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REVIEW

Role of ion channels in gastrointestinal cancer

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Abstract

In their seminal papers Hanahan and Weinberg described oncogenic processes a normal cell undergoes to be transformed into a cancer cell. The functions of ion channels in the gastrointestinal (GI) tract influence a variety of cellular processes, many of which overlap with these hallmarks of cancer. In this review we focus on the roles of the calcium (Ca^{2+}), sodium (Na^+), potassium (K^+), chloride (Cl^-) and zinc (Zn^{2+}) transporters in GI cancer, with a special emphasis on the roles of the KCNQ1 K^+ channel and CFTR Cl^- channel in colorectal cancer (CRC). Ca^{2+} is a ubiquitous second messenger, serving as a signaling molecule for a variety of cellular processes such as control of the cell cycle, apoptosis, and migration. Various members of the TRP superfamily, including TRPM8, TRPM7, TRPM6 and TRPM2, have been implicated in GI cancers, especially through overexpression in pancreatic adenocarcinomas and down-regulation in colon cancer. Voltage-gated sodium channels (VGSCs) are classically associated with the initiation and conduction of action potentials in electrically excitable cells such as neurons and muscle cells. The VGSC $Na_V1.5$ is abundantly expressed in human colorectal CRC cell lines as well as being highly expressed in primary CRC samples. Studies have demonstrated that conductance through $Na_V1.5$ contributes significantly to CRC cell invasiveness and cancer progression. Zn^{2+} transporters of the ZIP/SLC39A and ZnT/SLC30A families are dysregulated in all major GI organ cancers, in particular, ZIP4 up-regulation in pancreatic cancer (PC). More than 70 K^+ channel genes, clustered in four families, are found expressed in the GI tract, where they regulate a range of cellular processes, including gastrin secretion in the stomach and anion secretion and fluid balance in the intestinal tract. Several distinct types of K^+ channels are found dysregulated in the GI tract. Notable are hERG1 upregulation in PC, gastric cancer (GC) and CRC, leading to enhanced cancer angiogenesis and invasion, and KCNQ1 down-regulation in CRC, where KCNQ1 expression is associated with enhanced disease-free survival in stage II, III, and IV disease. Cl^- channels are critical for a range of cellular and tissue processes in the GI tract, especially fluid balance in the colon. Most notable is CFTR, whose deficiency leads to mucus blockage, microbial dysbiosis and inflammation in the intestinal tract. CFTR is a

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tumor suppressor in several GI cancers. Cystic fibrosis patients are at a significant risk for CRC and low levels of CFTR expression are associated with poor overall disease-free survival in sporadic CRC. Two other classes of chloride channels that are dysregulated in GI cancers are the chloride intracellular channels (CLIC1, 3 & 4) and the chloride channel accessory proteins (CLCA1,2,4). CLIC1 & 4 are upregulated in PC, GC, gallbladder cancer, and CRC, while the CLCA proteins have been reported to be down-regulated in CRC. In summary, it is clear, from the diverse influences of ion channels, that their aberrant expression and/or activity can contribute to malignant transformation and tumor progression. Further, because ion channels are often localized to the plasma membrane and subject to multiple layers of regulation, they represent promising clinical targets for therapeutic intervention including the repurposing of current drugs.

Key words: Ion channels; Gastrointestinal cancer; Colorectal cancer; Gastric cancer; Pancreatic cancer; Esophageal cancer; Hepatocellular carcinoma; Prognostic biomarker; Novel therapies; Clinical targets

Core tip: Ion channels play an essential function in the physiology of the GI tract. There is increasing evidence that they are dysregulated at all stages of gastrointestinal (GI) cancer, from early initiation to metastasis. This information provides for the use of ion channel expression as useful clinical prognostic biomarkers in GI cancer. Perhaps more importantly new therapeutic modalities targeting ion channels in the GI tract, including the potential to target their dysregulation in GI cancers are becoming increasingly feasible. This strategy includes the repurposing of existing drugs that are used to treat other ion channel pathologies, or other diseases altogether. This review seeks to provide an overview of the role of ion channels in GI cancers with an emphasis on the potential for new therapies that target them.

