatase (PTP)-PEST, PIN1-induced isomerization enhances the interaction of PTP-PEST with FAK and dephosphorylates FAK at the Tyr³⁹⁷ site^[99]. Dephosphorylation of FAK promotes migration, invasion and metastasis of Ras-induced transformed cells. In conclusion, PIN1 enhances cancer cell motility by controlling the EMT regulating proteins expression as well as focal adhesion protein activity.

PIN1 AS A NEW DRUG TARGET FOR HCC TREATMENT

The oncogenic role of PIN1 makes it an attractive target for the development of anticancer drugs. Most of the PIN1 inhibitors developed are small molecules that inhibit its isomerase activity by binding to its catalytic active site^[100] (Table 1). The first described PIN1 inhibitor is Juglone, which irreversibly inhibits the PIN1 PPIase activity^[101]. Juglone was found to suppress tumorigenicity in human cancer cells by inducing apoptosis^[102] and reducing number and size of colonies in human HCC cells in soft agar assay^[44]. However, in addition to its PIN1-inhibitory activity, Juglone also directly inhibits RNA polymerase II^[103], rendering it not suitable for clinical treatment of human cancers. Tatara *et al.*^[104] and Uchida *et al.*^[105] have also screened for additional PIN1 PPIase inhibitors using a chemical compound library. PiB and dipentamethylene thiuram monosulfide (DTM) were identified to exhibit specific inhibitory activity toward PPIase through binding to the active site of PIN1. Both PiB and DTM inhibit proliferation of colon carcinoma HCT116 cells by delaying or blocking cell cycle progression. Moreover, the same concentration of PiB inhibits proliferation of wild-type mouse embryonic fibroblasts (MEFs), but not *Pin1-null* MEFs, suggesting that PiB is more specific in suppression of cell proliferation through inhibition of PIN1. Recently, all-trans retinoic acid was also found to inhibit PIN1 activity and to suppress cell proliferation in breast cancer and acute promyelocytic leukaemia^[106]. However, the specificity of those PIN1 inhibitors remains a concern as some of them also possess parvulin-type PPIases inhibitory activity^[100]. In addition to directly inhibiting PIN1 activity, miRNA-mediated gene silencing may also be used to knock-down PIN1 expression in cancer cells. The first miRNA mimic MRX34 has already been tested in phase I clinical trial for HCC^[107]. PIN1 silencing by miR-200b/c or miR-296-5p may provide a new approach for HCC treatment.

The application of PIN1 inhibitors or miRNAs in the

Table 1 Potential PIN1 inhibitors for cancer treatment

Drug	Details	Status
Juglone	First PIN1 inhibitor	Preclinical
PiB	Irreversibly inhibits PIN1 PPIase activity Specifically inhibits PIN1 PPIase activity Inhibits colon cancer cell proliferation	Preclinical
Dipentamethylene thiuram monosulfide	Specifically inhibits PIN1 PPIase activity Inhibits colon cancer cell proliferation	Preclinical
All-trans retinoic acid	Binds PIN1 and inhibits its activity Inhibits breast cancer and APL cell proliferation	FDA approved for treatment of APL
miRNAs miR-200b/c miR-296-5p Sorafenib	Bind to the 3'UTR of PIN1 mRNA Suppress PIN1 expression in breast cancer and prostate cancer cells Multi-kinase inhibitor targeting Raf/Mek/Erk signalling pathway and tyrosine receptors Inhibits angiogenesis and growth of HCC tumours <i>in vivo</i> Inhibits phosphorylation of PIN1-interacting proteins (Mcl-1 and p70S6K) Improves overall survival and increases time to progression in HCC patients	Preclinical FDA approved for treatment of HCC
Bortezomib	Proteasome inhibitor Suppresses expression of PIN1 and its transcription factor E2F Inhibits HCC cell proliferation <i>in vitro</i>	FDA approved for treatment of multiple myeloma

APL: Acute promyelocytic leukaemia; FDA: Food and Drug Administration; HCC: Hepatocellular carcinoma; PPIase: Peptidyl-prolyl isomerase.

treatment of human cancers may be challenged by the fact that PIN1 is also expressed in normal cells for the regulation of cell division. PIN1 interacts with p53 to enhance protein stability and transactivation activity of p53 to promote cellular apoptosis in response to DNA damage^[16,24,108]. Depletion of PIN1 has also been reported to induce transformation and tumourigenesis of MEFs through stabilization of oncogenic proteins Myc and cyclin E^[21,22]. Therefore, it remains uncertain whether PIN1 inhibitors would have any adverse effect on normal tissues. To minimize the detrimental effects of PIN1 inhibitors on normal cells, a targeted delivery system may be employed to ensure specific drug delivery to HCC cells. More importantly, preclinical or clinical studies are necessary to examine the safety and effectiveness of the PIN1 inhibitors in cancer treatment.

Sorafenib is the only approved small molecular targeting agent for the treatment of advanced stage HCC. It is a multi-kinase inhibitor that blocks the Raf/Mek/Erk signalling pathway and inhibits several receptor tyrosine kinases, including VEGF receptor 2 and 3. In clinical studies, patients who received sorafenib had longer overall survival and time to progression^[109,110]. Recently, *in vitro* and *in vivo* studies have provided further evidence of the efficacy of sorafenib in suppressing the growth of HCC cells^[111,112]. Through inhibition of Raf/Mek/Erk signalling, sorafenib suppresses proliferation and enhances apoptosis of HCC cells. Moreover, sorafenib inhibits the angiogenesis and growth of patient-derived HCC tumour xenografts in mice. Sorafenib has also been shown to block the Erk-mediated phosphorylation of Mcl-1, which is required for interaction with PIN1 and subsequent protein stabilization^[76]. Indeed, sorafenib also inhibits the phosphorylation of another PIN1 target, p70S6K, which induces cell transformation through

enforced PIN1 expression. Therefore, inhibition of the phosphorylation of oncogenic PIN1 interacting partners may be an effective treatment strategy for PIN1-overexpressing tumours. Furthermore, proteasome inhibitor bortezomib (BZB) has been shown to suppress HCC cell growth through down-regulation of PIN1 and its transcription factor E2F^[113]. As a single agent, however, the benefit of BZB in advanced HCC patients is limited^[114]. Combination of BZB with other agents as treatment for HCC should be further evaluated.

CONCLUSION

HCC is an aggressive cancer associated with a poor prognosis. Conventional treatment options available for advanced HCC patients are very limited. Identification of important molecular targets in HCC may lead to the development of a new therapeutic approach. Dysregulation of PIN1 expression is associated with HCC and its expression is positively correlated with tumour size. Through phosphorylation-dependent prolyl isomerization, PIN1 functions as an amplifier to augment the oncogenic activities of its interacting proteins in hepatocarcinogenesis. The diverse oncogenic effects exerted by PIN1 overexpression in HCC render PIN1 as an attractive therapeutic target for treatment. In fact, several studies have already demonstrated that inhibiting PIN1 activity results in the suppression of cell growth and tumour development. Further studies and clinical trials are required to examine the safety and efficacy of PIN1 inhibition in the treatment of HCC.

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Reprogramming of glucose metabolism in hepatocellular carcinoma: Progress and prospects

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Abstract

Hepatocellular carcinoma (HCC) is one of the most lethal cancers, and its rate of incidence is rising annually. Despite the progress in diagnosis and

treatment, the overall prognoses of HCC patients remain dismal due to the difficulties in early diagnosis and the high level of tumor invasion, metastasis and recurrence. It is urgent to explore the underlying mechanism of HCC carcinogenesis and progression to find out the specific biomarkers for HCC early diagnosis and the promising target for HCC chemotherapy. Recently, the reprogramming of cancer metabolism has been identified as a hallmark of cancer. The shift from the oxidative phosphorylation metabolic pathway to the glycolysis pathway in HCC meets the demands of rapid cell proliferation and offers a favorable microenvironment for tumor progression. Such metabolic reprogramming could be considered as a critical link between the different HCC genotypes and phenotypes. The regulation of metabolic reprogramming in cancer is complex and may occur *via* genetic mutations and epigenetic modulations including oncogenes, tumor suppressor genes, signaling pathways, noncoding RNAs, and glycolytic enzymes *etc.* Understanding the regulatory mechanisms of glycolysis in HCC may enrich our knowledge of hepatocellular carcinogenesis and provide important foundations in the search for novel diagnostic biomarkers and promising therapeutic targets for HCC.

Key words: Hepatocellular carcinoma; Metabolic reprogramming; Aerobic glycolysis; Glucose metabolism; Noncoding RNAs

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Core tip: The reprogramming of glucose metabolism is one of the peculiar characteristics of cancer cells. This paper addresses the regulatory mechanism of glucose metabolism in hepatocellular carcinoma (HCC) and prospects its future application for HCC treatment.

Shang RZ, Qu SB, Wang DS. Reprogramming of glucose metabolism in hepatocellular carcinoma: Progress and prospects.

INTRODUCTION

Hepatocellular carcinoma is the second leading cause of cancer-related death in the world, responsible for approximately 700000 deaths annually^[1]. Although many treatment options have been developed and used in the clinic, including hepatic resection, local ablation, liver transplantation and molecular targeted therapies, patients' prognoses remain poor^[2]. Etiological studies of HCC revealed that hepatitis viruses, alcohol and aflatoxin might be the main risk factors for HCC^[3]. In different areas of the world, HCC caused by these risk factors alone or together exhibits great diversity in genotype and phenotype, which impede the research of HCC. One remarkable feature of HCC is the alteration of glucose metabolism, which may be a critical link between the different HCC genotypes and phenotypes. Thus, a thorough understanding of cancer metabolism may offer promising therapeutic strategies for HCC in the future.

As early as the 1950s, Otto Heinrich Warburg first characterized cancer cell metabolism. Cancer cells principally use the glycolysis pathway to metabolize glucose and generate ATP whether there is sufficient oxygen present. This phenomenon now referred to as the "Warburg effect" was described and lead to a wave of investigation of cancer metabolism over several decades^[4]. In the 1980s, the availability of ¹⁸F-deoxyglucose positron emission tomography (FDG-PET) pushed the study of tumor metabolism to the climax^[5]. Observations from FDG-PET scanning revealed that approximately 50%-70% ATP was generated by glycolysis in different tumor types^[6-8]. The application of FDG-PET was also recently involved in the detection and monitoring of metastasis and the recurrence of HCC and for prediction of patient's prognosis^[9-12]. Moreover, recent studies of metabolomics offer new mechanistic insights into aerobic glycolysis and provide promising individualized therapeutic strategies by targeting the Warburg effect for treatment of HCC^[13,14].

In this article, we will review the recent investigations of glucose metabolism in HCC and summarize the regulation methods of metabolic reprogramming. Moreover, we will describe the development of therapy by targeting cancer metabolism.

REPROGRAMMING OF GLUCOSE METABOLISM-RELATED ENZYMES AND TRANSPORTING PROTEINS IN HCC

As previously described, tumor cells rely on the aerobic glycolysis pathway to consume glucose and

generate ATP, which is a rapid but low-efficiency metabolic process^[15]. To meet the demands of energy, biosynthesis and redox for tumor progression, cancer cells reprogram their metabolic related enzymes and transporting proteins to facilitate increased glucose uptake, acceleration of glycolysis and metabolic end-product excretion (Figure 1).

The initial step of glycolysis is the transportation of glucose across the plasma membrane into the cytoplasm, which depends on the family of glucose transporters (GLUTs)^[16]. Much evidence has shown that GLUT1-4, particularly GLUT1, are often aberrantly expressed in different cancer types and significantly influence cancer glucose metabolism^[17-21]. Amann *et al.*^[22] observed that both mRNA and protein expression levels of GLUT1 were significantly up-regulated in HCC, and this plays a critical role in glucose transport, glycolysis and tumor progression in HCC cells. Daskalow *et al.*^[23] analyzed GLUT2 expression in 60 HCC samples and revealed the over-expression of GLUT2 in HCC. Another study demonstrated that positive GLUT2 predicts worse prognosis in HCC patients^[24]. To the best of our knowledge, studies of GLUT3 and GLUT4 in HCC have not been conducted.

Several glycolysis-related key enzymes have been demonstrated to participate glycolysis and carcinogenesis in HCC. Hexokinase (HK) family members catalyze the first key step of glycolysis in which glucose is phosphorylated to become glucose 6-phosphate (G-6-P). In the HK family, HK2 shows the highest affinity for glucose and is up-regulated in HCC and correlated with poor prognosis^[25]. PET-CT scans showed that over-expression of HK2 promotes the uptake of ¹⁸FDG in HCC cells^[26], which suggested that HK2 has a critical role in HCC glycolysis. The latest study showed that HKDC1, a newly discovered HK family member, was up-regulated in HCC with poorer prognosis and inhibited HCC cellular proliferating and migration *in vitro*, probably by repression of the Wnt/beta-catenin pathway^[27]. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) may also play an important role in HCC glycolysis. GAPDH used to be regarded as a stably expressed gene and was commonly used as a reference gene in the past. Recent studies have reported the aberrant expression of GAPDH in malignancies and raised the concern that it may play a role in tumor glycolysis^[28]. Gong *et al.*^[29] showed that increased expression of GAPDH promoted glycolysis and tumor progression in HCC. Moreover, GAPDH was able to affect glycolysis *via* regulating metabolism-related pathways such as the mammalian target of rapamycin (mTOR)-complex1 (mTOR-C1) signaling pathway^[30]. Pyruvate kinases (PKs) catalyze the last step of glycolysis to produce ATP and pyruvate, which regulates the influx of the glycolysis pathway together with HK and phosphofructokinase-1. PKs contain 4 isoforms (PKL, PKR, PKM1 and PKM2) that are encoded by the PKL and PKM genes. PKL and PKR are mainly expressed in liver cells and erythrocytes,

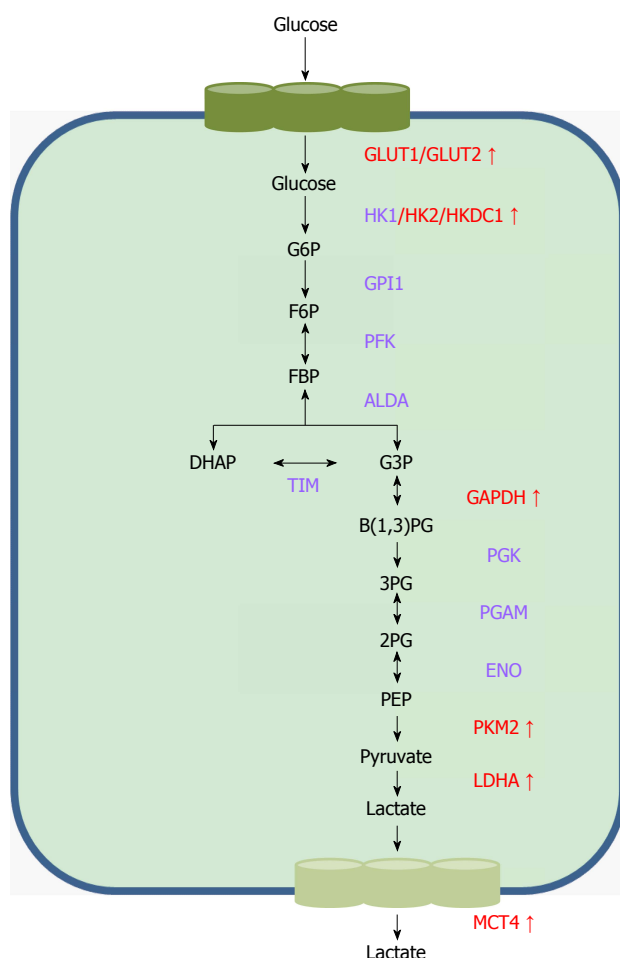


Figure 1 Reprogramming of glucose metabolism in hepatocellular carcinoma. Reprogramming of glucose metabolism-related enzymes and transporting proteins in HCC. The expression of GLUT1, GLUT2, HK2, HKDC1, GAPDH, PKM2, LDHA and MCT4 are up-regulated in HCC glycolysis pathway. GLUT: Glucose transporter; HK: Hexokinase; G6P: Glucose-6-phosphate; GPI1: Glucose-6-phosphate isomerase 1; F6P: Fructose-6-phosphate; PFK: Phosphofructokinase; FBP: Fructose-1,6-bisphosphatase; ALDA: Aldolase A, DHAP: Dihydroxyacetone phosphate; TIM: Triosephosphate isomerase; G3P: Glyceraldehyde-3-phosphate; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; PG: Phosphoglycerate; PGAM: Phosphoglycerate mutase; ENO: Enolase; PEP: Phosphoenolpyruvate; PKM2: Pyruvate kinase isoform M2; PFK: Phosphate fructose kinase; LDHA: Lactate dehydrogenase A; MCT4: Monocarboxylate transporter 4.

respectively, whereas PKM1 is constitutively expressed in normal cells. The over-expression of PKM2 was frequently observed in malignances and predicts worse prognosis^[31,32]. A recent study demonstrated that the expression of PKM2 is up-regulated in HCC and is a predictor of survival and recurrence^[33]. Dong *et al.*^[34] revealed the oncogenic role of PKM2 in HCC proliferation by its regulation of the expression of HIF-1 α and Bcl-xL. Another study further observed that PKM2 effects on cell growth depend on a glucose rather than glutaminolysis pathways by using PKM2 knockdown-sensitive HCC cells. Additionally, the switching from PKL to PKM2 was reported to promote the rate of glucose uptake and increase the oxidative stress in hepatocarcinogenesis^[35]. Lactate dehydrogenase (LDH) catalyzes the conversion of

pyruvate to lactate. Up-regulation of the LDHA subunit in cancers has been noticed due to its role in promoting glycolysis and reducing the oxygen dependency of cancer cells^[36,37]. A recent study has indicated that LDHA is up-regulated in HCC cells and promotes tumor growth and metastasis^[38]. A series of clinical studies assessed the serum levels of LDH in HCC patients who were treated with hepatic resection^[39,40], transarterial chemoembolization^[41,42] and sorafenib^[43,44] and found a similar conclusion that LDH may be an easily obtained biomarker for prognosis prediction and treatment selection for HCC patients.

Activation of the glycolysis pathway in cancer cells not only provides sufficient ATP for tumor progression but also produces acid by-products such as lactate. To avoid apoptosis caused by the accumulation of acids in cells, the monocarboxylate transporters (MCTs) are up-regulated in cancer cells to speed up the export of lactate into the extracellular milieu. Aberrant expression of isoforms MCT1, MCT2 and MCT4 was frequently observed in many cancers including colorectal carcinoma^[45], glioblastoma^[46] and gallbladder cancer^[47]. The role of over-expressed MCT4 in HCC has been illustrated. It is associated with HCC progression and poor prognosis^[48,49]. The latest study observed the reduced expression of MCT1 and MCT2 in HCC^[50]. However, the data from another study showed that MCT1 was over-expressed in HCC cells which facilitates the lactate exporting and promotes HCC glycolysis^[51]. Therefore, further studies are still needed to illuminate the specific role of MCT1 and MCT2 in HCC glycolysis and progression.

REGULATORY MECHANISM OF GLUCOSE METABOLIC REPROGRAMMING

Oncogenes and tumor suppressor genes involved in glucose metabolic reprogramming during carcinogenesis

Oncogenes are a number of important genes which are over-expressed or mutated in cancer cells that triggered the tumor initiation and maintained the tumor progression. Based on the biological functions, oncogenes are usually classified as growth factors, receptor tyrosine kinases, cytoplasmic tyrosine kinase, regulatory GTPase and transcription factors. The activation of oncogenes is complex and may be attributed to the genetic mutations and the tumor microenvironment. Hypoxic microenvironment is a crucial factor in the activation of some oncogenes. The lack of sufficient blood supply in rapidly proliferating tumor cells leads to hypoxia. HIF-1 is a key transcription factor that is activated in response to oxygen deprivation. In cancer cells, HIF-1 promotes glycolysis by activating glycolytic enzymes^[52]. Over-expression of HIF-1 was observed in HCC samples^[53] and was shown to promote cell proliferation and resistance to apoptosis by up-regulating FOXM1

Table 1 Oncogenes and tumor suppressor genes involved in glucose metabolic reprogramming during carcinogenesis

	Genes	Targets	Ref.
Oncogenes	HIF-1	HK1	[55]
		HK2	[55]
		GAPDH	[55]
		PKM	[55]
	Myc	LDHA	[58]
		GLUT1	[59]
		HK2	[60]
	CD147	MCT1	[62]
		GLUT1	[62]
Tumor suppressor genes	PIM1	GLUT1	[65]
		PKM2	[65]
	P53	GLUTs	[66]
	RRAD	GLUT1	[67,68]
		HK2	[67]

GLUT: Glucose transporter; HK: Hexokinase; PKM: Pyruvate kinase isoform M; LDHA: Lactate dehydrogenase A; MCT1: Monocarboxylate transporter 1; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; RRAD: Ras-related associated with diabetes.

expression^[54]. Hamaguchi *et al.*^[55] analyzed 22 glycolysis-related genes in HCC samples and identified 10 potential transcriptional targets of HIF-1 α including HK1, HK2, GAPDH and PKM. Interestingly, several studies showed that HIF-1 could be activated by Ras^[56] and membrane type-1 matrix metalloproteinase^[57] under normoxic conditions, which may provide new insights into cancer glycolysis regulation beyond the hypoxic microenvironment. Myc is another crucial oncogene involved in the Warburg effect. As a vital transcription factor, Myc was first linked with glucose metabolism through its transactivation of LDHA expression^[58]. A series of glycolytic enzymes were subsequently identified as direct targets of Myc, including GLUT1 and HK2^[59,60]. Moreover, the interplay between Myc and HIF-1 has also been observed, which indicates that Myc may play a complementary role in cancer metabolism under non-hypoxic conditions^[61,62]. CD147 (Basigin) is a transmembrane protein that is highly expressed in tumors. A number of studies have shown that CD147 is a "Warburg oncogene" due to its pivotal role in promoting glycolysis and inhibiting oxidative phosphorylation in cancer cells^[63,64]. In HCC, CD147 was reported to reprogram glucose metabolism by facilitating lactate export, mediated by MCT1, and promoting glucose uptake by up-regulating GLUT1 expression^[51]. Recently, some newly discovered oncogenes were also reported to play important roles in HCC glycolysis. For instance, PIM1 is involved in both aerobic and anaerobic glycolysis by targeting GLUT1 and PKM2^[65].

Likewise, tumor suppressor genes also have a great influence on cancer glycolysis. The role of the p53 tumor suppressor gene in cancer metabolism could be summarized as promoting oxidative phosphorylation and reducing glycolysis. The effect of p53 on glycolysis mainly depends on the reduced expression of glucose

transporters^[66]. Recently, we investigated the role of the tumor suppressor gene Ras-related associated with diabetes (RRAD) in HCC. We found RRAD could suppress the invasion, migration and aerobic glycolysis in HCC cells and identified GLUT1 and HK2 as potential targets for RRAD^[67]. Our results were recently verified by Yan *et al.*^[68] (Table 1).

Signaling pathways involved in glucometabolic reprogramming

AMPK pathway: The AMP-activated protein kinase (AMPK) is ubiquitously expressed in eukaryotes and acts as an energy status sensor and regulator of energy homeostasis^[69]. The activation of AMPK by energetic stress promotes the switching from glycolysis to oxidative phosphorylation. This switching inhibits the "Warburg effect" in rapidly proliferating cells, including tumor cells to spare glucose and restore energy homeostasis^[70]. At the same time, the activation of AMPK shuts down the synthesis of RNA, DNA, protein and lipid to inhibit the cell proliferation and growth. The downstream effect of AMPK activation on cancer metabolism has been well established. mTOR is a crucial downstream modulator of AMPK signaling in cancer cells. AMPK inhibits the activity of mTOR either directly or by reducing the activity of the mTOR-activating GTP-binding protein, Rheb, *via* activation of the Tuberous sclerosis complex 2^[71-73]. Inactivation of mTOR suppresses the expression of HIF-1 α , a key regulator of glycolysis, as mentioned previously^[52,74]. Recently, several reviews highlighted the regulatory role of AMPK on GLUT4 membrane translocation and GLUT1 activation in skeletal muscle cells and other normal cells^[69,75]. In HCC, AMPK signaling pathway was reported to participate in the ciliary neurotrophic factor induced GLUT4 translocation and glucose uptake^[76]. Considering the effect of AMPK on the inhibition of glucose uptake in transformed cells, further investigations are greatly needed to clarify the role of AMPK on glucose transporters and glycolytic enzymes in cancer cells.

PI3K/Akt/mTOR pathway: The PI3K/Akt pathway is one of the most frequently activated signaling pathways in human cancers including HCC. The PI3K/Akt pathway can be activated by mutated tumor suppressor genes, signaling from receptor tyrosine kinases, or by the PI3K components^[77]. The activation of the PI3K/Akt pathway is involved in cell proliferation, cell survival, cell cycle progression and cancer metabolism^[78]. Regulation of glucose metabolism by PI3K/Akt signaling is mediated by glycolytic enzymes. Firstly, PI3K/Akt promotes glucose uptake in cells by increasing the membrane translocation and expression of GLUT4^[79,80]. In addition, PI3K/Akt promotes glycolysis by activating HK and by the binding of HK2 to the voltage-dependent anion channel in mitochondria^[81,82]. Moreover, PI3K/Akt could regulate

Table 2 Noncoding RNAs regulate glucose metabolism by directly targeting enzymes and indirectly targeting glycolysis-related pathways

	Targets	noncoding RNAs	Ref.
Enzymes	GLUT1	miR-340	[85]
		miR-1291	[86]
		miR-22	[87]
		miR-144	[88,89]
	GLUT3	miR-195-5p	[90]
	GLUT4	miR-133	[91]
		miR-223	[92]
	HK2	miR-143	[93-95]
		miR-155/ miR-143	[96]
		miR-34a	[102,103]
	PKM2	miR-122	[98]
		miR-133-a/b	[99]
		miR-326	[100]
	PFK	miR-52s	[101]
Pathways	LDHA	miR-34a	[102,103]
	MCT1	miR-199a-3p	[104]
	AMPK	miR-451	[111-113]
		miR-195	[112]
		miR-125a	[114]
	PI3K/ Akt/ mTOR	miR-7	[115]

GLUT: Glucose transporter; HK2: Hexokinase 2; PKM2: Pyruvate kinase isoform M2; PFK: Phosphate fructose kinase; LDHA: Lactate dehydrogenase A; MCT1: Monocarboxylate transporter 1; AMPK: AMP-activated protein kinase.

glycolytic enzymes indirectly by regulating the expression of AMPK and HIF-1^[83,84].

Noncoding RNAs involved in glucose metabolism

Noncoding RNAs are functional RNAs that are not transcribed into proteins. In the past, noncoding RNAs have been regarded as the “noise” in transcription processes. However, accumulating evidence has suggested the indispensable role of noncoding RNAs in various biological processes including gene transcription and translation. Noncoding RNAs, especially microRNAs (miRNAs) and long noncoding RNAs (lncRNAs) are also reported to be involved in the Warburg effect. The regulatory mechanism of noncoding RNAs in aerobic glycolysis consists of the following two aspects: the regulation of glycolytic enzyme expression and the activation of glycolysis-related oncogenic pathways (Table 2).

Noncoding RNAs were reported to regulate glucose uptake in cancer cells by targeting expression of GLUTs. MicroRNA-340, which increases the glucose uptake and lactate secretion by increasing the expression of GLUT1, was decreased in oral squamous cell carcinoma^[85]. Yamasaki *et al.*^[86] evaluated the role of microRNA-1291 in renal cell carcinoma and found that reduced expression of miR-1291 promotes cancer cell proliferation and invasion and migration by direct targeting of SLCA1/GLUT1. Chen *et al.*^[87] demonstrated that miR-22 regulates GLUT1 expression and inhibits the proliferation and invasion of breast cancer. MicroRNA-144 was also reported to mediate

the metabolic shift by regulating GLUT1 expression in lung and ovarian cancers^[88,89]. Moreover, miR-195-5p inhibits the glucose uptake by down-regulating GLUT3 expression and thus reduces proliferation in bladder cancer cells^[90]. The expression of GLUT4 is also regulated by microRNAs, including miR-113^[91] and miR-223^[92]. MicroRNA-143 is a key regulator of HK2 in cancer. Studies have shown that miR-143 negatively regulates the expression of HK2 and thus modulates glycolysis in colon cancer^[93], lung cancer^[94] and head and neck squamous cell carcinoma^[95]. In breast cancer cells, HK2 was regulated by the miR-155/miR-143 cascade at the post-transcriptional level^[96]. Burchard *et al.*^[97] showed the up-regulation of miR-122 reduced lactate production and increased oxygen consumption in HCC. A subsequent study further demonstrated that miR-122 reduced the expression of PKM2 and thus repressed glycolytic activities^[98]. Other microRNAs, including miR-133a/b and miR-326, were reported to regulate PKM2 expression in cancers^[99,100]. PFK catalyzes the conversion from fructose-6-phosphate to fructose-1, 6-bisphosphate and is over-expressed in cancers. A recent study showed that the miR-52 family mediated the regulation of Tat-activating regulatory DNA-binding protein on PFK in HCC^[101]. Some microRNAs were able to regulate multiple glycolytic enzymes. For instance, miR-34a was reported to regulate key enzymes including HK1, HK2, GPI, LDHA and PDK1^[102,103]. Additionally, miR-199a-3p serves an important role in the aerobic glycolysis of testicular germ cell tumors by targeting MCT1 and PGK1^[104].

Noncoding RNAs were able to regulate cancer metabolism by interactions with oncogenes (tumor suppressor genes) and oncogenic pathways. lncRNA-p21 was first discovered as a p53-inducible lncRNA that mediates p53-related apoptosis in mouse cells^[105]. In cancer cells, the hypoxia-induced lncRNA-p21 was shown to be a direct transcriptional target of HIF-1 α and in turn promoted the stability of HIF-1 α by interfering with the VHL-HIF-1 α association. The hypoxic microenvironment and the reciprocal regulation of p21 and HIF-1 α constructs a positive-feedback loop leading to continual activation of GLUT1 and LDHA expression thus accelerating glycolysis in cancer cells^[106]. In another study published in 2011, Bruning *et al.*^[107] evaluated the interaction between HIF-1 α and miR-155 and proposed that miR-155 contributes to a negative-feedback loop for the degradation of HIF-1 α -dependent transcription, under continuous hypoxic conditions. The tumor suppressor gene p53 is one the most frequent targets of microRNAs and lncRNAs. MicroRNAs including miR-125b, miR-504 and miR-1228 can regulate p53 expression by directly binding to sites in p53 3'-UTR^[108,109]. It is worth noting that the over-expression of miR-1228 can negatively regulate p53 expression, and the down-regulation of p53, in turn, increases miR-1228 expression. This positive-feedback

loop contributes to the progression of HCC^[110]. Studies also showed that oncogenic pathways are regulated by noncoding-RNAs. Down-regulation of miR-451 was originally linked with cancer glycolysis through its contributions to the adaptation to glucose deprivation and its effect on the LKB1/AMPK pathway in glioma cells^[111]. A further study confirmed that the regulation of the LKB1/AMPK pathway by miR-451 is mediated by MO25 (an upstream modulator of AMPK)^[112]. Another study discovered a novel reciprocal negative-feedback loop that consists of OCT1, AMPK and miR-451 in glioblastoma multiforme. Briefly, under the conditions of glucose deprivation, the activation of AMPK inactivated OCT1, which subsequently reduced the level of miR-451, and conversely, sufficient glucose supply significantly increased miR-451 expression, which in turn impaired the activity of the AMPK pathway^[113]. Moreover, microRNAs can regulate the PI3K/Akt/mTOR pathway in HCC. Tang *et al.*^[114] reported that miR-125a suppress HCC progression by inhibiting the PI3K/Akt pathway. Fang *et al.*^[115] investigated the molecular mechanism of miR-7 in HCC growth and metastasis and revealed the regulatory role of miR-1 in the PI3K/Akt pathway *via* targeting PIK3CD, mTOR and p70S6K.

Advances in HCC therapy by targeting glucose metabolism

The metabolic shift from oxidative phosphorylation to aerobic glycolysis in HCC not only provides abundant ATP for sustaining tumor survival but also offers a favorable microenvironment for tumor progression. As one of the “hallmarks” of cancer, metabolic reprogramming relies on metabolic enzymes, thus providing many potential targets that could be exploited in HCC therapy.

Flavonoids (phloretin, silybin and quercetin) targeting GLUT

Flavonoids are safe and reliable agents that are extracted from natural products, which show a broad spectrum of biological activities with fewer side effects^[116]. Phloretin is a natural phenol which could be extracted from manchurian apricot and apple tree leaves. Studies showed the ability of phloretin to suppress cell proliferation and induce apoptosis by inhibiting glucose uptake in cancers^[117,118]. Wu *et al.*^[117] showed that the inhibition of GLUT2 by phloretin leads to apoptosis in HCC cells. Another study demonstrated that phloretin strengthens the anticancer effects of paclitaxel in HCC^[119]. Another natural compound, silybin, was identified as a GLUT inhibitor and showed a significant inhibitory effect on HCC growth *in vivo*^[120,121]. Moreover, a phase I clinical study of silybin-phosphatidylcholine has been conducted in advanced HCC^[122]. Quercetin is another bioactive flavonoid which has been proposed as a promising anticancer agent^[123]. The latest study showed that quercetin

might be a potential agent in HCC therapy that induced apoptosis and metabolic inhibition by competitively inhibiting GLUT1^[124].

2-Deoxy-D-glucose and 3-bromopyruvate targeting of HK

2-deoxy-D-glucose (2-DG) is a glucose analog that is frequently used in inhibiting glycolysis. The phosphorylation of 2-DG catalyzed by HK2 in turn noncompetitively inhibits the activity of HK2 and leads to the reduction of the glycolytic rate. Several studies showed increased apoptosis induced by 2-DG in cancer, including HCC^[125,126]. However, normal cells are only arrested in G1 phase of mitosis when treated with 2-DG^[127]. 3-bromopyruvate (3-BP) is a halogenated analog that suppresses the glycolytic pathway by directly inhibiting HK2 activity. A study performed on a rabbit VX2 tumor model of liver cancer showed that 3-BP induced more efficient damage to hepatoma cells compared with 2-DG. Apart from the inhibition of HK, this study also revealed that 3-BP inhibits HCC glycolysis by suppressing mitochondrial ATP synthesis^[128]. Based on these promising research achievements *in vitro* and *in vivo*, the orphan drug, 3-BP, has been designated for HCC by the FDA^[13] and was reported to prolong the lifetime and improved the quality of life of a patient with HCC^[14].

Metformin targeting AMPK pathway

Metformin, a first-line anti-diabetic drug, was linked to cancer prevalence and therapy because diabetes mellitus is a risk factor for cancer death in some cancer types. The association between diabetes and HCC was evaluated in large populations in the 1990s^[129,130]. Recent studies demonstrated that diabetes mellitus is an independent risk factor for HCC^[131,132]. The preventive effect of metformin in HCC has been established. Studies showed a decreased incidence of HCC in the type 2 diabetic patients who received metformin therapy^[133-135]. The results of a systematic review showed a direct anti-HCC effect of metformin in animal models^[136]. The mechanism of metformin in HCC prevention and therapy in type 2 diabetic patients is closely linked with the AMPK pathway. Metformin activates the expression of LKB1 and AMPK by increasing the energy stress levels inside cells. The activated AMPK pathway reduced IRS-1 and caused the inhibition of insulin/IGF-1 signaling, which is involved in carcinogenesis and cancer glycolysis regulation^[137]. Additionally, AMPK inactivated the downstream modulator, mTOR, which indirectly regulates glycolysis by targeting HIF-1 α , as previously described.

CONCLUSION

The reprogramming of glucose metabolism in cancer is a multi-factor and multi-step process, which can be

regulated by oncogenes, oncogenic signaling pathways, and even noncoding RNAs. The developments in the study of cancer metabolism greatly enriched the understanding of carcinogenesis and afforded numerous potential targets to hit the Achilles' heel of cancer^[138]. The agents that target glycolytic enzymes directly and glycolysis-related pathways indirectly showed some promising effects in HCC prevention and therapy in the laboratory. However, the limitation of glycolysis targeted anti-cancer therapy should be noted. As multiple enzymes catalyze multiple steps in the process, there is a complex compensatory mechanism in cancer metabolism. Therefore, the inhibitors that specifically target a single modulator of glycolysis may not have a prominent or persistent effect on cancer metabolism in the human body. In the future, the effects of combination drug therapy should be evaluated. Moreover, noncoding-RNAs, which target multiple glycolysis-related enzymes and pathways, are also needed to be carefully considered in future studies.

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Management of neuroendocrine carcinomas of the pancreas (WHO G3): A tailored approach between proliferation and morphology

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Abstract

Neuroendocrine carcinomas (NEC) of the pancreas are defined by a mitotic count > 20 mitoses/10 high power fields and/or Ki67 index > 20%, and included all the tumors previously classified as poorly differentiated endocrine carcinomas. These latter are aggressive malignancies with a high propensity for distant metastases and poor prognosis, and they can be further divided into small- and large-cell subtypes. However in the NEC category are included also neuroendocrine tumors with a well differentiated morphology but ki67 index > 20%. This category is associated with better prognosis and does not significantly respond to cisplatin-based chemotherapy, which represents the gold standard therapeutic approach for poorly differentiated NEC. In this review, the differences between well differentiated and poorly differentiated NEC are discussed considering both pathology, imaging features, treatment and prognostic implications. Diagnostic and therapeutic flowcharts are proposed. The need for a revision of current classification system is stressed being well differentiated NEC a more indolent disease compared to poorly differentiated tumors.

Key words: Pancreatic neuroendocrine tumors; Surgery; Neuroendocrine carcinomas; Chemotherapy; Prognosis; Metastases; Morphology; Proliferation

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Core tip: In this study, we reviewed the available literature for neuroendocrine carcinomas of the pancreas with a special focus on the differences between morphological poorly-differentiated and well-differentiated tumors. Although the quality of current evidence is suboptimal because of the retrospective design of the available studies, morphological well-differentiated tumors are associated with lower ki67 proliferative index, are less responsive to standard platinum-based chemotherapy and are associated with improved survival. The current category of neuroendocrine carcinomas should be revised taking into account these differences and new diagnostic criteria should be considered in order to clearly define poorly- and well-differentiated tumors.

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INTRODUCTION

The 2010 World Health Organization (WHO) classification of pancreatic neuroendocrine tumors (PNET) introduced a major change compared with the previous ones. In fact the 2010 WHO system identified three categories of PNET based on mitotic count and ki67 index^[1,2]. The category of neuroendocrine carcinoma (NEC) was defined by a mitotic count > 20 mitoses/10 high power fields (HPF) and/or Ki67 index > 20% , and included all the tumors previously classified as poorly differentiated endocrine carcinomas (PDECs) in the 2000 and 2004 WHO classification^[1-3]. Pancreatic PDECs are clinically aggressive tumors characterized by poorly differentiation with features suggesting endocrine differentiation, a high-proliferative rate and frequently abundant necrosis with prominent angioinvasion^[4-6]. The category of PDECs include two different entities, namely small cell and large cell endocrine carcinomas. Historically, poorly differentiated NECs have been considered as nearly equivalent to small cell lung cancer given the histological similarities observed between the two diseases^[7-10]. As a consequence, some of the treatment recommendations for pancreatic PDEC are based on the small cell lung cancer literature and there are scant clinical data

regarding to pancreatic PDEC^[4-6].

Moreover, based on the WHO 2010 criteria, a morphological well-differentiated tumor showing > 20 mitoses/10 HPF or Ki67 index > 20%, is classified as NEC. Therefore, the WHO 2010 NEC category likely comprise all PD-NEC (WHO 2000) but also tumors morphologically classified as well-differentiated PNETs (WHO 2000) but with G3 features^[3]. This overlap between morphologically well- and poorly-differentiated tumors has strong clinical and therapeutic implications, since their biological behavior may significantly differ.

In the present paper we review the current knowledge on pancreatic NEC (PNEC) analyzing their clinical and pathological characteristics, treatment and prognosis, and evaluating potential pitfalls in their current classification.

EPIDEMIOLOGY AND CLINICAL FEATURES

Pancreatic NEC is rare tumors, accounting for about 5% of all PNENs. They usually arise in adults in the VIth decade of life with a male predominance^[11]. Some patients have associated paraneoplastic syndromes such as Cushing's syndrome, hypercalcemia and carcinoid syndrome. Unlike patients with well-differentiated NETG1/NETG2 who typically present with a relatively indolent disease process, most patients with NEC present with symptoms similar to ductal adenocarcinoma, including back pain, cachexia, weight loss and jaundice^[5].

A specific association between NEC and genetic syndromes such as MEN1 syndrome has not been established.

IMAGING AND STAGING

More than 70% of patients with pancreatic NEC present with metastatic disease or with locally-advanced tumors, and only 20% to 30% of patients are amenable of surgical resection^[3-5]. Appropriate diagnosis and staging is of paramount importance in order to establish the subsequent treatment. Pancreatic NEC, especially if morphologically PD, may metastasize virtually to every organ of the body, although the most common site of metastases is the liver. For this reason, the whole body should be studied with imaging techniques to rule out distant metastases.

Patients with a pancreatic solid mass should undergo high-resolution imaging techniques including multidetector computed tomography (MDCT) or magnetic resonance imaging (MRI)^[12-17]. Current guidelines suggest that total-body contrast-medium MDCT should be the preferred imaging modality^[12]. MDCT can give information regarding the local spread of the tumor, the presence of peripancreatic or distant lymphadenopathy, and of distant metastases. For the

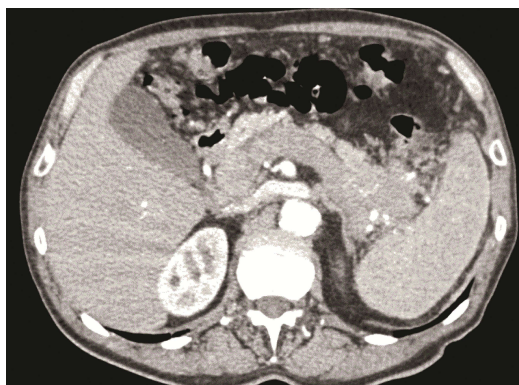


Figure 1 Pancreatic poorly differentiated neuroendocrine carcinomas of the pancreatic body-tail associated with neoplastic thrombosis of the splenic vein/portal vein, and with a lymphadenopathy along the stomach. The patients underwent left pancreatectomy with splenectomy, portal vein resection with portal vein thrombectomy and partial gastric resection.

purpose of local staging, it is important to assess the size of the tumor and localization within the pancreas, its relationship to the MPD and CBD, the major peripancreatic vessels (celiac trunk and its branches, superior mesenteric and splenic artery and vein, portal vein) and other adjacent structures. Pancreatic NET usually present as hyper vascularized lesions. This can be also the presentation of NEC, basically when there is a morphologically WD NEC with a relatively low ki67 (< 50%). On the other hand, morphologically PD NEC with higher proliferative index (ki67 > 50%) may present as a hypo vascularized mass frequently associated with the presence of necrosis (Figure 1)^[14-17]. This latter radiological presentation may resemble that of pancreatic ductal adenocarcinoma.

Nuclear medicine imaging is generally helpful in the imaging work-up of PNENs by using a PET camera. For PNEN imaging, two types of radiotracers are principally used: those related to receptor expression and those reflecting tumor metabolism^[13,18,19]. The first category includes somatostatin analogues (SSAs) labeled with the positron emitter 68Ga and the most often used preparations are 68Ga-DOTATOC, 68Ga-DOTANOC and 68Ga-DOTATATE besides somatostatin receptor scintigraphy imaging (Octreoscan®)^[18-20]. The use of 68Ga positron emission tomography (PET) or of Octreoscan® is helpful in order to confirm the endocrine nature of the lesion and PET is a useful tool complementary to CT for staging of regional and distant metastases^[18-21]. Of note, when a NEC is suspected, it is of paramount importance to perform both a 68Ga PET and ¹⁸FDG-PET. In fact ¹⁸F-fluorodeoxyglucose positron emission tomography (¹⁸FDG-PET) is preferred for tumor detection and staging as the somatostatin receptor (SSR) expression of these tumors is generally low or missing^[22-24]. Additionally, it has been demonstrated that a significant correlation exists between FDG-PET positivity and both Ki-67 and World Health Organization tumor grade.

Particularly when the Ki-67 is greater than 15%, the sensitivity of FDG-PET is greater than 92%^[22]. As such, these imaging modalities may be useful in distinguishing low- vs high-grade tumors. As a consequence, most morphologically WD NEC usually show a positivity for both 68Ga PET and ¹⁸FDG-PET, while in morphologically PD NEC with high ki67 index, there is almost exclusively a positivity for ¹⁸FDG-PET^[22-24]. Although PET imaging can be of help in differentiating poorly- and well-differentiated NEC, there are still many situations of uncertainty or of mild positivity of both 68Ga PET and ¹⁸FDG-PET; therefore all data from PET imaging should be always carefully integrated with clinical and pathological features.

Tissue biopsy is critical for a number of reasons^[5]. First, tissue biopsy confirms that the tumor is of neuroendocrine origin. Second, it provides further data regarding: (1) morphologic differentiation (*i.e.*, WD NEC vs PD NEC, small cells vs large cells PDEC); and (2) ki67 index evaluation. In order to provide these data, it is important to perform a fine-needle ago-biopsy (FNAB) rather than a FNA. These procedures can be carried out with endoscopic ultrasound of the primary pancreatic tumor, but FNAB may be performed on metastases as well. When several metastases are present, ¹⁸FDG positivity may be of help in order to select for biopsy those lesions with a higher metabolic activity, that are associated with a higher ki67 index.

PATHOLOGY AND PROGNOSTIC CORRELATION

As previously mentioned, NEC (WHO G3) is currently defined by a mitotic count > 20 mitoses/10 HPF and/or Ki67 index > 20^[2,3]. However, these tumors may be reported with a different terminology, including poorly differentiated carcinomas, high-grade neuroendocrine tumors, G3 neuroendocrine tumors, G3 NET, and well-differentiated neuroendocrine tumors with a high proliferative rate. Historically these tumors have been defined as PDEC, but with the 2010 WHO classification, the NEC category has become morphologically and biologically heterogeneous^[2-5]. In fact, at present, in the NEC category we may include:

Morphological PD NEC: these tumors were the previously classified PDEC^[1-3]. Morphological PD NEC are characterized by a high ki67 index, usually more than 50%-60%. They represent a group of very aggressive malignancies which show morphological and clinical features similar to those of the more frequent pulmonary PD NEC^[1-6]. Similarly to the lungs, they have traditionally been divided into the small cell (Figure 2A and B) and large cell (Figure 3A and B) subtypes, based on the morphological features of the neoplastic cells^[1,7]; various combinations of both small and large cells can be observed, and the term of "mixed

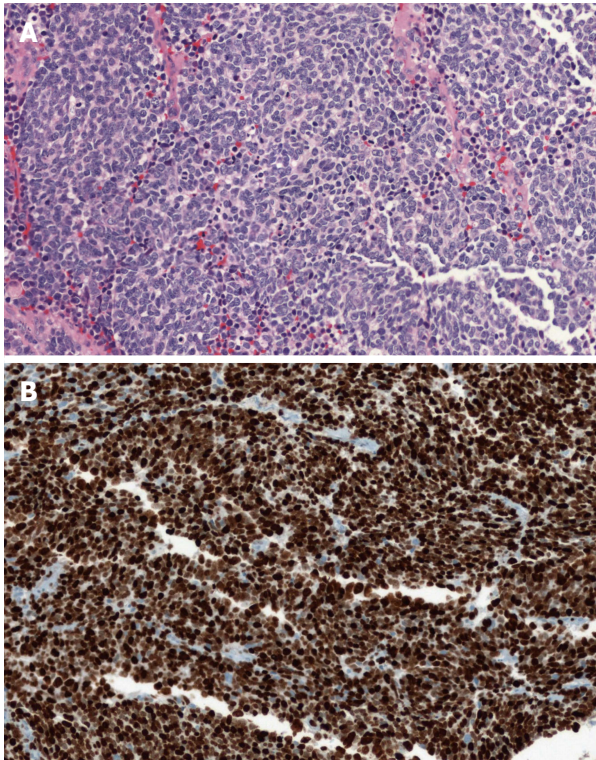


Figure 2 A small cell poorly differentiated neuroendocrine carcinoma of the pancreas (A) (haematoxylin-eosin stain) and a small cell poorly differentiated neuroendocrine carcinoma of the pancreas with high ki67 proliferative index (B, Ki67: 90%).

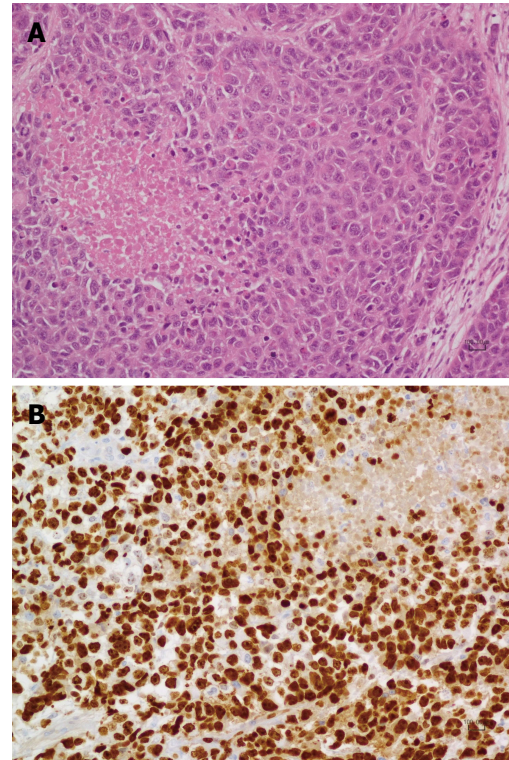


Figure 3 Shows a large cell poorly differentiated neuroendocrine carcinoma of the pancreas (A, haematoxylin-eosin stain) and a large cell poorly differentiated neuroendocrine carcinoma of the pancreas with its Ki67 proliferative index (B, Ki67: 80%).

type” has been proposed for this category.

Patient with morphologically PD NEC have a clinical behavior similar to that of small cell carcinoma or large cell neuroendocrine carcinoma of the lung, which is far worse than that of well-differentiated NETs. In the two largest series of pancreatic poorly differentiated NEC, the vast majority of patients had lymph node or distant metastases at presentation^[8]. Basturk *et al*^[8] reported a median survival of 11 mo (range 0 to 104 mo) with a five-year survival of 16% in a cohort of 44 patients. Crippa *et al*^[25] reported a similar survival in a cohort of 49 patients with PD NEC (median DSS: 12 mo). Of note patients with metastatic PD NEC succumb without treatment within weeks after diagnosis, and even with systemic chemotherapy the prognosis still remain severe with an expected survival of less than 6 mo^[8,9,25].

Morphological WD NEC: these tumors are well-differentiated NETs by a morphological point of view but they have a mitotic count > 20 mitoses/10 HPF and/or Ki67 index > 20% (Figure 4A and B). Of note, in this category there is also a small subset of patients with morphological well differentiated NET with less than 20 mitoses/10 HPF (G2 by mitotic count), but are associated with Ki-67 > 20%. Recently Basturk *et al*^[26] demonstrated that the clinical behavior of these grade-discordant NET was worse than grade-concordant G2

tumors (median survival 68 mo vs 54 mo), although the difference was not statistically significant.

Several studies have challenged the assumption that poorly differentiated histology and high tumor grade are equivalent^[5,27-29]. In fact when we consider morphological WD NEC, these tumors are associated with a markedly improved survival compared to morphological PD NEC. In a recent publication from our group, we found that patients with WD NEC had a significantly longer survival compared to those with PD NEC (43 mo vs 12 mo, $P = 0.004$)^[25]. Similar results were also reported by other Authors. Vélâyoudom-Céphise *et al*^[28] reported a median survival of 41 vs for WD NEC compared to only 17 mo for PD NEC. Basturk *et al*^[26] found a significantly improved survival for 19 patients with WD NEC compared to 43 PD NEC (median survival 54 mo vs 11 mo). Tang and colleagues showed a median disease-specific survival of 55 mo for WD NEC and of 16 mo for PD NEC.

The presence of low-/intermediate-grade and high-grade regions within the same NET is largely interpreted as well-differentiated NETs with progression to a more proliferative state (WD NEC)^[30]. Some tumors show features of well-differentiated NETs in some regions, including a low proliferative rate, but other regions or metastatic foci show a much higher proliferative rate along with more atypical cytological

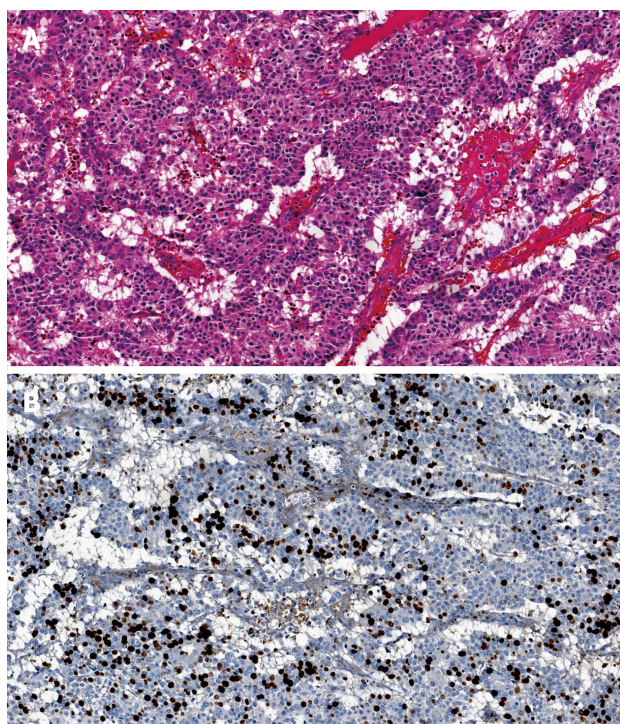


Figure 4 Morphological well differentiated neuroendocrine carcinoma of the pancreas (A, haematoxylin-eosin stain) and morphological well differentiated neuroendocrine carcinoma of the pancreas with Ki67 proliferative index of 30% (B).

features.

Unfortunately definitive histological diagnostic criteria are not clearly defined and accepted. Therefore further and larger studies are needed in order to better define and clarify histological diagnostic criteria and classification of both PD NEC and WD NEC.

MOLECULAR ALTERATIONS

Genomic investigations have found recurrent and mutually exclusive DAXX and ATRX mutations, which culminate in loss of corresponding protein expression in tumor cells, in approximately 44% of pancreatic WD-NETs^[31]. This genotype is specific for WD-NET and has not been seen in other pancreatic neoplasms, including PD-NECs^[7,30].

In contrast, pancreatic PD-NECs share some of the genotypic alterations of conventional pancreatic ductal adenocarcinoma including frequent gene mutations in TP53 and, less commonly KRAS, p16, and SMAD4, but these alterations were not found in pancreatic WD-NETs in several studies^[7,30].

Moreover, RB1 gene mutations and the associated loss of Rb protein expression are commonly observed in high-grade PD-NECs. Specifically, this mutation is found in more than 90% of small cell PD NEC while large cell subtype exhibit RB1 mutation in 50% to 60% of cases^[32,33]. On the contrary, RB1 and TP53 mutations have not been identified in WD-NETs^[7,30].

DIAGNOSTIC FLOWCHART

Figure 5 shows a diagnostic flowchart. In the suspect of a NEC or when there is a cytological diagnosis of NET with high-grade features, it is of paramount importance to perform a FNAB in order to collect tissue for a proper histological evaluation. The first evaluation should be a morphological one with the aim of classifying NEC in PD NEC or WD NEC. As previously discussed, the performance of a combined 68GaPET and of ¹⁸F-FDG PET may be of help in order to make a distinction between these two entities, although PET cannot fully discriminate between the two forms and data from PET imaging should be carefully integrated with other clinico-pathologic data. When morphological evaluation is uncertain or ambiguous, immunohistochemical studies should be considered. The loss of DAXX and ATRX are diagnostic for a WD NEC while the loss of Rb or an abnormal expression of p53 suggest the diagnosis of PD NEC.

MANAGEMENT OF POORLY DIFFERENTIATED NEUROENDOCRINE CARCINOMA

Figure 6 shows the potential therapeutic strategies for patients with PD NEC. After an accurate disease-staging, PD NEC can be classified in resectable, locally-advanced or metastatic tumors. Two recent studies have demonstrated that surgical resection of primary pancreatic tumor in resectable PD NEC is an independent predictor of survival^[25,34]. However, surgery alone is rarely curative, and the vast majority of patients with PD NEC undergoing resection will develop recurrence, being most recurrences distant and not local. For these reasons, adjuvant chemotherapy after curative resection of PD NEC should be considered, although no prospective studies are available to support this practice^[5]. Recent North American NeuroEndocrine Tumor Society guidelines recommend adjuvant therapy with 4 to 6 cycles of cisplatin or carboplatin plus etoposide^[6].

Recently the Nordic Neuroendocrine Tumor Group published the results of surgical treatments of patients with pancreatic NEC^[34]. They found, in a limited number of patients ($n = 14$) with localized non-metastatic disease, that surgical resection followed by adjuvant chemotherapy was associated with improved survival. However the median disease-free survival in this group was only 7 mo. Of note 13 out 14 patients developed early metastatic disease after resection, and this may be related to the presence of occult metastatic disease at diagnosis. In view of these results these Authors suggested that neoadjuvant chemotherapy may be also considered, but nowadays there are no evidence to support neoadjuvant chemotherapy in all patients

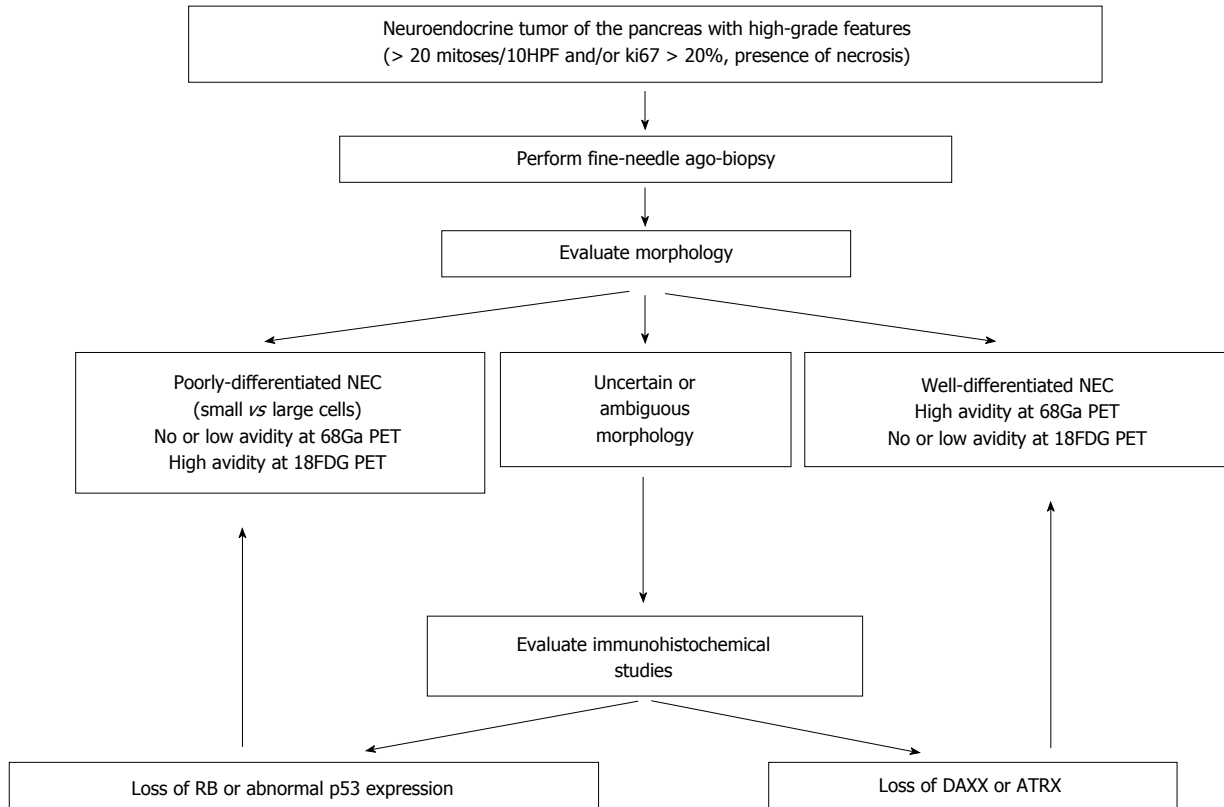


Figure 5 Diagnostic flowchart algorithm in patients with pancreatic neuroendocrine carcinomas.

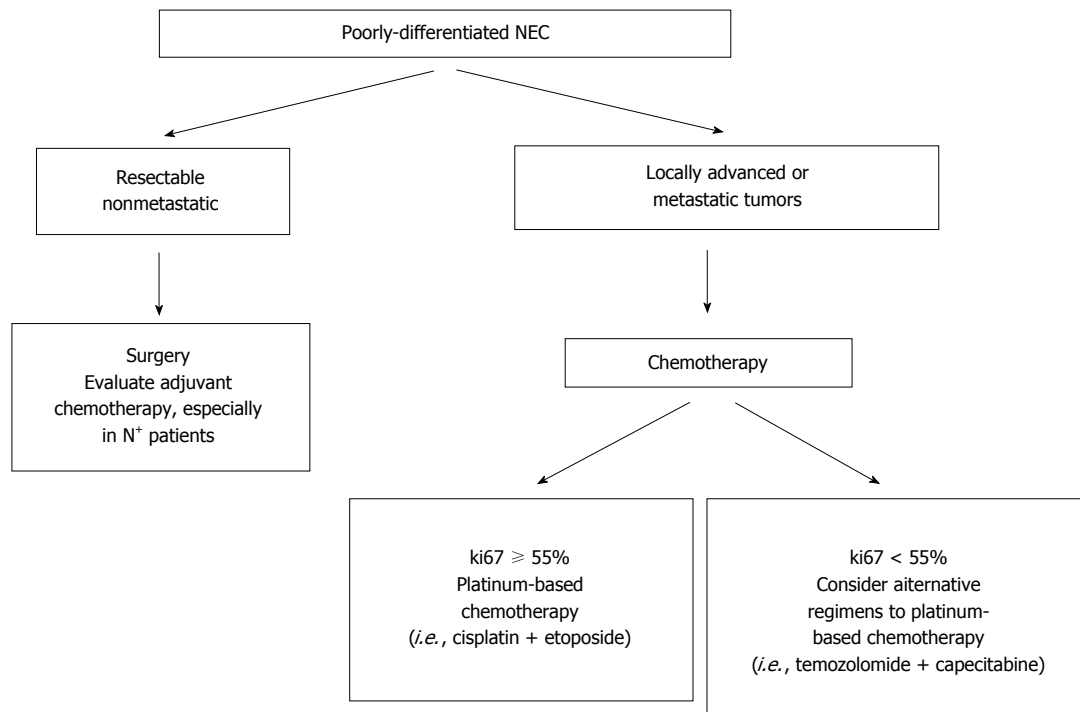


Figure 6 Different therapeutic options in patients with morphological poorly differentiated neuroendocrine carcinomas of the pancreas. ¹⁸F-DG-PET: ¹⁸F-fluorodeoxyglucose positron emission tomography.