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INTRODUCTION

In 2019, the American Cancer Society estimates that there will be more than 1.76 million new cases of cancer in the United States, accompanied by more than 607,000 cancer deaths[1]. Of these, the digestive system will have the highest number of new cases, and the second highest number of cancer deaths. The lifespan of cells in the gastrointestinal (GI) tract is very short. Propagated from stem cells, the epithelial cells of the stomach, small intestine, and colorectum are typically replaced in a matter of days and are some of the most replicative tissues in the body. This turnover is necessary due to the constant physical, chemical, and biological insults these tissues endure. This rapid proliferation increases the likelihood of cells in these tissues acquiring and accumulating oncogenic mutations.

The basic functions of the GI epithelium are: (1) to act as a physical barrier that selectively allows for the absorption of nutrients; while (2) excluding toxic or pathogenic substances; and (3) to excrete substances to aid in the digestion process. These functions require large quantities of water, ions, and nutrients to be transported across the epithelial layer. The significant driving force for this work is achieved through the use of ion gradients.

The unequal distribution of ions is required for the survival and function of any cell. This includes everything from concentration gradients across cellular and organellar membranes to gradients within the cytosol from one end to the other of a polarized cell. The distribution of ions is a consequence of the localization and activation of a variety of ion-specific channels, co-transporters, and pumps. Ion channels typically have a gating mechanism controlling when they are open or closed, and allow for the passive movement of select ions down their concentration gradient.

In contrast to this passive dissipation of gradients, pumps make use of ATP hydrolysis to actively set up gradients. Co-transporters, or secondary pumps, exploit the energy gained by moving one ion down its gradient to power the movement of another ion or molecule against its gradient. Precise regulation of these elements in response to changing environmental conditions and the subsequent changes in ion concentration or flux are necessary for a multitude of cellular processes including proliferation, motility, absorption/secretion, apoptosis and many others. The goal of this review will be to summarize what we know about the role of ion channel dysfunction as it pertains to cancer development within the GI epithelium. We focus on the four main classes of ion channels: potassium (K^+), chloride (Cl^-), sodium (Na^+), and calcium (Ca^{2+}) and zinc (Zn^{2+}) transporters and the major epithelial cancers that arise in the GI tract: colorectal cancer (CRC), gastric cancer (GC), esophageal cancer (EC), pancreatic cancer (PC) and hepatocellular carcinoma (HCC). For other important GI transporters and aquaporins and other types of cancers we refer readers to other specific reviews.

Nearly 20 years ago, Hanahan and Weinberg presented certain criteria that a normal cell must acquire to be transformed into a cancer cell[2]. These hallmarks of cancer have been expanded upon since that seminal paper, but their basic principles still remain[3]. The functions of ion channels influence a variety of cellular processes, many of which overlap heavily with these hallmarks of cancer. For this reason, cancer has been described as a channelopathy[4], with a recent review by Prevarskaya *et al*[5] asking whether cancer hallmarks are primarily oncochannelopathies. For example, Ca^{2+} channels play major roles in the control of cellular growth and proliferation, as well as the control of cell death[6]. K^+ and Cl^- channels are essential for the localized swelling and shrinking of different areas of a cell, necessary for cell migration[7]. Separate from their function as channels, studies of protein interactions with various ion channels have demonstrated their involvement in diverse processes such as cytoskeletal architecture and protein targeting[8]. It is clear, from the diverse influences of ion channels, that their aberrant expression and/or dysfunction can contribute to the transformation of normal cells into malignancy[4-5,9-15]. As discussed by Djamgoz *et al*[12] ion channels are expressed and dysregulated in all cancers throughout the multi-stage process, from initiation to metastasis. This is certainly true for the GI tract.