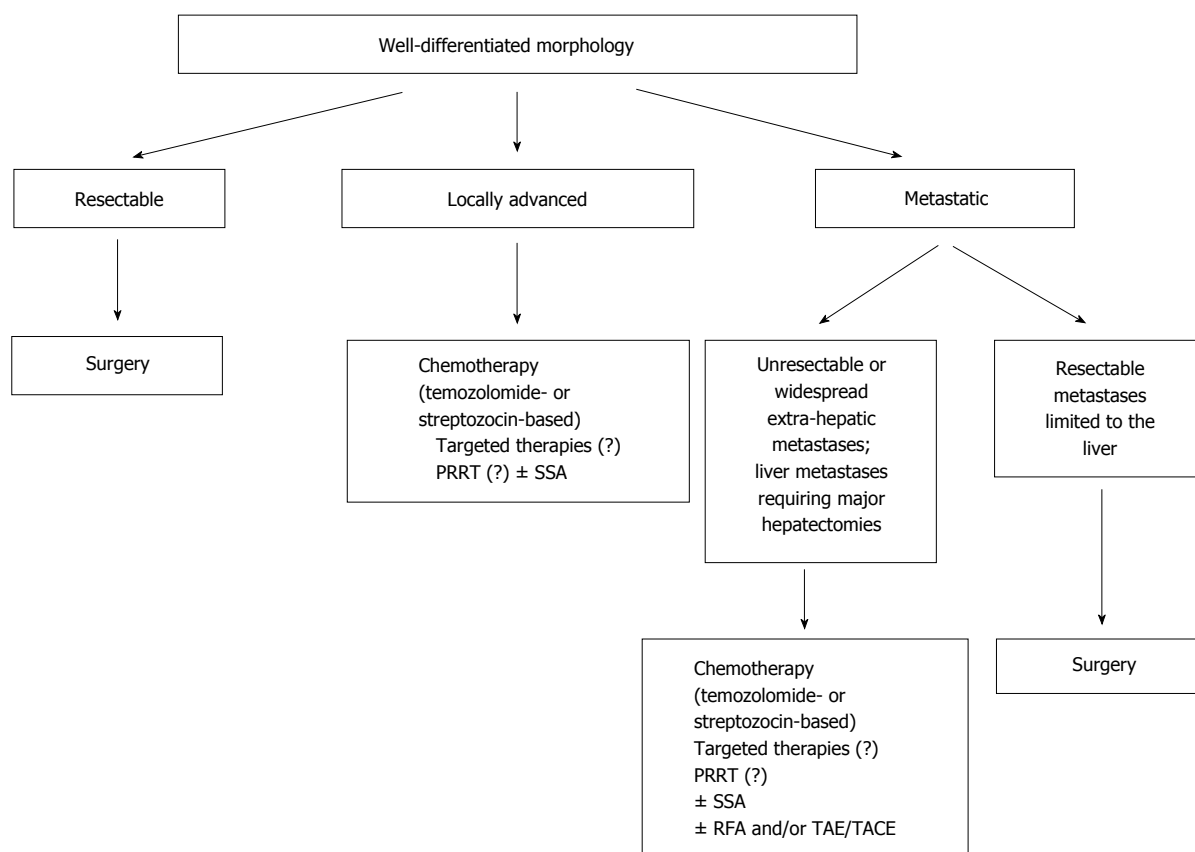


Figure 7 Management flowchart algorithm in patients with morphological well differentiated neuroendocrine carcinomas of the pancreas. PRRT: Peptide receptor radionuclide therapy; TAE: Transarterial embolization; TACE: Transarterial chemoembolization; SSA: Somatostatin analogues.

with resectable PD NEC^[5,34].

Patients with locally-advanced or metastatic PD NEC should undergo chemotherapy^[6]. The role of surgery in the setting of resectable pancreatic PD NEC with metastases limited to the liver is debated. Resection of primary NET in the presence of unresectable hepatic metastases is controversial, and most data come from retrospective and heterogeneous cohorts including mainly NET G1/G2^[35,36]. Some of these studies suggest possible benefit of primary tumor resection, but a bias toward more aggressive surgical approach in patients with better performance status or less advanced disease seems likely^[37,38]. For all these reasons, palliative resection of the primary pancreatic NEC in the setting of unresectable liver metastases is not recommended^[6,35]. Surgical metastasectomy is not recommended as well in the management of NEC^[6,39]. In fact in small series the median survival after partial hepatectomy for metastatic NEC from gastrointestinal tract including pancreas was 6 to 15 mo^[40,41]. Recently Partelli and coworkers demonstrated in a multicenter retrospective study that the presence of pancreatic neuroendocrine carcinoma G3 was the only factor independently associated with a poorer survival after resection in a cohort of 91 patients who underwent resection of primary NEN with ($n = 18$) or without ($n = 73$) hepatic resection^[42].

Ki67 index is important to establish the most appropriate chemotherapy regimen. In fact ki67 threshold of 55% was predictive for response to first-line platinum-based chemotherapy in different studies^[4-6,43,44]. Patients with PD NEC with ki67 > 55% had a response rate of 42%-67% to treatment with cisplatin/etoposide, while those with ki67 < 55% were less responsive to platinum-based chemotherapy (response rate: 15%). In these latter cases other agents including temozolomide proved to be more effective^[39,43,44]. Expected survival in patients with advanced PD NEC is less than one year, and performance status represents a significant prognostic factor^[9,25]. Patients with poor performance status does not receive chemotherapy in most cases but only best supportive care, reaching a median survival time of only 2 mo in such cases.

MANAGEMENT OF WELL DIFFERENTIATED NEUROENDOCRINE CARCINOMA

Figure 7 indicates the management flowchart for patients with WD NEC. In this setting the treatment may be more complex than in patients with PD NEC. In patients with resectable disease surgery with

curative intent must be considered. In patients with locally-advanced disease there is a wide range of possible therapies including temozolamide-based or streptozocin-based chemotherapy, peptide receptor radionuclide therapy, somatostatin analogues long-acting release and target therapies (*i.e.*, mTOR inhibitor everolimus)^[25,35,39]. Unfortunately little evidence-based data are available to guide therapy, and the decision to perform a treatment rather than another one should be individualized considering morphology, ki67, performance status and primary aim of the treatment (downsizing/staging).

In patients with resectable primary WD-NEC associated with resectable metastases limited to the liver, surgery can be considered with the aim of obtaining a curative resection, providing that no major hepatectomies are required^[35,39]. In patients with widespread metastatic disease and/or unresectable metastases limited to the liver, the benefit of a palliative resection of the primary pancreatic tumor is uncertain. In these patients there is a wide range of therapeutic options, including systemic therapies as well as liver-directed treatments. Again, there is a lack of strong evidence-based information in order to plan the most appropriate treatment or to determine the sequence of treatment to do. However, the less aggressive biological behavior of WD NEC as well as the different therapeutic options available can improve the prognosis of patients with WD NEC even in the metastatic setting.

CONCLUSION

Based on the current data, it is clear that the current WHO high-grade NEC category should be revised. In fact NEC constitute a heterogeneous group of neoplasms including WD NEC and PD NEC. Morphological WD NEC represents a subgroup with markedly improved survival while PD NEC are more aggressive tumors. This difference has significant implications for treatment and prognosis. A new classification of NEC is required considering both morphology and ki67 index. Specific and definite diagnostic criteria for histological diagnosis of PD NEC and WD NEC are also required.

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Basic Study

Apolipoprotein B100 is required for hepatitis C infectivity and Mipomersen inhibits hepatitis C

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Abstract

AIM

To characterize the role of apolipoprotein B100 (apoB100) in hepatitis C viral (HCV) infection.

METHODS

In this study, we utilize a gene editing tool, transcription activator-like effector nucleases (TALENs), to generate human hepatoma cells with a stable genetic deletion of *APOB* to assess of apoB in HCV. Using infectious cell culture-competent HCV, viral pseudoparticles, replicon models, and lipidomic analysis we determined the contribution of apoB to each step of the viral lifecycle. We further studied the effect of mipomersen, an FDA-approved antisense inhibitor of apoB100, on HCV using *in vitro* cell-culture competent HCV and determined its

impact on viral infectivity with the TCID₅₀ method.

RESULTS

We found that apoB100 is indispensable for HCV infection. Using the JFH-1 fully infectious cell-culture competent virus in Huh 7 hepatoma cells with TALEN-mediated gene deletion of apoB (*APOB KO*), we found a significant reduction in HCV RNA and protein levels following infection. Pseudoparticle and replicon models demonstrated that apoB did not play a role in HCV entry or replication. However, the virus produced by *APOB KO* cells had significantly diminished infectivity as measured by the TCID₅₀ method compared to wild-type virus. Lipidomic analysis demonstrated that these virions have a fundamentally altered lipidome, with complete depletion of cholesterol esters. We further demonstrate that inhibition of apoB using mipomersen, an FDA-approved anti-sense oligonucleotide, results in a potent anti-HCV effect and significantly reduces the infectivity of the virus.

CONCLUSION

ApoB is required for the generation of fully infectious HCV virions, and inhibition of apoB with mipomersen blocks HCV. Targeting lipid metabolic pathways to impair viral infectivity represents a novel host targeted strategy to inhibit HCV.

Key words: Apolipoprotein; Lipid; Hepatitis C virus; Gene silencing; Viral replication

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Core tip: Hepatitis C virus (HCV) circulates as a very-low-density lipoprotein (VLDL)-like lipoviral particle. Apolipoprotein B100 (apoB100) is the core protein of VLDL, but its role in HCV has remained incompletely characterized. Use of gene-editing with transcription activator-like effector nucleases permits the characterization of the role of apoB100 in HCV. We demonstrate that apoB100 is required for HCV infection. Loss of apoB100 results in the secretion of HCV virions with an altered lipid composition and limited ability to infect naive cells. Mipomersen, an FDA-approved antisense inhibitor of apoB100, has an anti-HCV effect and limits the viral infectivity.

Schaefer EAK, Meixiong J, Mark C, Deik A, Motola DL, Fusco D, Yang A, Brisac C, Salloum S, Lin W, Clish CB, Peng LF, Chung RT. Apolipoprotein B100 is required for hepatitis C infectivity and Mipomersen inhibits hepatitis C. *World J Gastroenterol* 2016; 22(45): 9954-9965 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v22/i45/9954.htm> DOI: <http://dx.doi.org/10.3748/wjg.v22.i45.9954>

INTRODUCTION

Hepatitis C virus (HCV) infection is a major global

health problem, affecting 185 million individuals worldwide and 3 million in the United States^[1,2]. While highly effective direct-acting antivirals (DAAs) for HCV have expanded therapeutic armamentarium against HCV, these drugs may be limited by genotype specificity, limited success in some subpopulations, and the potential for development of multiclass resistance in treatment failures. One successful strategy to address treatment failures is the targeting host factors required for HCV infection, which possess a high barrier to the development of virologic resistance^[3].

The development of direct-acting and host-targeted antiviral medications emerged on the heels of a vastly enhanced understanding of the HCV viral lifecycle, enabled by the development of robust *in vitro* models of HCV. Even prior to characterization beyond non-A non-B hepatitis, the virus was observed to physically associate with the low density fraction of human sera suggesting an association with human lipoproteins. Indeed, viral RNA could be precipitated with antibodies against apolipoprotein B100 and apolipoprotein E^[4-6]. More recent data has demonstrated that the virus circulates as a highly lipidated lipoviral particle (LVP), which contains both apoE and apoB, and the lipid composition of this LVP very closely resembles human very-low-density lipoprotein (VLDL)^[7,8].

Despite these observations, the exact role of apoB in HCV infection remains incompletely characterized. Data have been conflicting, with some pharmacologic studies suggesting an important role for apoB, but RNAi experiments suggesting that apoB does not play a function at all in HCV infection^[9-11]. An important limitation of these *in vitro* studies has been their use of hepatoma cells lines which are highly permissive to HCV, but which do not fully recapitulate the production of human VLDL.

Novel and specific gene editing tools have been developed to better understand gene function in cellular and animal models. One such tool is the use of transcription activator-like effector nucleases (TALENs), derived from plant nucleases, which can be specifically designed to bind target genomic sequences and result in loss of gene expression. This strategy generates stable cellular genetic deletions without requiring antibiotics or transfection, and has minimal off-target effects. We used this technique to generate a hepatoma cell line lacking *APOB* expression and found HCV infection to be inhibited in the absence of apoB^[12]. Following these findings, an additional study utilized zinc-finger nucleases and clarified that apoB and apolipoprotein E (apoE) both likely play a role in infectious HCV particle formation and that there is HCV core accumulation on lipid droplets without apoB and E expression. Further, additional data has additionally suggested that apoB is important for cell-free transmission of the virus^[13,14].

In this study, we characterize the specific contribution of human apolipoprotein B 100 to the HCV lifecycle and determine the effect of an FDA-approved

inhibitor of apoB on the virus. The cells used for this study were Huh7 human hepatoma cells which over-express the HCV entry co-receptor CD81. Huh7 cells do model human VLDL secretion^[15], and overexpression of CD81 renders them more permissive to HCV. Using these novel *APOB* knockout cells, we confirm that the loss of *APOB* inhibits HCV infection^[12] and that apoB expression is indispensable for HCV. Specifically, its absence results in virus that has a fundamentally altered lipidome and is significantly impaired in its ability to infect other cells. Further, and importantly, we demonstrate a novel use and potent and dose-dependent anti-HCV effect of an FDA-approved compound which inhibits apoB expression, mipomersen.

MATERIALS AND METHODS

Cell culture

TALEN-induced *APOB* KO Huh 7/CD81 cells were generated and maintained as previously described^[12]. All experiments were conducted in triplicate.

HCV infection

JFH1, a genotype 2a HCV isolate, and the Jc1e2FLAG JFH1 chimera were used for HCV infection. Naive cells were incubated with the virus for 4-6 h, after which the viral supernatant was removed. Virus of identical multiplicity of infection (MOI) was used for all experiments. Cell lysates were isolated for protein and/or RNA at the time points indicated. Lysate RNA was collected using RNEasy isolation kits (QIAGEN, Valencia, CA). Viral RNA was isolated from the supernatant using a viral nucleic acid isolation kit (Roche, Indianapolis, IN). LDL rescue was performed using commercially available LDL extracted from human plasma (Sigma, St. Louis, MO). In rescue experiments, LDL was delivered at a concentration of 2.5 pg/mL following infection with JFH1.

Immunofluorescence

Cells were cultured on coverslips and fixed with 4% PFA at indicated time points. Primary antibodies (LDL-R, Ab-Cam, Cambridge, MA, and HCV core hybridoma) were diluted in 10% Donkey Serum, and were stained with IgG Alexa Fluor 488 secondary antibodies (Life Technologies, Carlsbad, CA).

Pseudoparticle experiments

HCV E1 E2 and control VZV pseudoparticles were a kind gift of Dr. Francois-Loic Cosset (Lyon, France). Naive cells were plated in 96-well plate and exposed to HCVpp or VZV for 72 h. The infectivity was determined using immunofluorescence microscopy.

Replicon experiments

Genotype 2a (JFH-1) full genomic and subgenomic replicons were provided courtesy of Professor

Takaji Wakita (Tokyo, Japan). The constructs were electroporated into naive cells using the BioRad Gene PulseXcell electroporation system and then selected using G418 supplemented media for approximately three weeks. The G418-resistant colonies were then pooled and cultured on 10cm dishes. HCV levels were determined using RNA and protein isolation from cell lysates.

Infectivity

Virus generated in WT or KO cells was used to infect highly permissive Huh 7.5.1 cells. Dilutions were performed to normalize for HCV RNA titer in the supernatant. The tissue culture infectious dose (TCID50) per mL was performed using methods previously described^[16].

Lipid extraction

Jc1E2FLAG HCV particles were isolated and the lipid fraction of the purified Jc1E2FLAG HCV particles was extracted and purified using the methods previously described^[7]. The organic phase was collected for use in LC-MS analysis. Analyses of polar and non-polar lipids were conducted using an LC-MS system comprised of an Open Accela 1250 U-HPLC and a Q Exactive hybrid quadrupole orbitrap mass spectrometer (Thermo Fisher Scientific; Waltham, MA).

Mipomersen experiments

Huh 7/CD81 cells were treated with escalating doses of mipomersen as indicated, or mock, at the time of plating as well as 24 and 48 h following infection. Cell lysate and viral supernatant were harvested for analysis at 72 h following infection. Cell viability was monitored using Cell Titer Glo (Promega, Madison, WI).

Statistical analysis

Statistical analysis was performed using GraphPad Prism 5 software (GraphPad software, Inc, La Jolla, CA) and by a researcher trained in biostatistics. RT-QPCR data is represented as the mean with 95% confidence intervals. Two tailed student's *t*-test was used. A *P* value of < 0.05 was considered statistically significant.

RESULTS

APOB KO cells inefficiently support HCV infection

APOB KO cells were generated as previously described using TALEN-mediated gene knockout. The parent cells were Huh7 hepatoma cells over-expressing the HCV entry co-factor CD81 (Huh7/CD81). The *APOB*^{-/-} knockout (*APOB* KO) cells have a < 3% expression of *APOB* at the mRNA level and have no detectable apoB100 protein^[12].

As we have previously reported, *APOB* deletion impaired the ability of the hepatoma cells to support

HCV infection. Three days following infection with the fully infectious tissue culture-competent hepatitis C virus (HCVcc) strain JFH-1, we observed a 72% (95% CI: 67%-77%, $P = 0.01$) reduction in HCV RNA and no detectable core protein (Figure 1A). We then confirmed this finding in three additional knockout clones to ensure this finding was not a clone-dependent phenotype and found that HCV was inhibited in all studied clones (Figure 1B).

We next sought to determine whether restoring intracellular apoB100 to the *APOB* KO cells would, in turn, allow them to again support HCV infection. The large size of the full apoB100 protein (550 kDa) made plasmid-based transfection challenging; we therefore restored intracellular apoB expression *via* administration of LDL into the cell media, allowing for LDL-R mediated uptake of apoB. While it is held that apoB is degraded following LDL-R mediated endocytosis^[17], the fate of apoB in deficient cells is not known. We confirmed that reintroducing apoB protein into the *APOB* KO cells resulted in sustained intracellular levels of apoB *via* western blot and immunofluorescence (Figure 1C and D). Further, reintroduction of apoB led to a significant increase in the intracellular levels of HCV RNA and HCV core protein 72 h following the addition of LDL. These findings were confirmed with immunofluorescence staining, which demonstrated cytoplasmic expression of apoB100 protein following LDL exposure, and enhanced HCV core protein expression in the LDL-treated knockout cells (Figure 1C-E).

Taken together, these findings suggested that loss of *APOB* expression rendered the Huh7 hepatoma cells non-permissive to the full HCV lifecycle, a state that was reversed upon reintroduction of apoB into the cytoplasm.

Further, we confirmed that these changes were not due to impaired entry using HCV pseudoparticles, a model of HCV entry. We found that entry of HCV into the *APOB* KO cells was not impaired (Figure 2A), and that the cells were not deficient in known HCV entry factors (Figure 2B). We next sought to understand whether replication was affected in *APOB* KO cells.

HCV Replication is not impaired in *APOB* deficient hepatoma cells

HCV viral replication takes place on a highly specialized membranous web formed through the combined effects of both host and viral proteins, and which is closely apposed to cytoplasmic lipid droplets. While apolipoproteins have not been previously suggested to be directly involved in viral RNA replication, the lipid droplet is known to play an important role as a bridge between replication and early assembly. Approximately 20% of the total cellular HCV RNA is localized on the lipid droplet, where it has been demonstrated to co-localize with the HCV core protein and the non-structural protein NS5A^[18]. ApoB100 has

been described, in the absence of HCV infection, to localize to lipid droplets in a so-called "apoB crescent" while awaiting either lipidation or degradation^[19]. We therefore hypothesized that apoB100 might play a role in replication, potentially bridging replication and assembly, possibly through interactions with core, NS5A and the lipid droplet.

To determine whether apoB is required for HCV replication, we utilized both subgenomic and full genomic replicons derived from the genotype 2a JFH1 virus. The replicons are introduced into the cell by electroporation and model the intracellular steps of HCV propagation, but no live virus is secreted^[20]. The subgenomic replicon lacks the structural HCV proteins (E1, E2 and core), whereas the full genomic replicon produces all the HCV proteins. Both replicons were introduced into the *APOB* WT and *APOB* KO hepatoma cells as previously described^[21], and the intracellular HCV levels were determined after antibiotic-mediated selection for replicon-containing cells. We found no difference in HCV RNA between the *APOB* WT and KO cells using either cellular model, and detected no difference in the expression of core protein in the full genomic replicon model (Figure 3A and B).

To further verify these findings, we repeated these experiments in two additional replicon-based cell lines: the OR6 cell, which stably harbors a full length, genotype 1b HCV genome^[22], and pRep-Feo cells which contains a subgenomic, genotype 1b HCV replicon^[23]. Using RNAi-mediated gene silencing, we knocked down apoB100 expression, and detected no difference in HCV RNA in either cellular model (Figure 3C). These data provide firm evidence that apoB100 does not play an important role in the replication step of the HCV lifecycle.

There is decreased production of HCV virion in *APOB* KO human hepatoma cells

There are several lines of evidence demonstrating an association between the HCV virion and VLDL production. First, there is prior evidence for a protein-protein interaction between the HCV core protein and the microsomal triglyceride transfer protein (MTTP), a protein critical for the lipidation of apoB100, in the early stages of HCV assembly^[24]. Further, HCV follows the pathway of VLDL secretion^[25]. However, whether apoB100 is important for the production of fully infectious HCV virions has remained controversial^[26-28]. Some data have demonstrated that pharmacologic inhibition of MTTP blocks HCV assembly^[10], but inhibition of apoB100 itself has produced disparate effects on HCV LVP production^[26,29]. The parent cell line for the KO cells is competent for VLDL production, and the knockouts do not display decreased intracellular levels of either MTTP or apolipoprotein E (data not shown). We determined that the *APOB* KO cells have diminished HCV secretion.

We first examined the quantity of viral RNA released

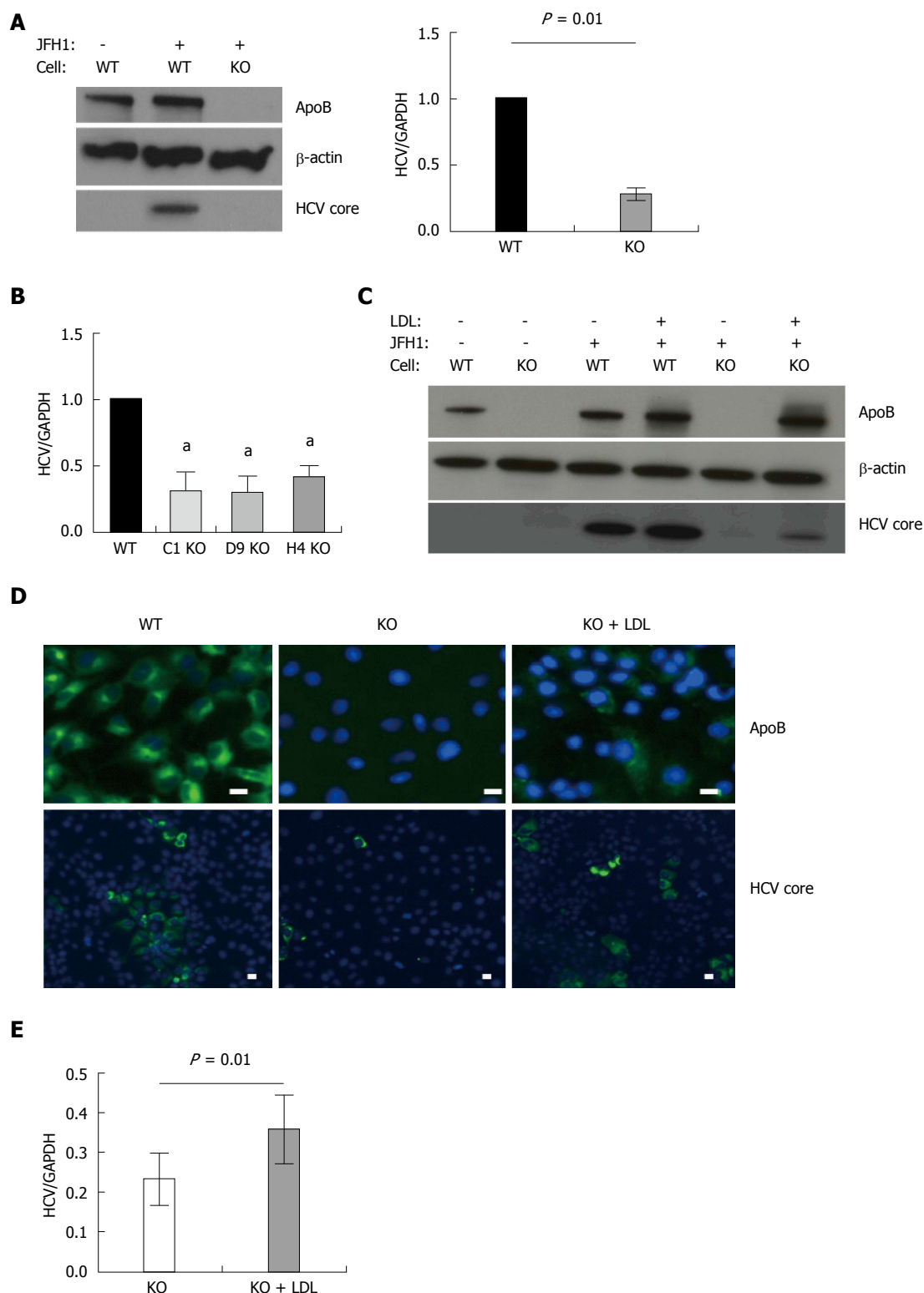


Figure 1 *APOB* genetic knockout Huh 7 hepatoma cells inefficiently support hepatitis C virus. **A:** Using TALEN genetic deletion of *APOB*, knockout huh7/CD81 (high) cells were generated (*APOB*^{-/-}, KO). We demonstrated that these cells inefficiently support hepatitis C virus (HCV) infection with the fully infectious tissue culture HCV virus, HCVcc (JFH1). There was markedly decreased JFH1 72 h following infection both at the level of HCV core protein and RNA. Data is shown as mean with 95% confidence interval, normalized to WT; **B:** To confirm this was not a clone-specific effect, three additional clones were infected with the JFH1 virus and intracellular HCV RNA assessed 72 h following infection. We again found a decrease in HCV RNA in the *APOB* KO clones ($^aP < 0.05$); **C, D:** Rescue experiments were performed by treating cells with human LDL at the same time as infection with the JFH1 virus. We found partial restoration of intracellular apoB expression at 72 h following exposure (**D**), and there was a partial restoration of HCV infection, with increased expression of HCV core protein (**C**), HCV RNA (**E**), and IF demonstrating increased HCV core expression in the LDL-treated cells (**D**). Scale bar: 20 μ m.

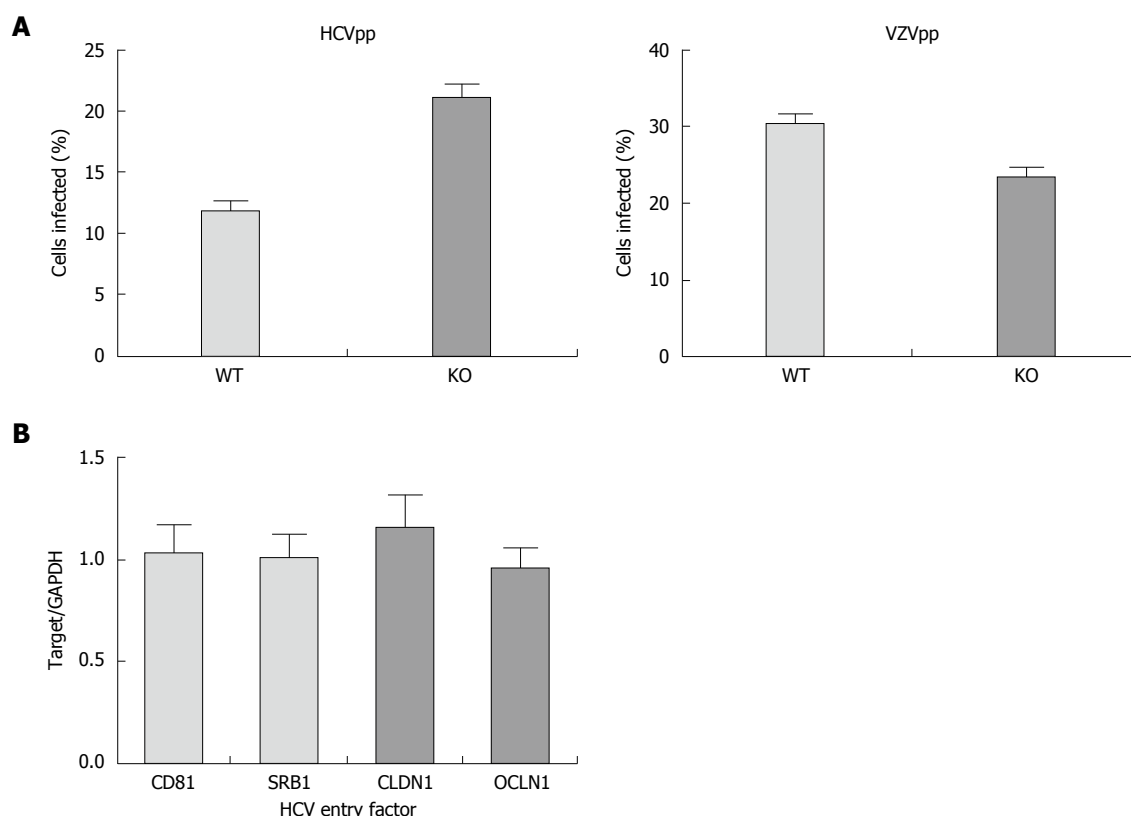


Figure 2 Hepatitis C virus entry is not impaired in *APOB* KO cells. A: Using an hepatitis C virus (HCV) genotype 1b pseudoparticle (HCVpp), cells were exposed to the pseudoparticle and incubated for 72 h at which time the degree of HCV entry was assessed using GFP expression; a control VZV pseudoparticle (VZVpp) was also assessed. We that entry of neither HCV nor VZV was impaired in the *APOB* KO cells; B: We confirmed that the expression levels of known HCV entry factors CD81, SRB1, CLDN1, OCLN1 were preserved in the KO cells (normalized to WT cell expression level, 1).

into the supernatant 72 h following the infection of the *APOB* WT and KO with JFH-1. There was a substantial and significant reduction in the quantity of HCV RNA released from the KO cells (Figure 4A).

We then sought to determine whether the virus that was successfully produced by the *APOB* KO cells differed from wild type virus with respect to its ability to infect naive cell (infectivity). To do this, we exposed uninfected, highly permissive, Huh 7.5.1 cells to virus generated by either *APOB* WT (WT virus) or *APOB* KO (KO virus) cells. We normalized the quantity of virus used for inoculation by the viral RNA titer (to account for the decreased levels of HCV RNA detected from the KO cells), and assessed infectivity using the previously described TCID₅₀ method^[16]. We demonstrated that the KO virus was significantly less infectious in the highly permissive Huh7.5.1 cells than the WT Virus (Figure 4A).

Lipids play an important role in flavivirus production and entry, and have been implicated in replication of the dengue virus^[30–32]. Dengue has a lipid-rich envelope, and interruption of cholesterol biosynthesis impairs its replication and infectivity^[33]. To determine whether the diminished infectivity observed in the KO HCV virus was related to a defect in lipid metabolism that is extendable to other flaviviridae, we examined viral production and infectivity of the Dengue virus

using the apoB cells.

After infecting WT and KO cells with dengue virus and assessing the levels of viral RNA 72 h following inoculation, we found no difference in the ability of the *APOB* KO cells to support dengue infection (Figure 4B). We analyzed the viral supernatant and additionally demonstrated that there was no decrease in DNV RNA produced by the KO cells. Further, after normalizing for RNA titers, there was no decrease in dengue viral infectivity (Figure 4C). We thus concluded that the perturbations observed following deletion of *APOB* are not generalizable to dengue virus, and may be specific to HCV.

HCV virions produced by *APOB* KO human hepatoma cells have a fundamentally altered lipidome

The density of viral particles has been described to be an important predictor of viral infectivity, and the lipid composition is the major determinant of buoyant density. The lipid composition of the HCVcc produced by Huh 7.5.1 cells has recently been characterized^[7]; however, the buoyant density of Huh 7.5 cells and tissue culture-generated virus is lower than that observed in human serum^[34,35], and Huh 7.5 and Huh 7.5.1 cells have not been demonstrated to recapitulate the human VLDL machinery, unlike Huh7 cells^[15]. Virus generated by VLDL-competent Huh7/CD81

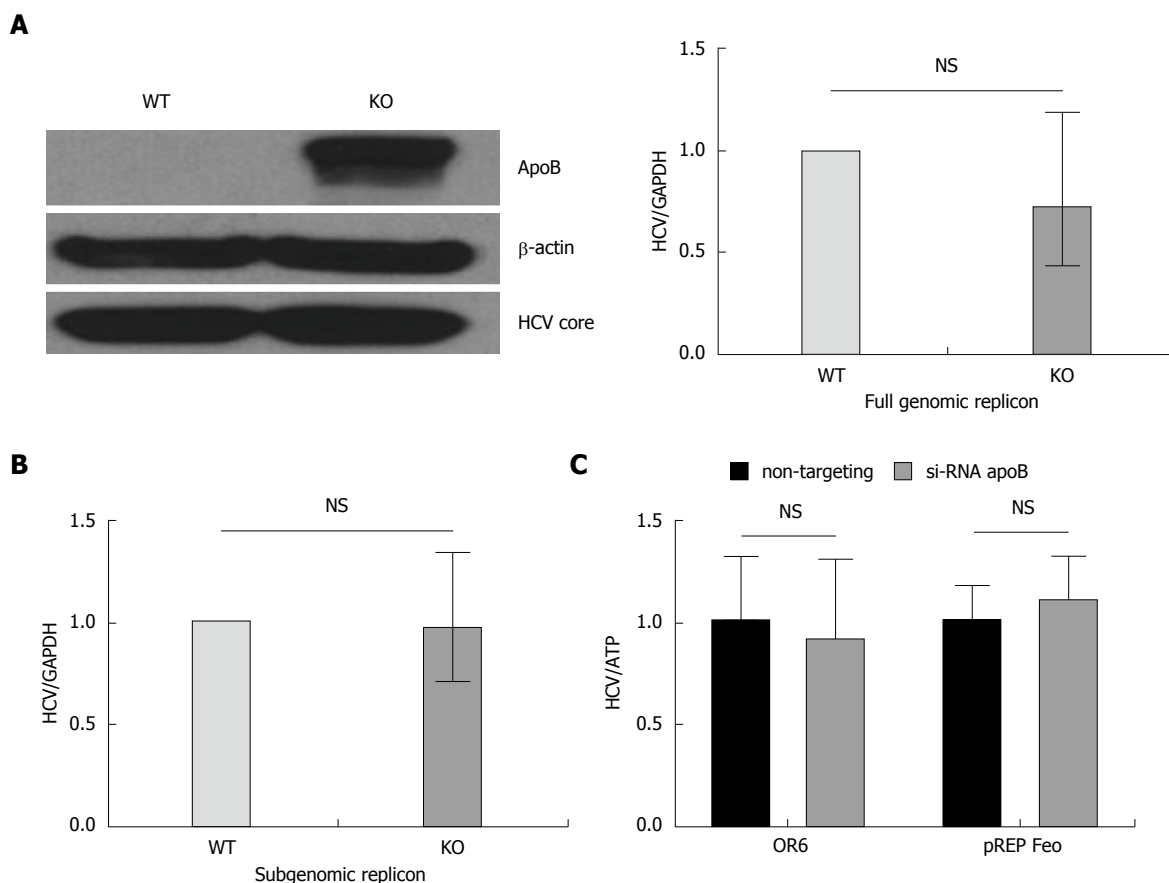


Figure 3 Loss of apoB does not impair replication in *APOB* KO cells. A: A full genomic replicon derived from the JFH1 hepatitis C virus (HCV)cc strain was electroporated into WT and KO cells which were then selected in culture based on antibiotic resistance. We demonstrated that there was no decrease in intracellular HCV core or RNA in the KO compared to WT cells; B: A subgenomic replicon, which lacks expression of the E1, E2 and core proteins, was also electroporated into the cells and again there was no difference in HCV RNA. Data is shown as mean with 95%CI, normalized to WT; C: To confirm these findings, we used replicon cells with a full genome of a different viral genotype (OR6, 1b and pREP-Feo, 1b) and subsequently knocked down apoB expression using RNAi. There was no difference in HCV replication in the setting of apoB knockdown. Data is shown as mean ± SD.

cells is more likely to reflect the human LVP, and we hypothesized that the observed impaired infectivity reflected an altered lipid composition of the KO virus.

Prior to characterizing the lipidome, we sought to confirm the presence of apoE in the virus, since apoE has also been well-described to be important for generation of viral particles and viral infectivity^[36]. Using an ELISA-based assay, we determined that the KO virus, as expected, had barely detectable apoB100 secreted into the media. We also determined, however, that there was a modest, but significant, reduction in the amount of apoE (Figure 5A). This reduction however was far less than the observed decrease in infectivity, suggesting that apoE reduction is not the primary cause of the loss of infectivity.

To characterize the lipidome of HCVcc produced in WT and KO cells, we utilized a JFH-1 derived, JC-1 virus with a FLAG-tag on the N-terminus of the E2 protein (JC1E2FLAG). After harvesting JC-1 virus generated in the *APOB* WT and *APOB* KO cells, we performed affinity purification of HCV virion. The purified virus was then analyzed using liquid chromatography/mass spectrometry (LCMS) to

determine the lipid composition of the virions.

KO virus had a profoundly altered lipidome compared to WT virus. Specifically, triacylglycerols and diacylglycerols comprised approximately 15% of the lipid composition in the WT virus, compared to close to 30% in the KO virus. Most strikingly, the primary lipids (accounting for approximately 40% of all lipids) of WT virus were cholesterol esters, which was consistent with prior data; however, the KO virus was completely lacking in cholesterol esters (Figure 5B). This perturbation alone may account for a substantial amount of the impaired infectivity of the KO virus: indeed, two of the known HCV entry receptors, SRB1 and the Niemann-Pick-C1-Like-Receptor1 (NPC1L1) are described to recognize cholesterol esters^[37,38].

We therefore concluded that apoB plays a critical role in HCV infection, primarily due to its deleterious effect on generation of infectious virus. Without apoB, production and secretion of new virion is impaired, and those virions which are secreted have a markedly diminished infectivity, which is likely related to the depletion of cholesterol esters from the lipidome.

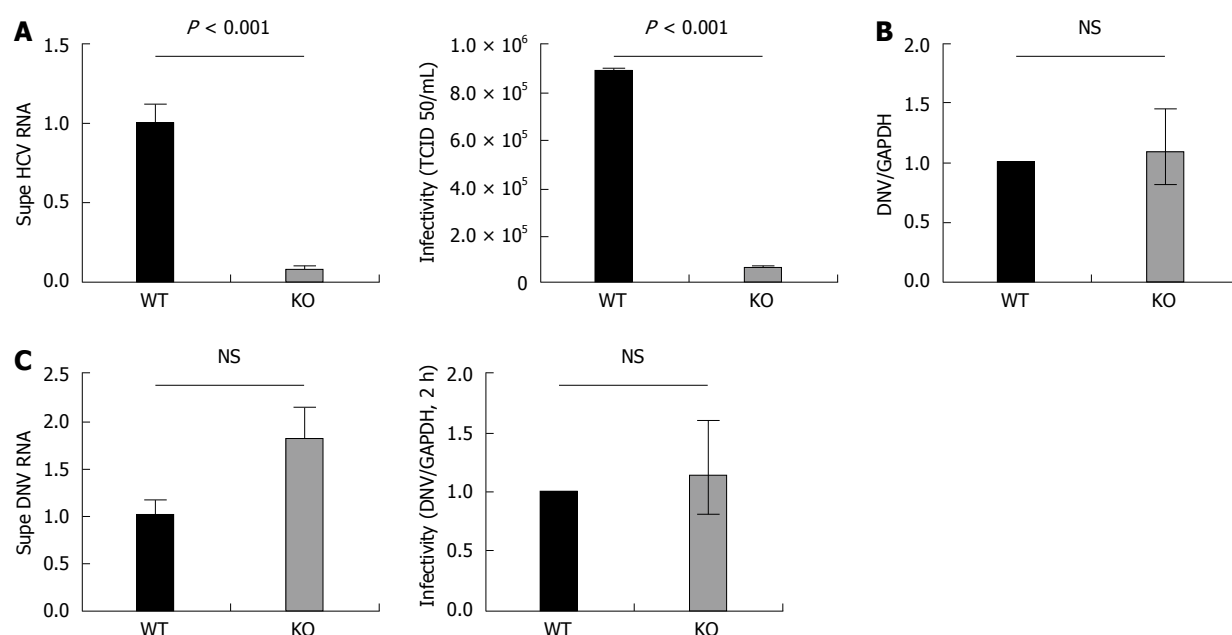


Figure 4 *APOB* KO cells have diminished hepatitis C virus production and KO virus has impaired infectivity, but *APOB* KO has no effect on dengue viral infection. A: 72 h following infection with JFH1 hepatitis C virus (HCV)cc, supernatant was harvested and viral RNA was isolated and quantified using qPCR. The supernatant harvested from infected KO cells had significantly lower levels of HCV RNA compared to WT supernatant. Data are normalized to WT RNA titer and standard error of the mean shown. After normalizing for the differences in HCV RNA by diluting the WT virus, the infectivity of the two viruses in highly HCV-permissive Huh 7.5.1 cells was then determined using the TCID₅₀ method. The KO generated virus had significantly reduced infectivity in the permissive Huh 7.5.1 cells; B: Dengue replication: WT and KO cells were infected with Dengue virus, and the level of intracellular RNA was assessed at 72 h. No difference was observed between WT and KO cells. Data is shown as mean with 95% confidence interval, normalized to WT; C: Supernatant of dengue-infected cells was harvested at 72 h and dengue viral RNA quantified. RNA levels were slightly higher in the supernatant of the KO cells, but the difference was not significant. A similar trend was observed with the infectivity of the KO generated dengue virus, assessed at DENV RNA at 2 h following infection.

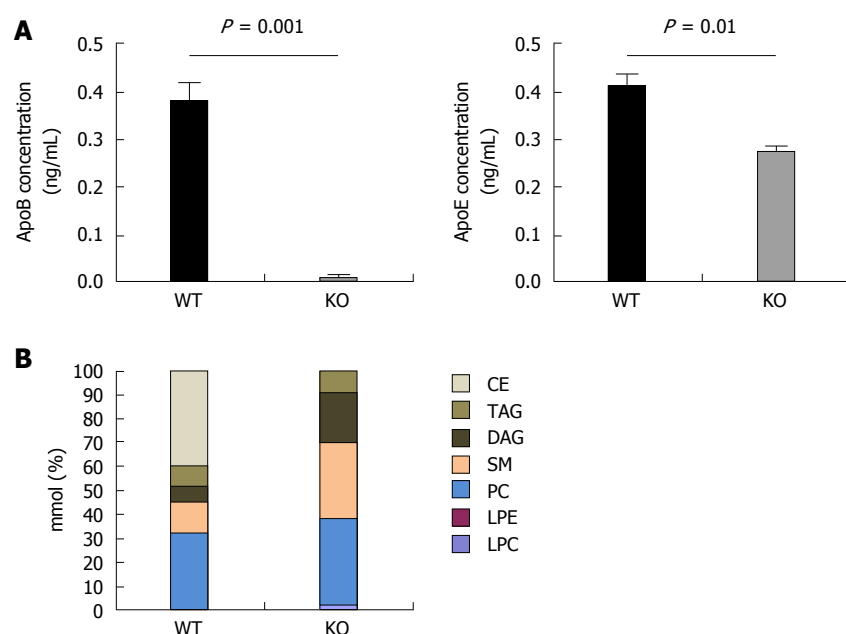


Figure 5 Hepatitis C virus produced by *APOB* KO cells has a fundamentally altered lipidome. A: The concentrations of apoB and apoE were determined of the viral supernatant using ELISA. ApoB levels were barely detected in the viral supernatant of the KO cells. ApoE concentrations were also found to be lower in KO compared to the WT cells; B: Hepatitis C virus (HCV)cc generated in *APOB* KO cells has an altered lipidome. Using a Jc1E2FLAG HCV tissue culture fully infectious virus, viral particles were purified using affinity purification particles were extracted and the lipidome was purified using liquid chromatography - mass spectrometry (LC-MS). LC-MS demonstrated that the viral particles generated in the *APOB* knockout cells are completely depleted in cholesterol esters. CE: Cholesterol and cholesterol esters; TAG: Triacylglycerols; DAG: Diacylglycerols; SM: Sphingomyelins; PC: Phosphatidylcholines; LPE: Lysophosphatidylethanolamines; LPC: Lysophosphatidylcholines.

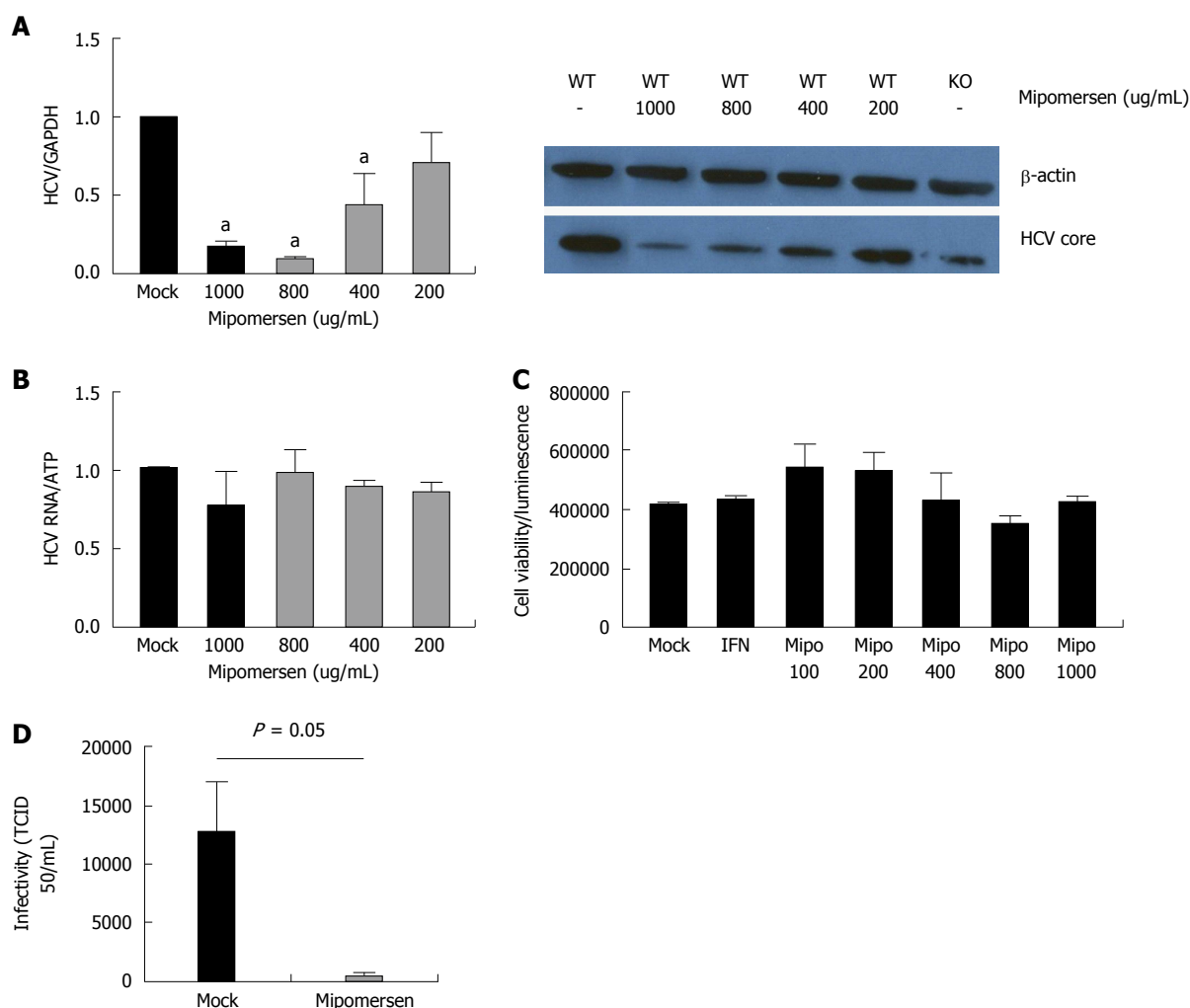


Figure 6 Mipomersen exerts a dose-dependent and potent anti-hepatitis C virus effect. A: Huh7/CD81 high cells were treated with escalating doses of mipomersen from 200 ug/mL to 1000 ug/mL and then infected with JFH1. At 72 h following infection, there was a dose-dependent inhibition of hepatitis C virus (HCV), demonstrated at both the RNA and protein (HCV core) level. Data are shown as means with 95%CI ($^*P < 0.05$); B: OR6 genotype 1b replicon cells were treated with mipomersen for 72 h and no effect was observed on HCV replication; C: There was no negative impact of escalating doses of mipomersen on cell viability; D: Infectivity of the virus was assessed using the TCID₅₀ method. Virus generated in cells treated with mipomersen had a significant reduction in infectivity compared with virus generated in mock-treated cells. Data shown as mean TCID₅₀/mL \pm SEM.

An FDA-approved anti-sense oligonucleotide against apoB inhibits HCV virion infectivity

Based on these findings, we hypothesized that a drug targeting apoB expression or synthesis would have potent anti-HCV effect. Mipomersen is an antisense oligonucleotide against apoB, and inhibits apoB synthesis at the level of transcription from mRNA. It has been demonstrated to significantly reduce production of apoB-containing lipoproteins, most notably circulating LDL, in phase III clinical trials^[39], and is currently approved by the FDA for the treatment of homozygous familial hypercholesterolemia. For its treatment of hypercholesterolemia, it is delivered as a once-weekly subcutaneous injection and has been demonstrated to have no significant drug-drug interactions.

We treated Huh7/CD81 WT cells with mipomersen at escalating doses and infected the cells with HCVcc (JFH1). At 72 h following infection, there was an 80%-85% reduction in intracellular HCV RNA with the

higher mipomersen doses (800 and 1000 μ g/mL), and we observed a dose-response effect. We confirmed these findings at the level of HCV core protein expression (Figure 6A). To confirm that the inhibition of HCV did not occur at the level of HCV RNA replication, assessed the effect of mipomersen on the OR6 replicon model, and confirmed that mipomersen had no effect on viral replication (Figure 6B). We observed no significant effect on cell viability with escalating doses of mipomersen (Figure 6C).

The mechanism by which mipomersen exerts its antiviral effect was confirmed using infectivity assays. HCVcc was generated in either mock-treated or mipomersen-treated (800 μ g/mL) Huh 7 cells and TCID₅₀ of the virus assayed. We determined that the mipomersen-treated virus was significantly less infectious, with a greater than 2-log reduction in TCID₅₀/mL (12536 vs 442 TCID₅₀/mL, $P < 0.05$). Mipomersen exerts its potent anti-HCV effect by impairing the infectivity of the virus (Figure 6D).

DISCUSSION

This is the first study to comprehensively characterize the role of apolipoprotein B100, the core protein of human VLDL and LDL, in each step of the HCV lifecycle and to demonstrate that inhibition of apoB100 by an FDA-approved therapeutic has potent antiviral effect. Similarities between circulating HCV and VLDL have long been observed, yet whether human apolipoprotein B100 is central to the viral lifecycle has remained uncertain, in part due to the limitations imposed by the techniques and cellular models used to date. This study leverages recent advances in genome editing (TALEN), allowing for specific and complete gene knockout, combined with a cell line (Huh-7/CD81) which recapitulates human VLDL synthesis, but also supports HCV to fully assess the role of apoB in HCV.

We have established that apoB is not required for entry or replication, but rather that loss of apoB renders the Huh7 hepatoma cells deficient in their ability to support HCV by generating a virus that has a fundamentally altered lipidome and is significantly impaired in its ability to infect naive cells. We determined by mass spectrometry that, in addition to being deficient in apoB, the generated lipoviral particles are significantly depleted in cholesterol esters. It has previously been described that cholesterol esters are required to maintain the association between HCV and host lipoproteins^[40], and are required for viral infectivity.

Depletion of the virion of cholesterol esters may additionally lead to impaired viral entry. SR-BI, a receptor which recognizes lipoproteins, is a well characterized co-entry receptor for HCV^[41-43], and it has been previously characterized that both lipoproteins and cholesterol esters are important for the uptake of HDL by SR-BI^[44]. Similar findings have been demonstrated in SR-BI-mediated HCV entry^[45]. Thus, the KO virus lacking in cholesterol esters, may in part have diminished infectivity due to loss of SR-BI-mediated uptake.

Finally, we demonstrated the *in vitro* efficacy of mipomersen, an FDA-approved drug for the treatment of familial hypercholesterolemia, which inhibits apoB synthesis using antisense technology. ApoB is therefore a practical and viable pharmacologic target in anti-HCV therapy, with an inhibitor already in clinical use. We showed that treatment with mipomersen significantly inhibited infection with HCV in a dose-dependent fashion. Further, similar to what we observed in the KO cells, we demonstrated that mipomersen exerts its anti-HCV by generating virus which is significantly less infectious than untreated virus. This highlights the possibility of a class of host-targeted antivirals which would serve as infectivity inhibitors, generating virus that is crippled in its ability to infect naive cells. The major limitation of this study is that it is limited to *in*

vitro experimentation in cell lines. This study did not include *in vivo* experiments, and additional studies to determine the effect of mipomersen *in vivo* are needed.

While the drugs to treat HCV have become highly effective as direct acting antivirals are emerging into clinical practice, the most difficult-to-treat patients, particularly those who have been treated with multiple DAA classes, may still require alternate regimens and strategies. In this regard, the “real world” experience with these novel regimens is not yet proven, and development of resistance may be a concern when the drug regimens are adopted for broader use.

Targeting host factors required for the virus is regarded as a promising avenue for the development of adjunctive therapies, since this strategy has been shown to impose a higher barrier to the development of viral resistance. ApoB targeting with an FDA-approved inhibitor would present an attractive target. Here we demonstrate the *in vitro* efficacy of mipomersen against HCV, and suggests an additional, readily targeted host factor required for HCV. Further, we demonstrate that blocking apoB alters viral infectivity by perturbing the lipidome required to generate fully infectious virus. The use of a drug to block this pathway highlights a novel approach towards the treatment of HCV. While we did not see effect against dengue virus, drugs which inhibit the infectivity of a virus by altering the cholesterol composition may pose a novel new strategy to combat refractory HCV and other emerging viral diseases.

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COMMENTS

Background

The hepatitis C virus (HCV) circulates in humans as a lipoviral particle (LVP) which has a very similar composition to very-low-density lipoprotein (VLDL). Despite this known relationship, the role of apolipoprotein B 100 (the core protein of VLDL) in the HCV lifecycle has remained uncertain.

Research frontiers

This study utilized genome editing technology (TALEN) to characterize the role of apoB100 in the HCV lifecycle.

Innovations and breakthroughs

The use of genome editing allowed for careful examination of several critical steps in HCV infection without the use of lipid-based or viral transfection for gene knockdown. This permitted lipidomic analysis of HCV lipoviral particles, demonstrating that apoB is critical to maintain the “VLDL”-like composition and infectivity of the lipoviral particle. Further, this study demonstrated that an FDA-

approved antisense inhibitor of apoB blocks HCV infection *in vitro*.

Applications

The findings highlight the critical role of lipids and lipoproteins in HCV infection, and specifically in the infectivity of the lipoviral particle. Targeting host lipid metabolic pathways may be useful in resistant or difficult-to-treat HCV infection in humans.

Terminology

TALEN refers to transcription activator-like effector nucleases, which can be designed to target host genomic sequences and generate stable genetic deletions without transfection, antibiotics. These have been shown to generate stable knock-out models with minimal off-target effect.

Peer-review

The paper presents work linking hepatic apoB100 secretion with hepatitis C virion assembly and secretion, studied *in vitro* with Huh7/CD81 cells in which apoB expression was deleted. The work makes the point that apoB co-expression is required for infectious HCV particle formation. Further work will be required to be sure that this applies to processing of HCV *in vivo*.

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Case Control Study

Portal hypertension in polycystic liver disease patients does not affect wait-list or immediate post-liver transplantation outcomes

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Informed consent statement: Informed consent was gained from all study participants who were enrolled onto the transplant database.

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Abstract

AIM

To establish the impact of portal hypertension (PH) on wait-list/post-transplant outcomes in patients with polycystic liver disease (PCLD) listed for liver transplantation.

METHODS

A retrospective single-centre case controlled study of consecutive patients listed for liver transplantation over 12 years was performed from our centre. PH in the PCLD cohort was defined by the one or more of following parameters: (1) presence of radiological or endoscopic documented varices from our own centre or the referral centre; (2) splenomegaly (> 11 cm) on radiology in

absence of splenic cysts accounting for increased imaging size; (3) thrombocytopenia (platelets $< 150 \times 10^9/L$); or (4) ascites without radiological evidence of hepatic venous outflow obstruction from a single cyst.

RESULTS

Forty-seven PCLD patients (F: M = 42: 5) were listed for liver transplantation (LT) (single organ, $n = 35$; combined liver-kidney transplantation, $n = 12$) with 19 patients (40.4%) having PH. When comparing the PH group with non-PH group, the mean listing age (PH group, 50.6 (6.4); non-PH group, 47.1 (7.4) years; $P = 0.101$), median listing MELD (PH group, 12; non-PH group, 11; $P = 0.422$) median listing UKELD score (PH group, 48; non-PH group, 46; $P = 0.344$) and need for renal replacement therapy ($P = 0.317$) were similar. In the patients who underwent LT alone, there was no difference in the duration of ICU stay (PH, 3 d; non-PH, 2 d; $P = 0.188$), hospital stay length (PH, 9 d; non-PH, 10 d; $P = 0.973$), or frequency of renal replacement therapy (PH, 2/8; non-PH, 1/14; $P = 0.121$) in the immediate post-transplantation period.

CONCLUSION

Clinically apparent portal hypertension in patients with PCLD listed for liver transplantation does not appear to have a major impact on wait-list or peri-transplant morbidity.

Key words: Polycystic liver disease; Portal hypertension; Liver transplantation

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Core tip: Clinically apparent portal hypertension is common in patients with polycystic liver disease, however it appears that this finding does not affect wait list or post-transplantation outcomes in the short-term.

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INTRODUCTION

Polycystic liver disease (PCLD) is an autosomal dominant condition that has 2 forms - either occurring in isolated form or combined with cysts in extra-hepatic organs^[1,2]. Of those patients who have PCLD associated with extra-hepatic cysts, 80%-90% have renal cysts and can develop progressive renal impairment ultimately leading to end-stage renal failure (ESRF). In those with only polycystic kidney

disease (PCKD) at initial diagnosis, 30% can thereafter develop liver cysts within 30 years of diagnosis^[3]. Genetic mutations have been identified in patients with PCLD with downstream protein processing defects leading to proliferation in cyst-lining epithelia, fluid secretion into cysts, extracellular membrane remodelling around cysts and finally neovascularization of the cysts^[4-8]. Eighty percent of patients can remain asymptomatic or present with mild abnormalities in liver function blood tests^[9], whereas some patients can become symptomatic with enlargement of cysts and their mass/pressure effects on adjacent organs. Liver cysts can become infected with a mortality of 2%^[10] or even in rare cases rupture with severe pain, haemodynamic instability and/or rarely death.

Treatment options for PCLD can include medical therapies, interventional radiology, surgical fenestration/resection or liver transplantation (LT), whilst renal replacement therapy (RRT) or renal transplantation can be performed for PCKD-associated ESRF in those with PCLD. Transplantation remains an effective curative treatment for PCLD and is indicated if disabling symptoms leading to decreased performance status or quality of life^[11]. In the United states, LT for PCLD falls into the Model for End-stage Liver Disease (MELD) exception guidelines^[12] whilst United Kingdom guidelines (NHSBT 2009:4.1.2.3) state that a LT can be performed in the PCLD setting if "intractable symptoms due to mass of liver or pain unresponsive to cystectomy, or severe complications secondary to portal hypertension"^[13].

Development of portal hypertension (PH) in PCLD can be a significant concern in advanced disease manifested by splenomegaly, ascites [without necessarily signs of hepatic venous outflow obstruction (HVOO)] or variceal formation. PH in the context of PCLD was described in 35% of patients from a European cohort^[14] and often can be notoriously difficult to treat^[15], however the clinical course and outcome of such patients once listed for LT has not yet been clearly identified. The aim of this study was thus to establish the impact of clinically apparent PH on wait-list and immediate post-transplantation outcomes specifically in patients with PCLD listed for LT.

MATERIALS AND METHODS

A single centre retrospective study was performed in a LT centre (Queen Elizabeth Hospital, Birmingham, United Kingdom). The study protocol was approved by the Queen Elizabeth Hospital local clinical audit committee (Reference CAB:04870-12). Patients with PCLD listed consecutively over a 12-year period (January 2000 and December 2012) were included. All PCLD referred to our centre or undergoing follow-up (irrespective of transplantation or not) were identified from a pre-existing transplant database which included all parameters of listed patient's illness, liver function, and if applicable listing criteria and operation/intensive

Table 1 Comparison of features of portal hypertension between the groups requiring single liver transplant and those requiring combined liver/kidney transplant (note some patients had > 1 manifestation)

	Single organ liver (<i>n</i> = 15 with portal HBP)	Combined liver/kidney (<i>n</i> = 4 with portal HBP)	<i>P</i> value
Ascites	8	3	0.582
Varices ¹	1	0	0.745
Splenomegaly ²	7	2	0.585
Thrombocytopenia ³	3	2	0.379

¹Confirmed endoscopically or radiologically on CT; ²Confirmed on imaging without evidence of splenic cysts; ³Platelet count < 150 × 10⁹. HBP: Hypertension.

care unit (ICU) spell details (including data on blood product usage and follow-up). Details on nutritional status except listing weight were not routinely available.

The decision to start patients on RRT whilst on the waiting list was made by the local Nephrology team and decision to perform combined liver/kidney transplantation was made together by the Hepatology and Nephrology teams at a multi-disciplinary transplantation listing meeting. Patients were listed for LT based on symptoms after vigorous multi-professional assessment process. Validated symptom questionnaires were not used as part of this study, rather cases were discussed on an individual basis by the clinical team during the transplantation assessment process. Symptoms were normally secondary to liver cysts causing intractable pain, poor quality of life, malnutrition, recurrent liver cyst infection, or those symptoms secondary to the development of PH. The transplant assessment process involved standard radiological assessment by the surgical team *via* Ultrasound (US), computed tomography (CT) and/or magnetic resonance imaging (MRI). Liver volumetric measurement data was not performed during this time period.

PH in the PCLD cohort was defined by the one or more of following parameters: (1) presence of radiological or endoscopic documented varices from our own centre or the referral centre; (2) splenomegaly (> 11 cm) on US, CT or MRI in absence of radiological splenic cysts accounting for increased imaging size; (3) thrombocytopenia (platelets < 150 × 10⁹/L); or (4) ascites without radiological evidence of HVOO from a single cyst. Patients were assessed and listed for a combined kidney/liver transplant if they had concomitant ESRF with the main measurement of renal function being the estimated glomerular filtration rate (eGFR), determined using the Modification of Diet in Renal Disease Study 4-variable equation^[16]. This was then confirmed with an isotopic GFR where required or recommended by the nephrology team. Chronic kidney disease (CKD) was defined as eGFR

< 60 mL/min/1.73 m² on at least 2 occasions and sustained^[17].

Normally distributed continuous variables and non-parametric continuous variables were compared using the Student's *t*-test and Mann-Whitney test, respectively. χ^2 analysis or Fisher's exact test were used for comparison of categorical data. Patient survival after transplantation was estimated using Kaplan-Meier plots with log-rank test for differences. Patients were censored at time of last known follow-up. Data was analysed using the SPSS 21 package (SPSS Inc, Chicago, IL, United States). All values are expressed as mean and standard deviation, median and inter-quartile range (IQR) and number and percent (%) as appropriate. *P* < 0.05 was considered statistically significant at all times.

RESULTS

Patient demographics and portal hypertension manifestations

A total of 75 patients were identified with PCLD attending our centre during the 12-year period. Of this overall cohort 32 patients (42.5%) had signs in keeping with clinically apparent PH. Of the overall cohort (*n* = 75), 47 patients (62.7%) were listed for LT (35 patients listed for single organ and 12 patients listed for combined liver/kidney). Of the 35 patients listed for single organ liver transplantation 15 had PH (42.9%) and of the 12 listed for combined liver/kidney transplant 4 had PH (33.3%) (*P* = 0.410). There were no differences in how PH was manifested between the groups (Table 1) irrespective if receiving a single organ liver transplant or combined liver/kidney transplant.

When comparing the PH group and those without (non-PH group - Table 2), at listing, baseline characteristics were similar between the groups. Characteristics were similar with regards to age (PH 50.6 years, non-PH 47.1 years, *P* = 0.101) and gender (*P* = 0.683). The patients' liver synthetic function was similar between the PH group and non-PH groups (Bilirubin *P* = 0.965, INR *P* = 0.173) and United Kingdom Model for End-Stage Liver Disease (UKELD) (PH 48, non-PH 46, *P* = 0.344)/MELD scores (*P* = 0.344/PH 12, non-PH 11, *P* = 0.422) were similar between the groups. 84.2% of patients with PH had CKD stage III-V compared to 67.9% of patients without (*P* = 0.179). 19.2% and 7.1% of PH and non-PH patients were dialysis dependent respectively (*P* = 0.317).

Outcomes on the list

Thirty-four patients were transplanted by the time of data analysis (72.3%). Two patients with PH died prior to transplantation (sepsis and progressive liver disease) and 1 patient without PH (progressive liver disease). Of the remaining 10 patients not transplanted at time of data analysis, 9 were still active on the waiting list and 1 patient had been removed from the

Table 2 Comparison of patient demographics between patients with and without portal hypertension

Listing parameter	Portal hypertension (<i>n</i> = 19)	Non-portal hypertension (<i>n</i> = 28)	<i>P</i> value
Age (yr)	50.6 (6.4)	47.1 (7.4)	0.101
Female gender	17 (89.5)	25 (89.3)	0.683
Bilirubin (μmol/L)	8 (6-11)	8 (6-12)	0.965
INR	1.1 (1.0-1.2)	1.1 (1.0-1.1)	0.173
Creatinine (mmol/L)	106 (104-436)	119 (92-201)	0.508
Platelet count (× 10 ⁹)	177 (147-242)	234 (198-242)	0.012
Sodium (mmol/l)	139 (137-143)	140 (138-142)	0.483
MELD score	12 (9-21)	11 (8-16)	0.422
UKELD score	48 (44-50)	46 (45-48)	0.344
Chronic kidney disease ¹	16 (84.2)	19 (67.9)	0.179
Dialysis dependant	3 (15.8)	2 (7.1)	0.317

¹Chronic kidney disease defined as stage III-V^[17]. INR: International normalized ratio; MELD: Model For End-stage Liver Disease; UKELD: United Kingdom Model for End-Stage Liver Disease.

Table 3 Intensive care unit requirements and hospital stay between the portal hypertensive and non-portal hypertensive groups in patients receiving a single organ liver transplant

	Portal hypertensive group (<i>n</i> = 8)	Non-portal hypertensive group (<i>n</i> = 14)	<i>P</i> value
Intra-operative			
RCC(units)	3 (1-4)	4 (2-7)	0.238
FFP(units)	10 (1-18)	8 (7-14)	0.973
Plts (units)	0	3 (0-10)	0.145
ICU stay (d)	3 (3-4)	2 (2-4)	0.188
Hospital stay (d)	9 (7-13)	10 (7-12)	0.973
RRT in ICU immediately post-op	2/8 (25%)	0/14 (0)	0.121

FFP: Fresh frozen plasma; ICU: Intensive care unit; Plts: Platelets; RCC: Red cell concentrate; RRT: Renal replacement therapy.

waiting list (as had declined a transplant after being listed). The median time from listing to transplantation for PH patients was 72 d (IQR 34-524) and for non-PH patients was 139 d (IQR 48-390) ($P = 0.466$).

In the single organ LT patients ($n = 22$), the median time from listing to transplantation for patients with PH was 49 d (IQR 16-426) compared to 139 d (IQR 53-345) ($P = 0.188$) for patients in the non-PH group. In the combined liver/kidney transplant patients ($n = 12$), the median time from listing to transplantation for PH patients was 289 d (IQR 58-551) and for those in the non-PH group 210 d (IQR 16-579) ($P = 0.933$). Overall, when the length of time of the list was compared, there were no significant differences found in the median time on the list between the PH group ($n = 12$) [72 d (IQR 34-524)] and the non-PH group ($n = 22$) [139 d (IQR 48-390), $P = 0.466$]. On follow up 3 patients died on waiting list (2 with PH).

ICU spells/requirements and hospital stays

In the patients who underwent LT alone, there was no difference in the duration of ICU (PH group 3 d; non-

Table 4 Intensive care unit requirements and hospital stay between the portal hypertensive and non-portal hypertensive groups in patients receiving a combined liver/kidney transplant

	Portal hypertensive group (<i>n</i> = 4)	Non-portal hypertensive group (<i>n</i> = 8)	<i>P</i> value
Intra-operative			
RCC (units)	13 (4-19)	9 (3-19)	0.933
FFP (units)	15 (7-23)	14 (5-18)	0.683
Plts (units)	10 (3-10)	10 (0-20)	0.683
ICU stay (d)	5 (3-7)	7 (3-41)	0.368
Hospital stay (d)	16 (12-18)	15 (12-49)	0.808
RRT in ICU immediately post-op	3/4 (75%)	3/8 (37.5%)	0.273

FFP: Fresh frozen plasma; ICU: Intensive care unit; Plts: Platelets; RCC: Red cell concentrate; RRT: Renal replacement therapy.

PH group 2 d, $P = 0.188$) and hospital stay (PH group 9 d; non-PH group 10 d, $P = 0.973$). There was no difference in frequency of RRT (PH group 2/8; non-PH group 1/14, $P = 0.121$) with similar observations made in the patients who underwent combined liver-kidney transplantation (data not shown). There were no differences found (in the single organ liver transplant or the combined liver/kidney group) when transfusion requirements were assessed between the PH groups and the non-PH groups (Tables 3 and 4). The duration of ICU spells post-transplantation were similar [3 d in PH group vs 2 d in non-PH group ($P = 0.188$)]. Also overall hospital stays were similar between the groups (9 d in PH group vs 10 d in non-PH group, $P = 0.973$).

DISCUSSION

Patients with PCLD can have variable courses of their disease with the majority of patients remaining asymptomatic. As the liver cysts grow, patients can start to develop symptoms due to local mass effect such as: right upper quadrant pain, early satiety and post-prandial fullness (due to pressure effects on adjacent stomach). Patient can also develop shortness of breath (due to liver volume burden), and direct compression of the portal vein/inferior vena cava. Symptoms also may be due to complications of the cysts such as haemorrhage, infection or rupture. Treatment can be broadly divided into medical, radiological and surgical. Medical treatments include somatostatin analogues which reduce the secretion of fluid into the cysts and inhibit cholangiocyte proliferation^[18-22] and have been shown to be effective in reducing liver volume when compared to placebo and effective in improving symptoms^[23-25]. Radiological treatments can include interventional radiological arterial embolization or injection sclerotherapy of cysts, whilst surgical techniques include cysts fenestration, resection or transplantation. The choice of surgical technique often is dependent on factors such as: symptoms, cyst characteristics, volume of normal liver parenchyma and

also patency of hepatic/portal veins - with the surgeons often using Schnellendorfer *et al.*^[26] or Gigot *et al.*^[27]'s classification to aid with decisions. LT remains an effective curative treatment for patients with PCLD with indications varying but indicated if decreased performance status or quality of life^[11,28] then LT can be offered. LT has been shown to improve domains of quality of life in a series of 36 patients^[29] with 11% of patients in this study having PH. Recent Australian national guidelines^[30] summarised that treatment of liver cysts should be directed at reducing liver volume when the patients were highly symptomatic, with options including sclerotherapy, fenestration, segmental resection and transplantation (Level 1D evidence).

PH in chronic liver disease is the established key event leading to such complications such as ascites and variceal formation. PH results from mechanical obstruction due to fibrosis or regenerative nodules resulting in increased resistance to flow. In PCLD this may be the case secondary to flow distortion due to large cysts and thus their compressive effects leading to increased intrahepatic resistance. In cirrhosis and PH a hyperdynamic circulation develops in response to changes in haemodynamics, manifested as high cardiac output with low systematic vascular resistance and arterial hypotension^[31]. In our centre portal pressure measurements are not routinely performed and could be deemed a criticism of this retrospective study however the technique to gain the hepatic venous pressure gradient (HVPG) measurement can indeed be difficult in patients with PCLD due to distorted anatomy^[32] with lack of reporting of such measurements in other PCLD studies where PH has been assessed^[14]. Varices in non-PCLD patients are more likely to develop if the HVPG is > 10 mmHg^[33] however the role of HVPG measurements in the PCLD patient cohort requires further clarification in future studies. In young patients with PCLD who have early signs of PH, congenital hepatic fibrosis should also be considered as a potential cause of PH^[34].

In our study we sought to explore if any differences in outcome in PCLD patients with PH once listed for LT to those who did not. To our knowledge our study is the 1st paper to analyse such subgroups in this manner in patients listed LT. The overall number of patients in such studies in PCLD has not been large and indeed those with PH described. In a review of 9 studies in patients with PCLD^[35-43] a median of 8 patients (range 3-17) was found - this compares to 47 patients listed for LT in our single-centre study. Mortality rates in the series studied ranged from 0%-50% on follow up and the number of patients with PH however was not clear. In one such multicenter study^[14], 58 patients were pooled together from 75 centres via the European Liver and Intestinal Association (ELITA) - with 35% patients having PH (compared to 42.9% of patients listed for LT in our study - a single centre). By analysing in such a manner, we have established to seek if there was any difference in patients who had clinically apparent PH as

a consequence of PCLD on their outcome once listed for transplantation. Ascites in the context of HVOO can be exudative due to high permeability of the dilated sinusoidal walls to proteins^[44]. Patients with HVOO can also however present with transudative ascites, abdominal pain and hepatomegaly in 90%-96% of cases^[34]. By taking established markers of clinically apparent PH and applying it to the PCLD cohort, we attempted to stratify the patients and assess for any differences in outcome once listed for LT. The 2 groups appeared to be well matched patient groups at time of listing between the PH and non-PH groups especially when assessing their liver synthetic function (Table 2), again suggesting clearly the mechanism in developing PH in this cohort is different to those patients with cirrhosis who can develop PH and synthetic liver dysfunction with progressive disease. Patients with PCLD often have preserved liver function as reflected by low UKELD and MELD scores - thus PCLD patients fall within MELD^[12] exception guidelines and also the variant syndrome United Kingdom listing criteria^[13]. When comparing the groups who had single LT compared to those having a combined kidney/liver transplant there appeared to be no significant differences in the type/manifestation of clinically apparent PH that these 2 groups had. The advent of PH importantly did not appear to affect the immediate post-transplantation course of the patients, with similar requirement for blood product use between the groups and similar post-operative ICU and hospital stays. PH in the context of transplantation for cirrhosis does often cause a need for blood product requirement with tendency to bleed from the high pressure portal circulation or the coagulopathy associated with cirrhosis. This however did not appear the case in this cohort.

There are shortcomings however of this retrospective study that should be noted with the main one the perceived small numbers of patients over a long-period of time. It could be argued that in PCLD studies large cohorts are not a regular finding but our numbers are comparable if not larger than already published series^[35-43]. In a multicentre European study only 58 patients were gained from 75 sites (0.77 per site)^[14] thus making our cohort a relatively fair one for a single centre. To answer the question in a more robust manner, larger multicentre databases could be generated or analysed with well defined criteria for clinically apparent PH to replicate our study on a larger setting. Another criticism may be the apparent lack of portal pressure studies not a routine practice in our centre however the difficulties of this have been mentioned previously. Another criticism may be the amalgamation of those patients with ascites along with those with other features as mentioned above of PH. Without HVPG measurements in this group, we think it is difficult to tease out specific mechanisms of ascites formation in this cohort of patients. Where possible, patients protein levels of ascites were checked how-

ever was not always commonplace over the whole 12 years of this study, and patients with ascites due to PCLD can have a mixed picture when analysing protein content as mentioned^[34,44]. By grouping these together who have clinically significant ascites (thus impaired quality of life and function) who often required large volume paracentesis (with the potential for introduction of infection) we included them in our cohort as they may have had worse clinical outcomes. However even when this was done, both the PH group and non-PH group had similar outcomes with regards to survival and with operative/post ICU spell parameters. Also irrespective of the actual pathophysiological mechanism of ascites formation, once present and is not managed by conventional therapies, LT assessment would be considered in our centre due to the effects on the patient's quality of life. The study period was conducted before the advent of high resolution volumetric studies in our institution hence we did not comment on liver volumes or those of kidneys retrospectively, and their role in predicting outcome peri- or post-transplantation from our retrospective data set. Also owing to the retrospective nature of the data there was no data available on advanced nutritional aspects of the patients such as actual sarcopenic measurements with CT^[45] something that is now practiced in our centre. With either ascites or pure weight-related effect of the liver cysts, weight would not be an accurate measurement for such a cohort thus not commented upon. Data was also not retrospectively available if women (the majority of our cohort) were on the contraceptive oral contraceptive pill (COCP). The presence of PCLD has been shown to be related to the COCP usage in patients with PCKD^[46]. Another area to comment is the lack of validated symptom questionnaires in our study to assess improvements with transplantation. Kirchner *et al.*^[29] showed there was indeed a symptomatic improvement in domains related to health post-LT, however moving forward looking at symptom assessment improvement *via* validated questionnaires between groups with PH and not would be interesting. A final note is caution to the estimated 15-year post-transplantation survivals with no obvious difference found between the groups ($P = 0.138$). In a recent study from Neijenhuis *et al.*^[47] a disease specific questionnaire was developed and validated in a European and United States cohort. Moving forward, this questionnaire could be used in prospective studies involving the PH-component of PCLD.

In conclusion, this retrospective single-centre study has shown that clinically apparent PH in patients listed for LT is common, however also our data suggests that PH may not impact on wait-list and peri-/post-operative outcomes in our cohort of patients studied. To our knowledge this is the 1st study to assess a PCLD cohort in such a manner. The advent of PH and the complications in the PCLD cohort should be remembered by physicians and surgeons alike

especially when patients are being assessed for liver transplantation, however does not appear clinically apparent PH affects outcome once the decision has been made to transplant such patients, especially in the hands of skilled surgeons.

COMMENTS

Background

The advent of portal hypertension in polycystic liver disease is not well described with regards to its effects on outcomes in patients who have severe disease or are listed for curative liver transplant procedure

Research frontiers

It was hypothesized that patients with polycystic liver disease who are listed for liver transplantation may indeed have worse outcomes if they have established portal hypertension than those without.

Innovations and breakthroughs

This study is the first study in our knowledge to explore the impact of portal hypertension on outcomes in patients with polycystic liver disease providing evidence that the advent of portal hypertension does not affect wait-list or short-term post liver transplantation outcomes.

Applications

This study investigated the advent of portal hypertension of patients listed for liver transplantation with polycystic liver disease. The finding of portal hypertension should always thus be noted and treated appropriately, but does not confer poorer outcomes from the results of our study.

Peer-review

A large retrospective study that you list the many drawbacks but nevertheless it is very interesting data. It is the largest single center study on this topic and it does gain strength from this.

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Case Control Study

***CYP1A1*, *CYP2E1* and *EPHX1* polymorphisms in sporadic colorectal neoplasms**

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Abstract

AIM

To investigate the contribution of polymorphisms in

the *CYP1A1*, *CYP2E1* and *EPHX1* genes on sporadic colorectal cancer (SCRC) risk.

METHODS

Six hundred forty-one individuals (227 patients with SCRC and 400 controls) were enrolled in the study. The variables analyzed were age, gender, tobacco and alcohol consumption, and clinical and histopathological tumor parameters. The *CYP1A1**2A, *CYP1A1**2C, *CYP2E1**5B and *CYP2E1**6 polymorphisms were analyzed by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). The *EPHX1* Tyr113His, *EPHX1* His139Arg and *CYP1A1**2C polymorphisms were detected by real-time PCR. Chi-squared test and binary logistic regression were used in the statistical analysis. Haplotype analysis was conducted using the Haploview program, version 2.05.

RESULTS

Age over 62 years was a risk factor for SCRC development (OR = 7.54, 95%CI: 4.94-11.50, $P < 0.01$). Male individuals were less susceptible to SCRC (OR = 0.55, 95%CI: 0.35-0.85, $P < 0.01$). The *CYP2E1**5B polymorphism was associated with SCRC in the codominant (heterozygous genotype: OR = 2.66, 95%CI: 1.64-4.32, $P < 0.01$), dominant (OR = 2.82, 95%CI: 1.74-4.55, $P < 0.01$), overdominant (OR = 2.58, 95%CI: 1.59-4.19, $P < 0.01$), and log-additive models (OR = 2.84, 95%CI: 1.78-4.52, $P < 0.01$). The *CYP2E1**6 polymorphism was associated with an increased SCRC risk in codominant (heterozygous genotype: OR = 2.81, 95%CI: 1.84-4.28, $P < 0.01$; homozygous polymorphic: OR = 7.32, 95%CI: 1.85-28.96, $P < 0.01$), dominant (OR = 2.97, 95%CI: 1.97-4.50, $P < 0.01$), recessive (OR = 5.26, 95%CI: 1.35-20.50, $P = 0.016$), overdominant (OR = 2.64, 95%CI: 1.74-4.01, $P < 0.01$), and log-additive models (OR = 2.78, 95%CI: 1.91-4.06, $P < 0.01$). The haplotype formed by the minor alleles of the *CYP2E1**5B (C) and *CYP2E1**6 (A) polymorphisms was associated with SCRC ($P = 0.002$). However, the *CYP1A1**2A, *CYP1A1**2C, *EPHX1* Tyr113His and *EPHX1* His139Arg polymorphisms were not associated with SCRC.

CONCLUSION

In conclusion, the results demonstrated that *CYP2E1**5B and *CYP2E1**6 minor alleles play a role in the development of SCRC.

Key words: Single-nucleotide polymorphisms; Colorectal neoplasms; Cytochrome P-450 CYP2E1; Cytochrome P-450 CYP1A1; Epoxide hydrolases 1

Core tip: Sporadic colorectal cancer (SCRC) includes malignancies that occur in the colon and rectum. This type of cancer is the third most common cancer worldwide. The main etiological factors are age over 50 years and tobacco and alcohol consumption. The elimination of environmental carcinogens contained in tobacco, as well as alcohol, requires metabolic

activation mediated by xenobiotic-metabolizing enzymes (XMEs). The *CYP2E1**5B and *CYP2E1**6 polymorphisms were associated with SCRC, as well as the *CYP2E1**5B (C) and *CYP2E1**6 (A) haplotype (minor alleles). Polymorphisms in several genes encoding these XMEs may be involved in alterations in gene expression related to important processes of colorectal carcinogenesis such as inflammation and angiogenesis.

Fernandes GMM, Russo A, Proença MA, Gazola NF, Rodrigues GH, Biselli-Chicote PM, Silva AE, Netinho JG, Pavarino EC, Goloni-Bertollo EM. *CYP1A1*, *CYP2E1* and *EPHX1* polymorphisms in sporadic colorectal neoplasms. *World J Gastroenterol* 2016; 22(45): 9974-9983 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v22/i45/9974.htm> DOI: <http://dx.doi.org/10.3748/wjg.v22.i45.9974>

INTRODUCTION

Sporadic colorectal cancer (SCRC) includes malignancies that occur in the large intestine (colon) and rectum. This type of cancer is the fifth most common cancer in Brazil. In 2016, an estimated 34280 new cases of SCRC will be diagnosed in Brazil, according to a survey conducted by the National Cancer Institute (INCA)^[1]. This is the third most common cancer worldwide with an estimated 136100 new cases each year, mainly in developed regions. The overall mortality rate is estimated to be 694000 deaths, 8.5% of all cases. Fifty-two percent of these deaths occur in developing regions of the world^[2]. The main etiological factors related to SCRC are age over 50 years^[1] and tobacco^[3] and alcohol consumption^[4].

Tobacco and alcohol are environmental carcinogens responsible for the release of exogenous compounds, including reactive oxygenated intermediates (ROMs) represented by benzo[a]pyrene (BaP) N-nitrosamines, heterocyclic amines (HAs) and polycyclic aromatic hydrocarbons (PAHs). These compounds are metabolically activated in electrophilic forms before interaction with DNA, and they generate adducts and contribute to tumor initiation^[5].

The elimination of these environmental carcinogens requires metabolic activation mediated by xenobiotic-metabolizing enzymes (XMEs), such as cytochrome P-450 (CYP) and epoxide hydrolase (EPHX1). Polymorphisms in several genes encoding these XMEs are responsible for metabolism errors, which can contribute to the development of several cancer types^[5-7].

In the liver and intestine, Phase I oxidative enzymes convert the compounds to highly reactive metabolites by introducing one or more hydroxyl groups in the substrate, increasing its water solubility and converting it into a form that will be more easily expelled. These enzymes, including CYPs and EPHX1, are involved in cellular pathways required for the

carcinogenesis process, such as the metabolism of eicosanoids, the biosynthesis of cholesterol and bile acids, steroid synthesis, biogenic amine synthesis and degradation, vitamin D3 synthesis, hydroxylation of retinoic acid, and arachidonic acid metabolism^[5,6,8].

Single-nucleotide polymorphisms (SNPs) in genes encoding XMEs can modify the enzyme expression or function and, consequently, alter the activation or detoxification of carcinogenic compounds. The balance between metabolic activation and detoxification can affect the risk of cancer once DNA adducts play an important role in the carcinogenic process^[5,6].

SNPs in the *CYP1A1* and *CYP2E1* genes, which encode important XMEs, can lead to alterations of the function of these enzymes, resulting in the activation of carcinogens, which are involved in tumor initiation^[5]. These polymorphisms have been associated with colorectal cancer development^[9,10]. Among the polymorphisms, the main ones are *CYP1A1**2A (rs4646903), resulting in the substitution of thymine for cytosine (*T3801C*) in the poly (A) tail of the 3' untranslated gene region^[11,12]; *CYP1A1**2C (rs1048943), resulting from the transition of adenine to guanine (*A2455G*)^[13,14]; *CYP2E1**5B (rs3813867), with the substitution of guanine for cytosine at the -1293 nucleotide position^[12,15]; and *CYP2E1**6 (rs6413432), caused by the alteration of thymine to adenine at position 7632 of the gene^[16,17].

EPHX1 *Tyr113His* (rs1051740) and *EPHX1* *His139Arg* (rs2234922), functional polymorphisms of the *EPHX1* gene, have been well characterized^[18]. These polymorphisms are associated with the susceptibility to SCRC^[19,20]. The *EPHX1* *Tyr113His* polymorphism, located at position 337 in exon 3 of the *EPHX1* gene, is characterized by a substitution of the amino acid histidine for tyrosine at position 113 of the protein. This change leads to a decrease of approximately 40-50% of the enzyme activity and stability *in vitro*. The polymorphism *EPHX1* *His139Arg*, localized in exon 4 at position 416 of the *EPHX1* gene, results in the amino acid substitution of arginine to histidine at position 139 of the protein. These modifications increase the enzyme activity and stability by 25%^[18,21].

In the present study, we investigated the association between the *CYP1A1**2A, *CYP1A1**2C, *CYP2E1**5B, *CYP2E1**6, *EPHX1* *Tyr113His* and *EPHX1* *His139Arg* polymorphisms and SCRC risk, the interaction between these polymorphisms with tobacco and alcohol consumption, and the association of SCRC with sociodemographic factors.

tobacco and alcohol consumption, and family history of cancer or adenomatous polyps and lesions were collected using a standard interviewer-administered questionnaire. The ethnicity was not evaluated during this study because of the miscegenation of the studied population.

Study populations

Six hundred twenty-seven individuals (227 patients with sporadic colorectal cancer and 400 controls) were included in the study (Table 1). The recruitment of patients and controls, as well as the collection of peripheral blood and clinical and histopathological data, was performed between 2010 and 2013 at the Coloproctology Service of Hospital de Base/Sao Jose do Rio Preto Medical School, Sao Jose do Rio Preto, SP, Brazil. In the present study, it was not necessary for a follow-up of the individuals. The case group consisted of individuals with a clinical and histopathological diagnosis of SCRC. The exclusion criteria were patients with hereditary cancer and those previously treated with chemotherapy and/or radiotherapy. The control group consisted of healthy individuals, blood donors with no history of a cancer diagnosis and no family history of cancer in at least three previous generations and other diseases according to the criteria of the American Association of Blood Donors^[22].

We considered smoker individuals as those patients who consumed >100 cigarettes in a lifetime. We considered alcohol drinkers as those patients who consumed > 1 drink per week (one drink was defined as approximately 44 mL of liquor or 118 mL of wine or 350 mL of beer)^[23].

Tumors were TNM classified according to the following three criteria: the tumor extent (T), the presence of regional lymph node involvement (N) and the presence of distant metastasis (M)^[24]. T1 and T2 tumors were classified as smaller tumors, and T3 and T4 tumors were classified as larger tumors. Lymph node involvement was classified according to its absence (N0) and presence (N1, N2, N3). Tumors were classified as non-aggressive (stage I and II) and aggressive (stage III and IV) according to the clinical staging (TNM)^[25]. Information about TNM was impossible in all cases. The analysis of these parameters was performed in a smaller group. Therefore, for the analysis of tumor extension, only 200 samples were analyzed. For the analysis of regional lymph node involvement, 198 samples were analyzed. For the evaluation of aggressiveness, 114 samples were included in the analysis.

Nucleic acid extraction

DNA extraction was performed from peripheral blood leukocytes according to the procedure by Miller and collaborators with modifications^[26]. Quantification and the purity of DNA samples were determined by absorbance at a wavelength (λ) at 260 and 280 nm using the Picodrop Pico200™ spectrophotometer

MATERIALS AND METHODS

Approval and consent

After approval by the Ethics in Research Committee CEP/FAMERP, protocol No. 012/2012 (CAAE: 0237.0.140.00011), the individuals who agreed to participate in the study signed an informed consent form. Information about current and past occupations,

Table 1 Sociodemographic data of patients with sporadic colorectal cancer and controls *n* (%)

Variables	Control (<i>n</i> = 400)	Case (<i>n</i> = 227)	OR ¹	95%CI	<i>P</i> value
Gender					
Female	125 (31.3)	106 (46.7)	1.00 (reference)		
Male	275 (69.7)	121 (53.3)	0.55	0.35-0.85	< 0.01 ²
Age (mean)					
< 62	350 (87.5)	105 (46.3)	1.00 (reference)		
≥ 62	50 (12.5)	122 (53.7)	7.54	4.94-11.50	< 0.01 ²
Tobacco consumption					
Non-smokers	243 (60.8)	131 (57.7)	1.00 (reference)		
Smokers	157 (39.2)	96 (42.3)	1.12	0.73-1.70	0.60
Alcohol consumption					
Non-drinkers	218 (54.5)	127 (55.9)	1.00 (reference)		
Drinkers	182 (45.5)	100 (44.1)	1.44	0.93-2.24	0.10

¹Odds ratio (OR) adjusted for age, gender, tobacco and alcohol consumption and polymorphisms in the dominant model; ²Significant *P* values < 0.05.

Table 2 Description of the primers sequences and restriction enzymes for *CYP1A1**2A, *CYP2E1**5B and *CYP2E1**6 polymorphisms analysis

Polymorphisms	Sequence of primers	Restriction Enzyme T/t
<i>CYP1A1</i> *2A		MspI
Sense	5'-GA TGA AGA GGT GTA GCC GCT-3'	37 °C/3 h
Antisense	5-TAG GAG TCT TGT CTC ATG CCT-3'	
<i>CYP2E1</i> *5B		PstI
Sense	5'-CCA GTC GAG TCT ACA TTG TCA-3'	37 °C/3 h
Antisense	5'-TTC ATT CTG TCT TCT AAC TGG-3'	
<i>CYP2E1</i> *6		DraI
Sense	5'-TCG TCA GTT CCT GAA AGC AGG-3'	37 °C/3 h
Antisense	5'-GAG CTC TGA TGC AAG TAT CGC-3'	

(Thermo Scientific).

Polymorphism genotyping

The genotyping of *CYP1A1**2A (rs4646903), *CYP2E1**5B (rs3813867) and *CYP2E1**6 (rs6413432) polymorphisms was performed by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). The primer sequences used for amplification and the enzymes used to identify polymorphic sites are shown in Table 2.

EPHX1 Tyr113His (rs1051740) and *EPHX1* His139Arg (rs2234922) and *CYP1A1**2C (rs1048943) polymorphism genotyping was performed by real-time PCR. The reactions were established according to the manufacturer's protocol (Applied Biosystems) with specific primers and probes validated (TaqMan MGB-probes: Assay ID C__14938_30, C__11638783_30 and C_25624888_50, respectively). The reactions were performed using the Step One Plus™ Real-Time PCR System (Applied Biosystems).

Statistical analysis

Descriptive statistics included the mean values, standard deviation for continuous data and percentages for categorical data. The BioEstat software, version 5.0 was used to evaluate the Hardy-Weinberg equilibrium

(HWE). The software Minitab, version 16.0, was used to perform the normality test (similar to the Shapiro-Wilk method) of the variable age, and a binary logistic regression model was used to evaluate the association between the variables and SCRC and also to evaluate the association of polymorphisms with clinical and histopathological parameters after the adjustment for age, gender, and tobacco and alcohol consumption.

The SNPStats software (available at: < http://bioinfo.iconcologia.net/SNPstats_web>) was used to perform binary logistic regression to evaluate the association of polymorphisms with SCRC risk in the log-additive model (major allele homozygotes vs heterozygotes + minor allele homozygotes with weight 2), the dominant model (major allele homozygotes vs heterozygotes + minor allele homozygotes), the recessive model (major allele homozygotes + heterozygotes vs minor allele homozygotes), the codominant model (heterozygotes vs major allele homozygotes and minor allele homozygotes vs major allele homozygotes), and the overdominant model (major allele homozygotes vs heterozygotes + minor allele homozygotes), after adjustment for age, gender and tobacco and alcohol consumption. The SNPStats program was also used to evaluate the potential interaction between the polymorphisms and tobacco or alcohol consumption, adjusted for the other variables on SCRC risk. The results are presented as odds ratios (ORs) and 95%CI. Linkage disequilibrium between the polymorphism and haplotype frequencies was determined using the Haploview program, version 2.05. Results with a *P* value < 0.05 were considered statistically significant. The statistical review of the study was performed by a biomedical statistician.

RESULTS

The normality test was performed for the variable age, which had a normal distribution (*P* < 0.01). Table 1 shows the sociodemographic data of the SCRC patients and controls. Age over 62 years (mean age of the case group; OR = 7.54, 95%CI: 4.94-11.50, *P* < 0.01)

Table 3 Alleles frequencies of *CYP1A1**2A, *CYP1A1**2C, *CYP2E1**5B, *CYP2E1**6, *EPHX1* Tyr113His and *EPHX1* His139Arg polymorphisms in the sample of this study

Polymorphisms	Allele	Control <i>n</i>	Allele frequencies	Case <i>n</i>	Allele frequencies
<i>CYP1A1</i> *2A	T	617	0.77	383	0.84
	C	183	0.23	71	0.16
<i>CYP1A1</i> *2C	A	699	0.87	416	0.92
	G	101	0.13	38	0.08
<i>CYP2E1</i> *5B	G	751	0.94	381	0.84
	C	49	0.06	73	0.16
<i>CYP2E1</i> *6	T	710	0.89	345	0.76
	A	90	0.11	109	0.24
<i>EPHX1</i> Tyr113His	T	586	0.73	340	0.75
	C	214	0.27	114	0.25
<i>EPHX1</i> His139Arg	A	615	0.77	373	0.82
	G	185	0.23	81	0.18

and male gender (OR = 0.55, 95%CI: 0.35-0.85, $P < 0.01$) showed a statistically significant association with SCRC.

The allelic frequencies of the polymorphisms are shown in Table 3. The genotype frequencies are in HWE equilibrium in both groups for the *CYP2E1**5B, *CYP2E1**6, *EPHX1* Tyr113His and *EPHX1* His139Arg polymorphisms. For the *CYP1A1**2A and *CYP1A1**2C polymorphisms, only the case group is in HWE equilibrium (*CYP1A1**2A case: $\chi^2 = 3.08$ and $P = 0.08$, control: $\chi^2 = 4.97$ and $P = 0.03$; *CYP1A1**2C case: $\chi^2 = 3.40$ and $P = 0.06$; control: $\chi^2 = 8.59$ and $P = 0.003$). HWE analysis was performed in case-control studies to verify if the allele frequency is similar to the expected frequency throughout the generations and to allow the investigation of the association between an allele and pathological conditions.

The results of the association between the six polymorphisms with SCRC are shown in Table 4. *CYP2E1**5B and *CYP2E1**6 polymorphisms were associated with SCRC in all genotype models, except for the log-additive for *CYP2E1**5B because the minor allele was not represented in the control group. *CYP1A1**2A, *CYP1A1**2C, *EPHX1* Tyr113His and *EPHX1* His139Arg polymorphisms were not associated with SCRC.

In the present study, the interaction of the presence of polymorphisms and tobacco or alcohol consumption with the SCRC risk was not demonstrated (Table 5). We observed that heterozygous or homozygous polymorphic genotype carriers for the *CYP2E1**5B polymorphism showed an increased SCRC risk independent of tobacco consumption (non-smokers: OR = 2.69 and 95%CI: 1.41-5.10; smokers: OR = 2.68 and 95%CI: 1.33-5.41) or alcohol consumption (non-drinkers: OR = 3.07 and 95%CI: 1.63-5.80; drinkers: OR = 3.90 and 95%CI: 1.82-8.38). The same was observed for non-smokers (OR = 2.89; 95%CI: 1.7-4.93) or smokers (OR = 2.99, 95%CI: 1.58-5.64) and non-drinkers (OR = 3.1, 95%CI: 1.80-5.48) or drinkers (OR = 4.10, 95%CI: 2.18-7.72) carrying

heterozygous or homozygous polymorphic genotypes for the *CYP2E1**6 polymorphism.

Regarding the clinical and histopathological parameters of SCRC, the most common variables were tumor extension T3 and T4 (61.63%), the absence of lymph node involvement (52.91%) and the rectum as the primary site (52.09%). The polymorphisms were not associated with clinical and histopathological parameters (data not shown).

Haplotype analyses were conducted to evaluate the combined effect of the polymorphisms on SCRC development. The *CYP1A1**2A and *CYP1A1**2C polymorphisms in our study were in strong linkage disequilibrium [logarithm of odds (LOD) = 39.44; Lewontin's D' (D') = 0.711]. The haplotype CA (minor alleles for both polymorphisms) was not associated with SCRC ($P > 0.05$).

The *CYP2E1**5B and *CYP2E1**6 polymorphisms were also in linkage disequilibrium [logarithm of odds (LOD) = 10.15; Lewontin's D' (D') = 0.39]. The haplotype formed by minor alleles (CA) of both polymorphisms presented a higher frequency in the case group ($P = 0.002$).

The *EPHX1* Tyr113His and *EPHX1* His139Arg polymorphisms were not in linkage disequilibrium in the population studied (logarithm of odds (LOD) = 0.17; Lewontin's D' (D') = 0.124).

DISCUSSION

The results of the present study showed that individuals aged 62 years and older are more susceptible to SCRC, corroborating the data reported by previous studies, which established that age is a risk factor for this disease^[1,10,19,20]. We also observed that male subjects were less susceptible to SCRC, although the incidence of SCRC is similar between genders^[1,21].

In the present study, the *CYP2E1**5B and *CYP2E1**6 polymorphisms were associated with increased SCRC risk. The *CYP2E1* haplotypes formed by both minor alleles (CA) were also associated with SCRC. The *CYP2E1**5B^[10] and *CYP2E1**6^[27] polymorphisms can enhance the transcription of the *CYP2E1* gene and increase the level of enzyme activity. *CYP2E1* is involved in arachidonic acid metabolism, producing hydroxyeicosatetraenoic acids and epoxyeicosatrienoic acids, which have been implicated in inflammation and vascular endothelial growth factor-dependent angiogenesis^[28-30]. Furthermore, *CYP2E1* is involved in reactive oxygen species (ROS) production, which is related to angiogenesis induction and metastatic growth of tumor cells^[31]. Therefore, the increase in enzyme activity as a result of the *CYP2E1* polymorphism may contribute to an increased risk of cancer.

Studies have also shown an association between the polymorphic genotype of *CYP2E1**5B (CC)^[10,27,32,33] and the polymorphic genotype of *CYP2E1**6 (AA)^[10,34] and increased SCRC risk in Caucasians. However, in other studies, these polymorphisms were not

Table 4 Association of *CYP1A1**2A, *CYP1A1**2C, *CYP2E1**5B, *CYP2E1**6, *EPHX1* Tyr113His and *EPHX1* His139Arg polymorphisms with sporadic colorectal cancer

Models	Genotype	Control, <i>n</i> (%)	Case, <i>n</i> (%)	OR ¹ (95%CI)	<i>P</i> value	Genotype	Control, <i>n</i> (%)	Case, <i>n</i> (%)	OR ¹ (95%CI)	<i>P</i> value
CYP1A1*2A						CYP1A1*2C				
Codominant	T/T	246 (61.5)	165 (72.7)	1.00 (reference)	0.27	A/A	312 (78)	193 (85)	1.00 (reference)	0.13
	T/C	125 (31.3)	53 (23.3)	0.76 (0.49-1.18)		A/G	75 (18.8)	30 (13.2)	0.70 (0.41-1.20)	
	C/C	29 (7.2)	09 (4)	0.59 (0.25-1.39)		G/G	13 (3.2)	4 (1.8)	0.36 (0.10-1.31)	
Dominant	T/T	246 (61.5)	165 (72.7)	1.00 (reference)	0.13	A/A	312 (78)	193 (85)	1.00 (reference)	0.08
	T/C-C/C	154 (38.5)	62 (27.3)	0.73 (0.49-1.10)		A/G-G/G	88 (22)	34 (15)	0.64 (0.38-1.06)	
Recessive	T/T-T/C	371 (92.8)	218 (96)	1.00 (reference)	0.29	A/A-T/G	387 (96.8)	223 (98.2)	1.00 (reference)	0.12
	C/C	29 (7.2)	09 (4)	0.64 (0.27-1.50)		G/G	13 (3.2)	4 (1.8)	0.38 (0.10-1.38)	
Overdominant	T/T-C/C	275 (68.8)	174 (76.7)	1.00 (reference)	0.30	A/A-G/G	325 (81.2)	197 (86.8)	1.00 (reference)	0.23
	T/C	125 (31.2)	53 (23.3)	0.80 (0.52-1.23)		A/G	75 (18.8)	30 (13.2)	0.72 (0.42-1.24)	
Log-additive	T/T	246 (61.5)	165 (72.7)	1.00 (reference)	0.11	A/A	312 (78)	193 (85)	1.00 (reference)	0.05
	T/C	125 (31.3)	53 (23.3)	0.77 (0.55-1.06)		A/G	75 (18.8)	30 (13.2)	0.66 (0.43-1.00)	
	C/C	29 (7.2)	09 (4)			G/G	13 (3.2)	4 (1.8)		
CYP2E1*5B						CYP2E1*6				
Codominant	G/G	351 (87.8)	157 (69.2)	1.00 (reference)	< 0.01 ²	T/T	314 (78.5)	126 (55.5)	1.00 (reference)	< 0.01 ²
	G/C	49 (12.2)	67 (29.5)	2.66 (1.64-4.32)		T/A	82 (20.5)	93 (41)	2.81 (1.84-4.28)	
	C/C	0	03 (1.3)	-		A/A	04 (1)	8 (3.5)	7.32 (1.85-28.96)	
Dominant	G/G	351 (87.8)	157 (69.2)	1.00 (reference)	< 0.00 ²	T/T	314 (78.5)	126 (55.5)	1.00 (reference)	< 0.01 ²
	G/C-C/C	49 (12.2)	70 (30.8)	2.82 (1.74-4.55)		T/A-A/A	86 (21.5)	101 (44.5)	2.97 (1.97-4.50)	
Recessive	G/G-G/C	400 (100)	224 (98.7)	1.00 (reference)	-	T/T-T/A	396 (99)	219 (96.5)	1.00 (reference)	0.016 ²
	C/C	0	03 (1.3)	-		A/A	4 (1)	8 (3.5)	5.26 (1.35-20.50)	
Overdominant	G/G-C/C	351 (87.8)	160 (70.5)	1.00 (reference)	< 0.01 ²	T/T-A/A	318 (79.5)	134 (59)	1.00 (reference)	< 0.01 ²
	G/C	49 (12.2)	67 (29.5)	2.58 (1.59-4.19)		T/A	82 (20.5)	93 (41)	2.64 (1.74-4.01)	
Log-additive	G/G	351 (87.8)	157 (69.2)	1.00 (reference)	< 0.01 ²	T/T	314 (78.5)	126 (55.5)	1.00 (reference)	< 0.01 ²
	G/C	49 (12.2)	67 (29.5)	2.84 (1.78-4.52)		T/A	82 (20.5)	93 (41)	2.78 (1.91-4.06)	
	C/C	0	03 (1.3)			A/A	4 (1)	8 (3.5)		
EPHX1 Tyr113His						EPHX1 His139Arg				
Codominant	T/T	214 (53.5)	126 (55.5)	1.00 (reference)	0.84	A/A	235 (58.8)	153 (67.4)	1.00 (reference)	0.18
	T/C	158 (39.5)	88 (38.8)	0.95 (0.63-1.41)		A/G	145 (36.2)	67 (29.5)	0.79 (0.52-1.20)	
	C/C	28 (7)	13 (5.7)	0.80 (0.36-1.76)		G/G	20 (5)	7 (3.1)	0.42 (0.14-1.26)	
Dominant	T/T	214 (53.5)	126 (55.5)	1.00 (reference)	0.68	A/A	235 (58.8)	153 (67.4)	1.00 (reference)	0.15
	T/C-C/C	186 (46.5)	101 (44.5)	0.92 (0.63-1.36)		A/G-G/G	165 (41.2)	74 (32.6)	0.74 (0.50-1.11)	
Recessive	T/T-T/C	372 (93)	214 (94.3)	1.00 (reference)	0.60	A/A-A/G	380 (95)	220 (96.9)	1.00 (reference)	0.14
	C/C	28 (7)	13 (5.7)	0.81 (0.37-1.77)		G/G	20 (5)	7 (3.1)	0.45 (0.15-1.35)	
Overdominant	T/T-C/C	242 (60.5)	139 (61.2)	1.00 (reference)	0.88	A/A-G/G	255 (63.8)	160 (70.5)	1.00 (reference)	0.37
	T/C	158 (39.5)	88 (38.8)	0.97 (0.65-1.44)		A/G	145 (36.2)	67 (29.5)	0.83 (0.55-1.25)	
Log-additive	T/T	214 (53.5)	126 (55.5)	1.00 (reference)	0.59	A/A	235 (58.8)	153 (67.4)	1.00 (reference)	0.08
	T/C	158 (39.5)	88 (38.8)	0.92 (0.67-1.25)		A/G	145 (36.2)	67 (29.5)	0.74 (0.52-1.04)	
	C/C	28 (7)	13 (5.7)			G/G	20 (5)	7 (3.1)		

¹Odds ratio (OR) adjusted for age, gender and tobacco and alcohol consumption and polymorphisms in the dominant model; ²Significant *P* values < 0.05.associated with SCRC^[17,34-36].

The *CYP1A1**2A, *CYP1A1**2C, *EPHX1* Tyr113His and *EPHX1* His139Arg polymorphisms were not associated with SCRC risk in the present study. The literature has shown controversial results from the influence of these polymorphisms on SCRC development. Studies in Japanese^[37] and Lebanese^[35] populations, as well as a recent meta-analysis^[38], did not find an association between the *CYP1A1**2A polymorphism and this tumor type.

On the other hand, a study conducted in Asia showed that the *CYP1A1**2A and *CYP1A1**2C polymorphisms increase the SCRC risk in this population^[39]. The association of the *CYP1A1**2C with SCRC was also evidenced in a study conducted in Hungary^[32] and was confirmed in two meta-analyses, especially in Asians and Caucasians^[40,41]. Two other studies conducted in an Asian population, similar to our findings, did not observe the influence of the

*CYP1A1**2C polymorphism on SCRC^[37,42].

The genotype frequencies of *CYP1A1**2A and *CYP1A1**2C are in HWE equilibrium in only the case group. According to the literature, case-control studies with SNP analysis have shown HWE disequilibrium in patients or controls or in both groups^[43].

Regarding the *Tyr113His* and *His139Arg* polymorphisms of the *EPHX1* gene, our results are consistent with another study from North America that did not find a significant association between these polymorphisms and SCRC^[21]. Some studies have shown an association between SCRC and these polymorphisms^[19,20,36]. A meta-analysis showed that there are differences between studies of different populations that explain the contradictory results. The authors have observed that the allele frequencies of *EPHX1* polymorphisms and their effects on cancer risk are different depending on the population studied. Different ethnic compositions, inclusion criteria, the

Table 5 Interaction between CYP1A1*2A, CYP1A1*2C, CYP2E1*5B, CYP2E1*6, EPHX1 Tyr113His and EPHX1 His139Arg polymorphisms and tobacco or alcohol consumption on the risk of SCRC

	Tobacco consumption						Alcohol consumption					
	Non-smoker			Smoker			Non-drinker			Drinker		
	Control	Case	OR ¹ (95%CI)	Control	Case	OR ¹ (95%CI)	Control	Case	OR ¹ (95%CI)	Control	Case	P value
CYP1A1*2A												
T/T	156	96	1	90	69	1.12 (0.69-1.83)	137	92	1	109	73	0.35
T/C-C/C	87	35	0.87 (0.51-1.49)	67	27	0.65 (0.35-1.21)	81	35	0.88 (0.50-1.52)	73	27	0.95 (0.50-1.81)
CYP1A1*2C												
A/A	190	107	1	122	86	1.08 (0.68-1.69)	165	105	1	147	88	0.91
A/G-G/G	53	24	0.81 (0.43-1.52)	35	10	0.44 (0.18-1.06)	53	22	0.65 (0.34-1.25)	35	12	0.87 (0.38-2.00)
CYP2E1*5B												
G/G	215	94	1	136	63	0.90 (0.56-1.44)	190	85	1	161	72	0.68
G/C-C/C	28	37	2.69 (1.41-5.10)	21	33	2.68 (1.33-5.41)	28	42	3.07 (1.63-5.80)	21	28	3.90 (1.82-8.38)
CYP2E1*6												
T/T	189	72	1	125	54	0.96 (0.57-1.63)	171	70	1	143	56	0.78
T/A-A/C	54	59	2.89 (1.70-4.93)	32	42	2.99 (1.58-5.64)	47	57	3.14 (1.80-5.48)	39	44	4.10 (2.18-7.72)
EPHX1 Tyr113His												
T/T	129	75	1	85	51	0.89 (0.51-1.54)	122	75	1	92	51	0.57
T/C-C/C	114	56	0.84 (0.51-1.40)	72	45	0.92 (0.52-1.62)	96	52	0.83 (0.49-1.41)	90	49	1.36 (0.78-2.37)
EPHX1 His139Arg												
A/A	141	89	1	94	64	0.87 (0.52-1.44)	132	83	1	103	70	0.34
A/G-G/G	102	42	0.64 (0.38-1.09)	63	32	0.79 (0.44-1.44)	86	44	0.89 (0.52-1.53)	79	30	1.00 (0.54-1.85)

¹Odds Ratio (OR) adjusted for age, gender and tobacco or alcohol consumption.

quality of original studies, selection bias and study sample size may contribute to the discrepancy. In this meta-analysis, the EPHX1 Tyr113His polymorphism was not associated with SCRC risk, and the EPHX1 His139Arg polymorphism was associated with decreased SCRC risk^[44].

The present study did not show a potential interaction between the presence of the polymorphisms investigated and tobacco and alcohol consumption concerning SCRC risk. These results agree with the study that evaluated the interaction of these variables with the CYP1A1*2A, CYP1A1*2C, EPHX1 Tyr113His and EPHX1 His139Arg polymorphisms^[45-47]. On the other hand, an interaction was observed between the CYP1A1*2A, CYP1A1*2C polymorphisms (heterozygous) and tobacco consumption concerning SCRC risk^[39]. Huang and colleagues^[17] found an elevated risk of SCRC in individuals who were EPHX1 Tyr113His and EPHX1 His139Arg polymorphisms carriers and smokers.

To our knowledge, no study evaluated the interaction between CYP2E1*6 polymorphisms and tobacco and alcohol consumption concerning SCRC risk. Regarding CYP2E1*5B, an interaction between the CYP2E1*5B polymorphism and alcohol consumption concerning SCRC risk was described in the literature^[33]. Interestingly, the observation in the present study about the increased SCRC risk in the presence of CYP2E1*5B and CYP2E1*6 polymorphisms, independent of tobacco and alcohol consumption, reinforces the influence of these polymorphisms in the etiology of SCRC.

The most representative primary site in this study was the rectum, corroborating a previous report regarding the higher occurrence of primary SCRC at this anatomical location^[48]. The tumor extent and presence of lymph node involvement were not associated with the polymorphisms evaluated during this study. According to our knowledge, there are no studies evaluating the association between these clinical variables and CYP2E1 and EPHX1 polymorphisms in SCRC. The association between the CYP1A1*2A polymorphism and clinical and histopathological data were investigated in lung cancer. However, no association was found^[49].

The discrepancy between these studies may be the result of several variables, such as differences in gender, epidemiological factors and study design. Therefore,

Table 6 Comparison between the results of this study and the results of other studies presented in the discussion

Ref.	Country study	Sample size		Gender		Age		Tobacco consumption				Alcohol consumption				Polymorphisms			
		Case	Control	Female	Male	Mean (SD)		Non-smokers		Smokers		Non-drinkers		Drinkers		CYP1A1	CYP2E1	EPHX1	
						Case	Control	Case	Control	Case	Control	Case	Control	Case	Control				Case
Huang <i>et al</i> ^[39] , 2005	Bethesda, Maryland	772	777	237	241	535	536	-	-	-	-	-	-	-	-	-	-	Tyr113His ¹ , His139Arg ¹	
van der Logt <i>et al</i> ^[36] , 2006	Netherlands	371	415	159	247	212	168	42	64.0	-	-	-	-	-	-	-	*5B, *6	Tyr113His, His139Arg	
Kiss <i>et al</i> ^[32] , 2007	Hungary	500	500	278	278	222	222	64.1	63.8	-	-	-	-	-	-	-	*5B ¹	Tyr113His ¹ , His139Arg	
Yeh <i>et al</i> ^[42] , 2007	China	727	736	317	327	410	409	-	-	-	-	-	-	-	-	-	*2C ¹	-	
Yoshida <i>et al</i> ^[39] , 2007	Japan	66	121	26	48	36	73	67.3 ¹	67.3	35	55	261	61	-	-	-	*2A, *2C	-	
Morita <i>et al</i> ^[33] , 2009	Japan	685	778	259	288	426	490	60.2	58.6	-	-	-	-	272	264	413	468	*5B	
Hlavata <i>et al</i> ^[20] , 2010	Czech	495	495	206	230	289	265	57.2 ¹	55.5	243	195	220	169	-	-	-	-	Tyr113His, His139Arg	
Nisa <i>et al</i> ^[37] , 2010	Japan	685	778	259	288	426	490	-	-	299	326	386	452	-	-	-	*2A, *2C	-	
Northwood <i>et al</i> ^[21] , 2010	United Kingdom	317	296	911	122	226	174	62.5	62.0	-	-	-	-	-	-	-	*2C	Tyr113His, His139Arg	
Darazy <i>et al</i> ^[35] , 2011	Lebanon	57	70	-	-	-	-	60.3	62.8	-	-	-	-	-	-	-	*2A	*6	
Jin <i>et al</i> ^[40] , 2011	China	5336	6226	-	-	-	-	-	-	-	-	-	-	-	-	-	*2C ¹	-	
Sameer <i>et al</i> ^[30] , 2011	India	86	160	37	72	49	88	52.0	52.0	31	75	55	85	-	-	-	-	*5B ¹	
Liu <i>et al</i> ^[43] , 2012	China	6395	7893	-	-	-	-	-	-	-	-	-	-	-	-	-	-	Tyr113His, His139Arg ¹	
Silva <i>et al</i> ^[34] , 2012	Brazil	131	206	70	124	61	82	62.4	61.7	-	-	-	-	-	-	-	*5B ¹	-	
Zheng <i>et al</i> ^[41] , 2012	China	6673	8102	-	-	-	-	-	-	-	-	-	-	-	-	-	*2A ¹ , *2C	-	
Jiang <i>et al</i> ^[27] , 2013	China	5137	6330	-	-	-	-	-	-	-	-	-	-	-	-	-	*5B ¹ , *6	-	
Qian <i>et al</i> ^[17] , 2013	China	4592	5918	-	-	-	-	-	-	-	-	-	-	-	-	-	*6	-	
He <i>et al</i> ^[38] , 2014	China	6975	8651	-	-	-	-	-	-	-	-	-	-	-	-	-	*2A	-	
This Study	Brazil	227	400	125	106	2751	121	62.0 ¹	46.7	243	131	157	96	218	127	182	100	*5B ¹ , *6 ¹	Tyr113His, His139Arg

¹P value significant; the variable or polymorphism was associated with SCRC.

further studies are needed to better understand the factors involved in SCRC etiology. A summary of the comparison between our results and the literature data can be observed in Table 6.

In conclusion, our data demonstrate the influence of the CYP2E1*5B and CYP2E1*6 polymorphisms in SCRC development for the population studied. In addition, individuals aged 62 years and older are more susceptible to the SCRC. Male individuals are less susceptible. These results can contribute to the identification of biomarkers for SCRC and understanding of the mechanisms involved in colorectal carcinogenesis.

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COMMENTS

Background

Colorectal cancer is the third most common cancer worldwide and can be related to altered metabolism of carcinogens. Therefore, it is interesting to evaluate polymorphisms in genes related to this process, such as *Cytochrome P-450 (CYP450)* and *Epoxide hydrolase 1 (EPHX1)*. Polymorphisms in the genes encoding CYP1A1, CYP2E1, and EPHX1 may alter the levels of gene transcription and enzyme activity. This alteration can lead to DNA damage and the deregulation of mechanisms involved in colorectal cancer.

Research frontiers

Polymorphisms in the genes encoding CYP1A1, CYP2E1, and EPHX1 have been extensively studied in the susceptibility to diseases such as cancer. However, the literature presents conflicting results. Therefore, several studies are necessary to evaluate and confirm the real role among the factors that influence alterations in metabolic processes related during colorectal cancer.

Innovations and breakthroughs

For the first time, a study evaluated the haplotype formed by minor alleles of polymorphisms of the CYP2E1 and CYP1A1 genes in colorectal cancer development. The haplotype formed by minor alleles of polymorphisms CYP2E1*5B and CYP2E1*6 was associated with increased colorectal cancer risk.

Applications

Data showed that carriers of polymorphisms CYP2E1*5B and CYP2E1*6 constitute a risk group for sporadic colorectal cancer (SCRC). Thus, considering the high incidence of this cancer, it is important for the comprehension of the factors that lead to carcinogenesis for the development of preventive and therapeutic strategies for cancer management.

Terminology

CYP1A1: Cytochrome P-450 CYP1A1 (cytochrome P450 family 1 subfamily A member 1), gene located on chromosome 15 (NC_000015.10). CYP2E1: Cytochrome P-450 CYP2E1 (cytochrome P450 family 2 subfamily E member 1), gene located in chromosome 10 (NC_000010.11). EPHX1: Epoxide Hydrolases 1, gene located in chromosome 1 (NC_000001.11).

Peer-review

Fernandes *et al* have conducted a very good case control study examining the involvement of CYP1A1, CYP2E1, and EPHX1 polymorphisms in SCRC. They find age over 62, female gender, CYP2E1*5B and CYP2E1*6 polymorphisms associated with SCRC.

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Retrospective Cohort Study

Prognostic value of glycated hemoglobin in colorectal cancer

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Abstract

AIM

To investigate the clinical significance of routinely used glycemic parameters in a cohort of colorectal cancer (CRC) patients.

METHODS

Pre-treatment fasting blood glucose, insulin, HbA_{1c} and homeostasis model of risk assessment (HOMA-IR) were retrospectively evaluated in a case-control study of 224 CRC and 112 control subjects matched for sex, obesity and diabetes frequency and blood lipid profile.

Furthermore, the prognostic value of routinely used glycemic parameters towards progression-free (PFS) and overall survival (OS) was prospectively evaluated.

RESULTS

Fasting blood glucose, insulin, HOMA-IR and HbA_{1c} (all $P < 0.0001$) levels were higher in non-diabetic CRC patients compared with obesity-matched controls. All parameters were associated with increased CRC risk at ROC analysis, but no relationship with clinical-pathological variables or survival outcomes was observed for glycemia, insulinemia or HOMA-IR. Conversely, advanced CRC stage ($P = 0.018$) was an independent predictor of increased HbA_{1c} levels, which were also higher in patients who had disease progression compared with those who did not ($P = 0.05$). Elevated HbA_{1c} levels showed a negative prognostic value both in terms of PFS (HR = 1.24) and OS (HR = 1.36) after adjustment for major confounders, which was further confirmed in a subgroup analysis performed after exclusion of diabetic patients.

CONCLUSION

HbA_{1c} might have a negative prognostic value in CRC, thus suggesting that glycemic metabolic markers should be carefully monitored in these patients, independently of overt diabetes.

Key words: Colorectal cancer; Type 2 diabetes; Glycated hemoglobin; Insulin resistance; Prognostic value

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Core tip: The clinical significance of routinely used pre-treatment fasting blood glucose, insulin, HbA_{1c} and homeostasis model of risk assessment was investigated in a cohort of colorectal cancer (CRC) patients. Despite all four metabolic markers were elevated in non-diabetic CRC patients, only elevated HbA_{1c} levels were significantly associated with advanced CRC stage and disease progression, showing a negative prognostic value both in terms of progression-free (HR = 1.24) and overall (HR = 1.36) survival after adjustment for major confounders. These results suggest that glycemic metabolic markers, mainly HbA_{1c}, should be carefully monitored in CRC patients as they could provide important risk stratification information.

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INTRODUCTION

Type 2 diabetes (T2D) and colorectal cancer (CRC) are

among the major causes of morbidity and mortality worldwide, and their increasing prevalence represents a significant public health burden^[1-3]. Several epidemiological studies and meta-analyses of published trials suggested that there is a significant association between T2D and increased risk of CRC^[4-6], which is further suggested by the findings that combined treatment of metformin and 5-fluorouracil had a better anticancer effect than 5-fluorouracil alone, possibly by reverting epithelial-mesenchymal transition phenotype in cancer cells^[7].