POTASSIUM CHANNELS

K^+ channels play a major role in maintenance of plasma membrane (PM) potential. The action of the Na^+/K^+-ATPase transporter, H^+/K^+-ATPase transporter, and the NKCC cotransporter, coupled with the exit of K^+ ions from the cell down their electrochemical gradient maintains a net intracellular negative charge at the PM. This hyperpolarized membrane potential is then used to drive the active transport of various molecules against their gradient. This is especially important in the GI epithelium, which must continuously transport mass quantities of water, electrolytes, and nutrients. With 77 genes coding for K^+ channels, they are the largest, most diverse group of ion channels in the human genome. In addition, many of these genes have known splice variants, can be post-translationally modified, or form complexes with regulatory subunits. This immense variability in K^+ channel function highlights the importance of being able to fine-tune K^+ conductance and brings into question what other functions these channels might be serving. Generally, K^+ channels are classified as either voltage-gated, Ca^{2+}-activated, inward rectifier channels, or 2P-domain channels.

Whereas Na_v channels are classically associated with the rapid depolarization of excitable cells, voltage-gated K^+ (K_v) channels are responsible for re-polarization during an action potential. In non-excitable cells, such as those of the GI epithelium, the typical role of these channels is hyperpolarization of the PM. This negative membrane potential facilitates Ca^{2+} signaling and is required for regulation of intracellular pH and cell volume. Owing to these broad influences, K^+ channels are implicated in a variety of cellular and tissue functions including cell proliferation and differentiation, pigmentation, hearing and the mammalian cochlea, contractility, circadian rhythms, migration, wound healing, cell cycle progression, apoptosis, autophagy, metabolism, angiogenesis, stem cell dynamics, and carcinogenesis, including cancer cell proliferation, invasion, migration and metastasis[9,11,16-20]. Notably, the mechanisms underlying loss of control of K^+ channels are still not well understood[18].

KCNQ1

Very prominent among the K_v channels contributing to cancer risk is KCNQ1, which

demonstrates flexibility in gating permitting functional versatility[21]. In the GI tract KCNQ1 assembles with the β-subunits KCNE2 (gastric) or KCNE3 (intestinal) converting it from a voltage-dependent channel in the stomach into a voltage-independent, constitutively open channel in the intestine[22]. In gastric parietal cells, luminal KCNQ1/KCNE2 is essential for gastric acid secretion, working in conjunction with a H^+/K^+-ATPase. In the intestinal and colonic crypts, KCNQ1/KCNE3 is located basolaterally, and establishes the driving force for cAMP-mediated Cl^- secretion through CFTR, necessary for mucus hydration[23,24]. In the colon, blocking the activity of KCNQ1/KCNE3 nearly entirely abolishes cAMP-mediated Cl^- secretion, versus only about 50% in the small intestine, demonstrating a reliance on KCNQ1 in the colon[25].

KCNQ1 deficiency in humans and animal models

Humans carrying germline mutations in *KCNQ1* (Jervell and Lange-Nielsen and Romano-Ward syndromes) develop a range of pathologies, most notably cardiac arrhythmia (long and short QT), but also hearing loss, elevated gastrin levels, gastric hyperplasia and in some cases gastric neoplasia[26-30]. These phenotypes are well modeled in *Kcnq1* knockout mice that develop inner ear defects, imbalance, chronic gastritis, gastric hyperplasia, and gastric metaplasia[31,32].

KCNQ1 and GI cancer

There is strong evidence for *KCNQ1* functioning as a tumor suppressor in GI cancers. The first data came from *Sleeping Beauty* (SB) transposon mutagenesis screens for intestinal cancer in mice. *Kcnq1* was the third-ranked common insertion site (CIS) gene (just behind *Apc* and *Rspo2*, well-known Wnt/β-catenin pathway signaling genes), with a predicted loss of function and 14 unique mutation sites, among 77 CIS genes identified in the first SB screen published in Science in 2009[33]. *Kcnq1* was then identified as a CIS gene in three subsequent SB screens for intestinal cancer[34-36]. *Kcnq1*'s role was then confirmed in crosses of *Kcnq1* knockout mice to the Apc^{Min} mouse model of intestinal cancer where *Kcnq1*-deficiency significantly enhanced tumor phenotypes, including the development of invasive adenocarcinomas[37]. The role of KCNQ1 in human CRC was then investigated, finding that maintenance of KCNQ1 mRNA and protein levels was associated with significant disease-free and overall survival in stage II, III, and IV CRC[37,38]. Notably, for stage IV CRC patients following hepatic resections, maintenance of KCNQ1 protein expression conferred a 23-month survival advantage[37]. In other GI cancers *Kcnq1* was a CIS gene in two SB screens for PC[39,40], one SB screen for HCC[41] and in one SB screen for GC, with a predicted loss of function[42]. Additional evidence in GC is provided by the phenotype of *Kcnq1* knockout mice that develop gastric hyperplasia, metaplasia and occasional neoplasia[31,32] and in studies of human gastric cells where treatment of cells with atrial natriuretic peptide reduced cell proliferation by upregulating KCNQ1 expression[43]. In studies of HCC in human tissue and HCC cell lines, expression of *KCNQ1* was down-regulated by promoter hypermethylation associated with epithelial to mesenchymal transition (EMT), and poor patient prognosis[44]. Additionally, in HCC it was reported that KCNQ1 regulated and sequestered β-catenin *via* physical interactions at the PM[44].

Although *KCNQ1* deficiency is associated with poor outcome in CRC[37,38,45] and in HCC[44], the mechanisms underlying tumor suppression are not well understood. However, one clue is that KCNQ1 is localized to the base of the intestinal epithelial crypt which is the site of the stem cell compartment and the likely site of origin of CRC[46]. Functional significance of crypt localization was demonstrated by Than *et al*[37] who found that crypts isolated from *KCNQ1*-deficient colon epithelium displayed increased clonogenicity suggesting a possible selective advantage for tumor development. In addition, several studies demonstrate involvement of *KCNQ1* with the Wnt/β-catenin pathway[38,44,45,47]. The Wnt/β-catenin pathway is vitally important in intestinal epithelial physiology and pathophysiology, with deregulation of the pathway contributing to over 80% of CRCs as well as a large percentage of HCCs. An early study in *Xenopus* oocytes demonstrated that β-catenin up regulated KCNQ1-mediated currents by promoting its insertion into PM without any effect on transcription[47]. More recent studies have looked at the interactions of KCNQ1 and β-catenin specifically in GI cancers. Analysis of 386 human stage II and III CRC tumors found correlation between KCNQ1 membrane-associated protein and nuclear β-catenin protein expression, which was surprising as KCNQ1-low expression was associated with poor outcome[38]. It was proposed that *KCNQ1* may be down-regulated by promoter methylation in some cancers so that in the presence of β-catenin, as seen in most CRC, *KCNQ1* would be upregulated, but if in addition the promoter becomes methylated *KCNQ1* would be down-regulated. In contrast, a second study found that β-catenin directly negatively regulated KCNQ1 transcription

in several CRC cell lines[45]. This group also found that KCNQ1 promoted cell membrane localization of β-catenin. This had the effect of limiting oncogenesis both by preventing nuclear localization of β-catenin and by maintaining adherens junctions that prevent EMT. A third study reported that in HCC cell lines, *KCNQ1* expression was enhanced by treatment with a methyltransferase inhibiter suggesting that expression may be down-regulated by promoter methylation[44]. However, KCNQ1 appeared to sequester β-catenin at the cell membrane and to limit its transcriptional activity. The situation is further complicated by the lncRNA *KCNQ1OT1*, which through the recruitment of chromatin and DNA modifying proteins, silences multiple genes in the KCNQ1 region[48]. *KCNQ1OT1* has been associated with poor patient survival in several GI cancers, including CRC[49], EC[50], and HCC[51]. *KCNQ1OT1* itself is regulated by β-catenin. The Kugoh group has demonstrated that nuclear β-catenin activates the transcription of *KCNQ1OT1* through a TCF-1 binding site within its promoter region[52]. These studies suggest that multiple factors affect the interactions between KCNQ1 and β-catenin, and that the balance of these factors differs among cancers. But apparently loss of KCNQ1 activity, through whatever means, can provide a selective advantage to the tumor, as each of these studies demonstrates that *KCNQ1* is a tumor suppressor. Channel openers such as retigabine have been developed as treatments for diseases caused by KCNQ1-deficiency[53]. Thus, understanding the contribution of KCNQ1 to cancer progression could lead to new cancer therapeutic opportunities.