Among the mechanisms advocated to explain this association, hyperinsulinemia may play a pivotal role, acting as a potent cell mitogen capable of promoting colon cancer growth^[8], an hypothesis that is corroborated by indirect findings of a harmful effect of insulin use for CRC risk^[9,10]. Other proposed explanations for the increased risk include obesity-related insulin resistance (IR)^[11] and cytokine production^[12], which may directly influence CRC growth not only through promotion of angiogenesis^[13,14], but also by inducing host inflammatory response^[13,15] or decreasing natural killer (NK) lymphocyte cytotoxic activity and, thus, the immunological defense against cancer^[16].

Independently of the pathogenetic mechanism(s) advocated for the relationship between T2D and CRC, elevated levels of fasting glucose, fasting insulin, glycated hemoglobin (HbA_{1c}) and IR evaluated by homeostasis model of risk assessment (HOMA-IR) have all been associated with increased CRC risk^[17-30], but the results were often conflicting, with some studies suggesting a superiority of HbA_{1c}^[20,26] and others placing emphasis on fasting blood glucose, insulin or on the composite index HOMA-IR^[31].

Furthermore, whilst most of the studies have focused on the association between T2D or metabolic markers of impaired glucose metabolism and CRC risk, little is known on the impact of a deregulation of the glucose-insulin axis on CRC progression and survival. The few available data come from selected diabetic populations^[32], or obese individuals^[30], while additional indirect evidences can be derived, once again, from T2D patients with CRC treated with metformin, suggesting that it could moderately improve survival outcomes^[33-37].

Based on the above, we sought to investigate the potential prognostic value of routinely used glycemic parameters in non-diabetic CRC patients. To this purpose, we first designed a case-control study aimed at evaluating the behavior of glucose metabolism indexes in a population of CRC patients, representative of a general practice cohort, compared with non-cancer controls matched for sex, obesity and T2D frequency and blood lipid profile, in order to minimize possible metabolic-related confounders. Thereafter, based on the hypothesis that an association between pre-treatment fasting blood glycemic indexes (blood glucose, insulin, HbA_{1c} and HOMA-IR) and CRC-

related survival outcomes might exist independently of T2D, the prognostic value of routinely used glycemic parameters towards progression-free and overall survival was prospectively evaluated.

MATERIALS AND METHODS

Patients and sample collection

Starting from January 2007, the PTV Bio.Ca.Re. (Policlinico Tor Vergata Biospecimen Cancer Repository) and the Interinstitutional Multidisciplinary Biobank of the IRCCS San Raffaele Pisana (SR-BioBIM, Rome, Italy) are actively involved in the recruitment of ambulatory patients with primary or metastatic cancer, who are prospectively followed under the appropriate Institutional ethics approvals, as part of a Clinical Database and Biobank project. Among these, a cohort of 224 CRC patients was eligible for the study. Inclusion criteria for the CRC patients from whom serum samples were stored in our Biobanks were: age above 18 years, an Eastern Cooperative Oncology Group performance status (ECOG-PS) ≤ 2 and adequate hematological, hepatic and renal functions. History of alcohol or drug abuse, concurrent infectious or inflammatory diseases were all considered as exclusion criteria for the current analysis.

CRC was staged according to the TNM classification. Surgery was performed in 104 patients with primary CRC and 8 with resectable synchronous metastasis. The remaining 112 patients had relapsing/metastatic disease and entered the study prior to the start of chemotherapy. Among the non-metastatic population, 21/224 (10%) and 83/224 (38%) patients received neoadjuvant and adjuvant therapies, respectively. First-line chemotherapy was instituted in all patients with metastatic disease. Anti-cancer regimens used were all 5-fluorouracil-based in combination either with irinotecan ($n = 97$) or with platinum compounds ($n = 114$). Bevacizumab or cetuximab were administered in 68 (57%) and 36 (30%) metastatic CRC patients, respectively. Supportive drugs included erythropoiesis-stimulating agents ($n = 3$, 1%), granulocyte colony stimulating factors ($n = 7$, 2%) or corticosteroids ($n = 40$, 12%). No patient was lost at follow-up. Clinical features of CRC patients are summarized in Table 1.

As control group, 112 unrelated individuals (mean age 60 ± 13 , ranging from 31 to 83 years), paired for T2D rate (22%), obesity (BMI: 25.7 ± 4.4 ; 18% obese, 33% overweight) and blood lipid parameters were recruited in a 2:1 ratio from otherwise healthy individuals enrolled in the SR-BioBIM.

The study was performed in accordance with the principles embodied in the Declaration of Helsinki. All patients gave written informed consent, previously approved by our Institutional Ethics Committees.

Blood sampling and assessment of glycemic indexes

Fasting serum samples were obtained from each recruited subject, aliquoted and stored at -80°C in

Table 1 Clinical characteristics of colorectal cancer patients - comparison between patients with or without impaired glucose tolerance or type 2 diabetes

	IGT or T2D		P value
	NO ($n = 173$)	YES ($n = 51$)	
Age (yr)			
mean \pm SD (range)	64 \pm 10 (30-83)	66 \pm 8 (54-80)	0.127
Sex, n (%)			
Males	103 (60)	30 (59)	0.927
Females	70 (40)	21 (41)	
Body mass index			
mean \pm SD (range)	25.4 \pm 4.1 (17.2-38.4)	26.5 \pm 4.2 (17.2-38.4)	0.105
HOMA index			
Median (IQR)	2.80 (1.83-4.79)	6.48 (3.95-10.26)	< 0.0001
HbA _{1c}			
mean \pm SD (range)	5.72 \pm 0.36 (4.30-7.10)	6.87 \pm 1.25 (5.30-13.0)	< 0.0001
Blood lipids (mg/dL)			
Total cholesterol	187 \pm 42	189 \pm 48	0.782
HDL cholesterol	47 \pm 12	44 \pm 13	0.284
LDL cholesterol	113 \pm 38	117 \pm 41	0.618
Triglycerides	136 \pm 69	138 \pm 52	0.838
Site, n (%)			
Colon	126 (73)	33 (65)	0.293
Rectum	47 (27)	18 (35)	
Histological diagnosis, n (%)			
Adenocarcinoma			0.933
Mucinous	30 (17)	10 (20)	
Non-mucinous	140 (81)	40 (78)	
Others ¹	3 (2)	1 (2)	
ECOG performance status, n (%)			
0	150 (91)	40 (82)	0.129
1	14 (8)	9 (18)	
2	1 (1)	0 (0)	
Stage, n (%)			
I	3 (2)	1 (2)	0.875
II A	26 (15)	6 (12)	
II B	4 (2)	1 (2)	
III A	3 (2)	1 (2)	
III B	37 (21)	8 (15)	
III C	9 (5)	5 (10)	
Metastatic	91 (53)	29 (57)	

¹Including 3 non diabetic patients with undifferentiated, squamous and mixed neuroendocrine cancers (one each) and one type 2 diabetes (T2D) patient with undifferentiated cancer. CRC: Colorectal cancer; IGT: Impaired glucose tolerance.

the facilities of the PTV Bio.Ca.Re. or the SR-BioBIM. Samples from CRC patients were obtained at baseline prior to chemotherapy.

Routine chemistry studies, including fasting blood glucose (Hexokinase/Glucose-6-phosphate dehydrogenase-based methodology; Abbott Laboratories, Abbott Park, IL, United States), were performed on fresh samples within one hour from blood withdrawal on an ARCHITECT c8000 System (Abbott Laboratories). Fasting insulin levels were analyzed on serum samples using a fully automated Lumipulse G 600 II chemiluminescent enzyme immunoassay analyzer (Fujirebio Inc. Tokyo, Japan) according to the manufacturer's instructions.

The HOMA index (a marker of insulin resistance)

Table 2 Glycemic parameters in colorectal cancer patients and non-cancer controls

	CRC	Controls	P value
Overall population	n = 224	n = 112	
Fasting blood glucose (mg/dL)	106 ± 35 (65-145)	101 ± 39 (61-289)	0.219
Fasting insulin (μIU/mL)	14.2 (8.7-23.3)	9.0 (6.0-12.2)	< 0.0001
HbA _{1c} (%)	5.98 ± 0.83 (4.30-13.0)	5.63 ± 1.10 (3.90-9.70)	0.001
HOMA index	3.5 (2.0-6.1)	2.0 (1.3-3.3)	< 0.0001
Non-diabetic	n = 173	n = 87	
Fasting blood glucose (mg/dL)	100 ± 25 (65-254)	87 ± 12 (61-123)	< 0.0001
Fasting insulin (μIU/mL)	12.5 (8.5-18.6)	7.6 (5.1-10.9)	< 0.0001
HbA _{1c} (%)	5.72 ± 0.36 (4.30-7.10)	5.14 ± 0.42 (3.90-6.10)	< 0.0001
HOMA index	2.8 (1.8-4.8)	1.7 (1.0-2.5)	< 0.0001
Non-diabetic normoweight	n = 90	n = 51	
Fasting blood glucose (mg/dL)	97 ± 19 (65-175)	84 ± 16 (61-112)	0.0001
Fasting insulin (μIU/mL)	10.8 (8.1-17.5)	6.5 (4.3-10.0)	< 0.0001
HbA _{1c} (%)	5.69 ± 0.31 (4.70-6.60)	5.13 ± 0.40 (4.00-6.10)	0.0005
HOMA index	2.5 (1.8-4.0)	1.4 (1.0-2.4)	< 0.0001

Data are reported as mean ± SD (range) or median (interquartile range).

was retrospectively calculated for each participating subject from fasting blood glucose and insulin according to the formula: glucose (mg/dL) × insulin (μIU/mL)/405^[38].

HbA_{1c} levels were immediately measured on EDTA anticoagulated whole blood by the Tosoh G7 Automated HPLC Analyzer - HbA_{1c} Variant Analysis Mode (Tosoh Bioscience, Rivoli, TO, Italy), certified by the NGSP (National Glycohemoglobin Standardization Program) and traceable to the Diabetes Control and Complications Trial.

All measurements were ascertained while blinded to the sample origin and to study endpoint.

Statistical analysis

Sample size of the study was based on the agreement to inclusion criteria and willingness to provide informed consent rather than on sample size calculations. However, estimation was later performed and showed that, given the observed proportions for patients and control groups for HbA_{1c} values and using a type I error probability of 0.05, the recruited population yielded a statistical power greater than 95%.

Data are presented as percentages, mean ± SD, or median and interquartile range. Student's unpaired *t*-test and ANOVA test were used for normally distributed variables. Appropriate non-parametric tests (Mann-Whitney *U*-test and Kruskal-Wallis ANOVA and median test) were employed for all the other variables. The cut-off values were generated from continuous data by receiver operating characteristic (ROC) curve analyses performed by MedCalc Statistical Software version 13.1.2 (MedCalc Software bvba, Ostend,

Belgium; <http://www.medcalc.org>; 2014).

Progression free (PFS) and overall survival (OS) represented the study endpoints. PFS was calculated from the date of enrollment until relapse or progression of disease. OS was calculated from the date of enrollment until death from disease. If a patient had not progressed or died, PFS or OS were censored at the time of the last follow-up. Survival curves were calculated by the Kaplan-Meier method and the significance level was assessed according to the log-rank test using a computer software package (Statistica 8.0, StatSoft Inc., Tulsa, OK). Cox-proportional hazards analysis was performed by a free web-based application (<http://statpages.org/>) to evaluate the association between clinical-pathological variables and PFS. For administrative censoring, follow-up was ended the date of March 31st, 2016. All tests were two-tailed and only *P* values lower than 0.05 were regarded as statistically significant.

RESULTS

Of 224 prospectively recruited CRC patients, 51 (23%) had an established diagnosis of IGT (*n* = 15) or T2D (*n* = 36). In addition, 86 (38%) and 27 (12%) of the patients were overweight or obese, respectively. Fasting blood glycemic indexes (blood glucose, insulin, HbA_{1c}) and HOMA-IR were retrospectively reviewed in the overall population, demonstrating that fasting insulin (*P* < 0.0001), HOMA-IR (*P* < 0.0001) and HbA_{1c} (*P* < 0.001), but not blood glucose levels were higher in CRC patients compared to non-cancer controls matched for IGT/T2D or obesity (Table 2). Of interest, pre-treatment levels of all four metabolic markers, including blood glucose, were significantly higher in CRC patients compared with controls once diabetic or overweight/obese subjects were excluded from comparative analysis (Table 2).

ROC curves were, thus, generated from continuous variables measured in non-diabetic individuals. As summarized in Table 3, the areas under the curve for fasting blood glucose, insulin, HOMA-IR and HbA_{1c} were: 0.657, 0.728, 0.738 and 0.860, respectively. At the designated criterion values all three parameters were associated with an increased CRC risk, although HbA_{1c} yielded the best performance, being associated with a sensitivity and specificity > 80% and a 4.5 positive likelihood ratio (+LR) of CRC risk (Table 3).

Despite the discriminative power of glycemic indexes at ROC analysis, there were no significant associations between pre-treatment fasting blood glucose or insulin and clinical-pathologic variables. On the other hand, mean pre-treatment HbA_{1c} was higher in mucinous (5.9% ± 0.4%) compared with non-mucinous (5.7% ± 0.3%, *P* = 0.007) adenocarcinomas and showed a trend to increasing concentrations from early (5.6%) to both advanced (5.8%, *P* = 0.044) and metastatic (5.8%, *P* = 0.020) CRC stages in non-diabetic patients (Anova test:

Table 3 Receiver operating characteristics and Bayesian analysis of glycemic parameters

	Fasting blood glucose	Fasting insulin	HOMA-IR	HbA _{1c}
AUC (SE)	0.657 (0.04)	0.728 (0.03)	0.738 (0.03)	0.860 (0.03)
95%CI	0.596-0.714	0.670-0.781	0.680-0.791	0.812-0.900
Criterion	97 mg/dL	12.5 µIU/mL	1.7%	5.4%
Sensitivity	42%	50%	81%	82%
Specificity	85%	84%	55%	82%
+LR (CI)	2.79 (1.63-5.06)	3.09 (1.87-5.41)	1.81 (1.43-2.30)	4.46 (2.96-7.06)
-LR (CI)	0.69 (0.61-0.81)	0.60 (0.52-0.72)	0.35 (0.24-0.50)	0.22 (0.17-0.30)
P value ¹	0.0001	0.0001	0.0001	0.0001

¹Two-tailed Fisher Exact test. AUC: Area under the curve; +LR: Positive likelihood ratio; -LR: Negative likelihood ratio.

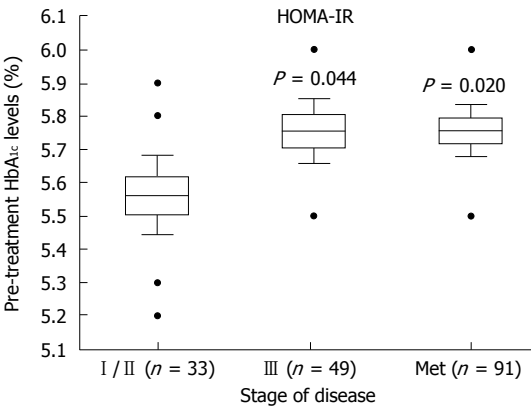


Figure 1 Box-plot of HbA_{1c} value distribution in non-diabetic colorectal cancer patients (*n* = 173) according to stages of disease. Solid lines: Mean values; boxes: Standard error; whiskers: 95%CI; closed circles: Outliers (Anova test including outliers: *F* = 3.9, *P* = 0.021).

F = 3.9, *P* = 0.021) (Figure 1). Thus, multivariate regression analysis (including age, sex, obesity, HOMA-IR, ECOG-PS and tumor site, histology and stage) was performed to assess possible determinants of HbA_{1c} in non-diabetic CRC patients. The final model by forward stepping showed that HOMA-IR (regression coefficient = 0.200, SE = 0.074, *P* = 0.008), mucinous features (regression coefficient = 0.196, SE = 0.072, *P* = 0.007) and advanced stage (regression coefficient = 0.177, SE = 0.074, *P* = 0.018) were all independent predictors of increased HbA_{1c}.

Overall, the mean time of follow up was 24.8 mo. Among patients with primary non metastatic CRC, 81/104 (78%) remained clinically free of disease, while 23/104 (22%) had progressive disease. Among patients with metastatic CRC, 3/120 (3%) had stable disease, 28/120 (23%) had a complete/partial response during chemotherapy, while 89/120 (74%) had CRC progression. Pre-treatment HbA_{1c} levels, but not fasting glycemia or insulinemia, were higher in patients who had CRC progression (5.8%) compared with patients in whom the disease did not progress (5.6%, *P* = 0.05).

To further evaluate its prognostic value, pre-treatment HbA_{1c} levels were divided into quartiles according to value distribution (median and interquartile

range) in the 224 CRC patients (Q1: ≤ 5.3%; Q2: > 5.3% and ≤ 5.7%; Q3: > 5.7% and ≤ 6.1%; Q4: > 6.1%). Overall, IGT/T2D or obesity were not associated to either PFS or OS at multivariate Cox proportional hazards survival analyses (data not shown). On the other hand, univariate Cox proportional hazards survival analyses showed that elevated pre-treatment HbA_{1c} levels had a negative prognostic value in terms of PFS [HR = 1.30 (95%CI: 1.09-1.54)] or OS [HR = 1.43 (95%CI: 1.16-1.76)]. No association with survival outcomes was observed for fasting blood glucose, insulinemia or the composite index HOMA-IR. These results were largely unmodified after adjustment for obesity or other variables known to be associated with PFS or OS, including disease stage, ECOG-PS, and tumor site and histotype [HRs for HbA_{1c} quartiles: PFS: 1.24 (95%CI: 1.01-1.53); OS: 1.36 (95%CI: 1.05-1.74)] (Table 4). Of interest, the negative prognostic value of HbA_{1c} in terms of OS was further confirmed in a subgroup analysis performed after exclusion of IGT/T2D patients (Table 4).

Figure 2A and C demonstrates the Kaplan-Meier PFS and OS curves for CRC patients stratified on the basis of pre-treatment HbA_{1c} levels. As shown, patients with HbA_{1c} in the highest quartile had a worse 5-year PFS and OS rate compared to patients with HbA_{1c} levels in the lower quartile. Similar results were confirmed in non-diabetic CRC (Figure 2B and 2D).

DISCUSSION

Although the presence of a causal link between T2D and CRC risk has been proposed since long time, the role of a deregulation of glucose metabolic axis in CRC prognosis is far less explored, and mostly relying on indirect findings on glucose lowering drugs in T2D patients with cancer^[33-37]. Furthermore, earlier studies have mainly used a “single marker approach” that does not take into account the complex synergistic interactions among the various metabolic features.

In the present study, we demonstrate the presence of increased pre-treatment fasting insulin and HbA_{1c} levels in a population of CRC patients representative of a general practice cohort, as compared to control subjects matched for obesity and T2D frequency.

Table 4 Cox proportional hazards survival regression analysis of the predictive value of clinical-pathological variables and glycemic indexes on progression-free and overall survival of colorectal cancer patients

Variable	Progression-free survival		Overall survival	
	HR (95%CI)	P value	HR (95%CI)	P value
Overall population (n = 224)				
Sex	0.96 (0.68-1.36)	0.809	0.82 (0.54-1.23)	0.336
Obesity ¹	0.95 (0.73-1.24)	0.701	0.88 (0.63-1.22)	0.448
Site	0.93 (0.62-1.41)	0.745	0.81 (0.49-1.34)	0.412
Histological diagnosis	0.90 (0.58-1.39)	0.629	0.88 (0.53-1.45)	0.604
Stage of disease ²	1.95 (1.11-3.42)	0.021	1.17 (0.58-2.34)	0.662
ECOG-PS	1.54 (0.96-2.47)	0.075	1.84 (1.05-3.23)	0.035
Type of treatment ³	1.37 (0.71-2.62)	0.346	1.94 (0.80-4.74)	0.145
Fasting glucose ⁴	0.99 (0.80-1.21)	0.892	1.06 (0.83-1.34)	0.657
Fasting insulin ⁵	0.90 (0.58-1.38)	0.619	1.01 (0.62-1.62)	0.981
Fasting HbA _{1c} ⁶	1.24 (1.01-1.53)	0.040	1.36 (1.05-1.74)	0.018
Non-diabetic patients (n = 173)				
Sex	1.05 (0.69-1.59)	0.824	0.79 (0.48-1.31)	0.355
Obesity ¹	1.11 (0.83-1.50)	0.482	0.91 (0.62-1.34)	0.640
Site	1.09 (0.67-1.76)	0.740	0.98 (0.54-1.79)	0.952
Histological diagnosis	0.91 (0.55-1.52)	0.728	0.88 (0.49-1.61)	0.687
Stage of disease ²	1.78 (0.93-3.40)	0.083	0.87 (0.39-1.93)	0.735
ECOG-PS	1.69 (0.98-2.92)	0.060	1.98 (1.02-3.85)	0.044
Type of treatment ³	1.46 (0.69-3.08)	0.321	2.93 (1.01-8.56)	0.050
Fasting glucose ³	0.94 (0.74-1.20)	0.625	0.91 (0.62-1.22)	0.531
Fasting insulin ⁵	1.01 (0.60-1.68)	0.976	0.91 (0.51-1.64)	0.755
Fasting HbA _{1c} ⁶	1.25 (0.97-1.62)	0.092	1.40 (1.03-1.92)	0.034

¹Coded as normoweight if body mass index (BMI) ≤ 25, overweight if BMI > 25 and ≤ 30, obese if BMI > 30; ²Coded as primary/metastatic; ³Coded as neoadjuvant/adjuvant/metastatic; ⁴Q1: ≤ 87 mg/dL; Q2: > 87 mg/dL and ≤ 97 mg/dL; Q3: > 97 mg/dL and ≤ 117 mg/dL; Q4: > 117 mg/dL; ⁵Q1: ≤ 8.7 μIU/mL; Q2: > 8.7 μIU/mL and ≤ 14 μIU/mL; Q3: > 14 μIU/mL and ≤ 23 μIU/mL; Q4: > 23 μIU/mL; ⁶Q1: ≤ 5.3%; Q2: > 5.3% and ≤ 5.7%; Q3: > 5.7% and ≤ 6.1%; Q4: > 6.1%. ECOG-PS: Eastern Cooperative Oncology Group performance status.

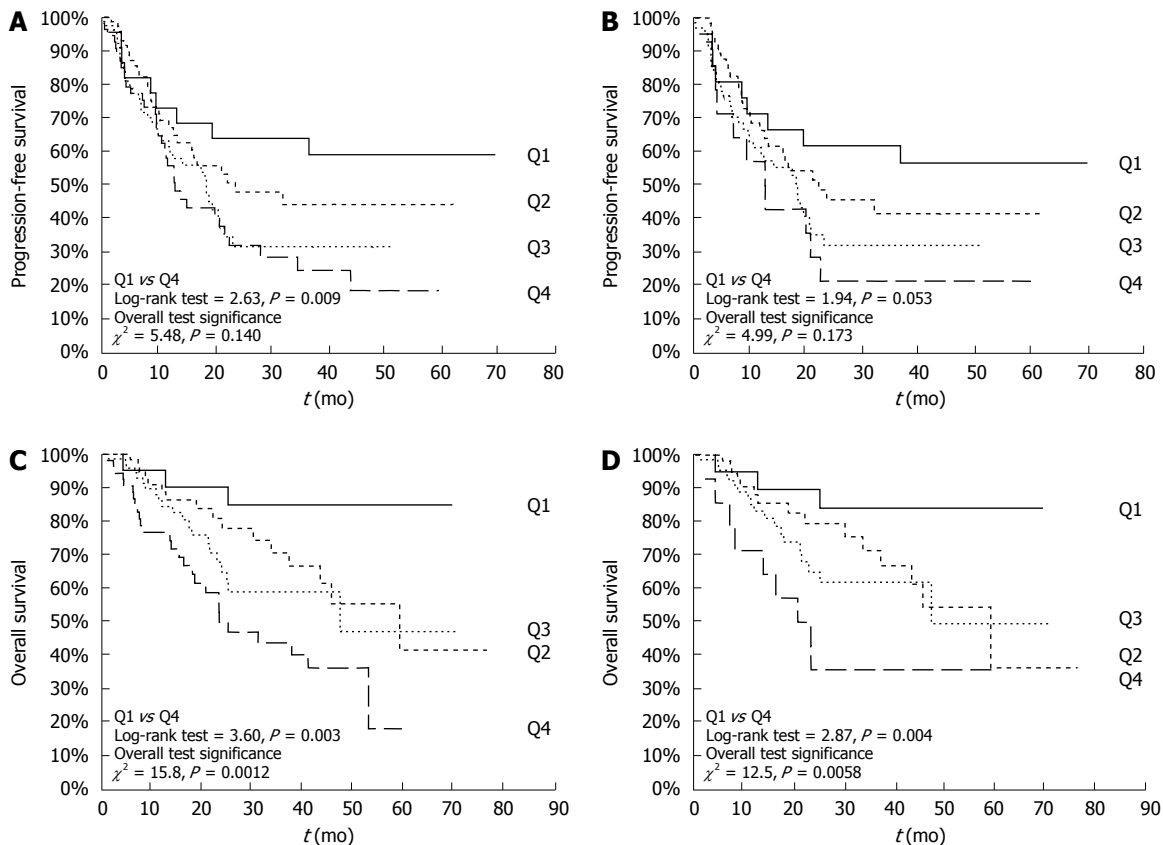


Figure 2 Kaplan-Meier curves of progression-free survival (A, B) and overall survival (C, D) of colorectal cancer patients. Comparison between patients stratified on fasting HbA_{1c} quartiles. First quartile (Q1): ≤ 5.3%; second quartile (Q2): > 5.3% and ≤ 5.7%; third quartile (Q3): > 5.7% and ≤ 6.1%; fourth quartile (Q4): > 6.1%. A, C: Overall population; B, D: Non-diabetic patients.

Furthermore, we provide evidence of an elevation of glycemic metabolic markers in normoweight non-diabetic CRC compared with matched control subjects.

Contrary to what reported for other tumors^[39,40], however, neither hyperglycemia nor hyperinsulinemia - and thus HOMA-IR - were associated to CRC clinical-pathological features, whereas pre-treatment HbA_{1c} levels were associated with advanced disease stage, especially of the mucinous histotype, in non-diabetic CRC patients. Most importantly, elevated pre-treatment HbA_{1c} levels acted as a negative prognostic factors for OS (HR = 1.43), independently of other well established prognostic factors (*e.g.*, stage or ECOG-PS). Indeed, patients with HbA_{1c} levels in the highest quartile (*i.e.*, > 6.1%) had an approximately three-fold increased risk of dying from disease (5-year survival rate 24%) compared to patients with HbA_{1c} levels in the lowest quartile (*i.e.*, < 5.3%; 5-year survival rate 85%).

These results are in agreement and extend previous reports in cancer patients with T2D, in whom HbA_{1c} levels were evaluated as a marker of glycemic control^[41,42]. In particular, Siddiqui *et al.*^[41] reported that poorly controlled T2D - defined as a HbA_{1c} level $\geq 7.5\%$ - was independently associated with a more advanced CRC stage at time of diagnosis and poorer 5-year survival, thus suggesting that in CRC patients with T2D, poor glycemic control is associated with a clinically aggressive cancer phenotype. Furthermore, no differences were observed in stage at presentation and 5-year mortality from CRC between well-controlled T2D and non-diabetic controls, suggesting that meticulous glycemic control might lead to a reduction in CRC mortality^[41]. These findings are only in apparent disagreement with data obtained in the present study. Indeed, Siddiqui *et al.*^[41] performed a comparative analysis between well-controlled T2D and non-diabetic CRC patients (both control groups in their study) without reporting the impact of increasing HbA_{1c} on survival in either group, thus its possible prognostic significance. Furthermore, in our study the negative prognostic value of HbA_{1c} on survival outcomes was assessed in a general practice cohort of CRC patients that included only a moderate rate (23%) of IGT/T2D patients. Accordingly, we did not categorized HbA_{1c} on currently accepted cut-off value for metabolic control, but on quartiles calculated on the basis of the value distribution in the overall population. Of interest, CRC patients in the highest HbA_{1c} quartile (> 6.1%) had the poorest survival. However, the small number of T2D patients enrolled did not confer enough power to perform a separate subgroup analysis. On the other hand, Boursi *et al.*^[42] reported no association between HbA_{1c} levels within 6 mo prior to cancer diagnosis and overall survival for CRC. This study is barely comparable with the results obtained in ours, since it enrolled only T2D patients in whom HbA_{1c} was evaluated as a marker of glycemic control.

More recently, Hope *et al.*^[43] reported the results

of a meta-analysis investigating the relationship between HbA_{1c} and cancer in people with or without T2D. Major finding was that the majority of studies that investigated HbA_{1c} levels in relation to CRC risk identified positive associations. In particular, CRC cases were found to have higher HbA_{1c} levels than control subjects; however, the possibility of reverse causality cannot be completely excluded due to many shortcomings in population selection (*e.g.*, different ethnicity) and lack of correction for CRC-related confounding factors known to increase HbA_{1c} levels such as iron deficiency anemia^[43]. Thus, data to support the role of HbA_{1c} either for the identification of people at risk of cancers or to provide some insight into the potential progression of the disease are still lacking.

On the other hand, and to best of our knowledge, this is the first report suggesting that HbA_{1c} may have a prognostic significance for PFS of non-diabetic CRC, in whom HbA_{1c} was capable of discriminating a subset of patients with a more favorable outcome, with a 3-year PFS rate of 56% in patients with HbA_{1c} levels in the lower quartile (< 5.3%) compared with 21% of those in the upper quartile (> 6.1%). These results are scarcely comparable with the currently available evidences, as there are no data on the prognostic value of HbA_{1c} in the PFS of non-diabetic CRC.

As reported above, HbA_{1c} is a common measure of glucose metabolism, as it reflects average glycemia over the previous three months^[44]. Accordingly, an elevated HbA_{1c} indicates poor long-term glycemic control and, possibly, chronic hyperinsulinemia. The data here reported are in agreement with the current knowledge that glycemic control is important in determining CRC development and progression. Moreover, the finding of a prognostic role of HbA_{1c} in non diabetic CRC patients could be explained by the common knowledge that, at the time of T2D diagnosis, patients usually have had hyperglycemia for more than a decade^[45]. Thus, it is conceivable to hypothesize that colorectal carcinogenesis may recognize, among its causative mechanisms, a deregulated glucose metabolism and that CRC might become manifest long before T2D diagnosis.

There are, of course, some limitations that need to be acknowledged. The most obvious resides in the relatively small sample size that might have weakened the statistical power. Another limitation resides in the fact that, as evidenced by Hope *et al.*^[43], data on iron deficiency anemia were missing also in our study. However, the fact that erythropoiesis stimulating agents were administered to three patients only, indicates a satisfactory hemoglobin asset in the overall population. On the other hand, the strength of our analysis was represented by the use of samples collected and processed using standard operating procedures in the context of two large Biobanks. In addition, tests were run by a single laboratory under ongoing quality control protocols, hence minimizing

the difference in sample analyses.

Based on the above, we may conclude that pre-treatment HbA_{1c} levels might have a negative prognostic value in CRC patients and, as such, should be carefully monitored, as they could provide important information in risk stratification. These results, however, should be regarded with caution and additional studies are required to prospectively evaluate their clinical value. Future investigations specifically designed to address the role of HbA_{1c} in the management of CRC, independently of T2D, may provide the rationale for lifestyle or glucose targeting dietary/pharmacologic interventions as a means of improving CRC outcomes.

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COMMENTS

Background

Although metabolic markers of impaired glucose metabolism have been often associated with increased colorectal cancer (CRC) risk, little is known on the impact of a deregulation of the glucose-insulin axis on CRC progression and survival. The few available data come from selected diabetic populations, or obese individuals, while indirect evidences can be derived from type 2 diabetes (T2D) patients with CRC treated with metformin, suggesting that combined treatment of metformin and 5-fluorouracil had a better anticancer effect than 5-fluorouracil alone.

Research frontiers

Future investigations specifically designed to address the role of HbA_{1c} in the management of CRC, independently of T2D, may provide the rationale for lifestyle or glucose targeting dietary/pharmacologic interventions as a means of improving CRC outcomes.

Innovations and breakthroughs

This study provides evidences that pre-treatment HbA_{1c} levels might have a negative prognostic value in CRC patients. Glycemic metabolic markers, mainly HbA_{1c}, should be carefully monitored in CRC patients, independently of T2D, as they could provide important information in risk stratification.

Applications

Clinicians should be alert to the potential risk of impaired glycemic control and advise CRC patients about lifestyle intervention, weight loss, and exercise as a part of their therapeutic plan. In the context of a precision medicine approach, HbA_{1c} guided incorporation of metformin might aid as adjunctive therapy in CRC management.

Terminology

HbA_{1c} is a glycation product resulting from hemoglobin's exposure to plasma glucose. As such, it provides an estimate of average blood glucose levels over the previous three months, as this is the lifespan of red blood cells. It is used in the diagnosis of T2D and as a marker of glycemic control.

Peer-review

The study is interesting and the observational results are intriguing.

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Retrospective Cohort Study

Elevated fibrinogen plasma level is not an independent predictor of poor prognosis in a large cohort of Western patients undergoing surgery for colorectal cancer

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Abstract

AIM

To evaluate the clinical significance of the preoperative fibrinogen plasma level as a prognostic marker after surgery for colorectal cancer.

METHODS

This retrospective study analysed 652 patients undergoing surgery for stage I-IV colorectal cancer between January 2005 and December 2012, at the Division of General Surgery A, University of Verona Hospital Trust, in whom preoperative fibrinogen plasma values were assessed at baseline. Fibrinogen is involved in tumourigenesis as well as tumour

progression in several malignancies. Correlations between preoperative plasma fibrinogen values and clinicopathological characteristics were investigated. Univariate and multivariate survival analyses were performed to identify factors associated with overall and tumour-related survival.

RESULTS

Among the 652 patients, the fibrinogen value was higher than the threshold of 400 mg/dL in 345 patients (53%). The preoperative mean \pm SD of fibrinogen was 426.2 ± 23.2 mg/dL (median: 409 mg/dL; range: 143-1045 mg/dL). Preoperative fibrinogen values correlated with age ($P = 0.003$), completeness of tumour resection, potentially curative *vs* palliative ($P < 0.001$), presence of systemic metastasis ($P < 0.001$), depth of tumour invasion pT ($P < 0.001$), nodes involvement pN ($P = 0.001$) and CEA serum level ($P < 0.001$). The mean fibrinogen value (\pm SD) was 395.6 ± 120.4 mg/dL in G1 tumours, 424.1 ± 121.4 mg/dL in G2 tumours and 453.4 ± 131.6 mg/dL in G3 tumours ($P = 0.045$). The overall survival and tumour-related survival were significantly higher in patients with fibrinogen values ≤ 400 mg/dL ($P < 0.001$). However, hyperfibrinogenemia did not retain statistical significance regarding either overall ($P = 0.313$) or tumour-related survival ($P = 0.355$) after controlling for other risk factors in a multivariate analysis.

CONCLUSION

Preoperative fibrinogen levels correlate with cancer severity but do not help in predicting patient prognosis after colorectal cancer surgery.

Key words: Colorectal cancer; Fibrinogen; Tumour markers; Prognosis; Colorectal surgery

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Core tip: Fibrinogen is involved in tumourigenesis and in tumour progression in several malignancies. Many studies, particularly from East, have shown a correlation between hyperfibrinogenemia and poor prognosis in patients with colorectal cancer (CRC). This study involves a large cohort of 652 Western patients underwent surgery for CRC. The analysis of our data demonstrates that preoperative fibrinogen plasma levels correlate with leading prognostic factors in patients undergoing surgery for CRC. Although long-term survival and tumour-related survival are worse in patients with hyperfibrinogenemia, these findings are not confirmed in multivariate analysis or after stratification of patients according to completeness of tumour resection and TNM stage.

Pedrazzani C, Mantovani G, Salvagno GL, Baldiotti E, Ruzzenente A, Iacono C, Lippi G, Guglielmi A. Elevated fibrinogen plasma level is not an independent predictor of poor prognosis in a large cohort of Western patients undergoing

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INTRODUCTION

Colorectal cancer (CRC) is the third most common cancer worldwide, with more than 1 million new cases and 600000 deaths per year^[1]. Many haemostatic derangements have been described in cancer progression and prognosis. Solid tumours, both in humans and animal models, entail a considerable amount of fibrinogen-related alterations. In particular, CRC is associated with a large range of fibrinolytic and procoagulant alterations, suggesting that fibrinogen plays an important role in tumour stroma formation^[2-4]. In fact, fibrin matrices promote the migration of endothelial cells, fibroblasts and macrophages, as well as neovascularization^[5-7]. Moreover, the products of fibrin degradation display chemotactic, angiogenic and immune-modulatory capacities^[8,9].

Increased plasma fibrinogen levels are associated with liver and lymph node metastasis, but not with peritoneal metastasis, in gastric cancer^[10,11]. Recent studies on CRC support the hypothesis that hyperfibrinogenemia may promote the enhancement of tumour volume^[12] and haematogenous metastasis^[13,14]. Several studies have demonstrated a significant contribution of high fibrinogen plasma levels in predicting the prognosis in various subsets of CRC patients^[15,16].

The aim of this retrospective study was to evaluate the clinical significance of preoperative plasma fibrinogen levels as a prognostic marker after colorectal cancer surgery.

MATERIALS AND METHODS

Inclusion criteria and population under study

The study population consisted of patients undergoing surgery for colorectal carcinoma at the Division of General Surgery A, University of Verona Hospital Trust, between January 2005 and December 2012. The inclusion criteria were age of 18 years or older, histological diagnosis of cancer, elective or urgent colorectal resection with absence of peritonitis or other acute infectious diseases, no pre-operative chemotherapy or radiotherapy, follow-up available for a minimum period of 30 mo, as well as preoperative assessment of fibrinogen levels. Informed consent was obtained from all the patients, and the study was approved by the local Ethics Committee with ID number: 42763 (CRINF-1034 CESC).

Between January 2005 and December 2012, 969 patients underwent surgery for colorectal carcinoma at the Division of General Surgery A, University of Verona Hospital Trust. Among them, 652 patients met the

inclusion criteria and were enrolled in the study.

Preoperative work-up and histopathological staging

Before surgery, all elective patients underwent preoperative staging by means of colonoscopy, thoracoabdominal CT scan and tumour markers (CEA, CA 19-9). Abdominal US was performed in selected cases when a CT scan was deemed unnecessary, whilst additional imaging modalities (*e.g.*, MRI, PET-CT, endoluminal US, *etc.*) were used when indicated (*e.g.*, rectal cancer or liver metastases). The preoperative assessment of urgent cases varied depending on clinical necessities.

All patients underwent preoperative routine laboratory tests, including coagulation profiles with assessment of plasma fibrinogen levels within two weeks of surgery.

The resected specimens were examined using routine histopathological analysis. Tumour staging was assessed according to the criteria established by the 7th Edition of the American Joint Committee on Cancer and the Union International Contre Le Cancer. Tumour differentiation; lymphatic, vascular and neural invasion; and inflammatory reactions were generally reported.

Preoperative assessment of fibrinogen levels

Blood samples were drawn by an expert phlebotomist in evacuated blood tubes containing 0.109 mol/L buffered sodium citrate (Terumo Europe NV, Leuven, Belgium). The blood tubes were left in an upright position at room temperature to allow complete blood stability and then centrifuged at $1500 \times g$ for 15 min. A second centrifugation was performed to obtain platelet-poor plasma, which was stored in aliquots at -70°C until measurement. At the time of measurement, the plasma aliquots were thawed in a water bath at 37°C and then left at room temperature for 1 hour. Fibrinogen was measured on an ACL TOP instrument (Instrumentation Laboratory, Milan, Italy) with the Clauss method and using proprietary reagents (HemosIL[®] Fibrinogen-C, Instrumentation Laboratory). The reference range of fibrinogen was 200-400 mg/dL.

Extent of surgery

The main goal of the surgery was the complete removal of the tumour (R0 resection), although palliative surgery was carried out in select cases to treat tumour-related complications. The extent of surgery was assessed considering the patient's performance status, tumour location and stage. Standard colorectal resection (*i.e.*, right hemicolectomy, extended right hemicolectomy, left hemicolectomy, anterior resection, low anterior resection, or abdominoperineal resection) with ligation of vessels at their origin was usually carried out to obtain the optimal management of nodal disease^[17], whereas limited colonic resection or stoma formation was performed in select cases (*i.e.*, palliative surgery, high-risk patients).

Follow-up and statistical analysis

All clinical and pathological data were retrospectively collected and stored in a digital dataset. The analysed variables included demographic, clinical, surgical and pathological characteristics. Survival and follow-up data were obtained by collecting outpatient clinical records or by contacting the patient or the family physician.

In the preliminary analysis, preoperative fibrinogen levels were normally distributed in the patient cohort. Different cut-off levels were considered to study the potential correlation with clinicopathological factors and survival. The upper limit of the reference range (*i.e.*, 400 mg/dL) was used as the predictive threshold.

To evaluate the significance of difference between cases with values above or below 400 mg/dL, a chi-square test or Fisher's exact test were used for categorical data and Student's *t*-test was used for continuous variables. Survival analysis was computed using the Kaplan-Meier method and compared by the log-rank test, with time of survival measured from the date of surgery to the date of death or most recent follow-up. Multivariate analysis was performed with the Cox regression model by taking into account the following risk factors: age (higher than median vs median or below), gender (male vs female), tumour location (rectum vs colon), type of surgery (urgent vs. elective), presence of residual tumour (R1, R2 vs R0), presence of systemic metastasis (M1a, M1b vs M0), pT category (pT2, pT3, pT4a, pT4b vs pT1), pN category (pN1, pN2 vs pN0), histological type (mucinous vs non-mucinous) and pre-operative fibrinogen plasma levels (fibrinogen value higher than 400 mg/dL vs fibrinogen value lower than this threshold). Statistical analysis was performed using SPSS software version 21.0 (IBM Corporation, Armonk, NY, United States), and the level of statistical significance was set at $P < 0.05$.

RESULTS

Fibrinogen plasma levels and clinicopathological variables

The preoperative mean \pm SD of fibrinogen was 426.2 ± 23.2 mg/dL (median: 409 mg/dL; range: 143-1045 mg/dL). Among the 652 patients, the fibrinogen value was higher than the threshold of 400 mg/dL in 345 patients (53%).

Mean \pm SD preoperative fibrinogen plasma levels for the 652 patients under study according to their clinicopathological variables are reported in Table 1. Fibrinogen value correlated with age ($P = 0.003$), type of resection (potentially curative vs palliative) ($P < 0.001$), presence of systemic metastasis ($P < 0.001$), CEA serum level ($P < 0.001$) as well as the pT ($P < 0.001$) and pN categories ($P = 0.001$).

Considering histological characteristics, patients with a mucinous histological type ($n = 113$) displayed

Table 1 Preoperative fibrinogen plasma levels (mg/dL) according to the main demographic and clinical characteristics of the 652 patients under study¹

	No. of patients	Fibrinogen plasma level		P value
		Mean value	± SD	
Age (yr)				P = 0.003
≤ 68.8	326	412.1	119.8	
> 68.8	326	440.3	125.1	
Gender				P = 0.479
Male	373	429.1	128.3	
Female	279	422.2	116.2	
Tumour location				P = 0.111
Colon	536	429.8	125.0	
Rectum	116	409.6	113.7	
Type of surgery				P = 0.053
Elective	618	424.0	120.1	
Urgent	34	466.0	167.9	
Resection				P < 0.001
Yes	640	423.8	121.3	
No	12	551.3	162.1	
Type of resection (R)				P < 0.001
R0	556	412.4	110.4	
R1	7	406.0	113.1	
R2	89	513.9	159.6	
Depth of invasion (pT) ¹				P < 0.001
pT1	86	373.7	97.1	
pT2	61	373.6	89.8	
pT3	259	421.5	111.1	
pT4a	141	442.0	143.9	
pT4b	93	482.1	118.6	
Node involvement (pN) ¹				P = 0.001
pN0	372	408.2	108.7	
pN1	166	445.0	142.6	
pN2	102	446.5	119.5	
Systemic metastasis (M)				P < 0.001
M0	534	412.1	109.6	
M1a	69	466.8	144.1	
M1b	49	522.3	170.5	
CEA serum level ²				P < 0.001
≤ 5 ng/mL	284	393.4	101.0	
> 5 ng/mL	130	485.7	129.6	

¹Depth of tumour invasion (pT) and nodal involvement (pN) were evaluated in 640 resected patients; ²CEA serum level was available for 414 patients.

higher fibrinogen values compared to non-mucinous histological types (467.1 ± 135.9 vs 417.6 ± 118.7 ; $P < 0.001$). The mean \pm SD was 395.6 ± 120.4 mg/dL in G1 tumours, 424.1 ± 121.4 mg/dL in G2 tumours and 453.4 ± 131.6 mg/dL in G3 tumours ($P = 0.045$). Conversely, vascular invasion ($P = 0.204$), lymphatic invasion ($P = 0.940$), neural invasion ($P = 0.183$) and presence of inflammatory reaction ($P = 0.067$) were not significantly associated with preoperative fibrinogen plasma levels.

Fibrinogen cut-off value (400 mg/dL) and clinicopathological variables

Considering the fibrinogen cut-off value of 400 mg/dL, a significant association was found with age ($P = 0.001$), type of resection ($P < 0.001$), depth of tumour invasion ($P < 0.001$), the presence of systemic metastases ($P = 0.001$), histological type ($P = 0.001$)

and CEA serum level ($P < 0.001$). Interestingly, fibrinogen values were found to be > 400 mg/dL in 74.2% of patients with macroscopic residual tumours after resection (R2) compared to 49.5% of potentially curative resections (R0) ($P < 0.001$). Similarly, fibrinogen values were found to be > 400 mg/dL in 71.4% of M1b patients compared to 65.2% of M1a patients and 49.6% of M0 patients ($P = 0.001$). Regarding the depth of tumour invasion, fibrinogen values were > 400 mg/dL in 32.6% of pT1 patients, 37.7% of pT2 patients, 54.8% of pT3 patients and 61.1% of pT4 patients ($P < 0.001$). Conversely, the extent of nodal involvement did not correlate with fibrinogen value. Fibrinogen plasma levels were found to be > 400 mg/dL in 50.3% of pN0 patients, 51.8% of pN1 patients and 61.8% of pN2 patients ($P = 0.017$).

Fibrinogen plasma levels and long-term survival

The 5-year survival and 5-year tumour-related survival rates of the study population were 64.4% and 75.2%, respectively. Five-year survival and 5-year tumour-related survival rates according to preoperative fibrinogen plasma levels are shown in Figure 1A. The 5-year survival rate was 72.4% for patients with values ≤ 400 mg/dL and 58.1% for patients with values > 400 mg/dL ($P < 0.001$). When considering tumour-related mortality, the 5-year survival rate was 81.2% for patients with values ≤ 400 mg/dL and 69.6% for patients with values > 400 mg/dL ($P < 0.001$).

Survival curves for patients undergoing potentially curative resection (R0) are shown in Figure 1B. Fibrinogen plasma levels were associated with overall survival ($P = 0.010$), whereas no significant difference was observed when tumour-related survival was considered ($P = 0.604$). In particular, 5-year tumour-related survival was 88.3% in patients with values ≤ 400 mg/dL and 88.7% for patients with values > 400 mg/dL.

Fibrinogen plasma levels and multivariate analysis

Table 2 shows the multivariate analysis (Cox regression model) adjusted for multiple factors. Age, the presence of systemic metastasis, the presence of residual tumour, pT category and pN category were confirmed as independent predictors of survival, whereas the fibrinogen plasma level was not [hazard ratio (HR) for fibrinogen value > 400 mg/dL compared to ≤ 400 mg/dL: 1.15 (95%CI: 0.86-1.54), $P = 0.355$]. Similar results were found for tumour-related survival [HR for fibrinogen value > 400 mg/dL compared to ≤ 400 mg/dL: 0.82 (95%CI: 0.54-1.21), $P = 0.313$]. Table 3 shows 5-year survival and tumour-related survival rates for Stage I, Stage II, Stage III and Stage IV tumours treated by potentially curative resection (R0).

DISCUSSION

The main findings of this study are: (1) preoperative

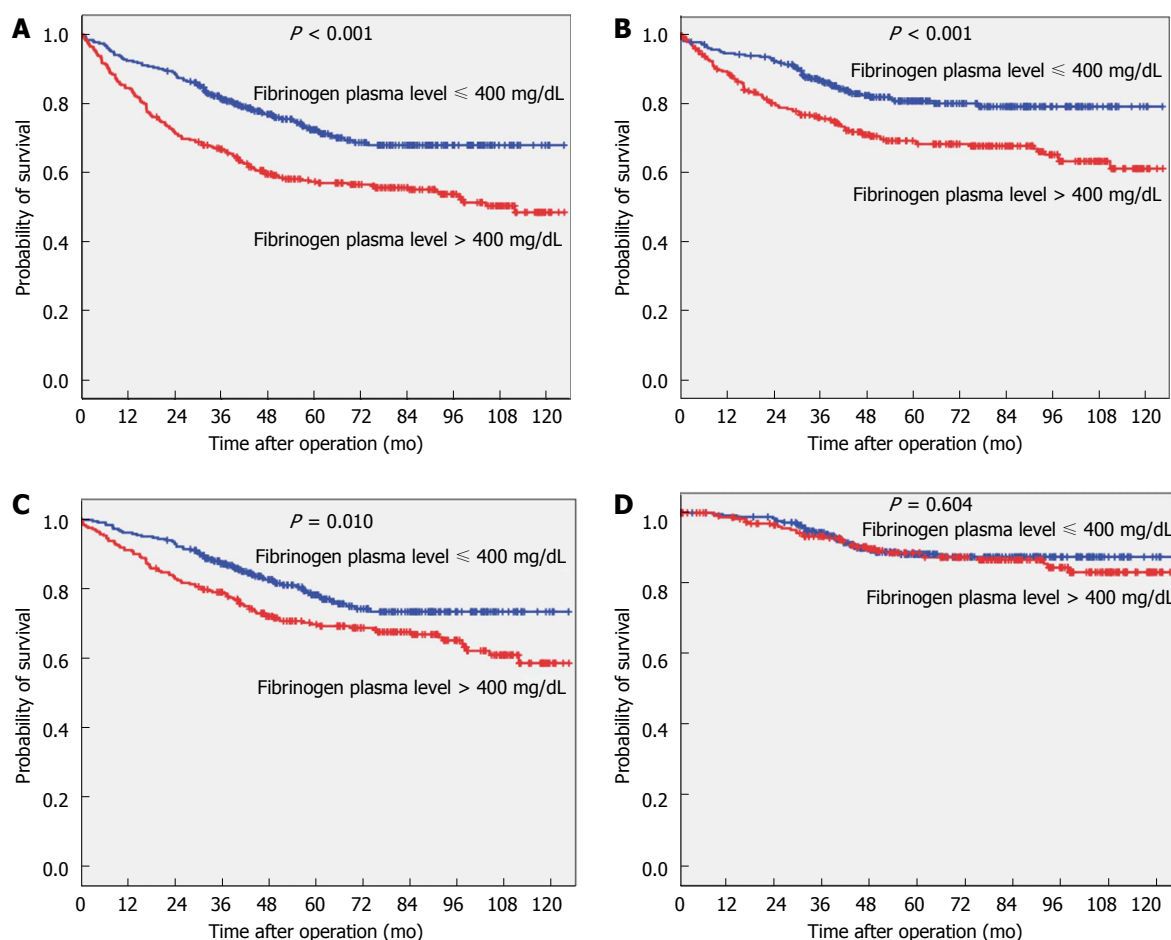


Figure 1 Kaplan-Meier estimates. A: Kaplan-Meier estimates of overall survival according to fibrinogen plasma levels in the 652 patients enrolled in the study; B: Kaplan-Meier estimates of tumour-related survival according to fibrinogen plasma levels in the 652 patients enrolled in the study; C: Kaplan-Meier estimates of overall survival according to fibrinogen plasma levels in the 556 R0 patients enrolled in the study; D: Kaplan-Meier estimates of tumour-related survival according to fibrinogen plasma levels in the 556 R0 patients enrolled in the study.

fibrinogen plasma levels correlate with the leading prognostic factors in patients undergoing CRC surgery; (2) long-term survival and tumour-related survival appear to be worse in patients with hyperfibrinogenemia; and (2) the prognostic value of the preoperative fibrinogen plasma level is not confirmed by multivariate analysis or by stratification of patients according to completeness of tumour resection (R0) and TNM stage.

It is now widely accepted that the outcome of cancer is mediated by an interaction between tumour-related factors and host factors, with chronic inflammation probably representing the main host-related factor. This explains why the correlation between inflammatory biomarkers and malignancies has been extensively studied^[18-20]. Fibrinogen is a protein synthesized by hepatocytes, playing a pivotal role in coagulation, thrombosis, wound healing, and platelet aggregation, as well as in inflammatory states^[21,22].

Although an increased plasma fibrinogen level is largely not specific and may occur in many physiological conditions (e.g., pregnancy or intense physical activity) and some pathological conditions (e.g., cardiovascu-

lar diseases, trauma and inflammatory diseases), a number of studies have demonstrated the existence of a correlation between high plasma fibrinogen levels and the development and progression of several tumours, including lung, pancreatic, gastric, and colorectal cancer^[23-26].

Several mechanisms have been put forward to explain the increase of fibrinogen plasma levels in patients with cancer. First, tumour cells may ectopically produce fibrinogen itself or other cytokines involved in inflammation, such as IL-6, which ultimately trigger the production of fibrinogen in the liver^[27]. Tumour growth is also frequently associated with hypercoagulability and hypoxia, with a subsequent increase in plasma fibrinogen levels^[28,29]. Finally, cancer-related tissue injury causes a systemic inflammatory response and, consequently, increases the level of plasma fibrinogen.

Several lines of evidence apparently demonstrate that fibrinogen participates in tumourigenesis, although the actual process is not yet completely understood. Fibrinogen may enhance tumour cell proliferation, migration and signalling through interaction with multiple integrin and non-integrin receptors. It may also promote tumour angiogenesis,

Table 2 Hazard ratio of death as a function of preoperative fibrinogen plasma level (mg/dL) for the 652 patients under study¹

	Survival		Tumour-related survival	
	HR (95%CI)	P value	HR (95%CI)	P value
Age (yr)		< 0.001		< 0.001
> 68.8 vs ≤ 68.8	2.55 (1.91-3.40)		2.13 (1.47-3.09)	
Gender		0.298		0.155
Male vs female	1.16 (0.88-1.53)		1.31 (0.90-1.89)	
Tumour location		0.368		0.928
Rectum vs colon	1.19 (0.82-1.72)		0.98 (0.59-1.61)	
Type of surgery		0.180		0.805
Urgent vs elective	1.42 (0.85-2.38)		0.92 (0.49-1.74)	
Type of resection (R)		< 0.001		< 0.001
R1 vs R0	1.98 (0.77-5.07)		4.39 (1.61-11.97)	
R2 vs R0	2.93 (1.82-4.71)		6.46 (3.63-11.49)	
Depth of invasion (pT) ¹		< 0.001		< 0.001
pT2 vs pT1	1.51 (0.59-3.86)		7.53 (0.88-64.83)	
pT3 vs pT1	1.97 (0.93-4.13)		6.03 (0.81-44.63)	
pT4a vs pT1	4.06 (1.90-8.68)		14.99 (2.02-111.57)	
pT4b vs pT1	2.88 (1.30-6.37)		15.55 (2.07-117.09)	
Node involvement (pN) ¹		< 0.001		0.135
pN1 vs pN0	1.33 (0.94-1.87)		1.51 (0.97-2.37)	
pN2 vs pN0	2.45 (1.69-3.54)		1.59 (0.94-2.67)	
Systemic metastasis (M)		< 0.001		< 0.001
M1a vs M0	2.62 (1.72-3.99)		4.72 (2.72-8.19)	
M1b vs M0	1.88 (1.06-3.30)		2.59 (1.32-5.06)	
Fibrinogen plasma value		0.355		0.313
> 400 vs ≤ 400	1.15 (0.86-1.54)		0.82 (0.54-1.21)	

¹Values in parentheses are 95%CI. Hazard ratio and P values were derived from Cox regression analysis, controlling for all other variables.

Table 3 Survival rates according to TNM stage as a function of preoperative fibrinogen plasma level (mg/dL) for the 556 R0 patients under study

	Survival		Tumour-related survival	
	5-yr rate	P value	5-yr rate	P value
Stage I		0.812		0.674
Fibrinogen ≤ 400 mg/dL	91.5		98.3	
Fibrinogen > 400 mg/dL	88.2		95.2	
Stage II		0.036		0.467
Fibrinogen ≤ 400 mg/dL	81.7		92.2	
Fibrinogen > 400 mg/dL	74.0		92.1	
Stage III		0.206		0.627
Fibrinogen ≤ 400 mg/dL	70.6		81.8	
Fibrinogen > 400 mg/dL	60.8		85.0	
Stage IV		0.566		0.213
Fibrinogen ≤ 400 mg/dL	30.0		31.8	
Fibrinogen > 400 mg/dL	40.4		61.2	

cooperating with growth factors such as vascular endothelial growth factor and fibroblast growth factors^[30]. High levels of fibrinogen receptors, such as $\alpha 5\beta 1$ and $\alpha v\beta 3$ integrins, also promote the stable adhesion of tumour cells to the endothelium of target organs and are largely expressed on malignant cells. Notably, a protective role for fibrinogen against natural killer (NK) cells seems to be involved in the haematogenous metastatic potential of tumour cells. Fibrinogen may hence suppress NK cell activity for cancer cell clearance, thus increasing the number of metastatic cells^[31]. Conversely, hyperfibrinogenemia does not seem to have a role in the metastatic

involvement of lymphatic tissue since the lymphatic fluid does not contain platelets and the lymphatic endothelium has peculiar characteristics compared to the vascular endothelium^[32].

A number of studies have shown a correlation between hyperfibrinogenemia and poor prognosis in patients with metastatic and non-metastatic CRC^[33,34]. In accord with previous data^[16,23], our experience, which represents one of the largest cohorts of CRC patients, confirmed the correlation between fibrinogen plasma levels and the most important prognostic factors, namely completeness of tumour resection ($P < 0.001$), the presence of systemic metastases ($P < 0.001$), pT category ($P < 0.001$), pN category ($P = 0.001$) and CEA serum level ($P < 0.001$). Similarly, long-term and tumour-related survival were associated with the presence of preoperative hyperfibrinogenemia. Unlike other studies, however, multivariate analysis and stratification of patients according to completeness of tumour resection (R0) and TNM stage failed to confirm the role of fibrinogen as an independent prognostic factor.

Son *et al*^[33] reported that preoperative hyperfibrinogenemia was significantly associated with shorter survival in 624 patients with non-metastatic CRC when considering stage II and III separately^[33]. Similar results were reported by Sun *et al*^[35] in 255 patients with CRC and Tang *et al*^[23] in 341 patients submitted to curative CRC surgery.

In previous studies, different cut-off values for preoperative plasma fibrinogen were used. Some

studies identified the mean value as a prognostic threshold^[23], others the median value^[16] or the 25th percentile^[33]. In our study, despite several threshold values being adopted (*i.e.*, mean value, median value, 25th and 75th percentile) to evaluate significance of difference in survival analysis, hyperfibrinogenemia was not found to be an independent prognostic factor in multivariate analysis or after stratification of patients according to completeness of tumour resection and TNM stage (data not shown). In our series, the median preoperative plasma fibrinogen value was 409 mg/dL, which is very close to the upper limit of 400 mg/dL.

In conclusion, this study represents the first analysis of the value of preoperative fibrinogen plasma level in a Western country to the best of our knowledge. The analysis of our data demonstrates that preoperative fibrinogen plasma levels correlate with leading prognostic factors in patients undergoing surgery for CRC. Although long-term survival and tumour-related survival are worse in patients with hyperfibrinogenemia, these findings are not confirmed in multivariate analysis or after stratification of patients according to completeness of tumour resection and TNM stage. It seems reasonable to suggest that evaluation of the preoperative fibrinogen level is not helpful for predicting the prognosis of patients with appropriate TNM staging.

COMMENTS

Background

Fibrinogen is involved in tumorigenesis as well as tumour progression in several malignancies. Previous studies have shown hyperfibrinogenemia to be correlated with main clinicopathological characteristics and prognosis after colorectal cancer surgery. Nonetheless, the effective clinical significance of preoperative plasma fibrinogen levels as a prognostic marker after colorectal cancer surgery has not yet been determined.

Research frontiers

This study represents the first analysis of the value of preoperative fibrinogen plasma level in a Western population and one of the largest cohort of patients.

Innovations and breakthroughs

This study based on a large Western cohort did not confirm hyperfibrinogenemia to be an independent prognostic factor in colorectal cancer patients.

Applications

Evaluation of fibrinogen plasma levels are routinely performed among preoperative blood tests. Its correlation with leading prognostic factors in patients undergoing surgery for colorectal cancer (CRC) is interesting and requires further studies.

Terminology

CRC is the third most common cancer worldwide. CRC is associated with a large range of fibrinolytic and procoagulant alterations and fibrinogen plasma levels could represent the expression of this relationship.

Peer-review

This paper tried to elucidate the role of fibrinogen plasma level in the prediction of CRC prognosis. The manuscript is well written and the data and table are clear.

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Retrospective Study

Inter- and intraobserver agreement in computed tomography enterography in inflammatory bowel disease

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Abstract

AIM

To evaluate intra- and interobserver agreement in imaging features in inflammatory bowel disease and comparison with fecal calprotectin (FC) levels.

METHODS

Our institutional computed tomography enterography (CTE) database was retrospectively queried to identify patients who underwent CTE from January 2014 to June 2015. Patient inclusion criteria were confirmed inflammatory bowel disease (IBD) and FC collected < 4 mo after CTE without any change in clinical treatment or surgical treatment during this interval. The exclusion criterion was poor image quality. Two blinded abdominal radiologists, with 12 and 3 years of experience analyzed the CTE regarding localization (small bowel, colonic, both, or no disease detected);

type of IBD (inflammatory, stenosing, fistulizing, > 1 pattern, or normal); and signs of active disease (present or absent). In 42 of 44 patients evaluated, routine CTE reports were made by one of the readers who re-evaluated the CTEs ≥ 6 mo later, to determine the intraobserver agreement. FC was considered a sign of disease activity when it was higher than 250 $\mu\text{g/g}$.