KCNE2 and KCNE3

Given its role as the β-subunit in the KCNQ1/KCNE2 channel in gastric tissue it is not surprising that deficiency for KCNE2 contributes to human GC cell growth and progression. This was supported by studies in *Kcne2* knockout mice that develop gastritis cystica profunda and neoplasia, pyloric polyadenomas and invasive adenocarcinomas, linked to upregulation of cyclin D1[54-58]. *Kcne3* (intestinal β-subunit) knockout mice also partially phenocopied loss of KCNQ1, demonstrating a disruption in intestinal Cl^- transport[46].

Human ether-a-go-go related gene 1

While KCNQ1 seems to have tumor-suppressive effects, another K_v channel, the human *ether-a-go-go* related gene 1 (hERG1) channel, has been implicated as an oncogene in various GI cancers including CRC[59-62], PC[62-66], EC[67-70], and GC[68,71-74]. In CRC, while hERG1 is not expressed in normal colonic mucosa, a distinct upregulation in expression is reported in adenocarcinomas, with the highest levels occurring in metastatic cancers, where hERG1 expression was associated with poor patient prognosis, and with little to no expression in adenomas. hERG1 expression levels and activity were positively correlated to cell migration using channel inhibitors and clones expressing various levels of the protein[59]. This role in cancer cell migration and invasion was later expanded upon with the discovery of hERG1's interactions with β1-integrins in PM complexes. hERG1 was shown to modulate β1-integrin mediated VEGF-A secretion through the recruitment of PI3K and AKT[72]. In PC hERG1 over-expression was reported in 59% of tumors[66] where it promoted cancer cell migration *via* modulation of F-actin organization[64]. hERG1 expression was also associated with lymph node involvement, tumor grade, TNM stage and poor patient prognosis[66]. One report linked hERG1 to dysregulation of the EGFR signaling pathway. In EC, hERG1 expression was associated with progression from Barrett's esophagus (BE) to EC, again associated with poor patient prognosis[70]. Finally, in GC, hERG1 over-expression was reported in 69% of cancers, where it promoted angiogenesis by mediating VEGF-A secretion *via* AKT-dependent regulation of HIF-1, and similarly, its expression was associated with poor patient prognosis[71-74].

EAG1

Another *ether-a-go-go* family member, EAG1, is also reported to function as an oncogene in several GI cancers, including CRC[18,75,76], EC[75], and HCC[16,62,75,77]. In all cases EAG1 is reported to be over-expressed, and associated with invasion of cancer cells and poor patient prognosis.

Other K^+ channels

At least nine other K^+ channels have been reported to be dysregulated in GI cancers. Those acting as oncogenes include KCNA5 in GC[73,78-80], and CRC[79]; KCNC1 in CRC[76]; KCND1 in GC[81]; KCNJ3 in PC[65,82]; KCNN4 in CRC[5,14,83], PC[65,84], and HCC; KCNS3 in CRC[85]; and KCNK9[5,86,87] in CRC. Those acting as potential tumor suppressors include KCNA3 in CRC[4,76,88] and PC[65,82,89], and KCNH5 in EC[90]. Of note, the pathophysiological actions of many of these channels seem to involve mechanisms that are independent of their channel action. An example is KCNS3/K_v9.3, an

electrically silent subunit in excitable cells, whose expression has been found increased in CRC and lung cancer cells, with a knockdown showing interference at the G0/G1 and G1/S cell cycle transitions[85]. Overall, the great majority of K^+ channels discussed here function as oncogenes (with KCNQ1 a prominent exception), consistent with a model of K^+ channel dysregulation/overexpression being necessary for cancer cell cycle and tumor progression. See Table 1 for a full listing of potassium ion channels and their role in GI cancers.

CHLORIDE CHANNELS

The functions of Cl^- channels in the GI tract include regulation of transepithelial transport (in particular major anions), volume control (osmoregulation), membrane potentials, lipid homeostasis, cell polarity, glucose and other metabolism, oxidative stress, inflammation, mucus, alterations in the microbiome, pH, cell motility, autophagy, mitochondrial dysfunction, apoptosis, cell polarity, cell-cell contact, stem cell function, and cellular immune responses[5,91-105]. All of these functions can be dysregulated in and contribute to malignant transformation, particularly in the GI tract due to its constant exposure to environmental influences.

CFTR

CFTR encodes a Cl^-, $HCO3^-$ anion channel found primarily on the apical surfaces of luminal epithelial cells. Mutations in *CFTR* are the cause of the hereditary life shortening disease cystic fibrosis (CF). Recently CFTR has been shown to be a tumor suppressor in CRC and CFTR deficiency has been implicated in several other cancers. Because the causal connection between CFTR and cancer has been most strongly established for CRC, this section of the review will focus primarily on CRC.

Normal functions of CFTR

The *CFTR* gene found on chromosome 7 encodes an mRNA of 6128 nucleotides[106]. The CFTR protein consists of two symmetrical halves. Each half contains 6 membrane spanning domains joined by an intracellular regulatory region. The membrane spanning domains assemble to form an aqueous pore that allows flow of Cl^- and $HCO3^-$ ions down their electrochemical gradients. In the intestine the flow of ions is from the cytoplasm to lumen. Ion specificity is provided by the amino acids lining the pore. Opening and closing is regulated by binding of ATP to two nucleotide binding domains in the regulatory region. ATP binding is mediated by cAMP activation of PKA which phosphorylates a site to open up the ATP binding domains. *In vivo* cAMP activity is commonly regulated by cholinergic stimuli. Outflow of ions creates osmotic pressure for the flow of water in the same direction so that CFTR indirectly determines the flow of water as well[107,108]. CFTR also regulates other ion channels (Na^+, K^+, Ca^{2+}, and other Cl^- channels). For example, CFTR indirectly regulates the cellular ionic environment by inhibiting activity of the Na^+ importing channel SCNN1, which has the effect of further encouraging outflow of water and also by supporting additional $HCO3^-$ transporters[109]. CFTR also interacts with other membrane proteins to maintain epithelial tight junctions and barriers to fluid flow, adjusts the levels of acidity in secretions, and participates in the transport of sphingosine-1 phosphate, a regulator of cell adhesion and a signaling molecule for inflammation[99]. CFTR also contains a cytoplasmic C-terminal PDZ-binding motif. This domain interacts with PDZ-containing proteins involved in regulating the actin cytoskeleton and intracellular signaling[110,111]. CFTR is expressed on the apical surface of the intestinal epithelium throughout the length of the intestine. In the small intestine CFTR expression decreases from duodenum to ileum with strongest expression in the crypts[112]. In addition, functional CFTR is found on the brush border of villus cells and rare CFTR-high expressing cells throughout the small intestine[113]. In the colon CFTR is expressed in a proximal to distal gradient with highest concentration in the proximal region and cecum. CFTR is most highly expressed at the base of the crypt where intestinal epithelial stem cells reside[112,114]. Overall, CFTR is a major determinant of ion and water homeostasis and because it is highly expressed at the base of the crypt it is in a position to influence the intestinal stem cell compartment.