RESULTS

Forty-four patients with IBD (38 with Crohn's disease and 6 with ulcerative colitis) were included. There was a moderate interobserver agreement regarding localization of IBD ($\kappa = 0.540$), type of disease ($\kappa = 0.410$) and the presence of active signs in CTE ($\kappa = 0.419$). There was almost perfect intraobserver agreement regarding localization, type and signs of active disease in IBD. The κ values were 0.902, 0.937 and 0.830, respectively. After a consensus between both radiologists regarding inflammatory activity in CTE, we found that 24 (85.7%) of 28 patients who were classified with active disease had elevated FC, and six (37.5%) of 16 patients without inflammatory activity in CTE had elevated FC ($P = 0.003$). The correlation between elevated FC and the presence of active disease in CTE was significant ($\kappa = 0.495$, $P = 0.001$).

CONCLUSION

We found almost perfect intraobserver and moderate interobserver agreement in the signs of active disease in CTE with concurrence of high FC levels.

Key words: Crohn's disease; Ulcerative colitis; Computed tomography; Fecal calprotectin; Inflammatory bowel disease activity

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Core tip: Evaluation of active inflammation in inflammatory bowel disease (IBD) is not simple and demands a multidisciplinary approach. A few studies have evaluated the interobserver agreement in computed tomography enterography (CTE) findings in patients with active inflammation in IBD. Intraobserver agreement was only evaluated in other imaging modalities. This study showed for the first time intraobserver agreement for CTE signs of active IBD and its correlation with fecal calprotectin (FC) levels. We found almost perfect intraobserver and moderate interobserver agreement in the characterization of signs of active disease in CTE, in concurrence with high FC levels in patients with IBD.

Horvat N, Tavares CC, Andrade AR, Cabral JCS, Leao-Filho HM, Caiado AHM, Ueda SKN, Leite AZA, Sipahi AM, Rocha MS. Inter- and intraobserver agreement in computed tomography enterography in inflammatory bowel disease. *World J Gastroenterol* 2016; 22(45): 10002-10008 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v22/i45/10002.htm> DOI:

INTRODUCTION

Inflammatory bowel disease (IBD) is considered an important healthcare problem worldwide, with a high morbidity and poor quality of life. The treatment of IBD is directed according to the analysis of clinical, endoscopic, laboratory and imaging features. The presence of active inflammatory disease plays a central role for tailored treatment planning. Nevertheless, the evaluation of active inflammation is not a simple task and demands a multidisciplinary approach.

Computed tomography enterography (CTE) has become an important imaging modality for evaluation of IBD due to its accessibility and reliability. CTE provides visualization of the entire gastrointestinal tract, allowing the differentiation in inflammatory, stenosing and fistulizing diseases, and enables the characterization of active disease^[1]. Imaging features of active inflammatory disease include mucosal hyperenhancement, wall thickening, mural stratification, prominent vasa recta (comb sign), mucosal ulcerations, enlarged mesenteric lymph node and mesenteric fat stranding^[2-5].

Magnetic resonance enterography (MRE) and CTE are equally accurate for assessment of disease activity^[6]. However, CTE is more widely available, especially in developing countries, less time consuming, and more reproducible in terms of image quality^[7]. Despite the need for intravenous contrast media and exposure to radiation, CTE is still widely used for evaluation of patients with IBD. The use of dose modulation can reduce CTE radiation dose, increasing the use of this method^[8].

Fecal calprotectin (FC) is a zinc- and calcium-binding protein that is found in bowel-activated neutrophils during mucosal damage, and is considered to be one of the most important biomarkers for evaluation of disease activity in IBD. It is a noninvasive and low cost method, which measures FC directly from stool samples. Increased FC levels have been found in IBD, with close correlation with endoscopic scores of inflammation^[5,9].

The aim of this study was to evaluate inter- and intraobserver agreement in detection of inflammatory signs in CTE, in comparison with FC levels.

MATERIALS AND METHODS

Study design

Institutional review board approval was obtained and the requirement for informed written consent was waived. Our institutional CTE database was retrospectively queried to identify patients who underwent CTE from January 2014 to June 2015.

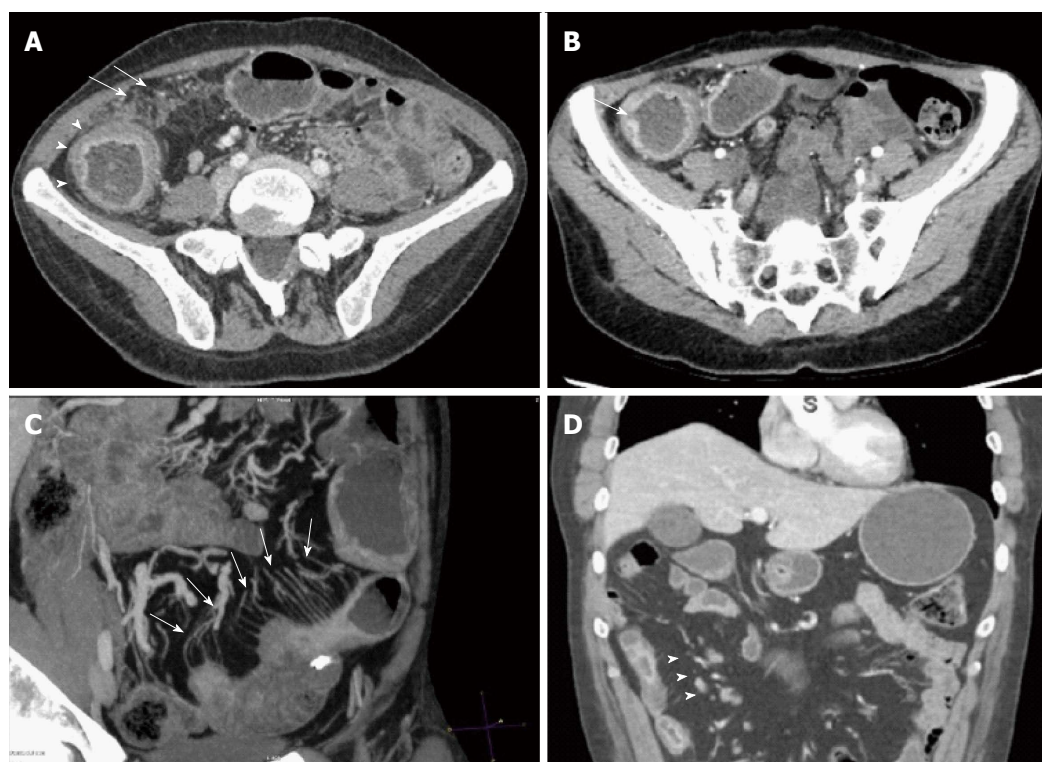


Figure 1 Computed tomography enterography shows signs of inflammatory activity in patients with inflammatory bowel disease. A: Mucosal hyperenhancement, wall thickening with mural stratification (arrowheads), mesenteric fat stranding (arrows); B: Mucosal ulcerations (arrow); C: Hypervascularity of the involved mesentery (comb sign) (arrows); D: Enlarged mesenteric lymph nodes (arrowheads).

Patient inclusion criteria for this study were confirmed inflammatory bowel disease and FC collected < 4 mo from the date of CTE, without any change in clinical treatment or surgical treatment during this interval. The exclusion criterion was poor image quality.

The CTE images of these patients were anonymized and reviewed by two abdominal radiologists (A.C.X. and C.D.Y. with 12 and 3 years of experience as an attending gastrointestinal radiologist) blinded for clinical, laboratory, endoscopic findings and previous reports of the patients. Despite the lower experience time in abdominal radiology, Reader 2 (A.C.X.) presented more experience in CTE.

In 42 of 44 patients evaluated, the routine CTE reports were made by Reader 2, who re-evaluated the CTEs ≥ 6 mo, to minimize the recall bias, in order to determine the intraobserver agreement.

CTE technique

CTE examinations were performed using a standardized clinical protocol on a 64-channel CT scanner (Brilliance, Philips Medical Systems, Eindhoven, the Netherlands; and Discovery HD 750, General Electric Healthcare, Waukesha, WI, United States). Patients fasted for ≥ 6 h and ingested 1.5 L of a polyethylene glycol solution in 50 min to distend the small bowel. Each patient received 10 mg intravenous N-butylhyoscine bromide, to reduce bowel peristalsis

and 8 mg intravenous ondansetron to reduce nausea and vomiting.

CTE images were acquired after intravenous injection of 2.0 mL/kg contrast agent (iopromide; Bayer, Berlin, Germany), containing 623 mg/mL iodine, at a rate of 4 mL/s, followed by 25 mL saline. Bolus-tracking software was used to trigger the arterial phase scans at 20 s after contrast enhancement of the upper abdominal aorta to an attenuation threshold of 150 HU. The enterographic phase was timed to start at 60 s after the start of contrast injection. Contrast-enhanced CT was performed using the following scanning parameters: 250 mA, 120 kVp, 0.5-s tube rotation time, and pitch 1.375. A 2.0-mm section thickness was used and images were reconstructed after every 1.5 mm.

Image evaluation

The two abdominal radiologists after a specific training analyzed the CTE in terms of localization (small bowel, colonic, both or no disease detected); type of IBD (inflammatory, stenosing, fistulizing, > 1 pattern, or normal); and signs of active disease (present or absent). Active disease was defined as the presence of ≥ 2 of the following findings: (1) mucosal hyperenhancement; (2) wall thickening with mural stratification; (3) hypervascularity of the involved mesentery (comb sign); (4) mucosal ulcerations; (5) enlarged mesenteric lymph node; and (6) mesenteric

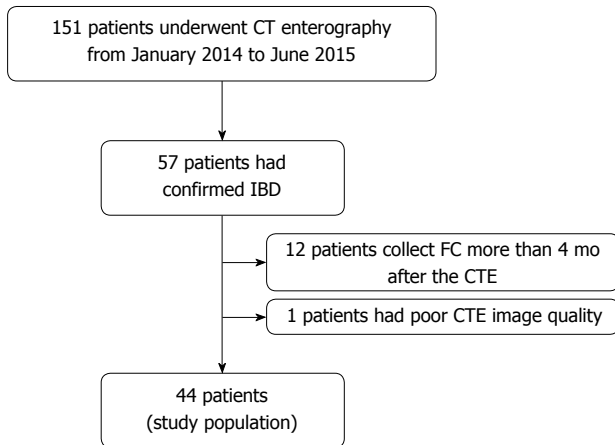


Figure 2 Selection of patients for the retrospective study with all the patients undergoing fecal calprotectin within 4 mo. CTE: Computed tomography enterography; IBD: inflammatory bowel disease.

fat stranding (Figure 1).

FC

Collected fecal samples used for FC measurements were stored and shipped on ice to Alvaro Laboratory (Cascavel, Brazil), where the FC levels were determined using a quantitative ELISA (BÜHLMANN fCAL® ELISA), using a standard method. The detection limits of this ELISA kit for FC range from 30 to 1800 µg/g. Levels above 250 µg/g were interpreted as disease activity.

Statistical analysis

The data were analyzed using the statistical program SPSS version 22.0 and MINITAB 16.0. The χ^2 test and Mann-Whitney test were used to compare variables between two groups. For all tests, $P < 0.05$ was considered statistically significant. Interobserver agreement was assessed using weighted κ with statistics. κ values were interpreted as follows: 0-0.20, slight agreement; 0.21-0.40, fair agreement; 0.41-0.60, moderate agreement; 0.61-0.80, substantial agreement; and 0.81-1.0, almost perfect agreement. The statistical methods of this study were reviewed by Mr. Valdecir Marvulle.

RESULTS

One hundred and fifty-one patients underwent CTE during the selected period. Fifty-seven patients had confirmed IBD. We excluded 13 patients: 12 from whom FC was measured > 4 mo after CTE and one patient who had poor CTE image quality. The final study population consisted of 44 patients (Figure 2). The median interval between CTE and FC measurement was 58.7 (range: 0-120) d.

Among 44 patients, 25 were women (56.8%), with a mean \pm SD age of 49 ± 25.4 years, with Crohn's disease (CD) ($n = 38$) and ulcerative colitis (UC) ($n = 6$).

Table 1 Patient characteristics ($n = 44$) n (%)

Variable	Value
Sex	
Male	19 (43.2)
Female	25 (56.8)
Age at CTE (yr)	49.0 ± 25.4
IBD	
Crohn's disease	38 (86.3)
Ulcerative colitis	6 (13.7)
Fecal calprotectin	
Minimum	30
Maximum	1800
Mean \pm SD	496 ± 706
> 250 µg/g (%)	30 (68.2)

CTE: Computed tomography enterography; IBD: Inflammatory bowel disease.

Table 2 Interobserver agreement in computed tomography enterography ($n = 44$)

CTE variables		Reader 2					% ¹	κ value	P value
Disease localization									
Reader 1	SB	C	B	ND	Total	65.9	0.54	< 0.001	
SB	10	0	8	0	18				
C	0	3	0	1	4				
B	0	0	9	0	9				
ND	2	4	0	7	13				
Total	12	7	17	8	44				
Type of IBD									
Reader 1	I	S	F	M	N	Total	54.5	0.41	< 0.001
I	9	1	0	2	1	13			
S	1	6	0	1	0	8			
F	0	0	1	2	0	3			
M	2	2	1	2	0	7			
N	5	0	2	0	6	13			
Total	17	9	4	7	7	44			
Signs of active disease									
Reader 1	Present		Absent		Total	70.4	0.419	0.002	
Present	19		2		21				
Absent	11		12		23				
Total	30		14		44				

¹Percentage of agreement. B: Both; C: Colon; CTE: Computed tomography enterography; F: Fistulizing; I: Inflammatory; IBD: Inflammatory bowel disease; M: More than one pattern; N: Normal; ND: No disease detected; S: Stenosing; SB: Small bowel.

Thirty patients (68.2%) had elevated FC (> 250 µg/g), and the mean FC value was 496 ± 706.31 µg/g (Table 1).

Localization of the disease was defined by Reader 1 in the small bowel in 18 patients (40.9%), four (9.1%) in the colon, nine (20.5%) in both, and no disease in 13 (29.5%). By Reader 2, the classification was: small bowel in 12 patients (27.3%), colon in seven (15.9%), 17 (38.6%) in both, and no disease in eight (18.2%). There was a moderate interobserver agreement regarding localization of the disease ($\kappa = 0.540$) (Table 2).

Regarding the type of IBD, Reader 1 classified 13 (29.5%) patients as inflammatory, eight (18.3%) as

Table 3 Intraobserver agreement in computed tomography enterography ($n = 42$)

CTE variables		Reader 2 (2 nd)					% ¹	κ value	P value
Disease localization									
Reader	SB	C	M	ND	Total	92.8	0.902	< 0.001	
2 (RE)									
SB	12	0	3	0	15				
C	0	7	0	0	7				
B	0	0	12	0	12				
ND	0	0	0	8	8				
Total	12	7	15	8	42				
Type of IBD (<i>n</i> = 44)									
Reader	I	S	F	M	N	Total	95.1	0.937	< 0.001
2 (RE)									
I	15	0	0	0	0	15			
S	0	8	0	0	0	8			
F	0	0	3	0	0	3			
M	2	0	0	6	0	8			
N	0	0	0	0	8	8			
Total	17	8	3	6	8	42			
Signs of active disease									
Reader	Present		Absent		Total	92.9	0.83	< 0.001	
2 (RE)									
Present	28		3		31				
Absent	0		11		11				
Total	28		14		42				

¹Percentage of agreement. 2nd: Second evaluation; SB: Small bowel; B: Both; C: Colon; CTE: Computed tomography enterography; F: Fistulizing; I: Inflammatory; IBD: Inflammatory bowel disease; M: More than one pattern; N: Normal; ND: No disease detected; RE: Routine evaluation; S: Stenosing.

stenosing, three (6.8%) as fistulizing, seven (15.9%) as > 1 pattern, and 13 (29.5%) as normal. Reader 2 classified 17 (38.6%) as inflammatory, nine (20.5%) as stenosing, four (9.1%) as fistulizing, seven (15.9%) as > 1 pattern, and seven (15.9%) patients as normal. The interobserver agreement regarding the type of IBD was moderate ($\kappa = 0.410$) (Table 2).

Reader 1 classified 21 (48%) patients as having active disease and Reader 2 classified 30 (68%) (Table 2). The weighted quadratic κ value for classifying the IBD as active or not was 0.419, indicating moderate agreement (Table 2).

There was almost perfect intraobserver agreement regarding localization, type and signs of active disease in IBD. The κ values were 0.902, 0.937 and 0.830, respectively (Table 3).

After a consensus between both radiologists regarding signs of active disease in CTE, we found that 24 (85.7%) of 28 patients who were classified as having active disease had elevated FC, and six (37.5%) of 16 patients without inflammatory activity in CTE had elevated FC ($P = 0.003$). The correlation between elevation of FC (> 250 $\mu\text{g/g}$) and presence of active disease in CTE was significant ($\kappa = 0.495$, $P = 0.001$). As such, using a Mann-Whitney test, the FC levels were significantly higher in patients deemed as having active disease in CTE ($P = 0.004$) (Figure 3).

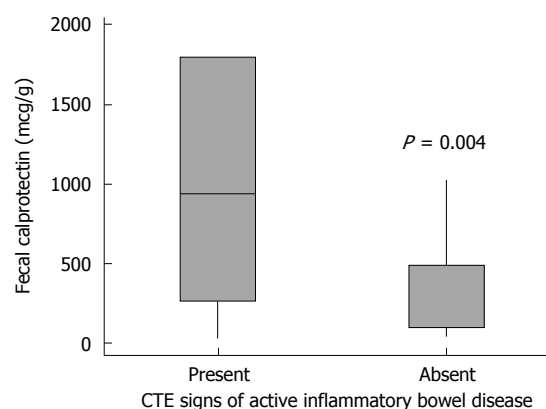


Figure 3 Fecal calprotectin levels in patients with and without signs of active inflammatory disease in computed tomography enterography. CTE: Computed tomography enterography.

DISCUSSION

Our study showed almost perfect intraobserver and moderate interobserver agreement in classifying IBD as active disease. We considered that the intraobserver was better than the interobserver agreement probably due to the greater experience in CTE of Reader 2. However, it must be considered that, despite the interval of ≥ 6 mo from routine evaluation to the second one, and previous anonymization of the patients' data, a recall bias might have occurred.

A few studies evaluated that the interobserver agreement for each CTE finding of active inflammation resulted in a moderate to substantial concordance, with κ values ranging from 0.43 to 0.83^[7,10]. Their interobserver agreement was higher for mural hyperenhancement^[10]. However, in clinical practice, the final interpretation of the radiologists usually is more relevant than the presence of each imaging feature alone.

Siddiki *et al.*^[11] evaluated the interobserver agreement regarding the final interpretation of the radiologists as active or inactive, which is similar to our study, and demonstrated a substantial interobserver agreement ($\kappa = 0.76$). One possible reason for a higher interobserver agreement is the fact that they classified the patients into four groups (definitely active, suspicious, inactive and absent) and then the suspicious subtype was considered as active for statistical analysis, which may have improved the concordance.

In contrast, we found almost perfect intraobserver agreement regarding localization, type, and inflammatory activity. To the best of our knowledge, this is the first study to evaluate intraobserver agreement in CTE, but there have been a few evaluations of other imaging modalities. De Franco *et al.*^[12] showed a substantial intraobserver agreement ($\kappa = 0.71$) in contrast-enhanced ultrasound parameters of active disease in patients with CD in the terminal ileum. Another MR enteroclysis study showed high

intraobserver agreement in the evaluation of each active criterion alone (κ ranged from 0.61 to 1.0)^[13].

The differences in interobserver agreement in our study in comparison with others may reflect the difference in CTE experience of the two radiologists, which, moreover, reflect the reality of most hospitals. This reinforces the need for objective and structured reports, such as magnetic resonance index of activity (MaRIA) used in MRE, which can improve the reproducibility of the reports, mainly between radiologists with different levels of experience in CTE^[14]. Moreover, the better intraobserver agreement strengthens the need for a multidisciplinary team with experience in IBD in all specialties, including radiology. IBD is a complex condition, with a high morbidity, in which the patients benefit from being treated in a reference hospital by an engaged team with reproducible results.

After a consensus between the radiologists, we found a significant correlation between active inflammatory disease on CTE and high levels of FC ($\kappa = 0.495$, $P = 0.001$). Our findings are in line with those of prior studies that demonstrated good correlation between high levels of FC with endoscopic scores and CTE^[15,16]. Arai *et al.*^[16] evaluated the correlation between FC, CTE and balloon-assisted enteroscopy in patients with IBD. The authors created a novel CTE score in which four imaging variables were evaluated in five predefined ileal-colonic segments, and each variable was scored from 0 to 4 per segment. The authors showed that the FC levels were well correlated with CTE score ($r = 0.4018$, $P = 0.0011$).

We also found that 85.7% of the patients who were classified as having active disease had elevated FC, opposed to 37.5% of patients without active inflammation on CTE who had elevated FC. FC is a biomarker that reflects intestinal mucosal damage, and using a cut-off point of 250 $\mu\text{g/g}$, as in our study, the sensitivity and specificity of detecting active inflammation in IBD are about 80%, when compared with endoscopy^[15-17]. However, some other studies have shown that FC presents a better sensitivity than specificity, which could explain the false-positive results^[4,18-20]. Furthermore, the best area under the curve was demonstrated in studies that correlated low FC levels with inactive disease^[21,22]. Additionally, other authors have shown that an increase in FC levels may precede the onset of inflammation^[23], but we did not follow-up the patients. The combination of these factors may have influenced these discordant results.

There were several potential limitations to our study. First, the small sample size and retrospective nature of the study, not allowing FC measurement and CTE to be performed on the same day. In addition, there was no correlation with the standard reference values, such as endoscopic or histological findings, and the interobserver agreement was only evaluated by one reader. Finally, we did not perform a follow-up of the patients with no inflammatory signs on CTE and

high FC levels. Therefore, further prospective studies with larger patient populations, with multireader evaluation and with other correlations (*e.g.*, laboratory, endoscopic and histological analysis) are needed to evaluate the role of each marker in the evaluation of patients with IBD.

In conclusion, we found almost perfect intraobserver and moderate interobserver agreement in the characterization of signs of active disease in CTE in concurrence with high FC levels in patients with IBD.

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COMMENTS

Background

The evaluation of active inflammation in inflammatory bowel disease (IBD) patients is not a simple task and demands multidisciplinary evaluation; being an important tool in patient management. Computed tomography enterography (CTE) provides visualization of the entire gastrointestinal tract, enabling the characterization of disease activity in IBD.

Research frontiers

A few studies have evaluated the interobserver agreement in CTE findings of active inflammation in IBD patients. However, the intraobserver agreement was only evaluated for other imaging modalities. In the present study, we aimed to evaluate the inter- and intraobserver agreement in the characterization of signs of active disease in CTE in comparison with fecal calprotectin (FC) levels.

Innovations and breakthroughs

This study evaluated for the first time intraobserver agreement in CTE signs of active IBD and their correlation with FC levels. The authors found almost perfect intraobserver agreement in the characterization of signs of active disease in CTE and significant correlation between active signs in CTE and high levels of FC.

Applications

This study strengthens the importance of CTE and FC in the evaluation of patients with IBD and reinforces the need for a multidisciplinary team with experience in IBD in all specialties, including radiology.

Terminology

The presence of active inflammatory disease plays a key role in tailored treatment planning in patients with IBD and CTE and FC which are important methods for evaluating IBD.

Peer-review

This is an interesting study. CTE is becoming a diagnostic modality for IBD recently due to its easy accessibility, especially for Crohn's disease. FC is confirmed correlation to the mucosal inflammation of the IBD.

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Retrospective Study

Heparin bridge therapy and post-polypectomy bleeding

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Author contributions: Kubo T designed the study and collected and analyzed the data; Kubo T and Yamashita K wrote the manuscript; Onodera K and Iida T provided analytical oversight; Arimura Y supervised the study; Nojima M conducted statistical analysis; Nakase H revised the manuscript for important intellectual content; all authors read and approved the final version.

Institutional review board statement: The Institutional Review Board of Sapporo Medical University approved this study.

Informed consent statement: All participants in this study provided their verbal informed consent prior to study enrollment.

Conflict-of-interest statement: The authors disclose no conflicts of interest.

Data sharing statement: The technical appendix, statistical code and dataset are available from the corresponding author at kubo-t@grape.plala.or.jp. Participants gave informed consent for data sharing.

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Abstract

AIM

To identify risk factors for post-polypectomy bleeding (PPB), focusing on antithrombotic agents.

METHODS

This was a case-control study based on medical records at a single center. PPB was defined as bleeding that occurred 6 h to 10 d after colonoscopic polypectomy and required endoscopic hemostasis. As risk factors for PPB, patient-related factors including anticoagulants, antiplatelets and heparin bridge therapy as well as polyp- and procedure-related factors were evaluated. All colonoscopic hot polypectomies, endoscopic mucosal resections and endoscopic submucosal dissections performed between January 2011 and December 2014 were reviewed.

RESULTS

PPB occurred in 29 (3.7%) of 788 polypectomies performed during the study period. Antiplatelet or anticoagulant agents were prescribed for 210 (26.6%)

patients and were ceased before polypectomy except for aspirin and cilostazol in 19 cases. Bridging therapy using intravenous unfractionated heparin was adopted for 73 patients. The univariate analysis revealed that anticoagulants, heparin bridge, and anticoagulants plus heparin bridge were significantly associated with PPB ($P < 0.0001$) whereas antiplatelets and antiplatelets plus heparin were not. None of the other factors including age, gender, location, size, shape, number of resected polyps, prophylactic clipping and resection method were correlated with PPB. The multivariate analysis demonstrated that anticoagulants and anticoagulants plus heparin bridge therapy were significant risk factors for PPB ($P < 0.0001$). Of the 29 PPB cases, 4 required transfusions and none required surgery. A thromboembolic event occurred in a patient who took anticoagulant.

CONCLUSION

Patients taking anticoagulants have an increased risk of PPB, even if the anticoagulants are interrupted before polypectomy. Heparin-bridge therapy might be responsible for the increased PPB in patients taking anticoagulants.

Key words: Post-polypectomy bleeding; Heparin bridge therapy; Colonic polypectomy; Anticoagulants; Antiplatelets; Endoscopic surgery

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Core tip: Post-polypectomy bleeding (PPB) is the most common complication of colon polypectomy. In this study, we demonstrated that patients taking anticoagulants have an increased risk of PPB, even if the anticoagulants are interrupted before polypectomy. Heparin-bridge therapy might be responsible for the increased PPB in patients who take anticoagulants.

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INTRODUCTION

Colorectal cancer (CRC) is the third most common cancer and ranks fourth as a cause of death worldwide^[1]. Endoscopic polypectomy is a safe and useful procedure to prevent CRC, reducing the CRC morbidity by 70%-80%^[2,3]. Post-polypectomy bleeding (PPB) is the most common complication of endoscopic polypectomy with reported incidences ranging from 0.65% to 8.6%^[4-6]. Risk factors for PPB include larger polyp size, right colon, pedunculated

type and anticoagulants^[6-9], although these are still controversial. Major guidelines recommend cessation of anticoagulants before polypectomy and heparin bridge therapy for high thrombotic risk cases^[10-12]. Nevertheless, a study demonstrated that the incidence of PPB was higher in patients taking anticoagulants, even if they were interrupted^[13]. Recently, another study suggested that heparin bridge therapy might be associated with a higher PPB rate in patients taking anticoagulants^[14]. Studies, including a meta-analysis, suggest that bridging therapy might be associated with high bleeding risk after invasive procedures including polypectomy in patients taking anticoagulants^[15,16]. A randomized double-blind placebo-controlled trial demonstrated that bleeding risk was higher in patients taking bridging therapy than in those without bridging and that thromboembolic risk was similar in both groups^[17]. The aim of this study was to elucidate the risk factors for PPB including antithrombotic agents and heparin bridge therapy.

MATERIALS AND METHODS

This is a case-control study based on medical records at Sapporo Medical University Hospital. All colonoscopic polypectomies, endoscopic mucosal resection (EMR) and endoscopic submucosal dissection (ESD) performed between January 2011 and December 2014 were included. Patient-, polyp- and procedure- related factors were obtained from the database. The patient-related factors included age, gender, comorbidity, antithrombotic agents (antiplatelet and anticoagulant). The polyp-related factors included location (right colon: cecum, ascending colon, and transverse colon; left colon: descending colon, sigmoid colon, and rectum), size, shape and number of resected polyp. The procedure-related factors were prophylactic clipping and resection method (polypectomy, EMR or ESD). PPB was defined as bleeding that occurred 6 h to 10 d after colonoscopic polypectomy and required endoscopic hemostasis. For such cases, a second-look colonoscopy was performed to identify the origin of the bleeding and endoscopic hemostasis was performed immediately.

The management of antithrombotic agents was based on the Japanese Gastroenterological Endoscopy Society guidelines published in 2005^[18]. All anticoagulants and antiplatelets were ceased before polypectomy except in high thrombotic risk cases. Aspirin and thienopyridines (ticlopidine and clopidogrel) were stopped 5-7 d before polypectomy and other antiplatelets such as cilostazol, dipyridamole or beraprost were ceased 24 to 48 h before the procedure. The anticoagulants used during the study period were warfarin, dabigatran and rivaroxaban. Warfarin was ceased 4-5 d before polypectomy and dabigatran and rivaroxaban were stopped 24 to 48 h prior to the procedure. All antiplatelets and anticoagulants were resumed 24 to 48 h from polypectomy. For high

Table 1 Prescription of antithrombotic agents

Anticoagulants	<i>n</i>
Warfarin	68
Dabigatran	12
Rivaroxaban	3
Antiplatelets	
Aspirin	93
Clopidogrel	35
Cilostazol	31
Ticlopidine	12
Others	28
Anticoagulants + antiplatelets	28
Dual antiplatelets	59
Triple antiplatelets	8
Heparin bridge	73

thrombotic risk patients, intravenous unfractionated heparin (UFH) was administered after ceasing anti-coagulants or antiplatelets. UFH was started 2-3 d before polypectomy at 10000 to 15000 U/d, which was adjusted by monitoring APTT. The UFH was stopped 4-6 h prior to polypectomy and resumed 2-6 h after the procedure.

The instrument used for polypectomy and EMR was a SnareMaster (Olympus medical, Tokyo, Japan) and normal saline was injected for EMR. The instruments used for ESD were a Hook knife (Olympus medical, Tokyo, Japan) or Flush Knife BT (Fujifilm, Tokyo, Japan). Glyceol® (Chugai Pharmaceutical Co., Ltd.) and hyaluronic acid was used for submucosal injection in ESD. An electrosurgical unit (VIO 300D; ERBE, Tübingen, Germany) was set according to the manufacturer's instructions and a mixed current was used for resection. As cold polypectomy was not adopted during the study period, all the procedures including polypectomy, EMR and ESD were performed using electrocautery (hot). PPB was treated endoscopically using soft coagulation, hemoclipping, or epinephrine injection.

Student's *t*-test was used for continuous variables and chi-square test or Fisher's exact test was used for categorical variables. First, a univariate analysis was performed for all possible risk factors. The significant variables were taken as potential risk factors and were included in the multivariate logistic regression model. All *P* values were two-sided and the results were considered significant when *P* values were < 0.05.

RESULTS

A total of 788 patients underwent polypectomy during the study period. Antithrombotic agents were prescribed to 210 (26.6%) patients; anticoagulants to 83 (10.5%), antiplatelets to 154 (19.5%), both to 28 (3.6%), dual antiplatelet agents to 59 (7.5%) and triple antiplatelet agents to 8 (1.0%) patients. Bridging therapy using intravenous UFH was adopted for 73 patients (9.3%) (Table 1). All anticoagulants and antiplatelets were ceased before polypectomy except

Table 2 Characteristics of the study cohort and polyps *n* (%)

		No bleeding	Bleeding	<i>P</i> value
No. of patients		759 (96.3)	29 (3.7)	
Age (yr, mean)		64 ± 14.3	62 ± 16.0	0.50
Gender	Male	458 (98.1)	9 (1.9)	0.35
	Female	301 (93.8)	20 (6.2)	
Polyp location	Right	239 (95.6)	11 (4.4)	0.93
	Left	336 (95.5)	16 (4.5)	
Polyp size	≥ 10 mm	338 (96.8)	11 (3.2)	0.43
	< 10 mm	361 (95.8)	16 (4.2)	
Polyp shape	Flat	266 (96.7)	9 (3.3)	0.73
	Sessile	416 (96.5)	15 (3.5)	
	Pedunculated	118 (95.2)	6 (4.8)	
No. of polyps resected	1	343 (96.6)	12 (3.4)	0.69
	≥ 2	416 (96.1)	17 (3.9)	
Prophylactic clipping	Yes	566 (95.8)	25 (4.2)	0.16
	No	193 (98.0)	4 (2.0)	
Resection method	Polypectomy or EMR	703 (96.6)	25 (3.4)	0.20
	ESD	56 (93.3)	4 (6.7)	
Antiplatelets	Yes	146 (94.8)	8 (5.2)	0.27
	No	613 (96.7)	21 (3.3)	
Anticoagulants	Yes	72 (86.7)	11 (13.3)	< 0.001
	No	687 (97.4)	18 (2.6)	
Heparin bridge	Yes	63 (86.3)	10 (13.7)	< 0.001
	No	696 (97.3)	19 (2.7)	
Antiplatelets + heparin bridge	Yes	32 (91.4)	3 (8.6)	0.11
	No	727 (96.5)	26 (3.5)	
Anticoagulants + heparin bridge	Yes	47 (82.5)	10 (17.5)	< 0.001
	No	712 (97.4)	19 (2.6)	

PPB: Post-polypectomy bleeding.

Table 3 Multivariate analysis of risk factors for Post-polypectomy bleeding

Variable	Odds ratio	95%CI	<i>P</i> value
Anticoagulants	4.227	1.126-15.872	0.033
Heparin bridge therapy	2.172	0.556-8.482	0.265
Anticoagulants+ heparin bridge	9.796	3.771-25.443	< 0.001

PPB: Post-polypectomy bleeding.

for aspirin or cilostazol in 19 cases. PPB occurred in 29 (3.7%) of 788 polypectomies performed. Four PPB patients required transfusion and none required surgery. None of the following were correlated with PPB: age, gender, polyp location, polyp size, polyp shape (flat vs sessile vs pedunculated), number of polyps resected, prophylactic clipping, resection method (polypectomy or EMR vs ESD), antiplatelets and antiplatelet plus heparin bridge therapy (Table 2). Anticoagulants, heparin bridge therapy, and anticoagulants plus heparin bridge therapy (meaning that anticoagulants were substituted by heparin before polypectomy) were significantly associated with PPB (Table 2).

The multivariate logistic regression analysis revealed that anticoagulants and anticoagulants plus heparin bridge therapy were independent risk factors for PPB whereas heparin bridge therapy alone was not (Table 3). The odds ratios of anticoagulants and anticoagulants

Table 4 Summary of 11 post-polypectomy bleeding cases taking anticoagulants

	Anticoagu-lants	Antiplatelets	Heparin bridge	Onset of PPB (POD)	PT-INR at PPB	Anticoagulants at PPB	APTT at PPB	Heparin at PPB
1	Warfarin	Clopidogrel	Yes	5	1.62	Yes	93.1	Yes
2	Warfarin	-	Yes	2	1.31	Yes	42.8	Yes
3	Warfarin	-	Yes	6	1.26	Yes	32.2	No
4	Warfarin	Aspirin	Yes	2, 5	1.20	Yes	29.4	Yes
5	Warfarin	-	Yes	5	1.25	Yes	73.1	Yes
6	Dabigatran	-	No	2	2.03	-	-	-
7	Warfarin	-	Yes	1, 2	1.31	No	45.2	Yes
8	Dabigatran	-	Yes	6	1.40	-	50.1	No
9	Warfarin	-	Yes	1, 6	1.23	No	33.5	Yes
10	Warfarin	Aspirin (Continued)	Yes	2	1.32	Yes	40.4	Yes
11	Warfarin	-	Yes	1	1.21	Yes	29.5	Yes

PPB: Post-polypectomy bleeding; POD: Post-operative day.

plus heparin were 4.2 (95%CI: 1.126-15.87, $P = 0.033$) and 9.8 (95%CI: 3.771-25.443, $P < 0.001$), respectively.

Eleven PPB cases that took anticoagulants are summarized in Table 4. Seven patients had atrial fibrillation, seven had valvular heart disease and one had cerebrovascular disease. Warfarin, dabigatran and antiplatelets were prescribed to 9, 2 and 3 patients, respectively. Anticoagulants and antiplatelets were ceased before polypectomy in all cases and heparin bridge therapy was carried out for 10 of 11 patients. Bleeding occurred 1 to 6 d after polypectomy. All PPB were successfully treated by endoscopy but re-bleeding occurred in 3 cases. Seven patients resumed anticoagulants before PPB but the PT-INR at PPB were within therapeutic range. Eight patients were still on heparin at PPB and APTT at PPB were elevated in 2 patients. A thromboembolic event occurred in a patient after ceasing anticoagulant treatment.

DISCUSSION

Our study demonstrated that anticoagulants and anticoagulants plus heparin bridge therapy might be independent risk factors for PPB despite periprocedural interruption. Several studies demonstrated a close correlation between PPB and anticoagulants^[5,13,14,19-21]. Sawhney *et al*^[5] demonstrated that resuming anticoagulants following polypectomy was strongly associated with severe delayed PPB. Witt *et al*^[13] also suggested the incidence of PPB was higher in patients receiving anticoagulation therapy, even though warfarin was interrupted for the procedure.

It has been recently suggested that heparin bridge therapy might be associated with PPB after ceasing antithrombotic agents^[15]. Inoue *et al*^[14] demonstrated that the incidence of PPB was significantly higher in a heparin bridge group than in a non-heparin bridge group (20.0% vs 1.4%, respectively). Ishigami *et al*^[22] also demonstrated that heparin-bridging therapy is associated with a high risk of PPB regardless of polyp size.

A meta-analysis^[15] and large-scale studies^[16,17] also suggest that heparin bridge therapy might increase bleeding after invasive procedures including polypectomy in patients taking anticoagulants. Notably, a randomized double-blind placebo-controlled trial demonstrated that the incidence of major bleeding was higher in a bridging group than in a no-bridging group whereas the incidence of arterial thromboembolism was similar in both groups (the BRIDGE trial)^[17].

Our study also demonstrated that anticoagulants and anticoagulants plus heparin-bridge therapy were independent risk factors for PPB. Anticoagulants were interrupted in all cases and PT-INR at PPB was below the therapeutic range in most cases. Of 11 PPB cases using anticoagulants, 10 underwent heparin bridge therapy and 8 were on heparin at the time of PPB. Heparin bridge therapy might be responsible for PPB in patients taking anticoagulants, though APTT at PPB was elevated in only 2 cases. Heparin might have a synergic effect with anticoagulants, which is not measurable using APTT or PT-INR.

Interestingly, antiplatelets plus heparin was not associated with PPB in our study. Previous studies demonstrated that aspirin is not a risk factor for PPB in conventional polypectomy^[19,20,23-25]. Younsfi *et al*^[23] demonstrated that there was no statistically relevant difference in prior aspirin use before polypectomy in a bleeding group and matched controls. Manocha *et al*^[25] demonstrated PPB rates of patients on aspirin and NSAIDs vs those not on aspirin or NSAIDs (3.2% vs 3.0%). In contrast, polypectomy on clopidogrel is likely to have increased risk for PPB^[8]. It might be prudent to postpone polypectomy for high thrombotic risk patients taking clopidogrel.

These results might reflect the mechanism of hemostasis: anticoagulants work on the secondary hemostasis process such as manufacturing of fibrin, while antiplatelet agents work on the primary hemostasis such as the cohesion of platelets. As the secondary hemostasis is stronger than the primary, anticoagulants including heparin might cause PPB more frequently than antiplatelets^[21].

The present study had several limitations. First, this study was a retrospective study conducted at a single institution. The second limitation was the small sample size. As PPB is a rare complication with incidences ranging from 0.65% to 8.6%^[4-6], the small sample size of our study might have led to the ambiguous conclusion. Despite these limitations, we believe that the results of this study may have important implications for clinical practice. A further study on a larger scale will be needed.

In conclusion, patients taking anticoagulants have an increased risk of PPB, even if anticoagulants are interrupted before polypectomy. Heparin-bridge therapy might be responsible for the increased PPB in patients taking anticoagulants. A prospective study to compare bridging with no bridging at polypectomy is warranted.

COMMENTS

Background

Post-polypectomy bleeding (PPB) is the most common adverse event of colonoscopic polypectomy. Past studies demonstrated risk factors for PPB but it is still controversial whether antithrombotic agents are associated with PPB. Major guidelines recommend ceasing anticoagulants before polypectomy and substituting by heparin (heparin-bridge) in high thrombotic risk cases.

Research frontiers

Recent studies suggest that heparin-bridge might increase bleeding after invasive procedure including polypectomy.

Innovations and breakthroughs

This study demonstrated that PPB increased in patients taking anticoagulants, despite they were ceased before polypectomy according to the guidelines. From the study results, the authors speculated that heparin-bridge might be responsible for PPB in patients taking anticoagulants.

Applications

When ceasing anticoagulants before polypectomy, no bridging might be better than heparin-bridge to reduce PPB. Prospective study is necessary to compare incidence of PPB as well as thrombotic events between 2 groups with and without heparin-bridge.

Terminology

In this study, PPB was defined as bleeding that occurred 6 h to 10 d after polypectomy and required endoscopic hemostasis.

Peer-review

The authors showed that the PPB was associated with heparin bridging therapy. Patients who took antiplatelets during heparin bridging therapy showed the high incidence of PPB. This study is new evidence about PPB.

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Retrospective Study

Clinical implications of doubling time of gastrointestinal submucosal tumors

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Informed consent statement: Patients were not required to give informed consent to the study because the analysis used anonymous clinical data that were obtained after each patient agreed to treatment by written consent. For full disclosure, the details of the study are published on the home page of Kitasato University School of Medicine.

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Abstract

AIM

To evaluate the efficacy of doubling time (DT) of gastrointestinal submucosal tumors (GIST).

METHODS

From April 1987 through November 2012, a total of 323 patients were given a final histopathological diagnosis of GISTs on surgical resection or endoscopic ultrasound-guided fine-needle aspiration (EUS-FNA) in Kitasato University East Hospital or Kitasato University Hospital. We studied 53 of these patients (34 with resected tumors and 19 with unresected tumors) whose tumors could be measured on EUS on at least two successive occasions. The histopathological diagnosis was GIST in 34 patients, leiomyoma in 5, schwannoma in 3, ectopic pancreas in 1, hamartoma in 1, cyst in 1, Brunner's adenoma in 1, and spindle-cell tumor in 7. We retrospectively calculated the DT of GISTs on the basis of the time course of EUS findings to estimate the growth rate of such tumors.

RESULTS

The DT was 17.2 mo for GIST, as compared with 231.2 mo for leiomyoma, 104.7 mo for schwannoma, 274.9

mo for ectopic pancreas, 61.2 mo for hamartoma, 49.0 mo for cyst, and 134.7 mo for Brunner's adenoma. The GISTs were divided into risk classes on the basis of tumor diameters and mitotic figures (Fletcher's classification). The classification was extremely low risk or low risk in 28 patients, intermediate risk in 3, and high risk in 3. DT of GIST according to risk was 24.0 mo for extremely low-risk plus low-risk GIST, 17.1 mo for intermediate-risk GIST, and 3.9 mo for high-risk GIST. DT of GIST was significantly shorter than that of leiomyoma plus schwannoma ($P < 0.05$), and DT of high-risk GIST was significantly shorter than that of extremely low-risk plus low-risk GIST ($P < 0.05$).

CONCLUSION

For GIST, a higher risk grade was associated with a significantly shorter DT. Small SMTs should initially be followed up within 6 mo after detection.

Key words: Gastrointestinal submucosal tumor; Doubling time; Submucosal tumor; Initial observational duration; Endoscopic ultrasonography; Endoscopic ultrasonography-guided fine needle aspiration; Fletcher's classification

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Core tip: The doubling time (DT) differed according to the type of submucosal tumors (SMTs), and gastrointestinal submucosal tumors (GISTs) were confirmed to have a significantly shorter doubling time than the other types of tumors. DT was 17.2 mo for GIST, as compared with 231.2 for leiomyoma, 104.7 for schwannoma. DT of GIST was significantly shorter than that of leiomyoma plus schwannoma ($P < 0.05$), and DT of high-risk GIST (3.9 mo) was significantly shorter than that of extremely low-risk plus low-risk GIST (24.0 mo) ($P < 0.05$). Even small SMTs less than 2 cm in diameter should initially be followed up within at least 6 mo after detection.

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INTRODUCTION

In Japan, gastrointestinal submucosal tumors (SMTs) are often detected on radiographic and conventional endoscopic examinations during health checkups. SMTs are covered by mucosa, and the majority of lesions are nonepithelial tumors arising from the submucosa or muscularis propria. The presence of SMTs can be detected on radiography and conventional

endoscopy, but qualitative diagnosis remains difficult on these imaging techniques. However, progress in endoscopic ultrasonography (EUS) and other diagnostic techniques has facilitated the qualitative evaluation of SMTs^[1,2]. In the differential diagnosis of gastrointestinal stromal tumors (GISTs), considered clinically important lesions, endoscopic ultrasound-guided fine-needle aspiration (EUS-FNA) plays a major role in deciding the treatment policy and is now widely used clinically^[3,4]. However, there remains room for improvement in the diagnostic performance of EUS-FNA for small lesions. Consequently, small SMTs yet to be definitively diagnosed are generally followed up once or twice per year^[5]. To our knowledge, very few studies have evaluated the doubling time of SMTs, an index of the rate of tumor growth, according to diagnosis. We estimated the doubling time of different types of SMTs and report our findings.

MATERIALS AND METHODS

Patients

From April 1987 through November 2012, a total of 323 patients were given a final histopathological diagnosis of gastrointestinal SMT on surgical resection or EUS-FNA in our hospital. We studied 53 of these patients (34 with resected tumors and 19 with unresected tumors) whose tumors could be measured on EUS on at least two successive occasions. Tumor-doubling time was estimated retrospectively. All examinations were carried out by endoscopists adequately experienced in EUS. Informed consent was obtained from each patient prior to the procedure. Regardless of the result, the good clinical practice was provided with consent of the patient. The longest and shortest tumor diameters were measured within the depicted range. The follow-up period was defined as the time between initial EUS and final EUS.

Endoscope

Radial scanning echoendoscopes (GF-UM20, GF-UM240, GF-UM2000, UM-DP20, and UM-DP12; Olympus Co., Tokyo, Japan) were used to perform EUS. EUS-FNA was performed with the use of convex array echoendoscopes (GF-UCT260, GF-UCT240, XGF-UCT160, GF-UC2000P; Olympus Co., Ltd.). The following puncture needles were used: 19-gauge needles (Wilson-Cook, Winston Salem, NC, United States), 22-gauge needles (NA-200H, Olympus Co., Ltd.), and 25-gauge needles (Echochip, Wilson-Cook). The aspiration pressure was 10 to 20 cc, and "in-and-out motion" was continued for 20 strokes (occasionally, 10 strokes). Puncture was performed 2 to 6 time (median, 3 times).

Measurement methods

Three-dimensional EUS is more accurate than 2-dimensional EUS for the measurement of tumor

Table 1 Demographic characteristics of all 53 patients

Characteristic	Value
Sex, No.	male/female: 26/27
Age, median (range), yr	63.0 (31-83)
Tumor location, No.	Esophagus/stomach/duodenum: 4/47/2

Table 2 Histopathological diagnosis

	Esophagus (n = 4)	Stomach (n = 47)	Duodenum (n = 2)	Total (n = 53)
GIST	4	45	0	49
Leiomyoma	0	34	0	34
Schwannoma	2	3	0	5
Spindle-cell tumor	1	2	0	3
Ectopic pancreas	1	6	0	7
Hamartoma	0	1	0	1
Intramural developmental cyst	0	0	1	1
Brunner's adenoma	0	1	0	1

GIST: Gastrointestinal submucosal tumors.

volume. However, commercially available three-dimensional echoendoscopes are probe type, making it difficult to measure large lesions. In the present study, we therefore measured the longest and shortest diameters within the range depicted on two-dimensional EUS. Few SMTs show a completely spherical growth pattern, and many grow in an oval fashion. In this study, however, we assumed that the tumors were spherical and used the mean value of the longest and shortest diameters as the tumor diameter to calculate tumor volume. The following equation was used to calculate doubling time: tumor growth rate (%) = $(V_1 - V_0) / V_0 \times 100$, in which V_0 is the tumor volume (mm^3) at baseline EUS ($\pi d_0^3/6$), V_1 is the tumor volume (mm^3) at the second or subsequent sessions of EUS ($\pi d_1^3/6$), d_0 is the tumor diameter (mm) at baseline EUS, and d_1 is the tumor diameter (mm) at the second or subsequent sessions of EUS.

The time courses of tumor growth rates were plotted on scattergrams with trend lines. The point at which the tumor growth rate became 100% was defined as the doubling time.

Definition of diagnosis

On immunostaining of specimens obtained by surgical resection or FNA, tumors that stained positive for CD34 or KIT were diagnosed as GIST. Leiomyomas were diagnosed if immunostaining was positive for smooth-muscle antibodies (SMA) and negative for CD34 and KIT. Schwannomas were diagnosed if the tumor stained positive for S-100 and negative for CD34 and KIT. Spindle-cell tumors were diagnosed if spindle-shaped cells were confirmed on hematoxylin-eosin staining, but immunostaining was precluded by an inadequate sample size on FNA. GISTs were classified

as extremely low risk, low risk, intermediate risk, and high risk on the basis of actual tumor diameters and mitotic figures in patients with resected tumors. In patients with unresected tumors, risk class was based on tumor diameter measured on EUS and mitotic figures.

Statistical analysis

For statistical analysis, the Mann-Whitney *U* test was used to compare doubling times. *P* values of less than 0.05 were considered to indicate statistical significance. SPSS statistical software, version 17.0 was used for statistical analysis.

RESULTS

The study group comprised 26 men and 27 women, with a median age of 63.0 years (range, 31 to 83). The tumor was located in the esophagus in 4 patients, the stomach in 47, and the duodenum in 2 (Table 1). The histopathological diagnosis was GIST in 34 patients, leiomyoma in 5, schwannoma in 3, ectopic pancreas in 1, hamartoma in 1, cyst in 1, Brunner's adenoma in 1, and spindle-cell tumor in 7 (Table 2). The median follow-up in the study group as a whole was 31.7 mo (range, 6.6 to 210). The median number of EUS procedures performed during follow-up was 3 (range, 2 to 13). The median tumor diameter (mean of the longest and shortest diameters) was 19.1 mm (range, 10 to 44.8 mm) on initial EUS and 25.3 mm (range, 13 to 52.1 mm) on EUS before tumor resection or EUS-FNA for final diagnosis (Table 3).

Tumor resection was performed in 29 of the 34 patients with GIST. Among the 5 patients with unresectable tumors, surgery was precluded by poor general condition due to other diseases (neurologic diseases) in 2 patients, follow-up observation was requested by 1 patient, and 2 patients dropped out of the study. Of the 5 patients with leiomyoma, 1 underwent resection, and 4 were followed up. Of the 3 patients with schwannoma, 2 underwent resection, and 1 was followed up. The patient with ectopic pancreas and the patient with Brunner's adenoma were followed up. Among the 7 patients with spindle-cell tumors, 5 were followed up, and 2 dropped out of the study.

In the patents with resected tumors and those with unresected tumors, the median follow-up was 24.9 mo and 36.5 mo, the median number of EUS sessions during follow-up was 3 and 4, the median tumor diameter at initial EUS was 19.5 and 19.0 mm, and the median tumor diameter on EUS before surgery or EUS-FNA was 28.0 and 22.8 mm, respectively. In patients with resected tumors, the median interval from final EUS to surgery was 3.8 mo (range, 22 d to 16.3 mo). The median longest tumor diameter of the resected specimens was 35 mm (range, 20 to 60 mm) (Table 3). None of the patients who underwent follow-

Table 3 Details of 53 patients

	Resected tumors (<i>n</i> = 34)	Unresected tumors (<i>n</i> = 19)	Total (<i>n</i> = 53)	GIST (<i>n</i> = 34)
Follow-up period, median (range), mo	24.9 (6.6-210)	36.5 (11.2-183.6)	31.7 (6.6-210)	27.3 (6.6-210)
EUS sessions, median (range)	3 (2-8)	4 (2-13)	3 (2-13)	3 (2-11)
Tumor diameter at initial EUS, median (range), mm	19.5 (10-30)	19.0 (11.5-44.8)	19.1 (10-44.8)	19.0 (10.9-44.8)
Tumor diameter before surgery or FNA, median (range), mm	28.0 (20-43.1)	22.8 (15.2-52.1)	25.3 (13.7-52.1)	26.7 (13.7-52.1)
Time from the final EUS to surgery, median (range)	3.8 mo (22d-16.3 mo)	-	-	-

GIST: Gastrointestinal submucosal tumors; EUS: Endoscopic ultrasound; FNA: Fine-needle aspiration.

Table 4 Details of 34 patients with gastrointestinal submucosal tumors

	Extremely low plus low risk (<i>n</i> = 28)	Intermediate risk (<i>n</i> = 3)	High risk (<i>n</i> = 3)	Total (<i>n</i> = 34)
Follow-up period, median (range), mo	31.0 (6.6-210)	47.3 (11.2-49.9)	12.4 (7.4-16.7)	27.3 (6.6-210)
Initial tumor diameter, median (range), mm	18.6 (10.9-30.0)	28.5 (20.0-44.8)	25.5 (14.0-27.3)	19.0 (10.9-44.8)
Doubling time, median (range), mo	24.0 (2.0-183.6)	17.1 (6.1-19.4)	3.9 (0.8-10.4)	17.2 (0.8-183.6)

Risk classification: Based on tumor diameter and mitotic figures.

Table 5 Tumors other than gastrointestinal submucosal tumors

	No. of patient	Follow-up period, median (range), mo	Doubling time, median (range), mo
Leiomyoma	5	47.1 (10.7-137.2)	231.2 (21.3-1303.8)
Schwannoma	3	50.1 (24.3-71.7)	104.7 (3.9-305.4)
Ectopic pancreas	1	66.5	274.9
Hamartoma	1	99.6	61.2
Intramural developmental cyst	1	29.5	49.0
Brunner's adenoma	1	30.6	134.7

up observation or who were observed after surgery died or had recurrence (excluding dropouts).

In the 34 patients with GIST, the median follow-up was 27.3 mo (range, 6.6 to 210), and the median tumor diameter at initial EUS was 19.0 mm (range, 10.9 to 44.8). The GISTs were divided into risk classes on the basis of tumor diameters and mitotic figures (Fletcher's classification). The classification was extremely low risk or low risk in 28 patients, intermediate risk in 3, and high risk in 3. The median follow-up period was 31.0 mo in patients with extremely low-risk and low-risk GISTs, 47.3 mo in those with intermediate-risk GISTs, and 12.4 mo in those with high-risk GISTs. The doubling time according to risk was 24.0 mo for extremely low-risk plus low-risk GISTs, 17.1 mo for intermediate-risk GISTs, and 3.9 mo for high-risk GISTs (Table 4).

The median doubling time for GISTs as a whole was 17.2 mo. In contrast, the doubling time was 231.2 mo for leiomyoma, 104.7 mo for schwannoma, 274.9 mo for ectopic pancreas, 61.2 mo for hamartoma, 49.0 mo for intramural developmental cyst, and 134.7 mo for Brunner's adenoma (Table 5). The doubling time of GISTs was significantly shorter than the doubling times of leiomyoma plus schwannoma ($P = 0.005$). When the doubling times of GISTs were compared according to risk class, the doubling time of high-risk

GISTs was significantly shorter than that of extremely low-risk plus low-risk GISTs ($P = 0.033$). Moreover, the doubling time of high-risk plus intermediate-risk GISTs was significantly shorter than that of extremely low-risk plus low-risk GISTs ($P = 0.047$). Doubling times did not differ significantly between high-risk and intermediate-risk GISTs or between extremely low-risk plus low-risk GISTs and intermediate-risk GISTs (Table 6). The growth rates of individual GISTs during follow-up and annual growth rates of GISTs according to risk class are shown in Figures 1 and 2, respectively.

We show some endoscopic and EUS findings of low grade GIST (Figure 3A-D), high grade GIST (Figure 4A-C) and ectopic pancreas (Figure 5A-C).

DISCUSSION

Many gastrointestinal SMTs are asymptomatic and incidentally detected on radiographic examinations during health checkups or endoscopic or computed tomographic examinations performed to evaluate other diseases. Few studies have estimated the incidence of gastrointestinal SMTs, but most arise in the stomach, and the detection rate on endoscopy was reported to be 0.36%^[3,6]. Tumorous lesions presenting with the characteristics of SMTs include mesenchymal tumors, lipomas, carcinoids, granular-cell tumors, glomus

Table 6 Comparison according to diagnosis

	Doubling time (mo), median	P value
GIST <i>vs</i> Leiomyoma + schwannoma	17.2 <i>vs</i> 204.2	0.005
High risk <i>vs</i> Intermediate risk	3.9 <i>vs</i> 17.1	0.127
Intermediate risk <i>vs</i> Extremely low + low risk	17.1 <i>vs</i> 24.0	0.423
High risk <i>vs</i> Extremely low + low risk	3.9 <i>vs</i> 24.0	0.033
High + intermediate risk <i>vs</i> Extremely low + low risk	8.2 <i>vs</i> 24.0	0.047

GIST: Gastrointestinal submucosal tumors.

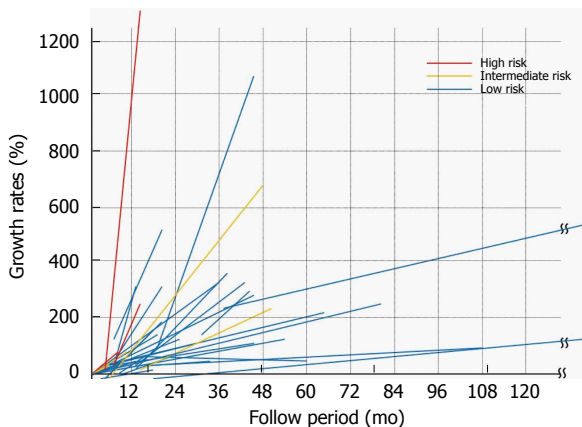


Figure 1 Growth rates of individual gastrointestinal submucosal tumors during follow-up.

tumors, and metastatic deposits. Nontumorous lesions include cysts, ectopic pancreas, Brunner's adenomas, and hamartomas^[1]. Conventional endoscopy only provides information useful for the local diagnosis of SMTs, whereas EUS can depict the local structure and internal characteristics of the gastrointestinal wall, thereby facilitating qualitative diagnosis^[7]. Although it is relatively easy to distinguish gastrointestinal mesenchymal tumors from tumors such as lipoma and cysts on EUS, it is difficult to differentially diagnose GISTs from leiomyomas and schwannomas, because all three of these lesions are depicted as hypoechoic tumors involving the fourth layer on EUS. The 2001 NIH GIST Consensus Meeting and the 2004 ESMO Consensus GIST Meeting proposed that GISTs are potentially malignant and recommended that surgical resection should be considered for all GISTs^[8-10].

Miettinen *et al*^[11] proposed a risk classification for GISTs, based on tumor diameter, mitotic figures, and location. They reported that tumors 2 cm or less in diameter have no risk of postoperative metastasis. However, metastasis has been associated with even small GISTs^[12]. It is therefore difficult to conclude that small tumors are benign. A histopathological diagnosis has an important role in formulating the treatment policy for SMTs. However, SMTs are covered by mucosa

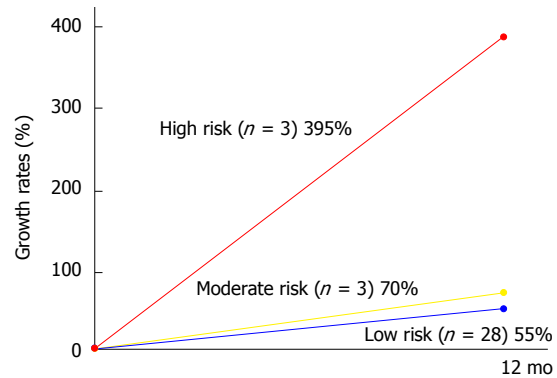


Figure 2 Annual growth rates of gastrointestinal submucosal tumors.

similar to that of the surrounding region, which often makes diagnosis challenging on conventional endoscopy with mucosal biopsy. EUS-FNA thus plays an important clinical role in the diagnosis of SMTs. In lesions measuring less than 2 cm, however, the rate of obtaining adequate specimens is generally low^[13]. There is also the risk of tumor seeding caused by lesion rupture on puncture with an aspiration needle. Moreover, it is difficult to obtain adequate tissue specimens for immunostaining or other examination techniques if adequate needle strokes cannot be taken. In general, EUS-FNA is indicated for lesions at least 2 cm in diameter. On the other hand, for lesions less than 2 cm in diameter with no findings suggesting malignancy, such as ulcer formation, irregular margins, or rapid growth^[14], follow-up observation once or twice per year has been recommended^[5]. However, with the exception of lesions showing distinct evidence of increasing size or an intragastric growth pattern, EUS is recommended for the follow-up of GISTs, particularly lesions showing an extragastric growth pattern precluding an accurate estimation of tumor size. EUS can be used to assess even small lesions and is simpler than computed tomography for the evaluation of small lesions.

Confirmation of differences in growth rate among specific types of SMTs during follow-up is expected to facilitate decision-making regarding the treatment policy. Similar to other types of tumors, a higher malignant potential of SMTs is generally assumed to be associated with a more rapid growth rate^[15]. To date, however, few studies have investigated the growth rates of different types of SMTs. A previous study estimated the doubling time of SMTs on computed tomography^[16]. To our knowledge, however, our study is the first to report the doubling time of SMTs on EUS. Because SMTs are generally oval tumors, EUS, which produces cross-sectional images, can be used to estimate the doubling time of most SMTs. However, some SMTs show a lobular growth pattern, making it difficult to accurately calculate the doubling time. In our study, we assumed that SMTs show a global growth pattern when we calculated the doubling

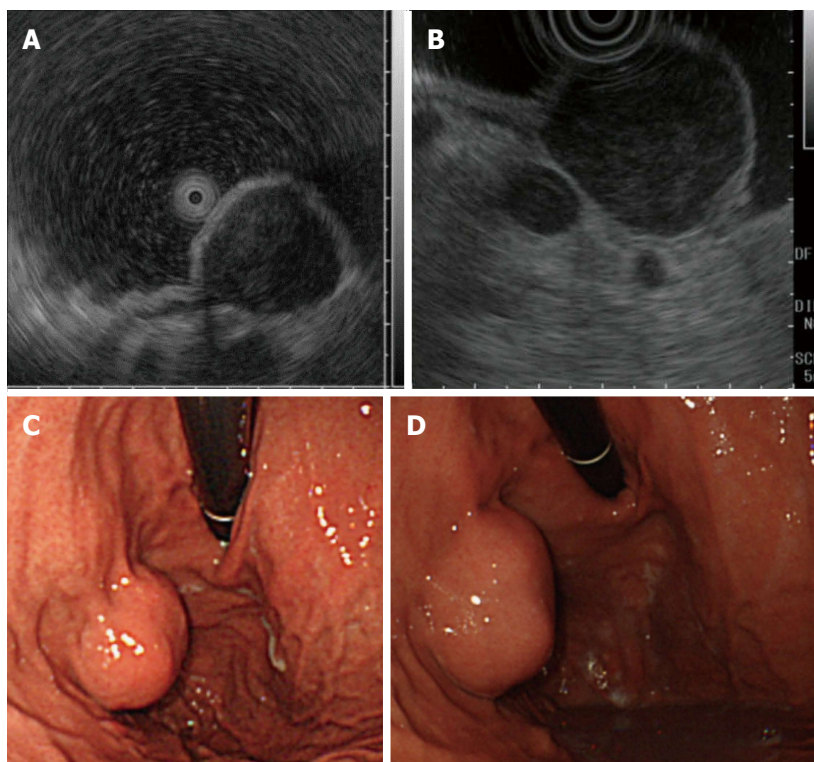


Figure 3 Gastrointestinal submucosal tumors low grade: Doubling time is 17.2 mo. A: EUS finding at baseline, tumor diameter is 22.0 mm; B: EUS finding at four years later, tumor diameter is 34.0 mm; C: Endoscopic finding at baseline; D: Endoscopic finding at four years later. There is almost no change in endoscopic findings in four years. EUS: Endoscopic ultrasound.

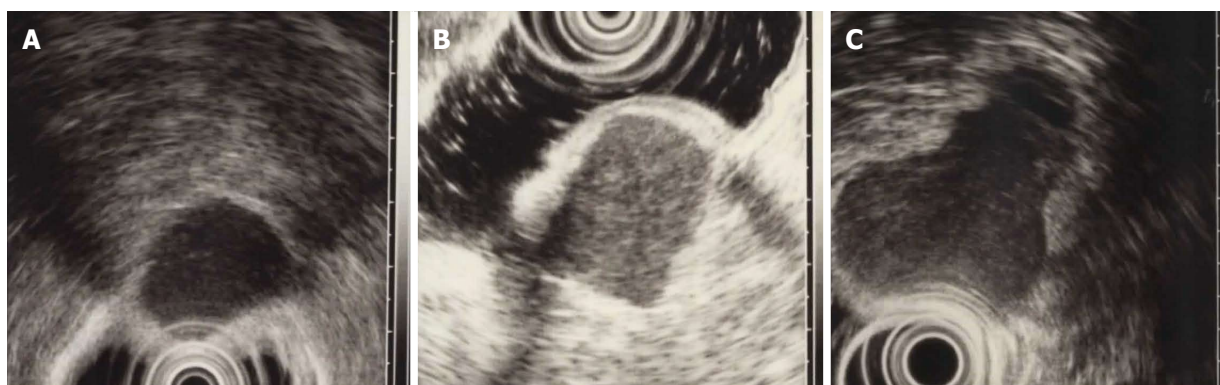


Figure 4 Gastrointestinal submucosal tumors high grade: Doubling time is 3.0 mo. A: EUS finding at baseline, tumor diameter is 25.5 mm; B: EUS finding at 6 mo later, tumor diameter is 28 mm; C: EUS finding at 12 mo later, tumor diameter is 38.5 mm. There are remarkable changes in one year. EUS: Endoscopic ultrasound.

time. The use of non-probe-type conventional three-dimensional EUS may allow tumor volumes to be more accurately estimated, but this issue must be addressed in future studies.

Our study confirmed that the growth rates of SMTs during follow-up differ according to the specific type of tumor. In particular, GIST had a shorter doubling time (17.2 mo) and a higher malignant potential than did the other types of SMTs. The difference in the doubling time between GISTs and mesenchymal tumors other than GIST (leiomyoma and schwannoma) may facilitate the differential diagnosis of GISTs from leiomyomas and schwannomas, all of which arise in the

fourth layer of the gastrointestinal wall. Among GISTs, a higher risk class tended to have shorter doubling times. Because our study group was small, further studies of larger numbers of patients are needed. In our study, the doubling times of intermediate-risk and high-risk GISTs were less than 6 mo. Initial follow-up examinations should be therefore performed within at least the first 6 mo after diagnosis, even for small SMTs less than 2 cm in diameter.

GISTs, leiomyomas, schwannomas, and other SMTs arising in the fourth layer that have a prolonged doubling time are considered to have low malignant potential. Small SMTs can therefore undergo follow-

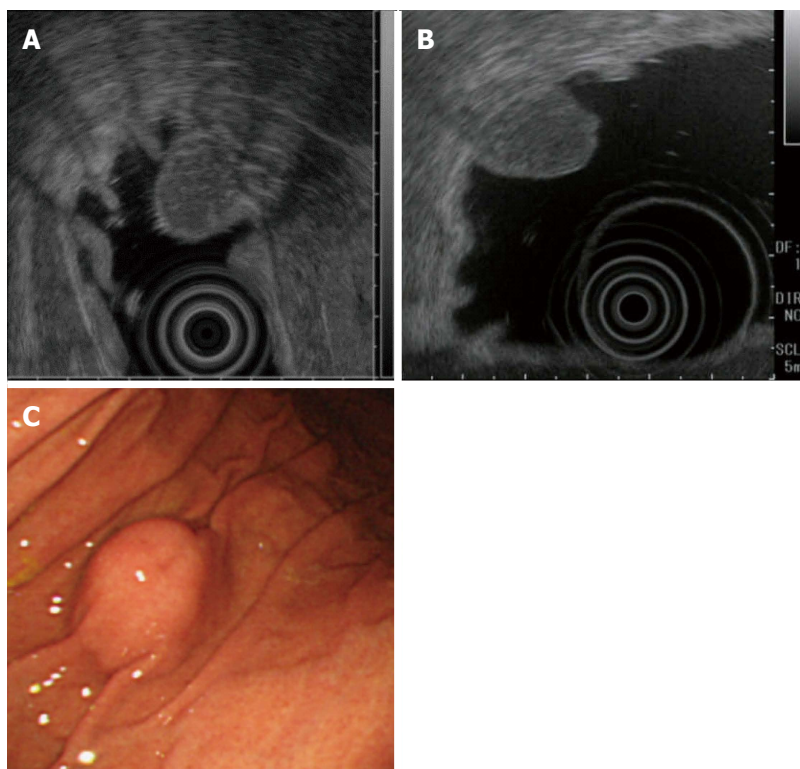


Figure 5 Ectopic pancreas. A: EUS finding at baseline, tumor diameter is 15.5 mm; B: EUS finding at five years later; C: Endoscopic finding. Ectopic pancreas has no change in five years. EUS: Endoscopic ultrasound.

up observation. Some extremely low-risk and low-risk GISTs have a longer doubling time than that of benign tumors, and we have encountered benign tumors with a shorter doubling time than that of GISTs. It is therefore important to obtain a histopathological diagnosis during follow-up, even for slowly growing tumors. Although considerable progress has been made in techniques and devices for EUS-FNA, the diagnostic accuracy is not 100%^[4,17]. Patients in whom a histopathological diagnosis cannot be made should therefore be closely followed up. In our study, the median tumor diameter in patients who underwent EUS-FNA was 22.8 mm, which was adequate for EUS-FNA. For SMTs 20 mm or more in diameter that cannot be diagnosed, EUS-FNA should be repeated, and close follow-up is recommended.

In our study, the risk class of GIST was diagnosed on the basis of mitotic figures in specimens obtained by EUS-FNA in patients who did not undergo surgery. Histopathologically, GISTs are heterogeneous masses, making it difficult to classify GISTs solely on the basis of specimens obtained by EUS-FNA^[18]. In our hospital, we aggressively perform EUS-FNA for lesions more than 2 cm in diameter as well as for lesions with heterogeneous contents suggestive of malignancy, even if the lesion diameter is less than 2 cm. If GIST is diagnosed, resection should be promptly performed, even if the tumor is small and shows few mitotic figures. For lesions that cannot be diagnosed and small lesions, the other techniques^[19-21] can be considered to obtain sufficient specimen.

Many SMTs are detected incidentally on upper gastrointestinal endoscopy, and many patients with small SMTs 1 to 2 cm in diameter are most likely followed up. The management of small lesions measuring less than 2 cm is often perplexing. Our study showed that the doubling time differed according to the type of SMT, and GISTs were confirmed to have a significantly shorter doubling time than the other types of tumors. In addition, a higher risk class of GIST was found to be associated with a significantly shorter doubling time. Our findings suggest that even small SMTs less than 2 cm in diameter should initially be followed up within at least 6 mo after detection. In a limited number of patients, surgery or EUS-FNA is indicated. High-risk GISTs that are large and symptomatic are usually surgically resected at the time of detection. Three of high-risk GISTs those were followed up are included in this study. This is valuable information because we're not able to follow up the high-risk GISTs after getting histopathological diagnosis recently. Our results demonstrated that SMTs showing evidence of rapid growth on follow-up EUS are likely to be high risk. The median doubling time for GISTs as a whole was 17.2 mo, but further studies of larger groups of patients are needed to confirm our findings.

COMMENTS

Background

Many gastrointestinal submucosal tumors (SMTs) are asymptomatic and

incidentally detected. Among SMTs, gastrointestinal stromal tumors (GISTs) are potentially malignant and should be resected surgically. Endoscopic ultrasound-guided fine-needle aspiration (EUS-FNA) plays an important clinical role in the diagnosis of SMTs. However, the management of small lesions is often difficult. Confirmation of differences in growth rate among specific types of SMTs is expected to facilitate decision-making regarding the treatment policy.

Research frontiers

EUS-FNA plays a major role in deciding the treatment policy. However, there remains room for improvement in the diagnostic performance of EUS-FNA for small lesions. Consequently, small SMTs yet to be definitively diagnosed are generally followed up once or twice per year.

Innovations and breakthroughs

To our knowledge, very few studies have evaluated the doubling time of SMTs, an index of the rate of tumor growth, according to diagnosis. The authors estimated the doubling time of different types of SMTs.

Applications

The doubling time of GIST was confirmed to be significantly shorter than that of other types of tumors. For GIST, a higher risk grade was associated with a significantly shorter doubling time. These findings suggest that small SMTs should initially be followed up within at least 6 mo after detection.

Terminology

EUS: An endoscopic procedure to obtain images of the chest and abdominal organs through the wall of the gastrointestinal tract. EUS-FNA: A technique to obtain specimens of chest and abdominal lesions by puncturing the gastrointestinal tract under real-time EUS guidance.

Peer-review

This is a very meaningful research regarding the growth rate of the SMTs, which may be a very important character for the evaluation. Authors of this paper describe their strategy regarding observational duration for tumor in small size around 2 cm by analyzing the doubling times of each SMT. Initial follow-up examinations remain unclear in major guidelines. Therefore, this result provides an important information in the management of small SMT.

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Retrospective Study

Preoperative evaluation of pancreatic ductal adenocarcinoma with synchronous liver metastasis: Diagnosis and assessment of unresectability

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Abstract

AIM

To identify predictors for synchronous liver metastasis from resectable pancreatic ductal adenocarcinoma (PDAC) and assess unresectability of synchronous liver metastasis.

METHODS

Retrospective records of PDAC patients with synchronous liver metastasis who underwent simultaneous resections of primary PDAC and synchronous liver metastasis, or palliative surgical bypass, were collected from 2007 to 2015. A series of pre-operative clinical parameters, including tumor markers and inflammation-based indices, were analyzed by logistic regression to figure out predictive factors and assess unresectability of synchronous liver metastasis. Cox regression was used to identify prognostic factors in liver-metastasized PDAC patients after surgery, with intention to validate their conformance to the indications of simultaneous resections and palliative surgical bypass. Survival of patients from different groups were analyzed by the Kaplan-Meier method. Intra- and post-operative courses were compared, including complications. PDAC patients with no distant metastases who underwent curative resection served as the control group.

RESULTS

CA125 > 38 U/mL (OR = 12.397, 95%CI: 5.468-28.105, $P < 0.001$) and diabetes mellitus (OR = 3.343, 95%CI: 1.539-7.262, $P = 0.002$) independently predicted synchronous liver metastasis from resectable PDAC. CA125 > 62 U/mL (OR = 5.181, 95%CI: 1.612-16.665, $P = 0.006$) and age > 62 years (OR = 3.921, 95%CI: 1.217-12.632, $P = 0.022$) correlated with unresectability of synchronous liver metastasis, both of which also indicated a worse long-term outcome of liver-metastasized PDAC patients after surgery. After the simultaneous resections, patients with post-operatively elevated serum CA125 levels had shorter survival than those with post-operatively reduced serum CA125 levels (7.7 mo *vs* 16.3 mo, $P = 0.013$). The survival of liver-metastasized PDAC patients who underwent the simultaneous resections was similar to that of non-metastasized PDAC patients who underwent curative pancreatectomy alone (7.0 mo *vs* 16.9 mo, $P < 0.001$), with no higher rates of either pancreatic fistula ($P = 0.072$) or other complications ($P = 0.230$) and no greater impacts on length of hospital stay ($P = 0.602$) or post-operative diabetic control ($P = 0.479$).

CONCLUSION

The criterion set up by CA125 levels could facilitate careful diagnosis of synchronous liver metastases from PDAC, and prudent selection of appropriate patients for the simultaneous resections.

Key words: CA125; Pancreatic ductal adenocarcinoma; Liver metastasis; Unresectability; Prognosis

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Core tip: The presence of liver metastasis from pancreatic ductal adenocarcinoma (PDAC) usually deprives patients of opportunities for resection of PDAC. We utilized a series of clinical parameters for pre-operative evaluation of PDAC with synchronous liver metastasis, including diagnosis and assessment of unresectability. The criterion set up by serum CA125 levels could facilitate the careful judgement of the occurrence of synchronous liver metastases from PDAC, and the prudent selection of appropriate patients for simultaneous resections for primary PDAC and synchronous liver metastasis, for the sake of prolonged survival and substantial reduction in morbidity and mortality.

Shi HJ, Jin C, Fu DL. Preoperative evaluation of pancreatic ductal adenocarcinoma with synchronous liver metastasis: Diagnosis and assessment of unresectability. *World J Gastroenterol* 2016; 22(45): 10024-10037 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v22/i45/10024.htm> DOI: <http://dx.doi.org/10.3748/wjg.v22.i45.10024>

INTRODUCTION

Pancreatic ductal adenocarcinoma (PDAC) is a highly aggressive and progressive malignancy with increasing incidence and death rates^[1,2]. Despite the steady improvement in survival for most cancers, progress has been limited for PDAC, for which the 5-year relative survival rate for all stages combined is 8%^[2]. The rate of resection for primary PDAC is only 10%-20%, and approximately 50% of new PDAC cases are discovered to have distant metastases^[3]. Some distant micro-metastases are undetectable at diagnosis through a thorough pre-operative imaging tests including positron emission tomography/computed tomography (PET/CT), and may only be confirmed by exploration during planned curative resection. Even those patients undergoing curative pancreatectomy are still at a 25%-50% risk of developing distant metastases^[4-6]. The dismal prognosis of PDAC with distant metastasis has been acknowledged by its 5-year relative survival rate of 1%^[7].

PDAC shows a remarkable preference for the liver to metastasize due to its portal venous blood draining and lymphatic spread. Weh *et al*^[8] summarized that the incidence of liver metastasis from PDAC ranged from 25% to 75%. About 12% of unsuspected liver metastases are not discovered until surgery, and liver metastasis reduces the survival of patients with PDAC to 5 mo^[9,10]. Currently, chemotherapy remains the mainstay of treatment for liver metastasis from PDAC, with two combination chemotherapy regimens-FOLFIRINOX (bolus plus infusional fluorouracil, leucovorin, irinotecan, and oxaliplatin regimen) and gemcitabine plus nanoparticle albumin-bound paclitaxel-emerging as new standards^[11-13].

The doctrine that the presence of liver metastasis from resectable PDAC contradicts a curative resection and indicates a palliative surgical bypass, deprives patients of an incremental benefit from simultaneous curative resections for primary and metastatic PDAC, even at a R1 status. An unconventional surgical option to curatively resect primary PDAC and synchronous liver metastasis may be merely justified by prolonged survival, a longer recurrence-free interval and, at least, no more surgical-related morbidity and mortality. Pancreaticoduodenectomy (PD) combined with additional organ resection has been indicated for locally advanced PDAC with the same safety as PD alone^[14]. Even if palliative PD can be performed instead, patients can benefit from significantly longer survival and low morbidity rate^[15,16]. Thus, simultaneous curative resections for primary PDAC and synchronous liver metastasis can also be advocated on highly individual basis. However, the threshold comprised of conventional clinical indexes has not been first established to pre-operatively distinguish

the occurrence of liver metastasis among patients with resectable PDAC. And, the criterion also needs to be set for selection for patients whom the simultaneous resections favor in a proper sense.

As the predictive accuracy of serum CA125 levels has been reported in a two-center clinical study where we were involved^[17,18], here we highlighted the relationships between serum CA125 levels and both synchronous liver metastasis from PDAC and unresectability of liver-metastasized PDAC, and focused on the long-term outcome of liver-metastasized PDAC patients after individualized surgeries indicated by serum CA125 levels.

MATERIALS AND METHODS

Patients

Sixty-nine patients with resectable primary PDAC and synchronous liver metastasis who underwent surgery at the Huashan Hospital between March 2007 and December 2015 were identified in a prospective database. Of these, 30 patients underwent simultaneous curative resections for primary PDAC and synchronous liver metastases, and 39 patients underwent palliative surgical bypass prior to gemcitabine-based chemotherapy due to unresectable liver metastases. All data collected was consented by these patients and approved by the Ethical Committee and Institutional Review Board. Only patients with histologically confirmed PDAC and liver metastasis who underwent surgery were included in the current study. Patients with unresectable primary PDAC, neuroendocrine tumor, cystadenocarcinoma, ampullary cancer, distal bile duct, and duodenal carcinoma as well as extrahepatic metastatic disease such as serosal implants or peritoneal metastases were not considered in the study. To investigate the predictors for synchronous liver metastasis, 138 patients with no evidence of distant metastases who underwent curative resection for primary PDAC alone were selected at the same period mentioned above for matching with the control group in a 1:2 fashion. These patients were matched as closely as possible to the baseline characteristics of the liver metastasis cohort.

Pre-operative evaluation

Routine pre-operative diagnostics consisted of a baseline history, physical examination and clinical laboratory tests and imaging tests. The tumor markers CA19-9, CA125 and CEA were used as serum diagnostic tools. Ultrasonography, computed tomography scanning and PET were performed in all instances. Pre-operative biliary drainage, endoscopic retrograde biliary drainage or percutaneous transhepatic cholangial drainage was indicated for jaundice.

Surgical procedures

Depending on the location of primary PDAC, curative resection was performed as pancreatoduodenectomy, or total pancreatectomy, or distal splenopancreatectomy, accompanied by lymphadenectomy. Selected patients underwent portal/superior mesenteric vein resection and artificial blood vessel replacement. The number and distribution of metastatic diseases, which were assessed by intra-operative ultrasonographic measurement once more to detect liver micro-metastases under suspicious conditions, determined the extent of liver resection. During the laparotomy, the abdomen was completely staged. Given that no acknowledged guidelines of surgery for liver metastasis from PDAC offered the use of reference to surgeons, the decision for resection was made by the intention to reach a R0 status in both the pancreas and the liver and a good performance status (American Society of Anesthesiologists ASA classification \leq III). The palliative Roux-en Y bypass was constituted by retrocolic end-to-side hepaticojejunal anastomosis and antecolic gastroenterostomy.

Data collection

The following data were assessed prospectively for each patient: demographics, pre-operative symptoms and previous history, histology of primary PDAC and synchronous liver metastasis, pre-operative treatments, blood parameters, operative details, post-operative course. Among them, plasma fibrinogen and platelets have been shown to play a possible role of both predictive and prognostic factors of distant metastasis^[19,20]. Blood neutrophil-lymphocyte ratio (NLR), lymphocyte-monocyte ratio (LMR), platelet-lymphocyte ratio (PLR) and prognostic nutritional index (PNI, albumin [g/L] + 5×total lymphocyte count [$\times 10^9$ /L]) have acted as inflammation-based indices to predict the clinical outcome of primary or metastasized cancers after surgery or chemotherapy^[21-25] as well as the association with metastasized cancer burden^[26-27]. Body-mass-index (BMI), NLR, LMR, PLR and PNI were obtained by calculation during the initial evaluation. All pathologic specimens were reviewed through intra-operative frozen section analysis or routine paraffin section analysis by two independent pathologists to unanimously confirm the diagnosis of primary PDAC and synchronous liver metastasis. Post-operative course included post-operative morbidity such as pancreatic fistula, and mortality defined as any death during hospitalization and within 30 d of surgery. Follow-up information was obtained through review of the medical records and direct contact with the patients. When the date of death was inaccessible, patients were censored at the last contact or record from hospitalization or oncological outpatient clinics.

Statistical analysis

Summary statistics were reported using mean or

Table 1 Clinicopathologic characteristics of 207 pancreatic ductal adenocarcinoma patients undergoing surgery

Parameter	No. of patients			
	Simultaneous resections (Group A) <i>n</i> = 30	Palliative surgical bypass (Group B) <i>n</i> = 39	Total (Group A + Group B) <i>n</i> = 69	Pancreatectomy alone (Group C) <i>n</i> = 138
Mean age \pm SD, yr	62.2 \pm 10.0	63.0 \pm 10.4	62.6 \pm 10.1	58.8 \pm 10.6
Sex (Female)	10	12	22	45
ASA				
I	11	10	21	37
II	19	27	46	97
III	0	2	2	4
IV	0	0	0	0
Primary tumor location				
Head/neck	15	38	53	106
Body/tail	15	1	16	32
Median primary tumor size [IQR], cm	4.0 (2.5-5.0)	-	-	3.0 (2.0-3.5)
Pathology (PDAC)	30	39	69	138
TNM stage				
I	0	0	0	22
II A	0	0	0	22
II B	0	0	0	94
III	0	0	0	0
IV	30	39	69	0
Primary tumor differentiation				
Well/moderate	13	-	-	71
Poor	17	-	-	67
Ki67 [IQR], %	20 (8-30)	-	-	30 (15-50)
Venous invasion	3	19	22	37
Lymph node invasion	14	-	-	82
Hepatic metastasis	30	39	69	0
Surgery for primary tumor				
Total pancreatectomy	1	0	1	5
Pancreaticoduodenectomy	11	0	11	95
Distal pancreatectomy	18	0	18	38
Palliative bypass	0	39	39	0

median values where appropriate. Student's *t*-test or analysis of variance was used for mean comparison of continuous variables distributed normally, whereas Mann-Whitney *U* test or Kruskal-Wallis *H* test was used to compare skewed continuous variables. Fisher's exact test or Pearson's χ^2 test was used to compare frequencies of categorical variables among groups. The cutoff value of fibrinogen, NLR, LMR, PLR, PNI and platelet was determined by widely accepted thresholds^[19,20,23,28], allowing comparison with the available literature. Serum CA19-9 level of 400 U/mL used for indicating distant metastasis of PDAC^[29,30] was adopted as a cutoff for logistic regression analysis and Cox regression analysis. According to receiver operating characteristic (ROC) curve, an optimal cutoff serum CA125 level of 38 U/mL was identified for analysis of predictors for synchronous liver metastasis, and 62 U/mL for assessment of unresectability for synchronous liver metastasis and overall survival for PDAC patients with synchronous liver metastasis. Predictors for synchronous liver metastasis from PDAC and unresectability for synchronous liver metastasis were estimated by logistic regression analyses. Prognostic factors for overall survival were estimated by Cox proportional hazards models. The Kaplan-Meier method was used to analyze the overall survival from the date

of surgery. Differences in survival were examined using the log-rank test. A two-sided *P* value of < 0.05 was considered to indicate a statistically significant difference. Statistical analyses were performed by utilizing SPSS statistics 20 (IBM corporation, Armonk, NY, United States).

RESULTS

Patient characteristics

Table 1 shows the clinicopathologic characteristics of 69 PDAC patients (group A and B) with liver metastasis, who were the focus of the study, and 138 PDAC patients (group C) with no distant metastases, who were enrolled as a matched group. In the case group, the majority of 69 patients were male (*n* = 47, 68.1%) with an overall mean age of 62.6 years. According to ASA grading system, 21 (30.4%) patients were evaluated as grade I, 46 (66.7%) as grade II, and 2 (2.9%) as grade III. The primary PDAC site was largely head or neck (*n* = 53, 76.8%). Primary PDAC displayed venous invasion in 22 (31.9%) patients and lymph node invasion in 14 (46.7%) patients.

Among these 69 patients, 30 patients (group A) underwent simultaneous curative resections for primary PDAC as well as synchronous liver metastasis,

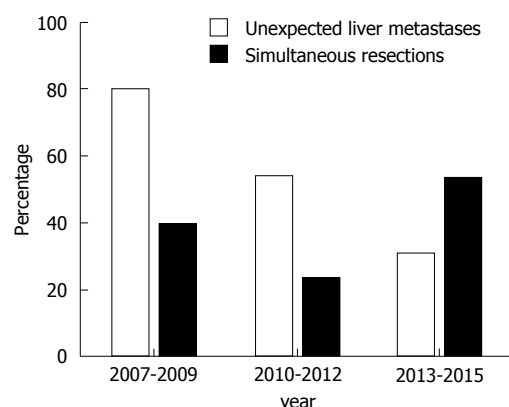


Figure 1 Trends in the occurrence of unexpected liver metastases identified during surgeries and implementation of the simultaneous resections among all cases across the study period.

and 39 patients (group B) underwent palliative surgical bypass. The curative resections for primary PDAC included PD ($n = 11$, 36.7%), distal pancreatectomy ($n = 18$, 60.0%) and total pancreatectomy ($n = 1$, 3.3%), with portal/superior mesenteric vein resection and artificial blood vessel replacement ($n = 3$, 2.2%). The mean age at the time of surgery was 62.2 years in group A, and 63.0 years in group B. Half of group A and 38 of group B suffered from adenocarcinoma of the head/neck of the pancreas. Fifteen of group A and 20 of group B were found to have unexpected liver metastases by direct-view or intra-operative ultrasonographic measurement during surgeries. The proportion of the successful simultaneous resections was triennially rising across the study period (Figure 1).

Most patients of the matched cohort (group C) were male ($n = 93$, 68.1%) with an overall mean age of 58.8 years. Thirty-seven (26.8%) patients were evaluated as ASA grade I, 97 (70.3%) as grade II, and 4 (2.9%) as grade III. As adenocarcinoma of the head/neck of the pancreas ($n = 106$) accounted for 76.8% of all resectable pancreatic adenocarcinoma, the majority of surgical options were PD ($n = 95$, 68.9%), and the rest were distal pancreatectomy ($n = 38$, 27.5%) and total pancreatectomy ($n = 5$, 3.6%). Thirty-seven (26.8%) patients underwent portal/superior mesenteric vein resection and artificial blood vessel replacement ($n = 3$, 2.2%) due to venous invasion. After lymphadenectomy, 82 of group C were found to have lymph node invasion.

Predictors for synchronous liver metastasis from PDAC

To determine which pre-operative factors are independent predictors for synchronous liver metastasis from PDAC, a univariate analysis was performed for preliminary screening of clinical parameters followed by a stepwise logistic regression analysis of the occurrence of synchronous liver metastasis from PDAC. In univariate analysis, there was a trend toward a higher incidence of no synchronous liver metastases in patients with CA19-9 > 400 U/mL ($P < 0.001$), CA125 > 38

Table 2 Predictors of synchronous liver metastasis from resectable pancreatic ductal adenocarcinoma

Parameter	Total (Group A + Group B) <i>n</i> = 69	Pancreatectomy alone (Group C) <i>n</i> = 138	<i>P</i> value (univariate)
Age, yr			
≤ 62	32	80	0.116
> 62	37	58	
Sex			
Male	47	93	0.916
Female	22	45	
BMI			
< 18 kg/m ²	6	11	0.196
18-25 kg/m ²	54	101	
> 25 kg/m ²	9	26	0.965
Smoke			
No	50	97	0.745
Yes	19	41	
ASA			
I	21	37	0.584
II-III	48	101	
CA19-9			
≤ 400 U/mL	40	116	< 0.001
> 400 U/mL	29	22	
CA125			
≤ 38 U/mL	20	121	< 0.001
> 38 U/mL	49	26	
CEA			
≤ 5 U/mL	42	112	0.002
> 5 U/mL	27	26	
Fibrinogen			
≤ 4.0 g/L	50	101	0.912
> 4.0 g/L	19	37	
NLR			
≤ 5	59	131	0.026
> 5	10	7	
PLR			
≤ 150	36	78	0.553
> 150	33	60	
PNI			
> 45	42	101	0.072
≤ 45	27	37	
Platelet			
≤ 250 × 10 ⁹ /L	53	108	0.813
> 250 × 10 ⁹ /L	16	30	
Jaundice			
No	34	78	0.431
Yes	35	60	
Albumin			
> 35 g/L	42	82	0.841
≤ 35 g/L	27	56	
Diabetes mellitus			
No	39	101	0.017
Yes	30	37	
Pancreatitis			
No	42	93	0.354
Yes	27	45	

U/mL ($P < 0.001$), CEA > 5 U/mL ($P = 0.002$), NLR > 5 ($P = 0.026$), and diabetes mellitus ($P = 0.017$) (Table 2). In multivariate analysis, both CA125 > 38 U/mL (OR = 12.397, 95%CI: 5.468-28.105, $P < 0.001$) and diabetes mellitus (OR = 3.343, 95%CI: 1.539-7.262; $P = 0.002$) were determined to independently predict synchronous liver metastasis from PDAC (Table 3). The area under the ROC curve (AUC) of serum CA125 level was 0.821 (95%CI: 0.752-0.891), with sensitivity of

Table 3 Multivariate analysis of predictors of synchronous liver metastasis

Parameter	Odds ratio	95%CI	P value
CA19-9			
≤ 400 U/mL			
> 400 U/mL	2.398	0.909-6.327	0.077
CA125			
≤ 38 U/mL			
> 38 U/mL	12.397	5.468-28.105	< 0.001
CEA			
≤ 5 U/mL			
> 5 U/mL	0.672	0.249-1.817	0.434
NLR			
≤ 5			
> 5	0.934	0.283-3.083	0.911
Diabetes mellitus			
No			
Yes	3.343	1.539-7.262	0.002

Table 4 Risk factors for unresectability of synchronous liver metastasis from pancreatic ductal adenocarcinoma

Parameter	Simultaneous resections (Group A) <i>n</i> = 30	Palliative surgical bypass (Group B) <i>n</i> = 39	P value (univariate)
Age, yr			
≤ 62	20	12	
> 62	10	27	0.004
Sex			
Male	20	27	
Female	10	12	0.821
BMI			
< 18 kg/m ²	2	4	0.664
18-25 kg/m ²	23	31	
> 25 kg/m ²	5	4	0.472
Smoke			
No	23	27	
Yes	7	12	0.494
ASA			
I	11	10	
II-III	19	29	0.326
CA19-9			
≤ 400 U/mL	22	18	
> 400 U/mL	8	21	0.026
CA125			
≤ 38 U/mL	11	9	
> 38 U/mL	19	30	0.221
CA125			
≤ 62 U/mL	21	11	
> 62 U/mL	9	28	0.001
CEA			
≤ 5 U/mL	22	20	
> 5 U/mL	8	19	0.066
Fibrinogen			
≤ 4.0 g/L	21	29	
> 4.0 g/L	9	10	0.688
NLR			
≤ 5	26	33	
> 5	4	6	0.811
PLR			
≤ 150	17	19	
> 150	13	20	0.513
PNI			
> 45	22	20	
≤ 45	8	19	0.066

Platelet			
≤ 250 × 10 ⁹ /L	26	27	
> 250 × 10 ⁹ /L	4	12	0.097
Albumin			
> 35 g/L	23	19	
≤ 35 g/L	7	20	0.021
Diabetes mellitus			
No	17	22	
Yes	13	17	0.983
Pancreatitis			
No	22	20	
Yes	8	19	0.066

Table 5 Multivariate analysis of risk factors for unresectability of synchronous liver metastasis from pancreatic ductal adenocarcinoma

Parameter	Odds ratio	95%CI	P value
Age, yr			
≤ 62			
> 62	3.921	1.217-12.632	0.022
CA19-9, U/mL			
≤ 400 U/mL			
> 400 U/mL	1.760	0.517-5.992	0.366
CA125, U/mL			
≤ 62 U/mL			
> 62 U/mL	5.181	1.612-16.665	0.006
Albumin			
> 35 g/L			
≤ 35 g/L	1.796	0.516-6.253	0.357

71.01% and specificity of 87.61% at the threshold of 38 U/mL.

Risk factors for unresectability of synchronous liver metastasis

Table 4 compares the clinical parameters between the curative group (group A) and the palliative group (group B), all of whom had primary PDAC and synchronous liver metastasis. In univariate analysis, the probability of unresectability was significantly increased when patients presented with age > 62 ($P = 0.004$), CA19-9 > 400 U/mL ($P = 0.026$), CA125 > 62 U/mL ($P = 0.001$) and albumin ≤ 35 g/L ($P = 0.021$). In multivariate analysis, one clinical index and one tumor marker, age > 62 (OR = 3.921, 95%CI: 1.217-12.632, $P = 0.022$) and CA125 > 62 U/mL (OR = 5.181, 95%CI: 1.612-16.665, $P = 0.006$), were found to correlate with increased unresectability when a 62-U/mL threshold of CA125 was used (Table 5). The AUC of serum CA125 level was 0.701 (95%CI: 0.576-0.826), with sensitivity of 71.79% and specificity of 70.00% at the threshold of 62 U/mL.

Prognostic factors for PDAC patients with synchronous liver metastasis

Following their respective surgeries, PDAC patients with synchronous liver metastasis had a decreased median survival compared to those with PDAC and no distant metastasis (7.0 mo vs 16.9 mo, $P <$

Table 6 Cox regression analysis of prognostic factors in pancreatic ductal adenocarcinoma patients with synchronous liver metastasis undergoing surgery

Parameter	<i>n</i>	Median OS (95%CI) (mo)	Univariate analysis	Multivariate analysis		
			<i>P</i> value	Hazard ratio	95%CI	<i>P</i> value
Age, yr						
≤ 62	32	9.988 (5.215-14.760)				
> 62	37	4.534 (3.294-5.774)	0.006	2.191	1.182-4.060	0.013
Sex						
Male	47	5.388 (2.454-8.322)				
Female	22	7.129 (5.820-8.439)	0.428			
BMI, kg/m ²						
< 18	6	4.008 (0.000-8.701)	0.939			
18-25	54	6.998 (5.147-8.849)				
> 25	9	7.721 (1.548-13.894)	0.548			
Smoke						
No	50	7.031 (4.606-9.455)				
Yes	19	5.979 (0.607-11.352)	0.317			
ASA						
I	21	9.988 (3.524-16.452)				
II-III	48	6.998 (3.803-10.193)	0.273			
Primary tumor location						
Head/neck	53	6.998 (4.910-9.086)				
Body/tail	16	7.129 (3.438-10.820)	0.762			
CA19-9, U/mL						
≤ 400	40	7.984 (6.454-9.513)				
> 400	29	4.008 (2.832-5.185)	0.042	1.398	0.773-2.527	0.267
CA125, U/mL						
≤ 62	32	9.035 (7.052-11.017)				
> 62	37	4.008 (2.801-5.215)	0.003	2.601	1.403-4.823	0.002
CEA, U/mL						
≤ 5	42	7.721 (6.340-9.102)				
> 5	27	5.191 (1.847-8.535)	0.320			
Fibrinogen, g/L						
≤ 4.0	50	7.129 (5.373-8.886)				
> 4.0	19	5.191 (2.575-7.807)	0.533			
NLR						
≤ 5	59	7.031 (5.123-8.939)				
> 5	10	4.008 (0.000-9.812)	0.495			
PLR						
≤ 150	36	7.031 (4.545-9.517)				
> 150	33	5.979 (3.287-8.672)	0.851			
PNI						
> 45	42	7.129 (6.237-8.022)				
≤ 45	27	5.848 (3.396-8.300)	0.890			
Platelet						
≤ 250 × 10 ⁹ /L	53	6.998 (5.018-8.978)				
> 250 × 10 ⁹ /L	16	7.097 (0.957-13.236)	0.993			
Jaundice						
No	34	7.721 (4.540-10.901)				
Yes	35	6.998 (3.593-10.403)	0.446			
Biliary drainage						
No	45	7.129 (5.245-9.014)				
Yes	24	5.027 (1.872-8.181)	0.878			
Bilirubin, μmol/L						
≤ 50	58	6.998 (4.918-9.078)				
> 50	11	7.031 (1.408-12.651)	0.448			
Albumin, g/L						
> 35	42	7.097 (4.893-9.300)				
≤ 35	27	5.027 (2.171-7.882)	0.799			
Diabetes mellitus						
No	39	6.998 (4.177-9.819)				
Yes	30	6.998 (4.424-9.572)	0.300			
Pancreatitis						
No	42	5.979 (4.876-9.186)				
Yes	27	6.998 (2.516-11.480)	0.789			

Table 7 Comparison of perioperative parameters in different cohorts of patients undergoing surgery

Parameter	No. of patients		P value
	Simultaneous resections (Group A) n = 30	Pancreatectomy alone (Group C) n = 138	
Mean operative time, min	344.3	380.5	0.494
Median blood loss, mL	400	400	0.780
Intra-operative RBC transfusion	12	61	0.691
Complication			
Pancreatic fistula	9	22	0.072
Any other	13	44	0.230
Biliary fistula	0	0	
Chylous fistula	1	3	
Delayed gastric emptying	4	10	
Intra-abdominal infection	6	28	
Gastrointestinal hemorrhage	0	1	
Cerebral infarction	1	0	
Pneumonia	1	2	
Post-operative diabetes mellitus			0.602
Dissolved	7	15	
New-onset	6	12	
Persistent	6	22	
Re-admission	2	4	
In-hospital mortality	0	0	
Hospital stay, d	18	19	0.479

pros and cons of. Accurate detection of liver metastases from PDAC and proper selection of patients who are likely to benefit from simultaneous curative resections for primary PDAC and synchronous liver metastasis are a great challenge for individualized therapy for PDAC. As pancreatectomy is performed with relatively high morbidity and mortality, assessment of unresectability of synchronous liver metastases under the circumstance of resectable primary PDAC, needs an objective standard using a series of pre-operative clinical parameters. In the present study, we identified a pre-operative serum signature of CA125 levels over 38 U/mL as one of the predictors for synchronous liver metastasis from PDAC. Serum CA125 levels over 62 U/mL were found not only to imply unresectability for synchronous liver metastasis, but also to indicate a poor survival for PDAC patients with synchronous liver metastasis. These suggest that PDAC patients with synchronous liver metastasis predicted by serum CA125 levels over 38 U/mL could be appropriate for and, more importantly, benefit from the simultaneous resections if serum CA125 levels range between 38 U/mL and 62 U/mL.

Since CA125 has been extensively used as a biomarker of various types of cancers, its diagnostic and prognostic values are gradually attracting great attention for PDAC. Recently it was reported in a two-center clinical study that elevated serum CA125 levels were more pronounced in patients with the metastasis-associated burden, especially liver metastasis^[17]. Elevated serum CA125 levels in patients with gastric adenocarcinoma were also observed with the presence of peritoneal metastases and lymph

node metastases^[31,32]. On the contrary to the similar elevated serum CA19-9 levels in all stages of PDAC, serum CA125 levels for PDAC with distant metastasis were shown to be higher than that for early or locally advanced PDAC^[33]. Our result indicated that patients with PDAC were more likely to have synchronous liver metastasis if serum CA125 level exceeded 38 U/mL instead of serum CA19-9 level higher than 1000 U/mL. Thus, it is inferred that serum CA125 levels are insensitive to primary PDAC.

In addition to differential diagnosis, serum CA125 levels reflected the extent of liver metastases as well. We found that the median serum CA125 levels of unexpected liver metastases was lower than that of detected liver metastases (52 U/mL vs 72 U/mL, $P = 0.009$). The median serum CA125 levels of liver metastases with fewer than 5 nodules smaller than 3 cm, or more than 3 nodules larger than 3 cm, or intermediate nodules was, respectively, 37 U/mL, 67 U/mL and 486 U/mL^[17]. Of note, even though patients received curative resection for primary PDAC and post-operatively displayed a decrease in serum CA19-9 level, an early distant metastasis and poor survival still troubled those who did not experience a decrease in serum CA125 levels^[17,18], as we observed. Furthermore, CA125 expression in PDAC was also found to directly correlate with tumor stage, grade and metastasis^[34,35], and to increase along with loss of differentiation of PDAC^[35], which denotes the tendency for distant metastasis^[36,37]. Primary PDAC expressed CA125 under the same intensity as metastatic lesions did, demonstrating the maintenance of PDAC for CA125 expression during the metastatic process^[35]. Therefore, we believe that CA125 is an effective pre-operative factor for monitoring synchronous liver metastasis from PDAC.

Given that CA19-9 can be influenced by obstructive jaundice or pancreatitis^[38] and cannot be detected due to lack of the Lewis antigen^[39], CA125 characterized by secretory stability is considered more suitable for objective judgement. Regarding the unresectability of cancer, CA125 has been widely utilized as the therapeutic strategy^[40-44]. Compared with CA19-9, the most common tumor marker evaluated in patients with PDAC, CA125 as a predictor for unresectability of primary PDAC had a superior ROC area of 0.81, with a cutoff level of 19.7U/mL^[45]. Moreover, elevated CA125 levels over the selected threshold could distinguish factually unresectable PDAC from equivocally resectable PDAC judged by multidetector CT^[45]. In the present study, we analyzed a series of clinical parameters, including tumor markers, and found that serum CA125 levels over 62 U/mL might signify unresectability of synchronous liver metastasis even if primary PDAC could be curatively resected at a R0 status. Considering that serum CA125 levels also implied the extent of liver metastasis^[17] and that the location and number of liver metastases determined

Table 8 Survival data from published studies with simultaneous resections of primary pancreatic ductal adenocarcinoma and synchronous liver metastasis

	Simultaneous resections		Palliative surgical bypass or chemotherapy		Pancreatectomy alone		P value
	Median (mo)	n	Median (mo)	n	Median (mo)	n	
Adam <i>et al</i> ^[57] (2006)	NA ¹	41	-	-	-	-	-
Yamada <i>et al</i> ^[58] (2006)	15.0	6	-	-	-	-	-
Gleisner <i>et al</i> ^[59] (2007)	5.9	17	-	-	-	-	-
Shrikhande <i>et al</i> ^[60] (2007)	7.9	10	-	-	-	-	-
De Jong <i>et al</i> ^[51] (2010)	17.7	NA	-	-	17.9	NA	0.730
De Jong <i>et al</i> ^[61] (2010)	13.0 ²	14	-	-	-	-	-
Dünschede <i>et al</i> ^[49] (2010)	8.0	9	11	5	-	-	-
Seelig <i>et al</i> ^[62] (2010)	11.8	4	-	-	-	-	-
Klein <i>et al</i> ^[50] (2012)	13.0	7	-	-	26.5	13	NA
Tachezy <i>et al</i> ^[63] (2016)	14.5	69	7.5	69	-	-	< 0.001

¹Five-year survival of 20% was provided; ²The median survival of 25 patients with pancreatic ductal adenocarcinoma (PDAC) and cholangiocarcinoma.

the feasibility and method of surgery^[46], our findings were quite deducible and rational. Taken together with predictability of synchronous liver metastasis by serum CA125 level over 38 U/mL, a narrow range of serum CA125 level from 38 U/mL to 62 U/mL denoted simultaneous resectability of primary PDAC and synchronous liver metastasis.

In spite of uneventful curative resections, prolonged survival does not necessarily belong to all patients. On one hand, it was demonstrated that resected patients with pre-operative serum CA125 levels over 18.4 U/mL survived less than half of the life time as those with lower serum CA125 levels (11.3 mo vs 25.3 mo)^[17]. More importantly, unlike CA19-9, no discrepancies of predictability by CA125 were found in PDAC patients with hyperbilirubinemia^[47]. It was determined in the two-center clinical study that the combination of CA19-9 over 1000 U/mL and either CA125 or CEA indicated a worse surgical outcome, with a median survival of 7.0 mo vs 18.2 mo for the validation cohort from our hospital^[18]. In addition, as a good response to curative surgery, decreasing CA125 levels after pancreatectomy were associated with longer survival time as well (40.8 mo vs 14.6 mo)^[17]. Our data also reflected that patients with elevated serum CA125 levels did not display a survival advantage following the simultaneous resections. Associated with the incidence of liver metastasis, co-expression of CA125 and mesothelin could signify unfavorable outcome in PDAC patients (19.0 mo vs 34.8 mo)^[48]. In this study, we showed that pretreatment serum CA125 level over 62 U/mL was useful for indicating a worse outcome for PDAC patients with synchronous liver metastasis. These imply that our surgical option for primary PDAC and synchronous liver metastasis determined by serum CA125 levels does have an impact on patient survival and that the simultaneous curative resections do improve clinical outcome. Aggressive therapeutic regimens may be more advantageous in patients with lower serum CA125 levels.

On the other hand, Dünschede *et al*^[49] claimed

shorter survival in patients with synchronous liver metastasis undergoing simultaneous resections than in those treated by gemcitabine (8.0 mo vs 11 mo) despite no statistical differences. However, resection for metachronous liver metastases instead of gemcitabine might extend survival in highly selected patients. Meanwhile, Klein *et al*^[50] reported that no similar survival was achieved by pancreatectomy and simultaneous liver resection for PDAC, albeit at a R0 status, compared with pancreatectomy for non-metastasized PDAC (13.0 mo vs 26.5 mo)^[50] (Table 8). On the contrary, we showed that the survival of PDAC patients with synchronous liver metastasis who underwent simultaneous curative resections (15.7 mo) was not only longer than that of those who underwent palliative surgical bypass alone (4.4 mo) but also similar to that of patients with non-metastasized PDAC who underwent curative pancreatectomy alone (16.9 mo). Such discrepancy with the previous two studies can be explained by inconspicuous residual lesion after liver resection misjudged by pre-operative or intra-operative assessment. In accordance with our data, De Jong *et al*^[51] demonstrated that overall survival appeared not to be different in the patients who underwent PD and liver-directed therapy compared with those with no evidence of liver metastasis who underwent PD (17.7 mo vs 17.9 mo). Therefore, our result of Cox regression analysis showing that serum CA125 levels less than 62 U/mL were independently associated with a prolonged survival, justified our criterion of serum CA125 level as appropriate for simultaneous resections for primary PDAC and synchronous liver metastasis, and certified its benefit for survival of patients with synchronous liver metastasis from PDAC.

Now that pancreatectomy itself is associated with significant morbidity and mortality, a simultaneous liver resection may carry the extraneous risks influencing overall survival, such as bile leak, hemorrhage, or liver abscess^[52,53]. The risk of developing a liver abscess reached nearly 40%-50%

for liver-directed therapy radiofrequency ablation of liver tumors in patients with a biliary tract procedure such as an enterobiliary anastomosis or biliary stenting^[54]. As for liver resection for metastasized PDAC, it is noteworthy that the construction of a biliary-enteric anastomosis during PD may be one of the induction factors of a liver abscess. The development of post-operative complications has been found to be detrimental to survival and to lead to early recurrence in PDAC patients^[55,56]. However, our study found no liver-specific complications caused by liver resection and no more severe pancreatic fistula caused by pancreatectomy, suggesting relative safety of simultaneous resections with similar morbidity compared with standard pancreatectomy alone.

The current study had several limitations. Despite a time span of 8 years, only a relatively small sample size of patients was identified as having primary PDAC and synchronous liver metastasis who underwent either simultaneous resections or palliative surgical bypass. As such, this study had limited statistical power. Meanwhile, there may have been selection bias in whether PDAC patients with synchronous liver metastasis were chosen for surgery. For example, if some PDAC patients with resectable tumor of body or tail of the pancreas and unresectable synchronous liver metastases did not present with biliary or upper digestive obstruction, they usually underwent gemcitabine-based chemotherapy instead of palliative surgical bypass first and were excluded from our study. In addition, the focus of our study was the impact of pre-operative factors on diagnosis of liver metastasis and selection of suitable patients for simultaneous resections, which overlooks the influence of intra-or post-operative factors on overall and recurrence-free survival. Furthermore, the role of serum CA125 levels in clinical prediction for metachronous liver metastasis from PDAC was not investigated and hence cannot provide guidance for all patients with liver metastasis from PDAC.

In conclusion, diagnosis and treatment of liver metastasis from PDAC must be individualized in the era of precision medicine because of its highly malignant biological behavior. Serum CA125 level over 38 U/mL predicts synchronous liver metastasis from PDAC, and serum CA125 level over 62 U/mL is associated with unresectability of metastatic disease burden. The criterion set up by serum CA125 levels facilitates the careful diagnosis of synchronous liver metastases from PDAC pre-operatively and the prudent selection of appropriate patients for simultaneous resections for primary PDAC and synchronous liver metastasis for the sake of prolonged survival and substantially reduced morbidity or mortality. Therefore, simultaneous resections for primary PDAC and synchronous liver metastasis are justified by prolonged survival in patients selected by serum CA125. It is foreseeable that the indication for the simultaneous resections for precisely diagnosed liver-metastasized PDAC will be

extended with the development of surgical techniques and thus more PDAC patients will have a clear survival benefit.

COMMENTS

Background

Approximately 50% of new pancreatic ductal adenocarcinoma (PDAC) cases are discovered to have distant metastases. The doctrine that the presence of liver metastasis from resectable PDAC contradicts a curative resection and indicates a palliative surgical bypass, deprives patients of an incremental benefit from simultaneous curative resections for primary and metastatic PDAC. Diagnosis of synchronous liver metastasis from PDAC and assessment of unresectability are still challenging to surgeons.

Research frontiers

The two-center clinical study we were involved in reported that elevated serum CA125 levels are more pronounced in patients with the metastasis-associated burden. On the contrary to the similar elevated serum CA19-9 levels in all stages of PDAC, serum CA125 levels for PDAC with distant metastasis were higher than that for early or locally advanced PDAC. Serum CA125 levels also imply the extent of liver metastasis, and the location and number of liver metastases determine the feasibility and method of surgery. Therefore, we hypothesized that CA125 might be an effective pre-operative factor for monitoring synchronous liver metastasis from PDAC, and that CA125 could predict unresectability of synchronous liver metastasis.

Innovations and breakthroughs

PDAC patients with synchronous liver metastasis predicted by serum CA125 levels over 38 U/mL might be appropriate for and, more importantly, benefit from simultaneous resections if serum CA125 levels range between 38 U/mL and 62 U/mL. The survival of PDAC patients with synchronous liver metastasis who underwent simultaneous curative resections was not only longer than that of those who underwent palliative surgical bypass alone but also similar to that of patients with non-metastasized PDAC who underwent curative pancreatectomy alone.

Applications

The criterion set up by serum CA125 levels facilitates the careful judgement of occurrence of synchronous liver metastases from PDAC, and the prudent selection of appropriate patients for simultaneous resections for primary PDAC and synchronous liver metastasis, for the sake of prolonged survival and substantially reduced morbidity or mortality.

Terminology

Liver metastasis is a remarkable preference of PDAC to disseminate due to its portal venous blood draining and lymphatic spread, and drastically reduces the survival of patients with PDAC. Simultaneous curative resection is an unconventional surgical option for synchronous liver metastasis, which requires further justification by prolonged survival, a longer recurrence-free interval and, at least, no more surgical-related morbidity and mortality.

Peer-review

This research is original and very interesting for publication.

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Retrospective Study

Clinical features of upper gastrointestinal serrated lesions: An endoscopy database analysis of 98746 patients

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Abstract

AIM

To analyse the clinical features of patients with the serrated lesions in the upper gastrointestinal tract (UGI) tract.

METHODS

Patients who underwent routine esophagogastroduodenoscopy (EGD) at the Digestive Endoscopy Centre of General Hospital, Tianjin Medical University between January 2011 and December 2015 were consecutively recruited. Patients with UGI serrated lesions were consecutively identified. The patients' demographics and histopathology were recorded. The colorectal findings for patients who underwent colonoscopy simultaneously or within six months were also extracted from the colonoscopy database. In addition, we analysed

differences in colorectal neoplasia detection between the study patients and randomly selected patients matched for age and gender who did not exhibit serrated lesions and who also underwent colonoscopy in the same period.

RESULTS

A total of 21 patients out of 98746 patients (0.02%) who underwent EGD were confirmed to have serrated lesions with predominantly crenated, sawtooth-like configurations. The mean age of the 21 patients was (55.3 ± 17.2) years, and 11 patients were male (52.4%). In terms of the locations of the serrated lesions, 17 were found in the stomach (including 3 in the cardia, 9 in the corpus and 5 in the antrum), 3 were found in the duodenum, and 1 was found in the esophagus. Serrated lesions were found in different mucosal lesions, with 14 lesions were detected in polyps (8 hyperplastic polyps and 6 serrated adenomas with low grade dysplasia), 3 detected in Ménétrier gastropathy, 3 detected in an area of inflammation or ulcer, and 1 detected in the intramucosal carcinoma of the duodenum. In addition, colonoscopy data were available for 18 patients, and a significantly higher colorectal adenoma detection rate was observed in the UPGI serrated lesions group than in the randomly selected age- and gender-matched group without serrated lesions who also underwent colonoscopy in the same period (38.9% *vs* 11.1%, OR = 5.091, 95%CI: 1.534-16.890, $P = 0.010$). The detection rate of advanced adenoma was also higher in the UPGI serrated lesions group (22.2% *vs* 4.2%, OR = 6.571, 95%CI: 1.322-32.660, $P = 0.028$).

CONCLUSION

Serrated lesions in the UPGI were detected in various mucosal lesions with different pathological morphologies. Moreover colonoscopy is recommended for the detection of concurrent colorectal adenoma for these patients.

Key words: Clinical features; Upper gastrointestinal tract; Serrated lesions; Colorectal adenoma; Colorectal cancer

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Core tip: In this retrospective study, the clinical features of the serrated lesions in the upper gastrointestinal tract (UPGI) were analysed. We found that serrated lesions in the UPGI occurred can be found in different mucosal lesions. Furthermore, a significantly higher colorectal adenoma detection rate was observed in the UPGI serrated lesions group than in the randomly selected age- and gender-matched group from our colonoscopy database, and the detection rate of advanced adenoma was also higher in the UPGI serrated group. Therefore, colonoscopy is recommended for the detection of concurrent colorectal adenoma in patients with UPGI serrated lesions.

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INTRODUCTION

A new "alternative" pathway by which adenocarcinomas develop from serrated lesions was recently described by *Jass and Smith*, and it may account for 10% to 30% of all cases of colorectal cancer (CRC)^[1-5]. According to the 2010 WHO classification, three subgroups including hyperplastic polyps (HP), traditional serrated adenoma (TSA), and sessile serrated adenoma/polyp have been divided, forming a heterogeneous group of colorectal lesions. However, detailed information about patients with serrated lesions in the upper gastrointestinal tract (UPGI) is very limited.

UPGI nonconventional adenomatous and nonadenomatous types of dysplasia, such as serrated adenoma and dysplasia, have recently been identified. In 1992, *Stolte et al*^[6] revealed a characteristic "hypertrophy" of the parietal cells that was induced by omeprazole, and produced a serrated internal gland profile. In addition, in 2001, *Rubio*^[7] reported the first case of serrated adenoma of the stomach. Since then, serrated dysplasia has been reported in patients with Ménétrier-like lymphocytic gastritis^[8], reactive gastropathy^[9] and even Barrett's esophagus^[10], and it is epitomised by hypereosinophilic cytoplasm, small oval-shaped nuclei and prominent luminal serration. Serrated adenomas characterized by branched villi exhibiting lateral saw-tooth indentations lined with dysplastic cells or "Christmas-tree-like" serrated configurations have also been detected in the esophagus^[11], the stomach^[12-20], the duodenum^[19,21-27], the pancreas^[28] and the gallbladder^[29]. Although serrated adenomas are rare, recent reports indicate that 53.4% (39/73) of traditional serrated adenomas in the UPGI are invasive carcinomas^[30]. Therefore, analysing the comprehensive clinical features of UPGI serrated lesions is still worthwhile.

Moreover, a meta-analysis was recently performed to assess patients at risk of developing colorectal polyps with upper digestive polyps, and the finding showed that the incidence of colorectal neoplasia was markedly higher in patients with UPGI polyps than in those without UPGI polyps^[31]. However the relationship between UPGI serrated lesions and colorectal neoplasia is not clear. Hence, in the present study, we analysed the clinical and pathological features of serrated lesions in the UPGI and also evaluated the colonoscopy findings in the study group.

MATERIALS AND METHODS

Design and patients

Patients who underwent a routine esophagogastroduodenoscopy (EGD) at the Digestive Endoscopy Centre of the General Hospital, Tianjin Medical University between January 2011 and December 2015 were consecutively recruited. Patients who underwent other types examinations, such as therapeutic endoscopy and emergent endoscopy, were not included. The patients' features, including their age, gender, body mass index (BMI), endoscopy indications, family history of cancer and the size and location of the lesions were extracted from the endoscopy reports and patient questionnaires.

In patients with histologically confirmed UPGI serrated lesions, colonoscopy was required simultaneously or within six months. Each patient was compared to 4 randomly selected age- and gender-matched controls without serrated lesions who also underwent a colonoscopy within the same time period. We also analysed the differences in colorectal neoplasia detection in each study patient and in the control group. Patients with a history of colonoscopy polypectomy or surgical resection of the colon or rectum and patients with a family history of CRC, polyposis syndromes or inflammatory bowel disease were excluded. Informed consents for EGD and colonoscopy were obtained from all of the participants before the procedure, and the researchers had access to the patients' identifying information. The study was approved by the Ethics Committee of the General Hospital, Tianjin Medical University.

Endoscopic procedure

Before undergoing the EGD, all of the patients were asked to undergo a fasting period of at least 12 h and water deprivation for 8 h. Polyethylene glycol lavage was prescribed for bowel preparation and watery diarrhea excretion prior to the procedure indicated adequate intestinal preparation for the colonoscopy. Experienced endoscopists carefully performed the colonoscopies while the patients were under anaesthesia. The cardiopulmonary function was monitored by an anaesthetist, and the patients were maintained under general anaesthesia with intravenous injections of propofol. Electronic gastroscopy (GIF-Q260, Olympus, Tokyo, Japan) and colonoscopy (Olympus CF-Q260, Olympus, Tokyo, Japan) equipment were used for all procedures.

Pathological evaluation

All of the biopsy specimens or resected lesions that collected during the EGD were fixed in 10% formalin within 1 h of removal and then fixed for a minimum of 4 h. All haematoxylin and eosin-stained sections used for the pathological assessment and classification were evaluated by experienced pathologists. Serrated

lesions in the UPGI exhibit clinically and molecularly diverse changes with common features, such as crypt luminal morphology characterized by glandular serration. In addition, advanced colorectal adenomas (AA) were defined as tubular adenomas > 10 mm in size, adenomas with villous histology, or high grade dysplasia. Multiple polyps were categorized according to the most advanced lesion.

Statistical analysis

All of the statistical analyses were performed with SPSS 17.0 (Chicago, IL, United States) for Windows. The means and standard deviations (SDs) were calculated for continuous variables. Categorical or constitute data were expressed as percentage. Risks of colorectal neoplasia between patients with serrated lesions in the UPGI and the control group in our database were compared *via* the χ^2 test or Fisher's exact test. The level of statistical significance was set at two-tailed $P < 0.05$.

RESULTS

General information on the study group

During the study period, 98746 routine EGDs were performed. A total of 21 patients with serrated lesions that exhibited predominantly crenated, sawtooth-like configurations were diagnosed. The mean age of these 21 patients with serrated lesions was (55.3 ± 17.2) years, and the proportion of males was 52.4% (11/21). The mean BMI of the patients was (24.9 ± 5.8) kg/m² and 13 patients (61.9%) had a BMI within the normal range. The proportions of patients with a history of smoking, alcohol use, and a family history of gastric cancer were 33.3% (7/21), 47.6% (10/21) and 4.8% (1/21), respectively. The indications for EGD included upper abdominal pain (23.8%, 5/21), nausea, vomiting and reflux (19.0%, 4/21), anemia and edema (19.0%, 4/21), positive fecal occult blood test (14.3%, 3/21), a history of gastric polyps (14.3%, 3/21) and dyspepsia (9.5%, 2/21) (Table 1).

Distribution of serrated lesions detected in UPGI

Table 2 presents the distribution of serrated lesions: 17 lesions were detected in the stomach (including 3 in the cardia, 9 in the corpus and 5 in the antrum), 3 were detected in the duodenum (2 in the duodenal bulb and 1 in the descending part) and 1 was detected in the lower esophagus.

Morphology of UPGI serrated lesions

The mean size of the UPGI serrated lesions was (11.7 ± 10.3) mm. The diameter was less than 20 mm in 18 patients, and more than 30 mm in 2 patients. The histopathological features of different serrated lesions were divided into four morphologies: (1) serrated hyperplasia (6/21) which was detected in

Table 1 General information on patients with serrated lesions in upper gastrointestinal tract

	<i>n</i> (%)
Total	21
Mean age (yr), mean \pm SD	55.3 \pm 17.2
Gender, male	11 (52.4)
Body mass index (kg/m ²), mean \pm SD	24.9 \pm 5.8
18.5-23.9	13 (61.9)
\geq 24.0	8 (38.1)
History of smoking	7 (33.3)
Alcohol consumption	10 (47.6)
Family history of gastric cancer	1 (4.8)
Indications for endoscopy	
Upper abdominal pain	5 (23.8)
Nausea, vomit and reflux	4 (19.0)
Anemia and edema	4 (19.0)
Positive fecal occult blood test	3 (14.3)
A history of gastric polyps	3 (14.3)
Dyspepsia	2 (9.5)

Table 2 Clinical features of serrated lesions in upper gastrointestinal tract

	<i>n</i> (%)
Size (mm), mean \pm SD	11.7 \pm 10.3
\leq 5	8 (38.1)
5-10	4 (19.0)
10-20	6 (28.6)
20-30	1 (4.8)
\geq 30	2 (9.5)
Distribution	
Esophagus	1 (4.8)
Cardia	3 (14.3)
Corpus	9 (42.9)
Antrum	5 (23.8)
Duodenum	3 (14.3)
Morphology	
Serrated hyperplasia	6 (28.6)
Hyperplastic polyps	8 (38.1)
Adenoma	6 (28.6)
Adenocarcinoma	1 (4.8)
Situation of serrated lesions in mucosal lesions	
Inflammation or ulcer	3 (14.3)
Serrated polyps	14 (66.7)
Ménétrier gastropathy	3 (14.3)
Duodenal cancer	1 (4.8)
Colonoscopy findings	18
No-polyp	4 (22.2)
Hyperplastic polyps	7 (38.9)
Non-advanced adenomas	3 (16.7)
Advanced adenomas	4 (22.2)
Tubular adenoma with high grade dysplasia	1 (5.6)
Tubulovillous adenoma	2 (11.1)
Adenoma > 10 mm	1 (5.6)

areas of inflammation or ulcer lesions (3/21) and Ménétrier gastropathy (3/21); (2) HPs (8/21); (3) serrated adenoma with low grade dysplasia (6/21); and (4) Serrated lesion (1/21), which was found in the intramucosal carcinoma of the duodenum. The typical pathological images and clinical features are shown in Figure 1 and Table 2.

Table 3 Colonoscopy findings in the patients with serrated lesions in upper gastrointestinal tract

Serrated lesions in upper gastrointestinal tract mucosal lesions (<i>n</i>)	Colonoscopy findings (<i>n</i>)
Inflammation or ulcer (3)	HPs (1)
HPs (8)	AA (2), NAA (1), HPs (2)
Serrated adenoma (6)	AA (2), NAA (2), HPs (2)
Ménétrier gastropathy (3)	HPs (2)
Duodenal cancer (1)	Absent

HPs: Hyperplastic polyps; AA: Advanced adenoma; NAA: Non-advanced adenoma.

Table 4 Prevalence of colorectal adenoma in patients with serrated lesions in upper gastrointestinal tract and the control group *n* (%)

	Patients with serrated lesions in UPGI (<i>n</i> = 18)	Average group (<i>n</i> = 72)	OR (95%CI)	<i>P</i> value
Colorectal adenoma	7 (38.9)	8 (11.1)	5.091 (1.534-16.890)	0.010
Non-advanced adenoma	3 (16.7)	5 (6.9)	2.680 (0.576-12.463)	0.195
Advanced adenoma	4 (22.2)	3 (4.2)	6.571 (1.322-32.660)	0.028

Prevalence of colorectal neoplasia in patients with UPGI serrated lesions

A total of 18 patients with UPGI serrated lesions (81.7%, 18/21) underwent a colonoscopy simultaneously or within six months, and 3 patients refused to undergo a colonoscopy. We then evaluated the colonoscopy findings of these patients. Three non-advanced colorectal adenomas (NAA) and 4 AAs (1 tubular adenoma with high grade dysplasia, 2 tubulovillous adenomas and 1 adenoma > 10 mm in size) were found, and all of these colorectal adenomas were detected only in patients with UPGI serrated adenomas or polyps. The remaining colonoscopy reports included 7 colorectal HPs and 4 patients without polyps. CRC was not detected found in 18 patients. The colonoscopy findings in the patients with UPGI serrated lesions are illustrated in Table 3. We also compared the detection rate of colorectal adenoma in the UPGI serrated lesions group with that in the control group in our colonoscopy database (Table 4). A total of 72 age- and gender-matched patients without serrated lesions who had presented to our centre for EGD and colonoscopy were randomly selected as the control group. A significantly higher colorectal adenoma detection rate was observed in the UPGI serrated lesions group than in the control group (38.9% vs 11.1%, OR = 5.091; 95%CI: 1.534-16.890; *P* = 0.010), and a higher detection rate of advanced adenoma was observed in the UPGI serrated lesions

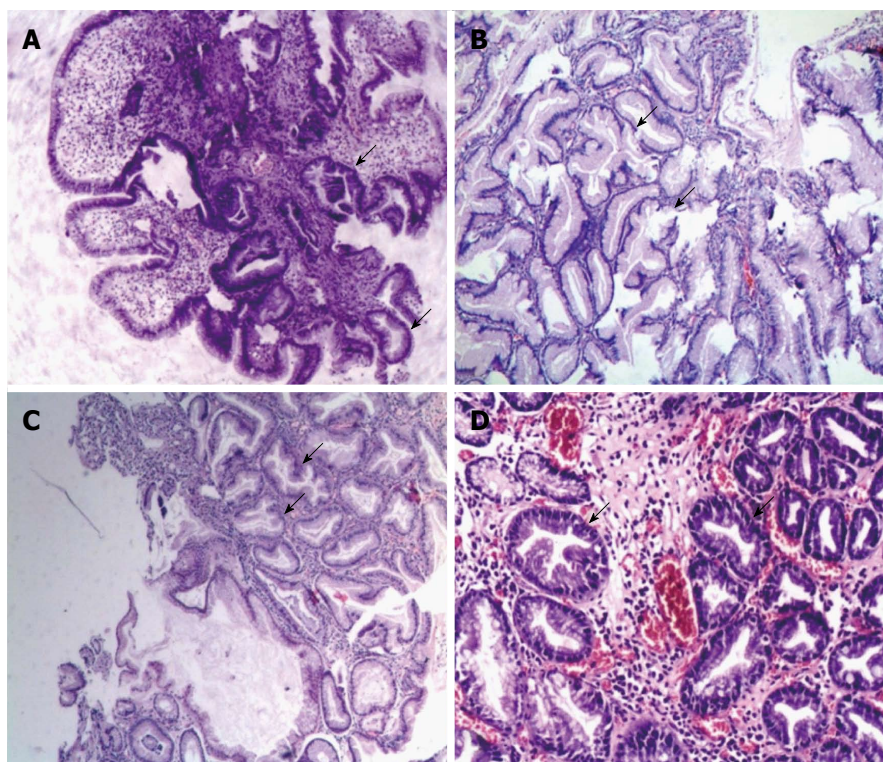


Figure 1 Typical pathological images of serrated lesions in upper gastrointestinal tract. Serrated lesions characterized by epithelial cells with luminal infolding and a serrated growth pattern were shown. A: Serrated hyperplasia in esophagitis ($\times 40$); B: Serrated hyperplasia in the Ménétrier gastropathy: marked foveolar hyperplasia and glandular cysts with serrated lesions in the stomach ($\times 100$); C: Hyperplastic polyp in the stomach: a serrated polyp without overt cytological atypia showed narrowed crypt bases that were predominantly lined with immature cells ($\times 100$); D: Serrated adenoma with low grade dysplasia in the duodenum: a serrated polyp with enlarged nuclei, a pencil-shaped, hyperchromaticity and nuclear stratification ($\times 100$).

group (22.2% vs 4.2%, OR = 6.571; 95%CI: 1.322-32.660; $P = 0.028$).

DISCUSSION

Since 1990, serrated polyps have been commonly found during colonoscopy and recognized as an important process in the development of CRC^[1,32,33]. Limited reports focused on the clinical features of UPGI serrated lesions because of the low prevalence of these lesions, and few articles have described serrated adenomas in the UPGI^[7-25]. Thus, serrated polyps have not been previously listed in the classifications of the upper digestive tract^[34-36]. The present study provides current information on serrated lesions in different UPGI diseases, including inflammation or ulcer, Ménétrier gastropathy, HPs, serrated adenomas, and adenocarcinoma, as well as the serrated profile found in cases of reactive gastropathy^[8,9]. We also found that nearly half of the UPGI serrated lesions were located in the gastric corpus, and 2/3 of the lesions were detected in polyps in the current study. In addition, we evaluated the colonoscopy findings of patients with UPGI serrated lesions, and found a significantly higher colorectal adenoma detection rate in the serrated lesions group than in the control group, thus colonoscopy may be recommended to exclude the presence of concurrent colorectal adenomas in

these patients.

This study provides the first description the detection and distribution of serrated lesions in the UPGI, and analysed the colonoscopy results of these patients compared with the control group. Simple and readily accepted methods (EGD and colonoscopy) were used, and the possibility of clinical heterogeneity was minimized because of the study setting, which was within a tertiary endoscopic centre. However, several limitations should be mentioned. First, relatively small sample size was used in the present study because of the rarity of serrated lesions, and a statistical analysis of the age, gender, BMI and family history of patients with different mucosal lesions could not be conducted. Second, this study was conducted in a tertiary endoscopic centre, therefore selection bias likely occurred. In addition, more rigorous studies with larger sample sizes from multiple clinical centres are necessary to determine whether patients with UPGI serrated lesions have a higher rate of colorectal adenomas and to ascertain whether these findings similar to those in previous reports^[31,37].

Activating mutations of the RAS-RAF-MAPK pathway have been reported to initiate and sustain lesions in the serrated pathway, and the presence of a positive CpG island methylation phenotype and DNA repair genes might play a major role in colorectal neoplastic progression^[5,38,39]. Compared with serrated

polyps of the colon, extracolonic serrated polyps are virtually undescribed and their genetic alterations are largely unknown. The pathological findings and analysis of the molecular alterations of 13 serrated neoplasms of the small intestine indicated that almost half of the neoplasms demonstrated high-grade dysplasia or were associated with an adenocarcinoma. However, the absence of the *BRAF*^{V600E} mutation does not support a role for the serrated neoplasia pathway in the development of these lesions, as it does in colorectal serrated polyps^[26]. Another report confirmed that oncogenic *KRAS* mutation was the most common abnormality in extracolonic serrated polyps, whereas a microsatellite instability and a CpG island methylator phenotype were less commonly^[19]. Rubio^[30] presented a TSA pathway of carcinogenesis in the UPGI, and 53.4% (39/73) of the UPGI TSAs reported in the literature are associated with invasive carcinomas, however, we only detected one case associated with duodenal cancer. The younger average age of the patients with serrated adenoma in our study (62.2 ± 11.4) than that in the past reports (66.4 ± 11.7) compared with that in previous may provide a suitable explanation for this phenomenon. Hence, the mechanism that causes these lesions to evolve into invasive carcinomas remains elusive.

In conclusion, serrated lesions in the UPGI, which represents a rarely described histological phenotype, were observed in various mucosal lesions with different pathological morphologies. Moreover, colonoscopy is recommended to exclude the presence of concurrent colorectal carcinomas in these patients. However, further studies are needed to clarify the clinical significance of these lesions.

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COMMENTS

Background

Recently, a new "alternative" pathway by which adenocarcinomas develop from serrated lesions was first described by Jass and Smith, and this pathway may account for 10% to 30% of all cases of colorectal cancer. However, information on upper gastrointestinal (UPGI) serrated lesions is limited. UPGI nonconventional adenomatous and nonadenomatous types of dysplasia, such as serrated adenoma and dysplasia, have been recently identified. Although serrated adenomas are rare, recent reports have indicated that 53.4% (39/73) of traditional serrated adenomas in the UPGI are invasive carcinomas. Therefore, analysing the comprehensive clinical features of the UPGI serrated lesions is still worthwhile.

Research frontiers

Colorectal serrated polyps are recognized as important contributors to colorectal cancer. However, detailed information on upper gastrointestinal serrated lesions is limited. The results of this study contribute to the analysis of the clinical features of serrated lesions in the UPGI, and the findings recommend colonoscopy for the detection of to find concurrent colorectal

adenomas in these patients.

Innovations and breakthroughs

In this article, the authors found that serrated lesions in the UPGI occur in different mucosal lesions, such as areas of inflammation and ulcers, hyperplastic polyps, serrated adenomas and Ménétrier gastropathy. Furthermore, a significantly higher colorectal adenoma detection rate was observed in the UPGI serrated lesions group than in the randomly selected age- and gender-matched group from our colonoscopy database, and the detection rate of advanced adenoma was also higher in the UPGI serrated lesions group. Therefore, colonoscopy is recommended for the detection of concurrent colorectal adenomas in patients with UPGI serrated lesions.

Applications

This study shows that serrated lesions in the UPGI occur in different mucosal lesions. Furthermore, patients diagnosed with serrated lesions in the UPGI, should undergo a colonoscopy to detect any concurrent colorectal adenomas.

Terminology

UPGI: Endoscopic examination that includes esophagus, stomach, ampulla and the descending part of the duodenum.

Peer-review

Although the serrated lesions in UPGI are rare in the population, it is very important to understand its clinical and pathological features as such lesions maybe related to invasive carcinoma in UPGI exhibited. Furthermore, authors found in this study that the serrated lesions in UPGI are associated with higher colorectal adenoma detection rate.

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Observational Study

Influence of capsaicin infusion on secondary peristalsis in patients with gastroesophageal reflux disease

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Abstract

AIM

To determine whether capsaicin infusion could influence heartburn perception and secondary peristalsis in patients with gastroesophageal reflux disease (GERD).

METHODS

Secondary peristalsis was performed with slow and rapid mid-esophageal injections of air in 10 patients with GERD. In a first protocol, saline and capsaicin-containing red pepper sauce infusions were randomly performed, whereas 2 consecutive sessions of capsaicin-containing red pepper sauce infusions were performed in a second protocol. Tested solutions including 5 mL of red pepper sauce diluted with 15 mL of saline and 20 mL of 0.9% saline were infused into the mid-esophagus *via* the manometric catheter at a rate of 10 mL/min with a randomized and double-blind fashion. During each study protocol, perception of heartburn, threshold volumes and peristaltic parameters for secondary peristalsis were analyzed and compared between different stimuli.

RESULTS

Infusion of capsaicin significantly increased heartburn perception in patients with GERD ($P < 0.001$), whereas repeated capsaicin infusion significantly reduced heartburn perception ($P = 0.003$). Acute capsaicin infusion decreased threshold volume of secondary peristalsis ($P = 0.001$) and increased its frequency ($P = 0.01$) during rapid air injection. The prevalence of GERD patients with successive secondary peristalsis during slow air injection significantly increased after capsaicin infusion ($P = 0.001$). Repeated capsaicin infusion increased threshold volume of secondary peristalsis ($P = 0.002$) and reduced the frequency of secondary peristalsis ($P = 0.02$) during rapid air injection.

CONCLUSION

Acute esophageal exposure to capsaicin enhances heartburn sensation and promotes secondary peristalsis in gastroesophageal reflux disease, but repetitive capsaicin infusion reverses these effects.

Key words: Capsaicin; Esophageal motility; Secondary peristalsis; Esophageal manometry; Gastroesophageal reflux disease

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Core tip: This clinical significance of this study is that acute esophageal infusion of capsaicin-containing red pepper sauce significantly enhances mechanosensitivity to distension-induced secondary peristalsis in patients with gastroesophageal reflux disease (GERD), which might be beneficial in reflux patient with hypomotility. Conversely, repeated esophageal exposure to capsaicin-containing red pepper sauce may reduce the efficiency of esophageal secondary peristalsis. Repeated capsaicin infusion may therefore reduce the protection of the esophagus by hampering the clearing of residue substance or refluxate in the esophagus, which may in turn prolong acid clearance in patients with GERD.

Yi CH, Lei WY, Hung JS, Liu TT, Chen CL, Pace F. Influence of capsaicin infusion on secondary peristalsis in patients with gastroesophageal reflux disease. *World J Gastroenterol* 2016; 22(45): 10045-10052 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v22/i45/10045.htm> DOI: <http://dx.doi.org/10.3748/wjg.v22.i45.10045>

INTRODUCTION

Secondary peristalsis is triggered by esophageal distension when food, liquid or air is retained in the esophagus after a failed primary peristaltic event or a reflux from the stomach^[1]. It is important to maintain an empty esophagus by clearing the bulk of the volume of the refluxate after a reflux event^[2]. In

order to prevent prolonged acid contact time in the esophagus^[3], secondary peristalsis helps normalize esophageal pH together with primary peristalsis and swallowed saliva^[2]. It is suggested in human esophagus that both mucosal and muscular mechanoreceptors are involved in triggering secondary peristalsis which arises from a reflex arc mediated by a vagal afferent pathway^[4,5].

Recent studies have demonstrated that patients with gastroesophageal reflux disease (GERD) have considerably abnormal secondary peristaltic response rates when compared with aged matched controls^[6]. By application of mid-esophageal injections of water and air, it was shown that GERD was characterized with defective triggering of secondary peristalsis^[6]. We have recently studied secondary peristalsis in patients with GERD using mid-esophageal air stimulation with different speeds^[7-9]. We notice that there is a substantial defect of activation of secondary peristalsis in a subgroup of GERD patients with significant esophageal dysmotility, indicating that increasing severity of failed primary peristalsis along with defective triggering of secondary peristalsis contributes to impaired esophageal clearance in patients with GERD^[7].

Despite its effect of enhancing secondary peristalsis when acutely administered to the esophageal mucosa^[10], we have recently demonstrated that repeated intra-esophageal infusion of capsaicin-containing red pepper sauce indeed inhibited secondary peristalsis in healthy adults with reducing induction of heartburn symptoms^[7]. The likelihood of secondary peristaltic response by abrupt air injection was increased by transient capsaicin infusion, but reduced by repeated capsaicin infusion. By characterizing esophageal desensitization as induced by repeated capsaicin infusion in modulation of secondary peristalsis in human esophagus, our recent work provides further insight in understanding the physiological basis of transient receptor potential vanilloid 1 (TRPV1) mediated chemosensitivity and mechanosensitivity in human esophagus^[7].

Therefore, the aim of this study was to test the hypothesis that heartburn perception and physiological characteristics of secondary peristalsis can differently be influenced by acute or repetitive intra-esophageal infusion of capsaicin-containing red pepper sauce in GERD patients.

MATERIALS AND METHODS

Subjects

In this prospective study, we enrolled consecutive patients with GERD who were previously diagnosed as having reflux disease by the presence of typical symptoms associated with positive endoscopy findings^[11] or documented abnormal acid exposure on 24-hour pH monitoring. All patients had typical reflux symptoms lasting for more than 6 mo. We excluded subjects with the following clinical conditions: (1) esophageal

strictures; (2) previous gastrointestinal surgery; (3) presence of systemic diseases that might interfere with esophageal motility; (4) chronic use of medications known to affect esophageal motility; and (5) intolerance and/or lack of cooperation with entire protocol. Prior to the study, all subjects did not use any medication that might affect gastrointestinal motility. This study was performed after approval by Research Ethics Committee of Hualien Tzu Chi Hospital, Buddhist Tzu Chi Medical Foundation, Hualien, Taiwan and written informed consent was obtained from the participants.

Esophageal manometry

Stationary esophageal manometry was performed using a Koenigsberg 4-channel probe (Sandhill Scientific, Inc., Highlands Ranch, CO, United States). The catheter with 4.5 mm in diameter includes a circumferential solid-state pressure sensor at 5 cm and three unidirectional pressure sensors at 10, 20, and 25 cm from the tip. The infusion port is in the mid esophagus with its location between 15 and 20 cm from the tip. Each subject had the catheter inserted transnasally into the esophagus up to a depth of 60 cm. Then, we used stationary pull-through technique to withdraw the catheter until that the most distal sensor was located in the high-pressure zone of the lower esophageal sphincter (LES). Data of the entire study were then recorded and stored on the computer. Swallowing was detected by the most proximal channel of the catheter, which was located in the pharynx in order to distinguish primary and secondary peristalsis.

Study design

After an overnight fast, secondary peristalsis was recorded 10 min after esophageal infusion of saline and capsaicin-containing red pepper sauce, or 2 sessions of capsaicin-containing red pepper sauce on two separate days. Tested solutions including 5 mL of red pepper sauce (Tabasco, McIlhenny Company, Avery Island, LA, United States), diluted with 15 mL of saline and 20 mL of 0.9% saline were infused into the mid-esophagus *via* the manometric catheter at a rate of 10 mL/min with a randomized and double-blind fashion. We wrapped the syringe in aluminum foil in order to mask characteristic colors of different infusions. Subjective symptoms including nausea, heartburn, stick and pain were evaluated with a visual analogue scale score (VAS) (0-100) shortly after each session of the infusion. The total amount of infused red pepper sauce suspension (5 mL of Tabasco) was equivalent to 0.84 mg of pure capsaicin^[10,12].

Secondary peristalsis was generated by the air injection into the esophagus conducted first by a slow air injection with an infusion pump attached to the manometric catheter at a rate of 0.25 mL/s. We measured total amount of volume tested with the pump machine based on the rate and time for air injection to induce secondary peristalsis. Secondary peristalsis

was then performed with rapid air injection, in which 1-mL volume was started and gradually increased by 1-mL increments until the activation of a secondary peristalsis or the volume of the injection reached 20 mL. The threshold volumes for air injections were measured as the minimal injection volume allowed for triggering a secondary peristaltic pressure wave^[13]. Then, secondary peristaltic response was determined by ten times of 20 mL of air injections. We determined overall secondary peristaltic response with an interval of 20 s, during which each subject refrained from any swallow. Each subject was allowed to take a dry swallow to clear any residual air inside the esophagus and to avoid any swallow during next air injection at the end of 20 s.

Data analysis

Successful secondary peristalsis was recognized if the pressure wave was greater than 12 mmHg in the proximal esophagus and was greater than 25 mmHg in the distal esophagus with normal propagation^[14]. The minimal latency of wave onset between two adjacent channels was 0.5 s. We analyzed the data in the same manner for both slow and rapid injections^[14]. For measuring esophageal wave amplitude (mmHg) and duration (s), the recording sites were located 5 cm above upper margin of the LES.

Statistical analysis

We assessed the normality of all data by D'Agostino's χ^2 test. All data including amplitude, duration, VAS score, and threshold volumes of secondary peristalsis were present as mean \pm SEM, and were compared by a paired *t*-test. Data for successful peristaltic response as induced by rapid air injection were analyzed and compared using the Wilcoxon signed-rank test and were shown as median with interquartile range. Data for peristaltic wave amplitude and duration were compared for distal esophagus. Statistical significance was determined if $P < 0.05$. Statistical analyses were performed with SPSS 19 for Windows (SPSS, Inc., IL, United States).

RESULTS

We studied 17 reflux patients who met the enrollment criteria and entered the study between Dec 1, 2012 and Nov 31, 2013. Ten patients (4 females, mean age 42 years, range 20-64) completed the entire protocol with different session of capsaicin-containing red pepper sauce infused to the esophagus without any complication. Of the patients with GERD, 8 patients with reflux esophagitis, LA grade A and 2 patients had normal endoscopy. The most frequent cause of exclusion from this study is intolerance to the protocol due to esophageal infusion with capsaicin-containing red pepper sauce.

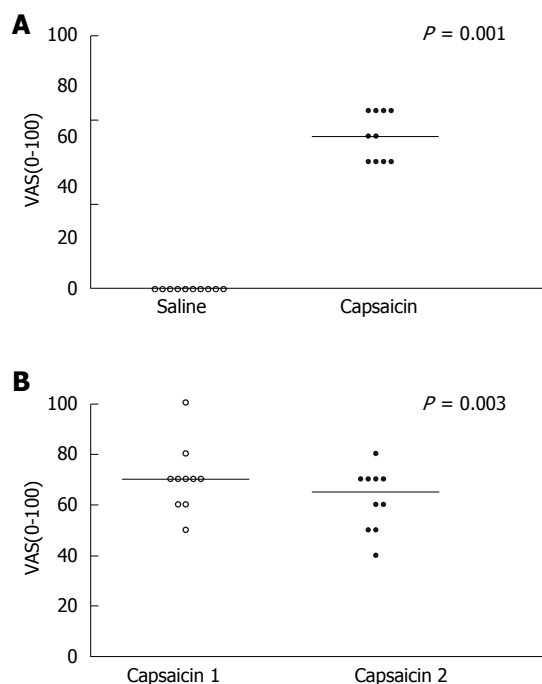


Figure 1 Influence of capsaicin-containing red pepper sauce on heartburn symptom. A: Capsaicin infusion induces a significant increase in the visual analog scale for heartburn symptom when compared with saline ($P < 0.001$); B: The visual analog scale for heartburn symptom is significantly decreased by repeated capsaicin infusion than first session of capsaicin infusion ($P = 0.003$). Values are expressed as mean \pm SE of the mean. Line represents the mean value.

Symptom perception and distension thresholds of secondary peristalsis

Infusion of capsaicin significantly increased the VAS score for heartburn symptom in patients with GERD when compared with saline infusion ($P < 0.001$) (Figure 1A). During 2 consecutive sessions of capsaicin infusion, the VAS score of heartburn symptom was significantly reduced after repeated infusion of capsaicin as compared with that after first capsaicin infusion ($P = 0.003$) (Figure 1B). When compared with saline infusion, infusion of capsaicin significantly reduced the threshold volume to activate secondary peristalsis during rapid air injection ($P = 0.001$) (Figure 2A), and a significant increase in the frequency of secondary peristalsis ($P = 0.01$) during rapid air injection (Figure 2B). Infusion of capsaicin increased the number of GERD patients with successive secondary peristalsis during slow air injection than saline infusion ($P = 0.001$) (Figure 3A), but the difference was not significant between first and second capsaicin infusions ($P = 0.18$) (Figure 3B). During 2 consecutive infusions of capsaicin infusions, there was a significant increase in threshold volume to generate secondary peristalsis after second infusion of capsaicin ($P = 0.002$) compared with that after first infusion of capsaicin during rapid air injection (Figure 4A). When compared with first infusion of capsaicin, second infusion of capsaicin significantly reduced the frequency of secondary peristalsis ($P = 0.02$) during rapid air injection (Figure 4B).

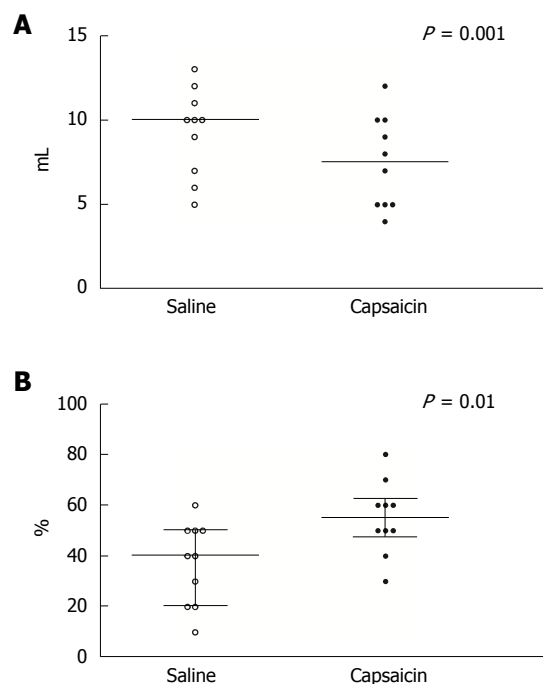


Figure 2 Influence of capsaicin-containing red pepper sauce on distension threshold to induce secondary peristalsis during rapid air injection. A: The threshold volume for inducing secondary peristalsis is significantly decreased after capsaicin when compared with saline infusion ($P = 0.001$); B: Secondary peristalsis is triggered more frequently after capsaicin infusion than saline infusion ($P = 0.01$). Values are expressed as mean \pm SE of the mean or median with interquartile range. Line represents the mean or median value.

Esophageal body peristalsis

Infusion of capsaicin did not change pressure amplitude or duration when compared with saline infusion during slow and rapid air injections (Table 1). Furthermore, during 2 consecutive sessions of capsaicin infusions, no significant difference was found between 2 capsaicin infusions for any peristaltic parameters of secondary peristalsis during slow and rapid air injections (Table 1).

DISCUSSION

This principal finding of this study is that acute esophageal infusion of capsaicin-containing red pepper sauce significantly enhances heartburn perception and mechanosensitivity to distension-induced secondary peristalsis in patients with GERD, which might be beneficial in reflux patient with impaired esophageal motility^[7]. However, secondary peristalsis is inhibited by repetitive esophageal infusion with capsaicin-containing red pepper sauce. Although this study supports the evidence that capsaicin sensitive afferents mediate heartburn symptom and secondary peristaltic thresholds, none of motility parameters of secondary peristalsis is influenced by acute or repeated esophageal infusion with capsaicin-containing red pepper sauce.

In this study, we found that heartburn symptoms in GERD patients were enhanced by rapid esophageal

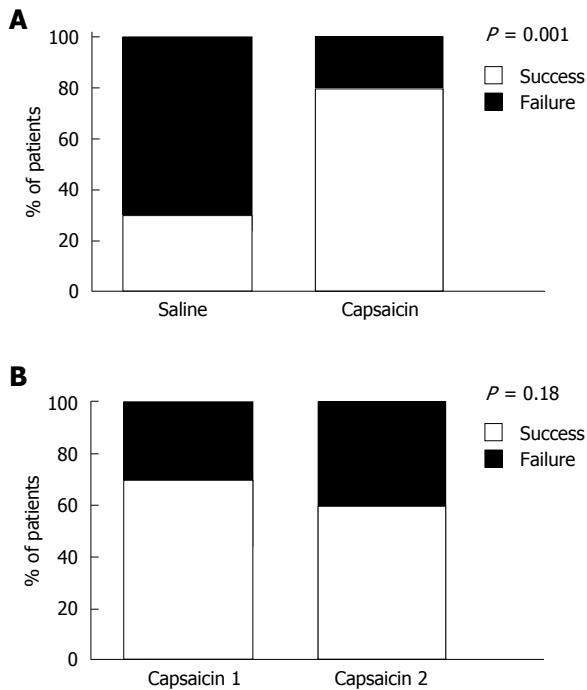


Figure 3 Influence of capsaicin-containing red pepper sauce on the prevalence of successive secondary peristalsis during slow air injection. A: The prevalence of patients with successive secondary peristalsis increases after capsaicin infusion than saline infusion ($P = 0.001$); B: There are no difference in the prevalence of patients with successive secondary peristalsis between first and second capsaicin infusions ($P = 0.18$).

infusion of red pepper sauce, but was suppressed by repeated infusion of red pepper sauce. Our findings are in line with previous study which demonstrated the activation of heartburn symptom in non-GERD subjects with intra-esophageal instillation of capsaicin at a dose equivalent to 0.84 mg^[10]. In addition, we have recently observed that repeated esophageal exposure to red pepper sauce reduced the intensity of heartburn symptom in healthy volunteers^[15]. The findings are in agreement with an earlier work in GERD patients that also noticed an analgesic effect in perceiving heartburn after repeated stimulation with the capsaicin^[16]. Together with these findings, it is conceivable that the perception of heartburn symptom is likely to be *via* TRPV₁ receptor as established in our previous work^[10]. Although sensitization of TRPV₁ receptor is important for mediating perception of heartburn symptom^[17,18], this receptor may also become desensitized after the continued presence of capsaicin^[19]. Capsaicin is known to be an intrinsic primary afferent neurons excitant and a neurochemical substance that initially activates and later desensitizes afferent pathways^[20]. It has been demonstrated in Pavlov's esophageal fistula dog that the cephalic phase of gastric secretion can be modulated in condition when the pharynx was bypassed^[21]. In this study, we found that repeated esophageal infusion of capsaicin selectively attenuated secondary peristalsis activated by rapid injection instead of slow air injection of the esophagus in GERD patients. Our findings are supported by the results of Lang *et al*^[5]

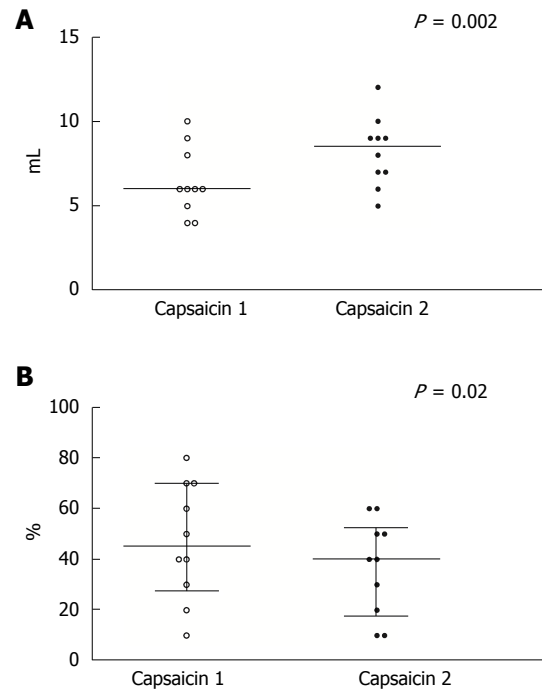


Figure 4 Influence of repeated capsaicin-containing red pepper sauce on distension threshold to induce secondary peristalsis during rapid air injection. A: The threshold volume for inducing secondary peristalsis is significantly greater after second infusion of capsaicin than first infusion of capsaicin ($P = 0.002$); B: Secondary peristalsis is triggered less frequently after second infusion of capsaicin than first infusion of capsaicin ($P = 0.02$). Values are expressed as mean \pm SE of the mean or median with interquartile range. Line represents the mean or median value.

who showed in animal model that repeated application of capsaicin selectively inhibited the reflexes activated by rapid distension rather than slow distension of the esophagus. Therefore, current findings reemphasize the notion that the reflexes generated by rapid distension of the esophagus are modulated by chemically sensitive esophageal mechanoreceptors while those reflexes induced by slow distension are likely to be mediated by chemically insensitive mechanoreceptors. That notion is evident in patients with GERD.

The discrepancy in the amplitudes of secondary peristalsis after capsaicin infusion between healthy controls and GERD patients can be explained due to the fact that patients with GERD are more likely to have relatively poor motility in term of ineffective motility. In patients with abnormal primary peristalsis, abnormal secondary peristalsis has been observed^[6]. It is suggested that the defect may occur in the efferent part of the motor pathway.

It is as yet not completely clear whether a desensitization of the esophagus can be induced by a repeated capsaicin infusion, although other studies have showed desensitization phenomenon in other human organs including the skin and nasal mucosa^[22,23]. Acute jejunal infusion of capsaicin induced burning sensations and pain without affecting sensitivity to balloon distension^[24,25], whereas other studies have shown that repeated administration of capsaicin is associated

Table 1 Effects of capsaicin-containing red pepper sauce on secondary peristaltic parameters

	Saline	Capsaicin	Capsaicin 1	Capsaicin 2
Amplitude of contractions (mmHg)				
Slow distension	82.9 (17.3)	82.6 (20.2)	95.9 (9.1)	105.1 (11.1)
Rapid distension	94.5 (11.5)	104.8 (15.2)	116.7 (22.0)	121.8 (17.1)
Duration of contractions (s)				
Slow distension	3.0 (0.3)	3.5 (0.4)	3.4 (0.5)	3.5 (0.7)
Rapid distension	3.24(0.4)	4.0 (0.7)	3.5(0.6)	4.0 (0.7)

Data are expressed as mean \pm SE of the mean.

with reduced sensitivity to balloon distension in the intestine^[26]. Conversely, there was no change in acid-induced esophageal mechanosensitivity to balloon distension after esophageal pretreatment with capsaicin^[27]. It is conceivable that the effect on mechanosensitivity of capsaicin, regardless of mode for esophageal distension, on mechanosensitivity may be variable according to differences regarding types of stimuli and study designs. We have previously demonstrated a desensitization effect on distension-induced secondary peristalsis by repeated capsaicin infusion of the esophagus in healthy subjects^[15]. In this study, we confirmed in a group of GERD patients that desensitization effect on secondary peristalsis can be accomplished by repeated esophageal capsaicin infusion.

It may be discussed whether repeated visceral exposure to capsaicin provides a complete esophageal desensitization^[28], in particular in humans. It has been reported that the durations of desensitization can last from several hours to weeks after capsaicin exposure in human studies^[22,29], and such a durable effect has been shown in upper gastrointestinal motility in healthy volunteers^[30]. In this work, the duration of the desensitization effects of repeated capsaicin administrations was studied only in a limited time period, which may impact the physiological significance and mechanisms how capsaicin-induced analgesia generates in the esophagus. Indeed, after repeated infusion of capsaicin in this study, local esophageal capsaicin concentration may reach about 10 μ mol/L, which may cause rapid degeneration of capsaicin-sensitive nerve endings^[31]. It is probably that the desensitization effect of repeated capsaicin infusion is due to the temporary loss of capsaicin-sensitive afferents in the esophagus. This needs to be clarified by further longitudinal studies.

This study has some clinical implications. Current data support an earlier notion that esophageal mucosa is sensitive to capsaicin stimulation which induces heartburn symptom and which can be reduced by repeated exposure to capsaicin-containing red pepper sauce^[32]. The fact that repeated esophageal exposure of capsaicin-containing red pepper sauce decreases heartburn symptom appears to have potential therapeutic benefit for relieving heartburn symptom in patients with symptomatic GERD, although further work is needed to confirm its clinical utility. From

the other hand, our study suggests that repeated esophageal exposure to capsaicin may inhibit secondary peristalsis and relevant reflex that may reduce the efficiency of esophageal transit and clearance as generated by secondary peristalsis^[13]. By doing so, repeated capsaicin infusion may indeed reduce the protection of the esophagus by hampering the clearing of residue substance or refluxate in the esophagus, which may in turn prolong acid clearance in patients with GERD.

There are some limitations in this study with regard to the issue of desensitization capsaicin-induced of secondary peristalsis in patients with GERD. First, we did not apply the novel technique of high resolution manometry and impedance, which allows better characterization of secondary peristalsis, which may be missed by conventional manometry due to its inferior capability of depicting the peristaltic activity. Second, it is still unclear whether complete desensitization can be achieved by current dose of capsaicin-containing red pepper sauce, although such dose has been successfully applied for studying heartburn and secondary peristalsis in human esophagus with a similar fashion to acid instillation^[7,8,10]. The effect of desensitization is achievable only when the dose causes subjective symptoms with maximal magnitude; however, this is not ethically plausible for *in vivo* study in human esophagus. Third, there are possibly 2 subgroups of GERD including mild erosive reflux disease and those with non-erosive reflux disease; however, we enrolled those patients with typical symptoms as well as good response to acid suppression therapy to get a more homogeneous patient cohort. Finally, the number of studied subjects is small due to intolerability to the procedure, which may lead to type II error.

In summary, acute esophageal infusion with capsaicin-containing red pepper sauce appears to exacerbate heartburn symptom and promote the efficiency of secondary peristalsis in patients with GERD. However, these effects are likely to be reduced with repetitive esophageal exposure to capsaicin-containing red pepper sauce. Our study supports the hypothesis that capsaicin sensitive afferents are responsible for modulating esophageal symptom and distension-induced secondary peristalsis in patients suffering GERD symptoms.

ACKNOWLEDGMENTS

A part of this study was presented as a presentation at the Digestive Disease Week® (DDW) 2014 in Chicago, Illinois and published as an abstract form in *Gastroenterology* (2014) 146 (5 Suppl. 1): S678.

COMMENTS

Background

Capsaicin-containing red pepper sauce improves esophageal secondary peristalsis in healthy adults.

Research frontiers

The authors determined whether acute and repetitive capsaicin-containing red pepper sauce suspension could influence heartburn perception and secondary peristalsis in patients with gastroesophageal reflux disease.

Innovations and breakthroughs

Acute esophageal infusion with capsaicin-containing red pepper sauce appears to exacerbate heartburn symptom and promote the efficiency of secondary peristalsis in patients with gastroesophageal reflux disease (GERD). However, these effects are likely to be reduced with repetitive esophageal exposure to capsaicin-containing red pepper sauce.

Applications

The authors found that capsaicin sensitive afferents are responsible for modulating esophageal symptom and distension-induced secondary peristalsis in patients suffering GERD symptoms.

Peer-review

This was a qualitative study with an original approach to establishing the effects of capsaicin infusion in patients with GERD. The study was well designed and the results are clearly described.

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Observational Study

Smoc2 potentiates proliferation of hepatocellular carcinoma cells *via* promotion of cell cycle progression

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Abstract

AIM

To determine the influence of Smoc2 on hepatocellular carcinoma (HCC) cell proliferation and to find a possible new therapeutic target for preventing HCC progression.

METHODS

We detected expression of Smoc2 in HCC tissues and corresponding non-tumor liver (CNL) tissues using PCR, western blot, and immunohistochemistry methods. Subsequently, we down-regulated and up-regulated Smoc2 expression using siRNA and lentivirus transfection assay, respectively. Then, we identified the effect of Smoc2 on cell proliferation and cell cycle using CCK-8 and flow cytometry, respectively. The common cell growth signaling influenced by Smoc2 was detected by western blot assay.

RESULTS

The expression of Smoc2 was significantly higher in HCC tissues compared with CNL tissues. Overexpression of Smoc2 promoted HCC cell proliferation and cell cycle progression. Down-regulation of Smoc2 led to inhibition of cell proliferation and cell cycle progression. Smoc2 had positive effect on ERK and AKT signaling.

CONCLUSION

Smoc2 promotes the proliferation of HCC cells through accelerating cell cycle progression and might act as an anti-cancer therapeutic target in the future.

Key words: Smoc2; Hepatocellular carcinoma; Cell cycle; Proliferation

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Core tip: In our study, we confirmed that Smoc2 was up-regulated in hepatocellular carcinoma (HCC) tissues and played an important role in regulating liver cancer cell proliferation. Besides, we verified that Smoc2 participated in promoting HCC cell proliferation mainly through regulation of cell cycle progression. We have not investigated the promotive role of Smoc2 in regulating cell proliferation, whether it is through cell cycle regulation only or involves regulation of cell apoptosis as well. Moreover, the exact mechanism of how Smoc2 regulates cell cycle remains unclear. The core contents of our study included Smoc2 promotion of HCC cell proliferation *via* accelerating cell cycle progression.

Su JR, Kuai JH, Li YQ. Smoc2 potentiates proliferation of hepatocellular carcinoma cells *via* promotion of cell cycle progression. *World J Gastroenterol* 2016; 22(45): 10053-10063 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v22/i45/10053.htm> DOI: <http://dx.doi.org/10.3748/wjg.v22.i45.10053>

INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common malignancies worldwide, with high mortality rate and low early diagnostic rate^[1]. HBV infection, alcohol abuse, aflatoxin exposure and HCV infection are identified as major causes of HCC. The current therapies available for HCC include surgery, interventional therapy, radio frequency therapy, radiotherapy, biological target therapy and so on^[2]. All of these treatments have certain curative effects, but have inherent limitations and adverse effects, especially for HCC patients at the advanced stage^[3]. Thus, it is urgent to find new treatment target for the sake of enhancing curative effect and reducing adverse effects, especially in advanced HCC patients.

Apart from the common etiologies of HCC listed above, certain oncogenes, cytokines, neurotransmitters, chemokines, extracellular secretory proteins and tumor microenvironment are thought to play important roles in origin and progression of HCC^[4]. Therefore, oncogenes and tumor microenvironment, which facilitate HCC progression, can be chosen as therapeutic targets for HCC treatment^[5].

The secreted protein acidic and rich in cysteine

(SPARC; alternative names: osteonectin; ON or basement membrane-40; BM-40) family is recognized as extracellular matrix proteins^[6]. A differential expression of SPARC in tumor tissue and its surrounding stroma compared to normal tissues has been reported for many different types of cancer^[7]. And, SPARC was found to be up-regulated in several solid tumors and to facilitate tumor metastasis^[8].

Secreted modular calcium-binding protein-2 (Smoc2) is a novel member of the SPARC family^[9]. Previous study confirmed that Smoc2 could promote cell cycle progression of human umbilical vein endothelial cells by inducing the expression of transcripts required for cell cycle^[10]. Other studies have shown that Smoc2 is necessary for DNA synthesis in the cell cycle and is likely to impact cell growth *in vitro* and *in vivo*^[11]. However, to the best of our knowledge, no study has been conducted on the role of Smoc2 in HCC.

Thus, the present study was designed to investigate the effect of Smoc2 on proliferation of HCC cells and the impact on cell cycle. Our study showed that Smoc2 promoted proliferation of HCC cells and accelerated cell cycle progression. The results suggested that Smoc2 might act as an anti-cancer therapeutic target for HCC treatment.

MATERIALS AND METHODS

Clinical samples

A total of 20 pairs of HCC tissues and corresponding non-tumor liver (CNL) tissues were obtained from the Liver Surgery Department of the corresponding hospital. All the human liver tissues were obtained with informed consent and the study was approved by the Ethical Committee of the corresponding hospital.

Immunohistochemistry

The paraffin-embedded human liver tissues were incised into 4-5 μ m thickness slices and then dewaxed using xylene and ethanol, in a stepwise manner. After dewaxing, the slices were rehydrated for subsequent staining. For immunohistochemistry (IHC) staining, the slices were treated with hydrogen peroxide and boiled for 15 min in citrate solution for antigen retrieval. When the slices had cooled naturally to room temperature, we added goat serum for blocking of unrelated antigens. Afterwards, the slices were incubated with Smoc2 antibody (Abcam) at 4 °C overnight. The following day, the slices were washed three times with phosphate buffer and incubated with horseradish peroxidase-labelled secondary antibody for 1 h at room temperature. The slices were then developed using DAB substrate liquid (Thermo) and dehydrated by ethanol, in a stepwise manner, and finally sealed with neutral balsam.

Western blotting assay

The tissue protein was isolated from human liver tissues using T-PER Tissue Protein Extraction Reagent

(Pierce Biotechnology) according to protocols provided by the manufacturer. The cell protein was lysed using lysis buffer that contained Tris-HCl, NaCl, Triton-X 100, MgCl₂, PMSF and so on. The cell lysate, which was used for cell signaling detection, was obtained by IP cell lysis solution (Beyotime Biotechnology). All the proteins were separated by SDS-PAGE and transferred to a nitrocellulose membrane under constant current condition. Then, the membrane was blocked using 5% bovine serum albumin at room temperature for 1 h. The nitrocellulose membrane was then incubated using antibodies for Smoc2 (Abcam), extracellular regulated protein kinase (ERK; Cell Signaling Technology, CST), phospho-ERK (CST), AKT (CST), phospho-AKT (CST), Src (CST), phospho-Src (CST), FAK (CST), phospho-FAK (CST) and GAPDH (Sigma) at 4 °C overnight. The next day, the nitrocellulose membrane was incubated with the fluorescence-conjugated secondary antibodies at room temperature for 1 h. All the fluorescence signals were captured and saved by the Odyssey imaging system (LI-COR). The images of western blots were analyzed using ImageJ software for gray value calculation.

Immunofluorescence staining

For cell staining, SMMC-7721 cells transfected with Smoc2 vector using lentivirus method were seeded on rounded slides in 24-well plates and incubated at 37 °C and a 5% CO₂ atmosphere overnight. The next day, cells were fixed using paraformaldehyde and washed three times by phosphate buffer before staining. Then, the slides were incubated with Smoc2 antibody (Abcam) for 75 min at room temperature. After that, the slides were washed three times using phosphate buffer and incubated with Alexa Fluor 488nm-conjugated secondary antibody. The nuclei were stained using DAPI (Sigma) and the immunofluorescence stain images were record using fluorescence microscope (Carl Zeiss).

Real-time quantitative PCR assay

Total RNA was isolated from fresh liver tissues using the Trizol Reagent (Takara). The isolated RNA was then immediately applied to the reverse transcription reaction. Primers used for quantitative PCR reaction were: forward primer, 5'-GCTCACGTTCTTGAGAGTCG-3'; and reverse primer, 5'-TGTAGCTGTGACACTGGACC-3'. The PCR reaction conditions were 30 s at 94 °C, 1 min at 57 °C and 1 min and 30 s at 72 °C for 35 cycles. The Amplitaq polymerase and related reagents were supplied by Takara.

Lentivirus transfection assay

The full-length cDNA cloning vector was purchased from GeneCopoeia and used for amplification template. The Smoc2 full-length cDNA was amplified with *NheI* and *BamHI* restriction sites using KOD-PLUS Neo polymerase. Then, the PCR-amplified cDNA was cloned

into the pcDNA3.1 vector using restriction enzymes and DNA ligase. The recombined vector was packed with VSV, REV and GAG vectors and transfected into 293T cells using the Lipofectamine 2000 Reagent (Invitrogen) to obtain virus supernatant. The virus supernatant was added into SMMC7721 cells and Huh7 cells with polybrene. The transfected SMMC7721 cells and Huh7 cells were cultured under puromycin condition and verified by western blot assay.

Small interfering (si)RNA interference assay

MHCC-97H cells and HCC-LM3 cells were transfected with siRNA duplexes against Smoc2 or with control RNA duplex oligonucleotides using Lipofectamine 2000, following the manufacturer's instructions. The Smoc2 interference target sequence was TTAAGAGGTTCTCTGCGAAA.

CCK-8 cell proliferation assay

MHCC-97H and HCC-LM3 cells transfected with Smoc2-SiRNA were seeded into 96-well plates at density of 3000 cells per 100 µL and cultured for 5 d *in vitro*. In addition, the SMMC-7721 and HCC-LM3 cells transfected with Smoc2 lentivirus were seeded into 96-well plates at density of 2000 cells per 100 µL and cultured for 5 d at 37 °C and in 5% CO₂ atmosphere. The CCK-8 reagent was added to each well at 10 µL volume and reacted for 1 h in light-free condition, at 37 °C, and in 5% CO₂ atmosphere. Then, the absorbance value was detected using a microplate reader. The absorbance value at 450 nm was recorded.

Cell cycle assay

The cells were seeded into 6-well plates and collected at logarithmic growth phase. The cells were cultured in serum-free condition overnight before collection. Liver cancer cells were collected and washed three times using phosphate buffer. Then, the cells were centrifuged at 1000 r/min for 5 min and resuspended in 1 mL phosphate buffer. Next, the cells were added to 9 mL 70% cool ethanol, slowly and carefully. The fixed cells were then stored at -20 °C for at least 24 h. For cell cycle assay, the fixed cells were centrifuged at 1000 r/min for 10 min and washed three times using cool phosphate buffer. Then, we added 0.5 mL RNase (50 µg/mL) to each tube and incubated for 20 min at 37 °C. Lastly, we added propidium iodide at 50 µg/mL to each tube and stained on ice for 30 min in light-free condition. The stained cells were detected using flow cytometry (BD Biosciences).

Statistical analysis

We used SPSS 16.0 software to analyze the statistical significance of differences in our study. Statistic differences were calculated using two-tailed Student's *t*-test. *P* < 0.05 was considered statistically significant and *P* < 0.01 was considered very statistically significant.

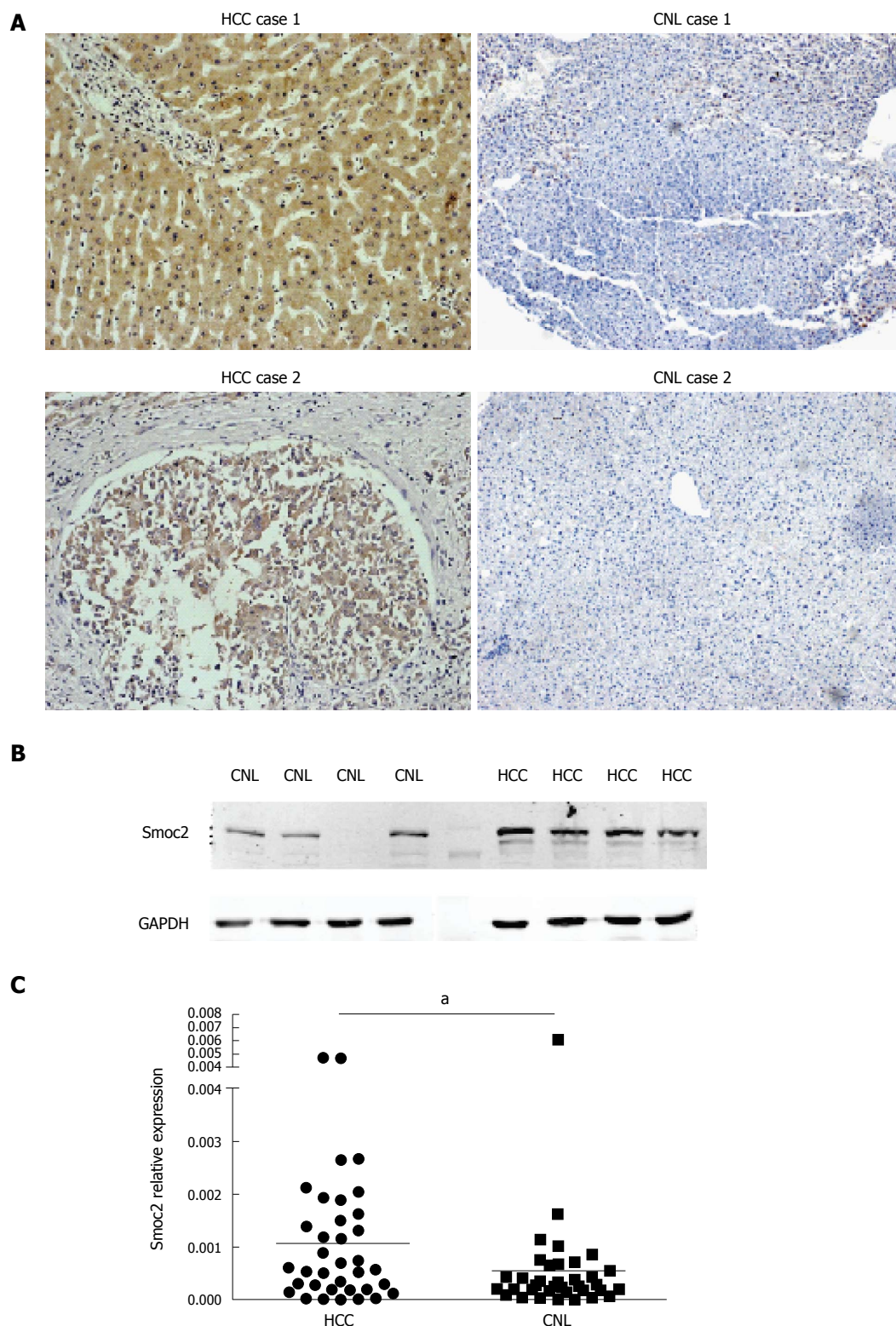


Figure 1 Smoc2 was up-regulated in hepatocellular carcinoma tissues compared with corresponding non-tumor liver tissues. A: Representative images of immunohistochemistry (IHC) staining assay; IHC images show that expression of Smoc2 was higher in hepatocellular carcinoma (HCC) tissues compared with corresponding non-tumor liver (CNL) tissues; B: Western blot assay show the expression of Smoc2 was higher in fresh HCC tissues than in CNL tissues; C: Quantitative real-time PCR assay showed that the relative expression of Smoc2 was higher in fresh HCC tissues than in CNL tissues. ^a $P < 0.05$.

RESULTS

Smoc2 was up-regulated in HCC tissues compared with CNL tissues

The expression of Smoc2 was significantly up-

regulated in HCC tissues, compared to CNL tissues, as evidenced by IHC (Figure 1A). IHC results showed that expression of Smoc2 was mainly located in the cytoplasm of HCC cells and the extracellular lesion of liver tissues. Western blot assay showed that protein

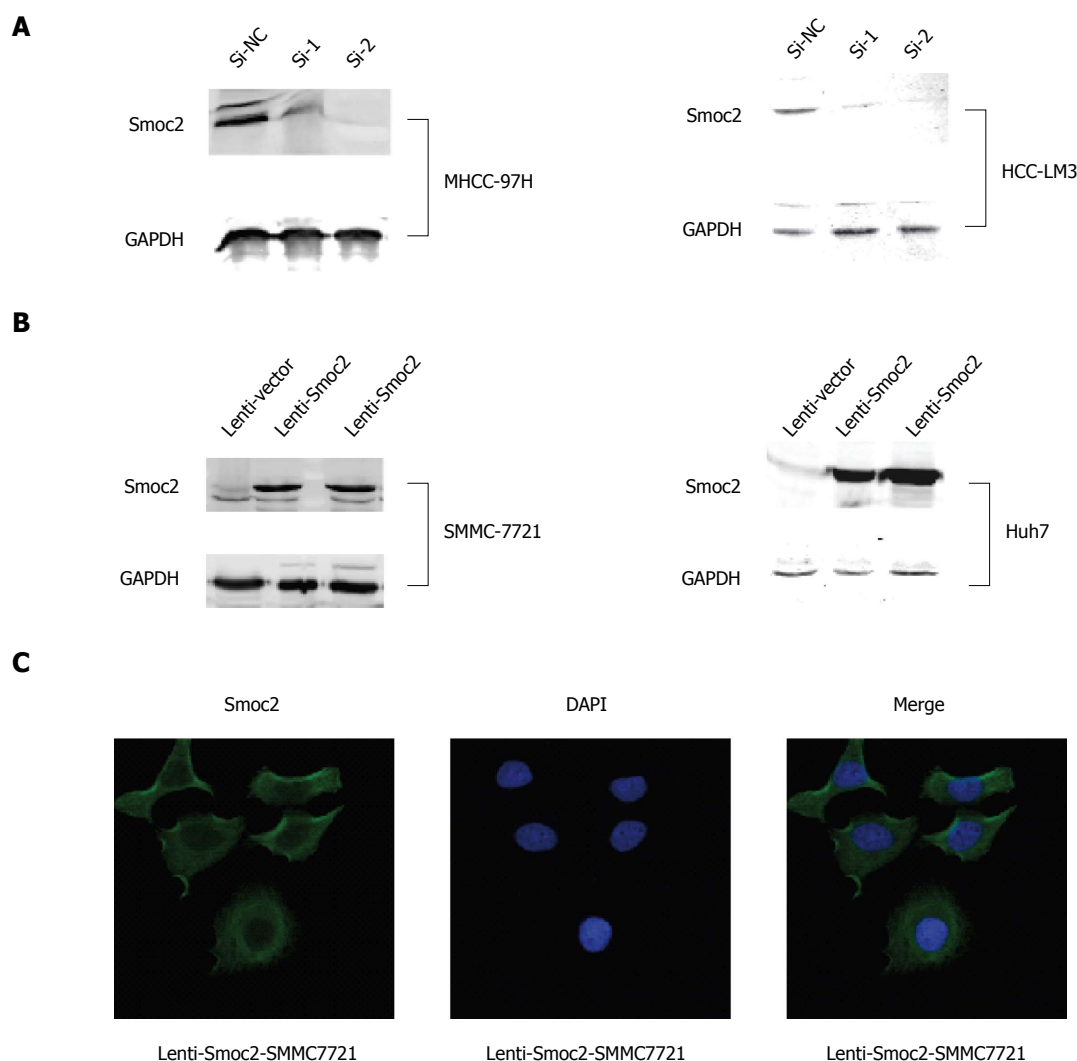


Figure 2 Western blot assay. A: Western blot assay showing that expression of Smoc2 in MHCC-97H cells and HCC-LM3 cells was inhibited by small interfering (si)RNA; B: Western blot assay showing that expression of Smoc2 in SMMC-7721 cells and Huh7 cells was significantly up-regulated by lentivirus transfection. Lenti-Smoc2 has overexpression of Smoc2 by lentivirus transfection; lenti-vector was the negative control of lentivirus transfection; C: Immunofluorescence staining showing overexpression of Smoc2 in SMMC-7721 cells; DAPI was used for nucleus staining.

expression level of Smoc2 was significantly higher in human HCC tissues, compared to CNL tissues (Figure 1B). The real-time quantitative PCR result indicated that mRNA expression level of Smoc2 in HCC tissues was remarkably higher than in CNL tissues. All the results above revealed that expression of Smoc2 was up-regulated in HCC tissues, compared to CNL tissues, at both protein and mRNA levels (Figure 1C).

Silencing Smoc2 by siRNA transfection and overexpressing Smoc2 by lentivirus transfection assay
We carried out siRNA transfection for silencing of Smoc2 in MHCC-97H and HCC-LM3 cells, and verified the silencing effect using western blot assay (Figure 2A). We induced overexpression of Smoc2 in SMMC-7721 and Huh7 cells using the lentivirus transfection method and identified the overexpressing effect using western blot assay (Figure 2B). The immunofluorescence stain results showed that expression of Smoc2 induced by

lentivirus transfection can be found in cytoplasm of SMMC-7721 cells (Figure 2C).

Silencing of Smoc2 inhibited HCC cell proliferation and overexpression of Smoc2 promoted HCC cell proliferation *in vitro*

CCK-8 assay directly reflects cell viability and can be used for evaluating cell proliferation. We collected MHCC-97H and HCC-LM3 cells transfected with Smoc2-siRNA at 48 h and detected the cell viability for 5 d *in vitro* using CCK-8 assay. The results showed that the proliferation capacity of MHCC-97H and HCC-LM3 cells transfected with Smoc2-siRNA were significantly worse than that in the control group (Figure 3A). Besides, the proliferative capacity in Smoc2 overexpressing SMMC-7721 and Huh7 cells was remarkably stronger than in control groups *in vitro* (Figure 3B). These results suggested that Smoc2 plays an important role in promoting HCC cells proliferation *in vitro*.

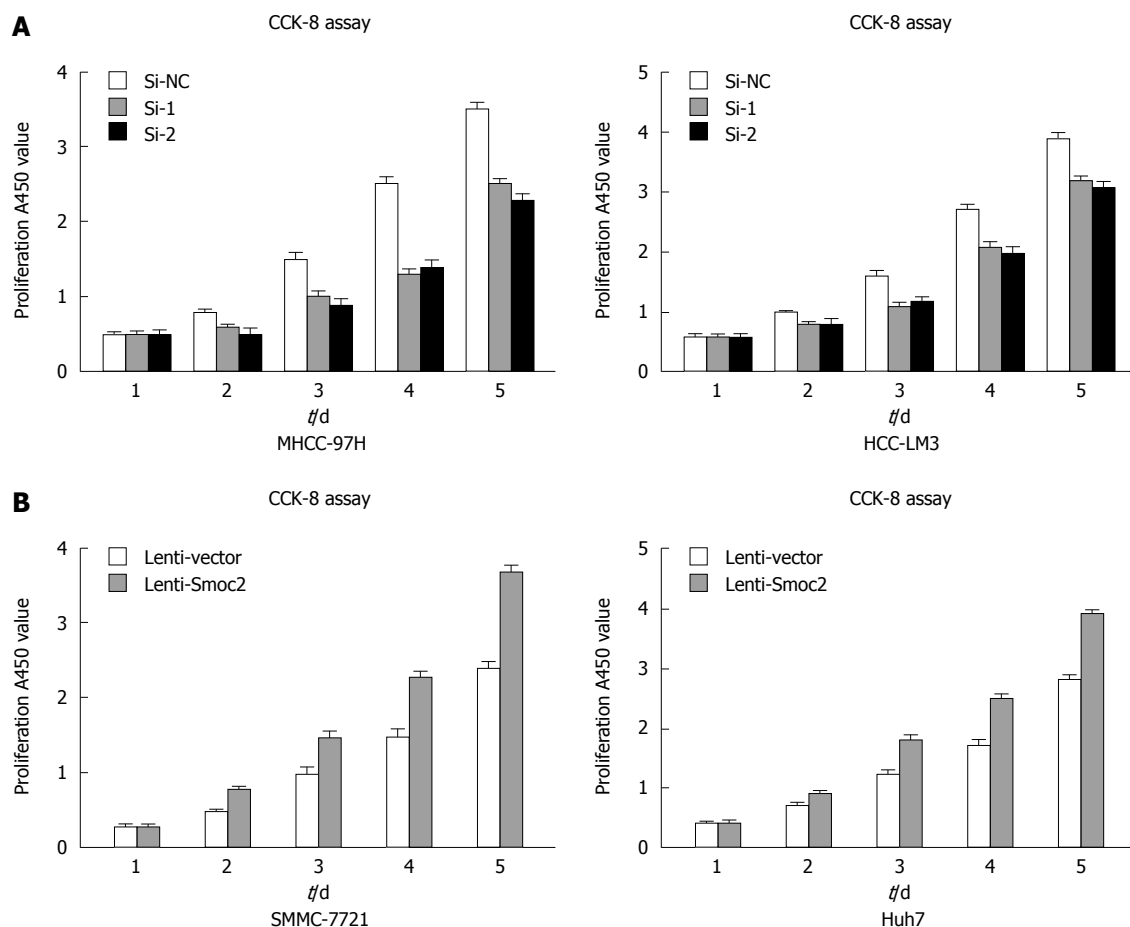


Figure 3 CCK-8 assay. A: CCK-8 assay was used for cell viability detection and reflected cell proliferation. The results show that the proliferation ability was inhibited by small interfering (si)RNA in MHCC-97H cells and HCC-LM3 cells; B: CCK-8 assay results show that the cell proliferation ability in SMMC-7721 cells and Huh7 cells was enhanced by lenti-Smoc2-virus transfection.

Overexpression of Smoc2 accelerated cell cycle progression by increasing the proportion of S phase cells

Cell cycle directly reflects cell proliferation capacity and is composed of G0/G1 phase, S phase and G2/M phase. The cell proliferation capacity can be reflected by proliferation index (PI) and S phase cell fraction (SPF). PI equates to the percentage of sum of S phase and G2/M phase cells in total cell cycle cells. SPF equates to the percentage of S phase cells in total cell cycle (G0/G1 + S + G2/M). Our results showed that both PI and SPF values in overexpressing Smoc2 cells were higher than those in the control group, which indicated the promotive role of Smoc2 in cell cycle progression and cell proliferation (Figure 4A and B; Figure 5A and B).

Smoc2 potentiated ERK, AKT and FAK signaling in HCC cells

The ERKs consist of ERK1 and ERK2, having molecular weights of 44 kDa and 42 kDa respectively^[12]. Phosphorylation of ERK allows translocation from the cytoplasm to the nucleus, and mediates transcriptional activation of Elk-1, ATF, NF- κ B, Ap-1, c-fos and c-Jun. Activation of ERK signaling participates in regulating

cell proliferation and differentiation, construction of the cell skeleton, and the processes of cell apoptosis and cell canceration^[13]. Our results showed that phosphorylation of ERK was up-regulated in Smoc2 overexpressed cells and down-regulated in Smoc2 siRNA interfered cells (Figure 6).

Signaling by AKT, also known as protein kinase B, plays an important role in regulating cell survival and apoptosis^[14]. Our results indicated that phosphorylation of AKT was up-regulated in Smoc2 overexpressed cells and down-regulated in Smoc2 siRNA interfered cells (Figure 6).

FAK, also known as focal adhesion kinase, and its activation is related to cell proliferation and cell cycle^[15]. Inhibition of FAK signaling can cause decrease of S phase cell ratio in cell cycle. Thus, inhibition of FAK signaling lead to inhibition of cell proliferation and enhancement of cell apoptosis. Our results illustrated that down-regulation of Smoc2 by siRNA transfection caused inhibition of FAK phosphorylation (Figure 6).

DISCUSSION

As one of the most frequent human cancers worldwide,

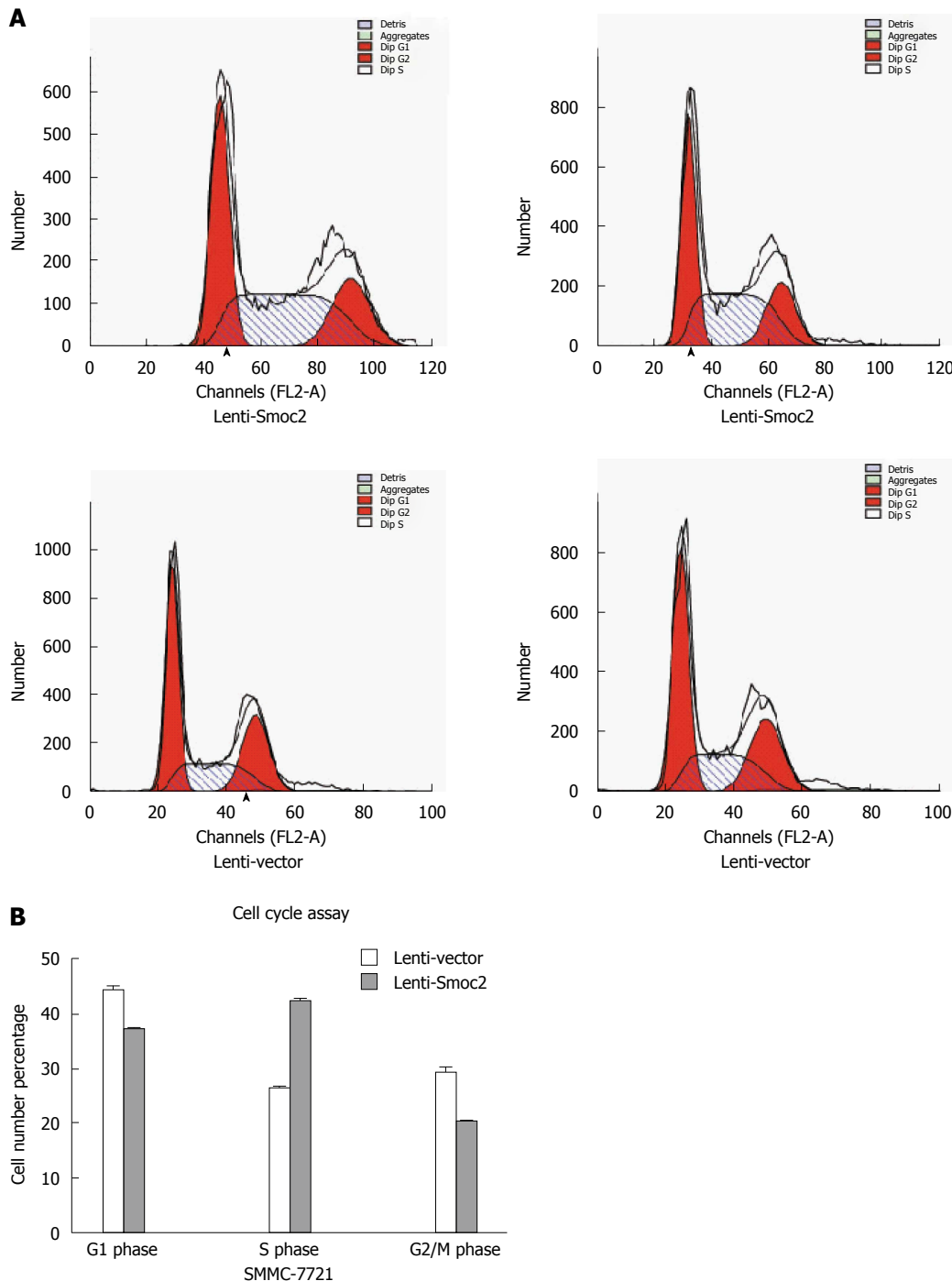


Figure 4 SMMC-7721 cells. A: Fluorescence-activated cell sorting assay for cell cycle detection. All the SMMC-7721 cells were stained by propidium iodide, which can be detected at 488 nm; B: The overexpression of Smoc2 caused increase of S stage cell proportion in SMMC-7721 cells, which indicated promotion of cell cycle progression.

the incidence and progression of HCC are affected by oncogenes and tumor microenvironment^[16,17]. Cytokines, chemokines, pH value, hypoxia, fibroblast and extracellular matrix proteins are important components of the tumor microenvironment^[18-21]. The tumor microenvironment plays an important role in regulating tumor progression^[22,23]. It has been reported that many extracellular matrix proteins exert significant influence on tumor progression. Thus, we focused on finding a new secretory protein which can

cause tumor development.

The *Smoc2* gene, encoding a protein belonging to the SPARC family and identified as an extracellular matrix protein, is up-regulated during embryogenesis and wound healing^[24]. The *Smoc2* gene encoded protein promotes extracellular matrix assembly and potentiates endothelial cell proliferation and migration^[25], and has pro-angiogenic activity^[26]. It was reported that the *Smoc2* gene is indispensable for VEGF-induced vascular endothelial cells mitogenesis

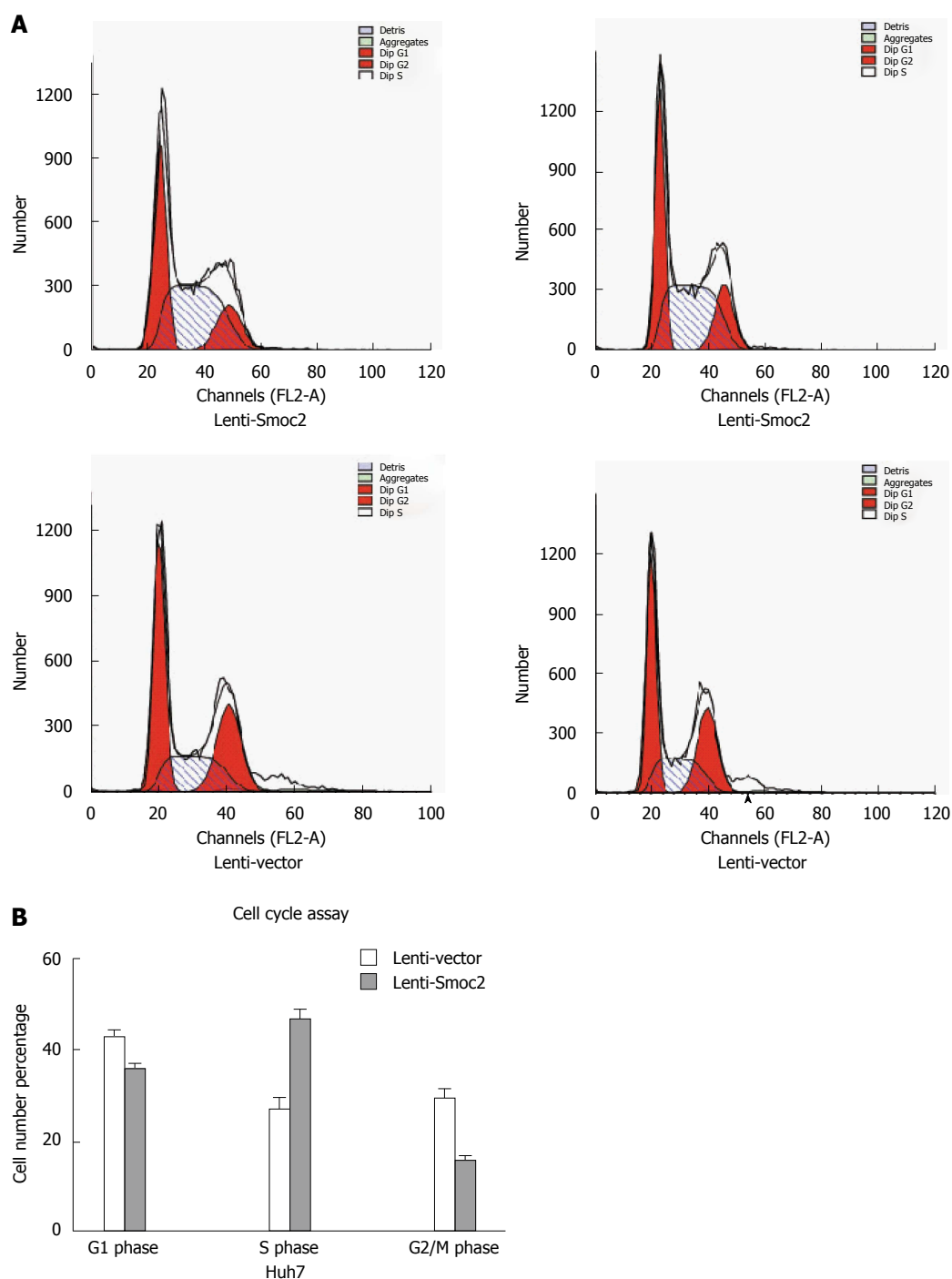


Figure 5 Huh7 cells. A: Fluorescence-activated cell sorting assay for cell cycle detection. All the Huh7 cells were stained by propidium iodide, which can be detected at 488 nm; B: The overexpression of Smoc2 caused increase of S stage cell proportion in Huh7 cells, which indicated the promotion of cell cycle progression.

and tube formation^[27,28]. Due to its promotive effect on angiogenesis, the *Smoc2* gene may represent a useful target for inhibition of tumor progress.

The cell cycle consists of G0/G1 phase, S phase and G2/M phase in mammalian cells^[29,30]. The cell cycle is mainly regulated by various kinds of growth factors, such as platelet-derived growth factor^[31,32]. Cyclin D1 expression is induced by mitogens and is crucial for G1 progression^[33]. Previous results showed that Smoc2 plays an important role in promoting G1/S progression through participating in the maintenance

of cyclin D1 expression^[34-36]. In other words, cyclin D1 can be considered as an effector of Smoc2-induced DNA synthesis^[37,38].

It was also demonstrated that Smoc2 was necessary for DNA synthesis in response to PDGF and other growth factors during the cell cycle^[39-41]. Our study demonstrated that Smoc2 had a promotive effect on HCC cell G1/S phase progression, as well as on cell proliferation. The S phase cell ratio was significantly elevated in Smoc2 overexpressing HCC cells, and this result was in accordance with those from a previous

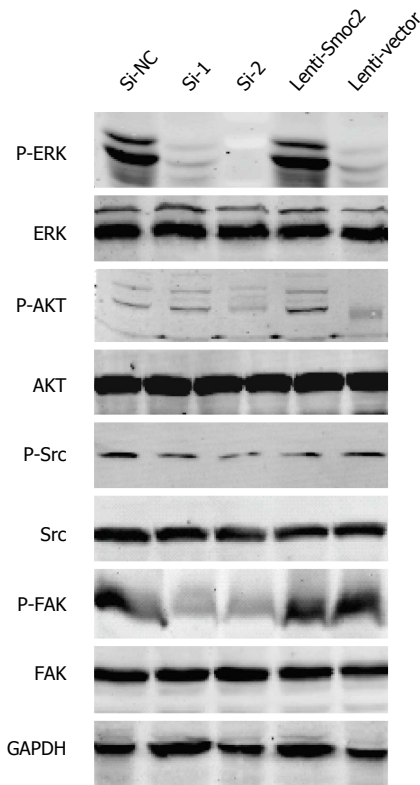


Figure 6 Phosphorylation of ERK up-regulated in Smoc2 overexpressing cells and down-regulated in cells with Smoc2 small interfering (si)RNA. Western blot assay showed the change of phospho-ERK, Src, FAK and AKT expression by Smoc2 interference or overexpression.

study using human umbilical vein endothelial cells transduced with Ad-Smoc2^[42,43].

Compared with Swiss 3T3 cells transfected with Sicontrol, the cells transfected with SiSmoc2 oligonucleotide duplexes showed no significant change in phospho-MAPK and phospho-AKT levels^[44-47]. Unlike the results in Swiss 3T3 cells, our results indicated that Smoc2 had promotive influence on MAPK/ERK and AKT signaling pathways in HCC cells. MAPK/ERK and AKT are identified as PDGF β receptor effector kinases and positively regulate the expression of cyclin D1^[48]. Therefore, we speculated that Smoc2 could potentiate PDGF-induced mitogenesis and the subsequent cell proliferation. Nevertheless, another study based on Swiss 3T3 cells showed that the promotion of Smoc2 in DNA synthesis was independent of PDGF-binding activity but dependent on integrin-activated protein kinase^[49].

Previous studies revealed that Smoc2 promotes growth factor-induced DNA synthesis and differs from SPARC, which was identified as an anti-mitogenic factor. It was reported that SPARC could bind various kinds of mitogens (such as VEGF, PDGF, FGF and so on) but inhibit the signaling mediated by these growth factors. Unlike SPARC, Smoc2 could facilitate growth factor-induced DNA synthesis^[50]. The exact mechanism of how Smoc2 promotes growth factor-induced DNA synthesis in HCC cells remains unclear and needs to be

studied further.

Our research focused on the proliferation promotion effect of Smoc2 in HCC cells. However, Smoc2 also has influence on cell migration. It has been reported that extracellular matrix protein Smoc2 can promote keratinocyte migration *in vitro*. Accordingly, the role of Smoc2 on HCC cell migration and invasion need to be further studied. The influences of Smoc2 on HCC cell biological behaviors may be extensive.

In conclusion, our results demonstrated that Smoc2 is an important regulator of cell mitogenesis, and plays a promotive role in growth factor signaling and cell cycle progression in HCC. Based on our study, Smoc2 might represent a promising therapeutic target for HCC treatment.

COMMENTS

Background

The secreted modular calcium-binding protein-2 (Smoc2) gene, belonging to the secreted protein acidic and rich in cysteine (SPARC) family, was identified as encoding an extracellular matrix protein and as being up-regulated during embryogenesis and wound healing. The Smoc2 gene encoded protein promotes extracellular matrix assembly and potentiates endothelial cell proliferation and migration, as well as has pro-angiogenic activity. Due to the promotive effect on angiogenesis, the Smoc2 gene can be chosen as a target for inhibition of tumor progression.

Research frontiers

Smoc2 is a novel member of the SPARC family. Current studies have confirmed that Smoc2 can promote cell cycle progression by inducing the expression of transcripts required for the cell cycle. Other studies have shown that Smoc2 is necessary for DNA synthesis in the cell cycle and is likely to impact cell growth *in vitro* and *in vivo*. These studies also revealed that Smoc2 plays an important role in regulating the cell cycle. However, to the best of our knowledge, no study has investigated the role of Smoc2 in hepatocellular carcinoma (HCC).

Innovations and breakthroughs

The expression of Smoc2 was significantly higher in HCC tissues, as compared to CNL tissues. Overexpression of Smoc2 promoted HCC cell proliferation and cell cycle progression. Down-regulation of Smoc2 led to inhibition of cell proliferation and cell cycle progression. Smoc2 had a positive effect on ERK and AKT signaling.

Applications

According to our studies, Smoc2 was identified as pro-proliferative gene in HCC. Besides, blockage of Smoc2 could result in inhibition of liver cancer cell proliferation. Therefore, Smoc2 might be chosen as a target gene for preventing HCC progression. In the future, a neutralizing antibody for Smoc2 could be produced and used in animal experiments to further test its efficacy for preventing HCC proliferation. Blockage of Smoc2, by using neutralizing antibody or another approach, may contribute to HCC treatment and improve therapeutic efficacy.

Terminology

The SPARC family was recognized as extracellular matrix proteins. SPARC has been found to be up-regulated in several solid tumor tissues and to facilitate tumor metastasis. Smoc2, a novel member of the SPARC family, was identified as an extracellular matrix protein.

Peer-review

This study is very interesting. The authors determined the influence of Smoc2 on HCC cell proliferation and aimed to find a possible new therapeutic target for preventing HCC progression. The authors detected expression of Smoc2

in HCC tissues and corresponding non-tumor liver (CNL) tissues using PCR, western blot, immunohistochemistry methods and down-regulation and up-regulation of Smoc2 expression using small interfering RNA and lentivirus transfection assay respectively. The effect of Smoc2 on cell proliferation and cell cycle was identified using CCK-8 and flow cytometry respectively. The common cell growth signaling influenced by Smoc2 was detected. The authors found that Smoc2 promotes the proliferation of HCC cells through accelerating cell cycle progression and might act as an anti-cancer therapeutic target in the future. Overall, this study is well designed and the manuscript is well written.

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Prospective Study

Low phase angle is associated with the development of hepatic encephalopathy in patients with cirrhosis

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Abstract

AIM

Evaluate the association between phase angle and the development of hepatic encephalopathy in the long-term follow-up of cirrhotic patients.

METHODS

This was a prospective cohort study. Clinical, nutritional and biochemical evaluations were performed. Mann-Whitney's *U* and χ^2 tests were used as appropriate. Kaplan-Meier curves and Cox proportional Hazards analysis were used to evaluate the prediction and incidence of hepatic encephalopathy.

RESULTS

Two hundred and twenty were included; the most frequent etiology of cirrhosis was hepatitis C infection, 52% of the patients developed hepatic encephalopathy (18.6% covert and 33.3% overt); the main precipitating factors were infections and variceal bleeding. Kaplan-Meier curves showed a higher proportion of HE in the group with low phase angle (39%) compared to the normal phase angle group (13%) ($P = 0.012$). Furthermore, creatinine and phase angle remained independently associated to hepatic encephalopathy in the Cox regression multivariate analysis [hazard ratio = 1.80 (1.07-3.03)].

CONCLUSION

In our cohort of patients low phase angle was associated with an increased incidence of hepatic encephalopathy. Phase angle is a useful nutritional marker that evaluates cachexia and could be used as a part of the integral assessment in patients with cirrhosis.

Key words: Malnutrition; Hepatic encephalopathy; Phase angle; Cachexia; Prognosis

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Core tip: Malnutrition in cirrhosis is a known risk factor for mortality and the development of complications. We used phase angle derived from bioelectrical impedance that reflects cachexia, the type of malnutrition seen in chronic diseases. We found an association between the presence of low phase angle and higher incidence of hepatic encephalopathy in the 48 mo of follow-up compared to the group with normal phase angle, demonstrating the importance of this complication. We proposed a nutritional marker that can predict the development of hepatic encephalopathy with the advantage of being a non-invasive, inexpensive and bedside method.

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INTRODUCTION

Hepatic encephalopathy (HE) is defined as a spectrum of neuropsychiatric abnormalities observed in patients with liver dysfunction, in the absence of other explanatory medical conditions; this disorder may significantly impair the daily function, quality of life and survival of patients with cirrhosis^[1]. The pathophysiology of HE is complex, and multiple mechanisms have been implicated in its development. Apart from hyperammonemia, other factors such as oxidative stress, inflammation, false neurotransmitters, genetic factors and cerebral hemodynamics are known to trigger HE^[2,3].

As complications arise from cirrhosis, the different stages of malnutrition follow including depletion of muscle mass sarcopenia, depletion of both muscle and fat mass and the release of inflammatory mediators cachexia^[4], affecting the normal functioning of different organs such as the brain. Some aspects of malnutrition share common mechanisms with those observed in patients with HE, including increased oxidative stress and systemic inflammation^[5]. This is especially important in cachexia, where loss of muscle and fat mass together with systemic inflammation contribute to detrimental general status^[6,7].

Loss of skeletal muscle is related to poor ammonia clearance by decreasing the amount of glutamine synthetase - a key enzyme related to ammonia scavenging - which catalyzes the conversion of ammonia and glutamate into glutamine^[8,9]. Substantial evidence has linked oxidative stress with malnutrition; in fact, the underlying mechanism includes low bioavailability of antioxidants, enhanced production of free radicals and impaired functioning of free radical scavenging enzymes^[10]. Finally, cachexia-driven inflammation and oxidative stress might further impair brain function leading to hepatic encephalopathy^[5].

Among the current available methods for the assessment of the nutritional status, bioelectrical impedance-derived phase angle (PhA), is regarded as an useful tool indicating the balance between cell hydration and body mass, which is finally translated into tissue homeostasis and nutritional status^[11]. Low values of PhA reflect poor nutritional status typically found in clinical settings such as cancer, human immunodeficiency virus and cirrhosis. The clinical application of PhA in patients with cirrhosis and HE includes the evaluation of cachexia^[12-14].

Therefore, the aim of this study was to evaluate the association between PhA and the HE development in the long-term follow-up of cirrhotic patients.

Ruiz-Margáin A, Macías-Rodríguez RU, Ampuero J, Cubero FJ, Chi-Cervera L, Rios-Torres SL, Duarte-Rojo A, Espinosa-Cuevas Á, Romero-Gómez M, Torre A. Low phase angle is associated

MATERIALS AND METHODS

This was a prospective cohort study. Patients attending Gastroenterology and Hepatology clinics at a third-level hospital (Instituto Nacional de Ciencias Médicas y Nutrición “Salvador Zubirán”, México City, México) between March 2009 and June 2010 were screened for study enrollment. This study was designed and conducted according to the principles of the Declaration of Helsinki and was approved by the local Institutional Ethics Committee (REF 1652). Informed consent was obtained from each participant.

Patients

We included patients with an age of 18 to 65 years, and diagnosed with cirrhosis based on the combination of clinical features, radiological imaging, presence of portal hypertension, compatible biochemical parameters, and/or confirmatory liver biopsy. Inclusion was not restricted to etiology of cirrhosis. Patients with acute or chronic renal failure, an episode of overt HE in the previous 6 mo, with current treatment for HE, major surgery within four weeks before recruitment, pregnancy, active alcoholism, acute disease such as infections, use of neuropsychiatric drugs, hypothyroidism and extremity amputation, were excluded.

Clinical assessment

All patients underwent clinical evaluation to evaluate the presence of ascites, edema, and hepatic encephalopathy. HE was classified according to West Haven criteria. If diagnosis of overt HE was made during follow-up, anti-ammonia treatment (lactulose, antibiotics, L-ornithine-L-aspartate) was prescribed, and resolution/improvement of HE manifestations was confirmed at a follow-up visit.

Biochemical tests including liver function tests, international normalized ratio (INR), creatinine, sodium, and ammonia levels were obtained within the first week of study enrollment. Disease severity was determined according to Child-Pugh (CP) and Model for End-Stage Liver Disease (MELD) scores^[15,16].

Bioelectrical impedance analysis and phase angle analysis

The BIA measurement was performed using RJL systems Quantum IV (Clinton Township, MI, United States) applying alternating electric currents of 800 μ A at 50 kHz with the aid of Ag/AgCl source and sensor electrodes to obtain R, Xc and phase angle. BIA was performed within the first week of enrollment after an overnight fasting in supine position with arms and legs abducted from the body. Source and sensor electrodes were placed on the dorsum of the hand and foot on the right side of the body, respectively. The BIA-derived PhA was obtained according to standard calculations.

Cachexia can be defined as loss of muscle and fat mass, accompanied by an inflammatory component

given by the presence of chronic disease^[6], PhA has been shown to be associated to inflammation as well as muscle and fat depletion^[11,14]. Cachexia was defined as when the patient presented a PhA value $\leq 4.9^\circ$ based on the specific cut-off for our population^[17] and specifically loss of muscle mass and fat mass that was obtained through a combined evaluation of PhA and vector analysis (RXc graph).

Statistical analysis

Kolmogorov-Smirnov test was performed to test data distribution. To compare groups *U* of Mann-Whitney and Chi-square test (χ^2) were used. Incidence of HE was assessed with Kaplan-Meier curves, using the log rank test to compare the curves, followed by a multivariate Cox regression analysis. To obtain the best regression model, backward elimination model was selected. Statistical analysis was carried out using GraphPad Prism® 5 and SPSS v21 (SPSS Inc., Armonk, New York, United States).

RESULTS

The total population consisted of 220 outpatients (60% females), with a mean follow-up time of 34 ± 9.8 mo. The main etiology of cirrhosis was hepatitis C virus (HCV) (36%), followed by non-alcoholic steatohepatitis (NASH) (18%), primary biliary cirrhosis (17%), alcohol (10%), autoimmune hepatitis (10%), and other causes (9%). A total of 35% of patients were categorized as CP A, 47% CP B, and 18% CP C.

The total population was then divided into two groups, depending on HE development during follow-up; there were no significant differences in age, BMI and mid-arm muscle circumference among groups; however CP, MELD score and its components, as well as phase angle, sodium, hemoglobin and ammonia were worse in the HE group (Table 1).

In total, during the 48 months of follow-up, 52% of the patients developed HE; from this 18.6% was covert HE and 33.3% was overt HE. From the total population 16.4% of the patients had more than one episode of HE during follow-up. Mean PhA was significantly lower in patients that showed persistent HE when compared to the group without persistent HE 4.6 ± 1.17 vs 5.2 ± 1.18 ($P = 0.017$).

When the analysis of HE development was stratified by nutritional status according to PhA \leq or $> 4.9^\circ$, there was a clear difference between groups. The incidence of HE was significantly higher in patients with cachexia, as evidenced by low phase angle, when compared to patients compared to well-nourished patients, 39% vs 13%, respectively ($P = 0.012$) (Figure 1).

The main precipitating factors of HE were infections different from spontaneous bacterial peritonitis (SBP) 26.7%, this included urinary tract infections and respiratory infections mainly, the second most common factor was variceal bleeding 23.3%, followed by con-

Table 1 Clinical and demographic characteristics of the study population according to the presence of hepatic encephalopathy

	HE (n = 115)	Non-HE (n = 105)	P value
Age (yr)	54.2 ± 10.3	51.8 ± 11.7	0.109
BMI (kg/m ²)	26.4 (21.3-30.7)	27.4 (24-30.5)	0.185
MAMC (cm)	22.3 (20.3-26.4)	24.4(21.1-26.98)	0.230
Phase angle (°)	4.6 (4.0-5.5)	5.3 (4.6-6.1)	0.000
Child-Pugh (points)	8 (7-10)	6 (5-8)	0.000
MELD score	13 (10-15)	10 (8-12)	0.000
Total bilirubin (mg/dL)	2.1 (1.3-3.4)	1.5 (1.0-2.6)	0.001
Albumin (mg/dL)	3.4 ± 0.6	2.9 ± 0.6	0.000
INR	1.3 (1.2-1.4)	1.2 (1.1-1.3)	0.005
Creatinine (mg/dL)	0.84 (0.67-1.09)	0.72 (0.63-0.83)	0.000
Sodium (mEq/L)	136 (133-139)	139 (136-141)	0.000
Hemoglobin (g/dL)	12.2 (10.3-14.3)	14.0 (12.1-15.3)	0.000
Ammonia (μ/dL)	83.9 (50.9-123.3)	56.3 (37.6-80.3)	0.000

Data presented as mean ± SD, median (P25-P75). HE: Hepatic encephalopathy; BMI: Body mass index; MAMC: Mid-arm muscle circumference; MELD: Model for End-Stage Liver Disease; INR: International normalized ratio.

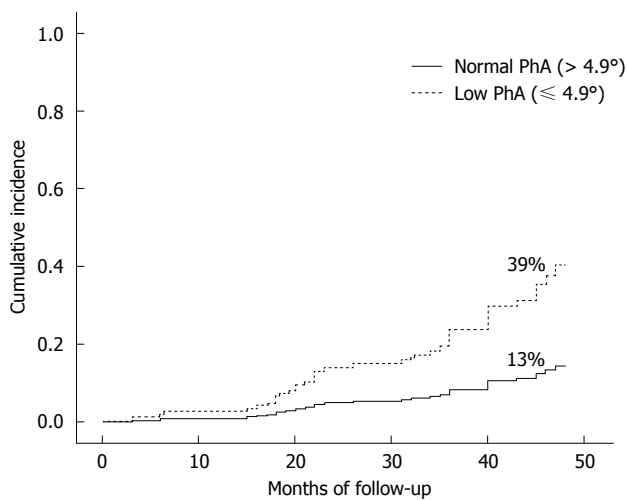


Figure 1 Development of hepatic encephalopathy according to phase angle during the follow-up period of 48 mo ($P = 0.012$). PhA: Phase angle.

stipation 15.6% and SBP 12.2%. Together, infections, non-SBP and SBP, comprised 38.9% of these factors (Figure 2).

The precipitating factors were then evaluated to search for differences between the group with low PhA and normal PhA; in the group with low PhA infections were higher (26.3% vs 16.3%), and specifically from the group of patients with SBP as precipitating factor 70% had low PhA, although this did not reach statistical significance ($P = 0.332$ and $P = 0.166$).

For the multivariate analysis a stepwise Cox regression using Backward elimination method for variables with P values > 0.05 was used; several variables known to be associated to HE were initially included in the univariate analysis their association to the development of HE where PhA, sodium, hemoglobin, ammonia and creatinine were statistically significant; however, after multivariate adjustment, only PhA and creatinine remained independently associated to HE (Table 2).

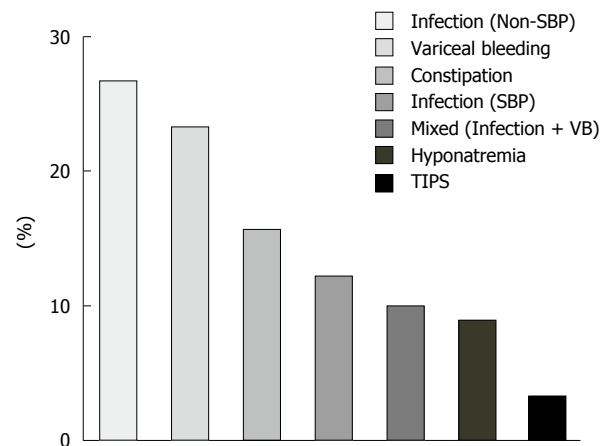


Figure 2 Precipitating factors of hepatic encephalopathy in the cohort. SBP: Spontaneous bacterial peritonitis; VB: Variceal bleeding.

DISCUSSION

The importance of nutritional status in cirrhosis has been widely recognized; it was included in the original description of the Child-Turcotte-Pugh score, and, since then, the influence of nutritional status in cirrhosis and its complications has been studied in different clinical scenarios^[18].

Through the years, the need for improved methods for the nutritional assessment in patients with cirrhosis has become evident, mainly due to the bias induced by fluid retention and decreased hepatic synthetic function^[19].

In this study, we defined malnutrition, and specifically cachexia, based on phase angle, a direct marker obtained from BIA, previously validated as a marker of nutritional status related to mortality in cirrhosis^[17].

The use of this nutritional marker has some advantages over other available methods including reproducibility, ease of use, low cost and accuracy. Low values of PhA have been linked to disrupted

Table 2 Characteristics associated with hepatic encephalopathy in the Cox regression model

Univariate analysis	HR	95%CI	P value	β
Phase angle ($\leq 4.9^\circ$)	1.597	1.081-2.358	0.019	0.468
Sodium (mEq/L)	0.941	0.906-0.977	0.002	-0.061
INR	2.270	1.154-4.465	0.018	0.820
Hemoglobin (g/dL)	0.897	0.836-0.962	0.002	-0.109
Ammonia (μ /dL)	1.006	1.002-1.010	0.005	0.006
Creatinine (mg/dL)	4.112	1.954-8.654	0.000	1.414
Total bilirubin (mg/dL)	1.031	0.966-1.100	0.362	0.030
Multivariate analysis				
Phase angle ($\leq 4.9^\circ$)	1.806	1.076-3.031	0.025	0.591
Sodium (mEq/L)	0.957	0.912-1.003	0.065	-0.044
Creatinine (mg/dL)	4.116	1.573-10.767	0.004	1.415

2-Log-likelihood: Block 0 = 557.59, Block 1 = 540.42, $P = 0.000$. HR: Hazard ratio; INR: International normalized ratio.

cell membrane and cell death, as well as changes in hydration status in different clinical settings^[11,12]. Based on these facts, in patients with cirrhosis and HE, a low PhA might reflect low-grade edema and astrocyte swelling^[20]. On the other hand, a low PhA translates directly into poor nutritional status, indicating mainly cachexia. The importance of recognizing cachexia in cirrhosis resides in the three components inherent to this condition: loss of muscle and adipose tissue as well as inflammation. Both sarcopenia and inflammation are important pathophysiological mechanisms related to the development and progression of HE^[21]. In this cohort of patients, anthropometric measurements such as triceps skinfold thickness and mid-arm muscle circumference showed no difference between the patients with and without hepatic encephalopathy.

The results of our study show an association between the presence of cachexia and the subsequent development of HE. There are no studies showing the long-term effect of cachexia and the risk of developing HE, although malnutrition and HE has been evaluated in some studies. One cross-sectional study found an association between the presence of HE and muscle depletion, using handgrip muscle strength and other nutritional markers^[21-23]. Another study found higher proportion of HE in patients with sarcopenia evaluated through computed tomography (CT) compared to patients without sarcopenia (60% vs 49%, $P = 0.1$), however, no follow-up data was available^[24].

Malnutrition could influence the development of HE in different ways. The brain, liver, gut and muscle play an important role in maintaining ammonia metabolism in cirrhosis and might be directly affected by malnutrition^[2]. For instance, the muscle function could be damaged since ammonia scavengers through the conversion from ammonia and glutamate into glutamine by the enzyme GS. The disruption of the intestinal barrier in cirrhosis and further increase in intestinal permeability and endotoxin levels, are involved in increased cytokine production, inflammation, and risk of infections^[25]. Cachexia has been linked to dysbiosis which can further contribute to increased ammonia production, damage to the intestinal epithelial cells, and

increased inflammation, ultimately leading to HE^[26].

The main precipitating factors of HE episodes in this cohort were infections, followed by variceal bleeding. It has been established that malnutrition in any condition is related to a higher incidence of infections^[27], thus, it can very likely be a possible explanation of the higher incidence of HE in malnourished patients, besides altered ammonia detoxification.

In the univariate analysis, biochemical parameters including sodium, INR, hemoglobin, ammonia and creatinine, as well as PhA were significantly related to the development of HE, and after backward multivariate adjustment, only creatinine and PhA remained significant. Altogether these data support the long-term effect of malnutrition in the development of HE.

The main benefits of this study are the prospective design and the long-term follow-up, which is essential to evaluate the causal effect of cachexia in the development of hepatic encephalopathy, thus it is not only a cross-sectional association, but also a validated and easily reproducible nutritional marker. There were also limitations in this study, individual components of malnutrition (fat mass, muscle mass) cannot be specifically addressed, since only the direct measurements were used, due to the limitations of conventional BIA using prediction equations to calculate indirect markers. Likewise, the cut-off value is specific of our population; therefore it needs to be tested in different populations.

In our cohort of patients low PhA was associated with an increased incidence of HE. PhA is a useful nutritional marker that evaluates cachexia, and could be used as a part of the integral assessment in patients with cirrhosis. Whether improving nutritional status can prevent or delay the development of episodes of HE warrants further investigation.

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COMMENTS

Background

Malnutrition is seen in 40%-90% of the patients with cirrhosis and has been associated to the progression of complications and higher mortality. Some mechanisms such as oxidative stress and systemic inflammation that are involved in the pathophysiology of hepatic encephalopathy (HE) are increased in patients with malnutrition, therefore malnutrition could be associated to HE.

Research frontiers

Some studies have shown the cross-sectional association between malnutrition and HE, however, the long-term effect of malnutrition on the subsequent development of HE has not been fully addressed. Furthermore there is a growing need of a reliable method, capable of easily diagnosing malnutrition that allows multiple measurements over time and that is also related to prognosis.

Innovations and breakthroughs

In this manuscript we propose phase angle as a nutritional marker that reflects malnutrition and specifically cachexia. The authors were able to demonstrate that phase angle has the ability to predict the development of HE. Phase angle is an easily accessible marker that is obtained from a bedside method; therefore, it is useful even in patients with high grades of HE, which is not achieved by the conventional methods of nutritional assessment.

Applications

Phase angle could be easily implemented both in outpatients and hospitalized patients with cirrhosis given that it is derived from a portable, non-invasive method which makes it completely suitable for daily clinical practice; it can be performed safely as often as needed with a very low cost compared to other accurate methods such as computed tomography-scan. And finally this marker could be part of the medical and nutritional follow-up helping to evaluate the effect of any dietary or nutritional interventions.

Terminology

In physical science, phase angle is defined as the arc tangent of the reactance and resistance obtained with bioelectrical impedance. In clinical terms, phase angle is a nutritional prognostic marker that is related to the integrity of cell membranes and tissue quality. Cachexia can be defined as loss of muscle and fat mass, accompanied by a pro-inflammatory component present in chronic diseases.

Peer-review

This manuscript addresses a clearly defined and relevant issue in the field of Gastroenterology, the process of malnutrition in general, and cachexia in particular and its association with hepatic encephalopathy. The focus is on the reliability of phase angle data directly generated through a noninvasive, cost-effective, reproducible method being able to predict successfully the development of HE which is an improvement over the current situation in managing these patients.

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Dangerous dietary supplements: *Garcinia cambogia*-associated hepatic failure requiring transplantation

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Abstract

Commercial dietary supplements are marketed as a panacea for the morbidly obese seeking sustainable weight-loss. Unfortunately, many claims cited by supplements are unsupported and inadequately regulated. Most concerning, however, are the associated harmful side effects, often unrecognized by consumers. *Garcinia cambogia* extract and *Garcinia cambogia* containing products are some of the most popular dietary supplements currently marketed for weight loss. Here, we report the first known case of fulminant hepatic failure associated with this dietary supplement. One active ingredient in this supplement is hydroxycitric acid, an active ingredient also found in weight-loss supplements banned by the Food and Drug Administration in 2009 for hepatotoxicity. Heightened awareness of the dangers of dietary supplements

such as *Garcinia cambogia* is imperative to prevent hepatotoxicity and potential fulminant hepatic failure in additional patients.

Key words: Dietary supplements; Fulminant hepatic failure; Drug-induced liver injury; Liver transplantation; Hydroxycitric acid; Weight-loss supplements

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Core tip: The current regulatory practice for over-the-counter dietary supplements in addition to celebrity endorsements of these products unfounded claims has resulted in a significant increase in the use of dietary supplements for weight loss. Unfortunately, several such products have previously been demonstrated to be serious health risks. Here we present one of the first known cases of fulminant hepatic failure associated with one such popular weight loss supplement, *Garcinia cambogia*.

Lunsford KE, Bodzin AS, Reino DC, Wang HL, Busuttil RW. Dangerous dietary supplements: *Garcinia cambogia*-associated hepatic failure requiring transplantation. *World J Gastroenterol* 2016; 22(45): 10071-10076 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v22/i45/10071.htm> DOI: <http://dx.doi.org/10.3748/wjg.v22.i45.10071>

INTRODUCTION

Dietary supplements are an increasingly recognized cause of acute liver injury and fulminant hepatic failure. Under the Dietary Supplement Health and Education Act of 1994, supplements, unlike prescription and over-the-counter medications, require proven toxicity prior to FDA sanctions^[1]. The Drug Induced Liver Injury Network (DILIN) identifies dietary supplements among the most common causes of drug-induced hepatotoxicity. Nearly a quarter of cases suffer irreversible liver damage, resulting in potential liver transplant (4%) and death (6%)^[2]. Evaluation of dietary supplement-induced hepatotoxicity is difficult due to wide formulaic variations, and ineffectual federal manufacturing oversight allows contamination by aflatoxins, microorganisms, pesticides, heavy metals, and synthetic drugs. These contaminants have known hepatotoxicity and may contribute to detrimental effects^[3]. In addition, following formal FDA citation, a supplement may be remarketed after minor reformulation and/or rebranding.

Of particular note has been hepatotoxicity associated with several different brands of "fat busters". Commercial fat-burning dietary supplements are widely marketed as "miracle-cures" for obesity on major network television shows with celebrity endorsements. Supplements are advertised to stimulate

weight loss by increasing the body's basal metabolic rate; however, campaigns are bereft of associated side effects (reviewed in^[3,4]). Multiple companies manufacture supplements of the same name with different composition, contaminants, and concentrations of active ingredients, potentially resulting in variable hepatotoxicity. Effort made by the FDA to collect data regarding toxicities through "Safety Reporting Portal" in the MedWatch system^[5] is reliant upon consumer and industry reporting compliance. As a result, recognition of toxicities may arise slowly.

Garcinia cambogia (*G. cambogia*) containing products are currently one of the most highly marketed group of weight-loss supplements commercially available. The supplement is derived from the rind of the fruit of the *Garcinia cambogia* tree, which is native to southwestern India. It has gained significant acclaim for its weight-loss benefits through mainstream talk shows and medical media celebrity spokespeople. Here, we report the first known case of fulminant hepatic failure associated with dietary intake of a "pure" *Garcinia cambogia* supplement.

CASE REPORT

A 34-year old Hispanic male presented with nausea, vomiting, abdominal pain, and dark urine. Testing revealed elevated transaminases and bilirubin; however, imaging failed to demonstrate cirrhosis or anatomic abnormality. Hepatitis work-up, including testing for viral hepatitis, hemochromatosis, Wilson's disease, and autoimmune hepatitis, was unremarkable with exception of an elevated Ferritin level of 7089 mg/dL. Genetic testing for hemochromatosis was negative. Medical history was only positive for occasional social alcohol use, and drug toxicology testing was negative. He denied use of energy drinks, herbs, Chinese teas, or muscle milk. He was advised to discontinue alcohol use, which he did, and his symptoms initially seemed to abate.

Six weeks later, the patient developed asterixis, jaundice, and confusion. Follow-up imaging was concerning for rapid onset of cirrhosis or infiltrative hepatocellular carcinoma. He was transferred to our center for further evaluation. On admission, transaminases were elevated with aspartate aminotransferase (AST) 624 U/L, alanine aminotransferase (ALT) 520 U/L and total bilirubin of 34.7 mg/dL. International normalized ratio (INR) remained elevated despite multiple infusions of fresh frozen plasma and vitamin K. Factor V and VII activities were 18% and < 6%, respectively. Magnetic resonance imaging (MRI) with Eovist contrast demonstrated interval development of heterogeneous, enhancing nodularity with portal venous washout, unlikely to be an infiltrative tumor process.

A full repeat hepatitis work-up was performed (Table 1). No definitive cause of acute liver failure could be identified; however, some findings were equivocal. Autoantibody titers demonstrated a positive anti-

Table 1 Work-up for causes of acute liver failure in reported patient

Laboratory test	Result	Reference range
Hepatic function panel		
Aspartate aminotransferase	624 U/L (H)	7-36 U/L
Alanine aminotransferase	520 U/L (H)	4-45 U/L
Alkaline phosphatase	156 U/L (H)	31-103 U/L
Bilirubin, total	34.7 mg/dL (H)	0.2-1.1 mg/dL
Bilirubin, conjugated	14.8 mg/dL (H)	0.0-0.2 mg/dL
Albumin	3.6 g/dL (L)	3.7-5.1 g/dL
Total Protein	5.8 g/dL (L)	6.2-8.6 g/dL
Coagulation factors		
Prothombin time	37.9 s (H)	9.1-11.9 s
INR	3.5 (H)	< 1.2
Factor VII activity	< 6% (L)	> 50% activity
Factor V activity	18% (L)	> 50% activity
Tumor markers		
CEA	2.3 ng/mL	< 3.1 ng/mL
CA 19-9	235 U/mL (H)	0-35 U/mL
AFP	51.1 ng/mL (H)	1.6-4.5 ng/mL
AFP-L3	19.0% (H)	0.5%-9.9%
PIVKA	4.4 ng/mL	< 6.3 ng/mL
Viral serologies		
Hepatitis A, IgM	Nonreactive	Nonreactive
Hepatitis A, IgG	Reactive ¹	Nonreactive
Hepatitis B surface antigen	Nonreactive	Nonreactive
Hepatitis B surface antibody, quantitative	< 10 IU/L	< 10 IU/L
Hepatitis B core antibody, total	Nonreactive	Nonreactive
Hepatitis C antibody screen	Nonreactive	Nonreactive
Hepatitis C RNA quantitative PCR	Not Detected	Not detected
Hepatitis E antibody, IgG	Not Detected	Not detected
Hepatitis E antibody, IgM	Not Detected	Not detected
CMV antibody immune status	Positive ¹	Negative
CMV DNA quantitative PCR	Not Detected	Not detected
Liver tissue CMV <i>in situ</i> hybridization	Negative	Negative
EBV-VCA IgM	Negative	Negative
EBV-VCA IgG	Positive ¹	Negative
EBV DNA quantitative PCR	Not Detected	Not detected
Liver tissue EBV <i>in situ</i> Hybridization	Negative	Negative
Adenovirus DNA Quantitative PCR	Not Detected	Not Detected
Liver tissue adenovirus <i>in situ</i> hybridization	Negative	Negative
Herpes Simplex 1 IgM screen	Negative	Negative
Herpes Simplex 2 IgM screen	Negative	Negative
Liver Tissue HSV 1 and 2 <i>in situ</i> hybridization	Negative	Negative
RPR	Nonreactive	Nonreactive
Autoantibody titer		
Antinuclear antibody	Positive ¹	Negative
Antinuclear antibody titer	1:40 ¹	< 1:20
Smooth muscle antibody	< 1:20	< 1:20
Liver kidney microsome antibody IgG	< 20.0 U	< 20.0 U
Soluble liver antigen autoantibody	< 20.1 U	< 20.1 U
Wilson's disease evaluation		
Copper, RBC	0.71 mg/L	0.53-0.91 mg/L
Copper, serum	95 µg/dL	70-140 µg/dL
Ceruloplasmin	22 mg/dL	17-48 mg/dL
Copper, 24-h urine	1055 µg/d (H)	3-50 µg/day
Quantitative liver copper	47 µg/g tissue	10-55 µg/g tissue
Hemochromatosis evaluation		
Total iron	243 µg/dL (H) ²	23-202 µg/dL
Iron binding capacity	< 308 µg/dL (L) ³	240-520 µg/dL
Transferrin	163 mg/dL (L)	198-386 mg/dL
Ferritin	3254 ng/mL (H)	8-350 ng/mL
Alpha-1-antitrypsin	91 mg/dL	83-199 mg/dL
Acetaminophen	< 10 µg/mL	10-20 µg/mL

¹Indicates positive result; ²(H) indicates value above the reference range; ³(L) indicates value below the reference range. AFP: Alpha-fetoprotein; EBV: Epstein Barr virus; CEA: Carcinoembryonic antigen; CMV: Cytomegalovirus; CA 19-9: Carbohydrate antigen 19-9; AFP-L3: Lectin-reactive AFP percentage; HSV: Herpes simplex virus; INR: International normalized ratio; PCR: Polymerase chain reaction; PIVKA: Protein induced by vitamin K absence.

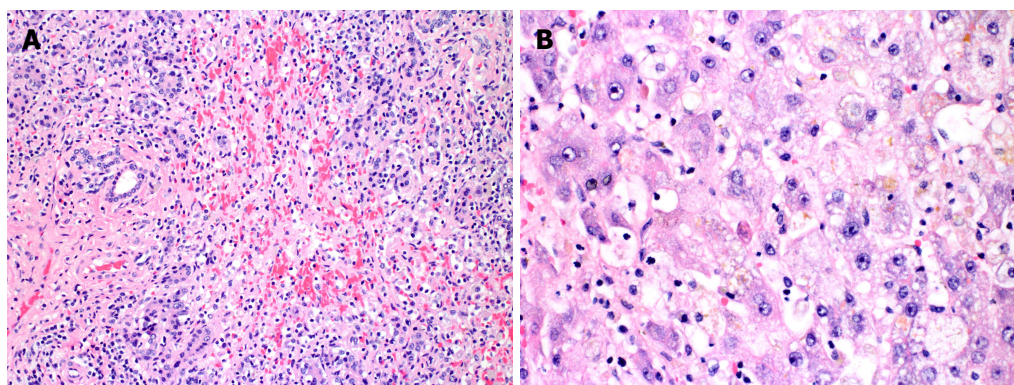


Figure 1 Histopathologic evaluation of explanted liver with *Garcinia Cambogia* associated fulminant hepatitis. A: Histopathologic examination demonstrates large areas of panacinar necrosis with complete hepatocyte dropout, collapsed lobules, florid ductular reaction, and predominantly lymphocytic infiltrates (hematoxylin-eosin stain, original magnification $\times 200$); B: Non-necrotic areas demonstrate hepatocyte ballooning, cholestasis, and mild lymphocytic infiltration. Occasional apoptotic hepatocytes (acidophil bodies) were present (hematoxylin-eosin stain, original magnification $\times 400$).

nuclear antibody, but no other positive autoantibodies. Evaluation of Wilson's disease demonstrated normal ceruloplasmin and copper levels; however, 24-h urine copper was elevated. Serum ferritin but not transferrin was elevated.

Liver biopsy was performed and demonstrated submassive necrosis with collapse of the hepatic architecture involving about 70% of the liver parenchyma. Mild lymphocytic inflammatory infiltration and minimal canalicular cholestasis were seen. No viral inclusions or other infectious agents were identified by histology or immunohistochemistry. No evidence of granuloma, tumor, or features of cirrhosis were demonstrated. Periodic acid-Schiff (PAS) stain with diastase was negative for alpha-1 antitrypsin globules. Iron stain showed only mild iron deposition in Kupffer cells and hepatocytes. Quantitative tissue copper was within normal limits (Table 1). Findings were felt to be potentially related to drug-induced liver injury.

After extensive questioning, the patient divulged intake of *Garcinia cambogia*, purchased through the Internet retailer Swanson Vitamins. He imbibed two 80 mg capsules of "Garcinia Cambogia 5:1 Extract" three times daily before meals for five months preceding initial presentation. Since not advised against intake, he continued the supplement after initial presentation. He denied any other medications or supplements and reported no alcohol intake for two months.

The patient's status declined and his mental status deteriorated. He was listed status 1A for liver transplantation. He received an orthotopic liver transplant from an ABO-identical brain dead donor and has recovered without incident. Histopathologic examination of the explanted liver demonstrated near total hepatic necrosis with massive hepatocellular dropout and mixed inflammatory cell infiltrates, consistent with severe drug-induced liver injury (Figure 1).

DISCUSSION

Americans spent approximately 59.8 billion dollars

on weight loss products in 2014^[6], and an estimated 10.1% of obese Americans have purchased over-the-counter supplements for weight-loss. Unfortunately, utilization does not correlate with sustained weight-loss^[7]. One product that has been heavily marketed as a "revolutionary fat buster" and a "magical ingredient" to promote weight loss is *G. cambogia*. This extract from the rind of the *G. cambogia* fruit is currently contained in 655 currently marketed products according to the Natural Medicine Comprehensive Database^[8]. These include "purified" supplement pills, multivitamins, and even energy drinks with widely disparate compositions, dosage, and potential contaminants.

The particular brand of *Garcinia cambogia* in this case was "Swanson Premium Brand Garcinia Cambogia 5:1 Extract," reported to contain 80mg of a 5:1 concentrate of *G. cambogia* (equivalent to 400mg of standard preparation). Other listed ingredients include rice flour, gelatin, magnesium stearate, and silica. The company reports that they do no assay for the hydroxycitric acid concentration, the fruit derivative reportedly responsible for weight-loss benefits of *Garcinia cambogia*^[9]. This is of note since hydroxycitric acid is the main derivative thought to be responsible for the weight-loss benefits of *Garcinia cambogia*. Mechanistically, it acts to prevent citric acid metabolism resulting in inhibition of de novo fatty acid synthesis^[10].

G. cambogia was also a main active ingredient in the weight-loss supplement Hydroxycut[®] (Iovate Health Sciences, Inc., Oakville, ON), which has known hepatotoxicity^[11-16]. In May 2009, the FDA issued a consumer warning recalling all Hydroxycut[®] products due to 23 hepatotoxicity cases. Prior to 2004, the formulation also contained ephedra, which was removed following the FDA ban. However, ten of 23 cases of hepatotoxicity, including the patient death, occurred after the 2004 reformulation to remove ephedra^[17]. *G. cambogia* was present in Hydroxycut[®] following the 2004 reformulation, but additional cases of hepatotoxicity occurred and a second FDA warning resulted in a second recall

in 2009. The supplement was again reformulated and remarketed. *G. cambogia* is absent from the currently marketed formulations of Hydroxycut®.

Although *G. cambogia* has been suggested as the putative cause of the banned supplement's hepatotoxic effects^[16], there is no definitive evidence. The majority of *G. cambogia* formulation associated with hepatotoxicity have been mixed supplements where a definitive causal relation could not be drawn. However, in the past several months, several cases of *G. cambogia* associated acute liver failure have been reported^[18,19], reinforcing the toxic potential of this particular supplement. Agreement upon the actual liver toxicity of *G. cambogia* has been mixed, and the majority of evidence is drawn from rodent models^[20]. The product can induce liver inflammation, fibrosis, and oxidative stress in mice. In one such study, supplement intake increased collagen deposition, elevated liver function tests, induced inflammatory cytokines, and stimulated oxygen free-radicals^[21]. However, supplement advocates cite rodent models in which *G. cambogia* demonstrates hepatoprotective effects, including decreased hyperlipidemia and hepatic oxidative stress in rats fed with a high-fat diet^[22].

This is one of the first reported cases of acute liver failure specifically associated with a "purified" supplement of *G. cambogia*. The patient had histologic evidence of drug-induced liver injury in the absence of other medication or alcohol use. Viral, autoimmune, and genetic (*i.e.*, hemochromatosis and Wilson's disease) causes of acute liver failure were definitively ruled-out, and *G. cambogia* intake was the only apparent risk factor. Unfortunately, independent laboratory evaluation of the supplement was not performed, which could have identified potential contaminants and verified manufacturer reported composition. While evidence from a case report rarely offers indisputable proof of causality, this case, in conjunction with known cases of hepatotoxicity and liver failure associated with other *G. cambogia*-containing supplements warrants a high index of suspicion.

Conditions predisposing patients to liver toxicity associated with *Garcinia cambogia* and like products remain unidentified. Acute liver failure from supplement ingestion appears relatively rare compared to their widespread use. Certain patients may have genetic predisposition or pre-existing liver damage, compounding hepatotoxicity. Cytochrome P450 is most commonly responsible for hepatic metabolism of drugs, and genetic polymorphisms in cytochrome P450 genes have previously been shown to result in toxic accumulation of certain drugs or metabolites. For example, toxicity associated with weight-loss supplements containing *N*-nitrofenfluramine has been associated with cytochrome CYP2C19 phenotypes^[23]. Mitochondrial injury, suggesting of toxic accumulation of *N*-nitrofenfluramine, was associated with a poor metabolizer phenotype; while, mitochondrial injury was absent in extensive metabolizers of the drug. One

extensive metabolizer developed hypersensitivity-associated hepatitis related to drug ingestion; however, mitochondrial injury was absent. Toxicity to *G. cambogia* may have incomplete penetrance due to a similar dependence upon genetic polymorphisms. Alternatively, injury may be more likely as a second hit in the setting of pre-existing liver damage. At our institution, a second case of *G. cambogia*-associated acute liver failure was identified; however, the patient had a remote history of heavy binge drinking with final pathology suggestive of early fibrosis. Drug-induced hepatotoxicity could not be definitively diagnosed due to this history, but the association with ingestion of large quantities of *G. cambogia* was suspicious, given that the degree chronic liver disease insufficiently accounted for her acute hepatic failure.

While additional research is necessary to further identify the link between *Garcinia Cambogia* and severe liver damage, public warning to potentially deadly side effects is necessary. This case emphasizes the need for direct questioning regarding dietary supplement intake in any case of acute hepatic injury. Manufacturers compliance with current regulations regarding contaminants is insufficient to preclude consumer toxicity, and increased public awareness of these dangers is crucial. Current regulation and oversight of the dietary supplement market should be scrutinized to improve supplement purity and identification of dangers. Endorsements by medical media celebrities and claims of "miracle results" should be carefully monitored for veracity. This case bears concerning similarity to those of Hydroxycut-associated liver failure, suggesting that although the product name may change, deadly side effects remain the same.

COMMENTS

Case characteristics

The patient initially presented with symptoms of nausea, vomiting, abdominal pain, and symptoms progressed to include confusion, coagulopathy, and jaundice.

Clinical diagnosis

Evaluation for viral, genetic, and antibody mediated causes of hepatitis were largely negative with histological evidence of near total hepatic necrosis on biopsy.

Differential diagnosis

Alternative autoimmune, viral, and genetic causes of acute liver failure must be excluded. In the absence of these, careful questioning regarding medications, herbal supplements, and energy drinks must be undertaken.

Laboratory diagnosis

Negative laboratory evaluation for autoimmune, viral, and genetic causes of acute liver failure in the presence of elevated liver function tests and coagulopathy should raise clinical suspicion for drug-induced liver injury.

Imaging diagnosis

Magnetic resonance imaging with Eovist contrast demonstrated interval development of heterogeneous, enhancing nodularity with portal venous

washout, unlikely to be an infiltrative tumor process.

Pathological diagnosis

Explant pathology demonstrated near total hepatic necrosis with massive hepatocellular dropout and mixed inflammatory cell infiltrates.

Treatment

Patient received liver transplantation with complete resolution of symptoms.

Related reports

Acute liver failure and severe hepatotoxicity has been associated multiple dietary supplements utilized for weight loss. These include the supplement Hydroxycut[®], which was removed from the market by the FDA and has undergone several reformulations.

Term explanation

Garcinia cambogia - tree grown in southwestern India. Extracts from the rind of the fruit from this tree are high in hydroxycitric acid and are marketed as weight-loss supplements.

Experiences and lessons

Careful questioning of any patient presenting with liver function abnormalities or acute liver failure should prompt questioning regarding dietary supplement and energy drink consumption. Patients with acute liver failure should be promptly referred to a transplant center for treatment.

Peer-review

This article presents one of the first known cases of hepatotoxicity associated with intake of the dietary supplement *Garcinia cambogia*. Reviewers felt the article was timely and well written. They requested additional information regarding dosage of the supplement as well as some additional discussion regarding its effects.

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