Cystic fibrosis

Inactivating germline mutations in *CFTR* cause the hereditary life shortening disease CF. CF is the most common autosomal recessive hereditary disease among Caucasians[115]. The most severe clinical manifestations are pulmonary inflammation and obstruction leading ultimately to pulmonary failure[116]. However, CFTR is expressed in many extra-pulmonary tissues including the linings of the pancreatic and biliary ducts where its loss ultimately leads to CF-related diabetes and CF-related

Table 1 Potassium channels

Gene name (s)	Cancer	Role	Functional activity
KCNA3/Kv1.3	Colorectal	Unclear	One report that Kv1.3 is frequently hypermethylated and expression down-regulated in CRC; a different report that Kv1.3 is upregulated in human and mouse colon carcinomas[4,76,88]
	Pancreatic	Tumor suppressor	Expression down-regulated by promoter hypermethylation; promotes metastasis[65,82,89]
KCNA5/Kv1.5	Gastric	Oncogene	Expression up-regulated; silencing in GC cells inhibits proliferation; alters drug resistance[73,78-80]
	Colorectal	Oncogene	Expression up-regulated[79]
KCNC1/Kv3.1	Colorectal	Oncogene	Expression up-regulated[76]
KCND1/Kv4.1	Gastric	Oncogene	Expression up-regulated[81]
KCNE2/MiRP1	Gastric	Tumor suppressor	Expression down-regulated; deficiency promotes tumor progression; knockout mice develop gastritis cystic profundal and neoplasia, pyloric polyadenomas; invasive adenocarcinomas; upregulation of cyclin D1; down-regulated in gastric cancer tissues and cell lines; overexpression in cell lines suppressed growth in soft agar and mouse tumor xenografts[54-58]
KCNH1/EAG1/Kv10.1	Colorectal	Oncogene	Up-regulated; one report showed 75% of CRC tumors positive for Eag1; another report found overexpression in 3.4% of adenocarcinomas[18,75,76]
	Esophageal	Oncogene	Expression up-regulated; associated with depth of invasion; independent negative prognostic factor[75]
	Hepatocellular	Oncogene	Expression up-regulated[16,62,75,77]
KCNH2/hERG1/Kv11.1	Colorectal	Oncogene	Expression up-regulated; triggers angiogenesis and tumor progression via inducement of PI3K/AKT signaling and HIF1-induced activation of VEGF-A; associated with invasiveness, poor prognosis for stage I and II; up-regulation in *ApcMin* and AOM mouse models enhanced cancer phenotypes[59-62]
	Pancreatic	Oncogene	Expression up-regulated in 59% of pancreatic cancers; promotes migration of cancer cells by modulation of f-actin organization; associated with lymph node involvement, tumor grade, TNM stage, poor patient prognosis; linked to EGFR pathway; down-regulated by miR-96; overexpressed in pancreatic cancer cell lines[62-66]
	Esophageal	Oncogene	Expression upregulated; promotes progression from Barrett's esophagus to esophageal cancer; associated with poor patient prognosis[67-70]
	Gastric	Oncogene	Expression up-regulated; stimulates angiogenesis by promoting VEGF-A secretion *via* AKT-dependent regulation of HIF1; positive in 69% of gastric cancers; associated with poor patient prognosis[62,71-74]
KCNH5/EAG2/Kv10.2	Esophageal	Tumor suppressor	Expression down-regulated by promoter hypermethylation[90]
KCNJ3/Kir3.1	Pancreatic	Oncogene	Expression up-regulated[62,82]
KCNN4/Kca3.1	Colorectal	Oncogene	Expression upregulated; promotes EMT[5,14,83]

	Pancreatic	Oncogene	Expression up-regulated[65,84]
	Hepatocellular	Oncogene	Expression up-regulated[62]
KCNS3/Kv9.3	Colorectal	Oncogene	Silencing causes inhibition of proliferation of HCT15 CRC cells[85]
KCNK9/K2p9.1/Task3	Colorectal	Oncogene	Expression up-regulated[5,86,87]
KCNN3/Kca2.3/SK3	Colorectal	Oncogene	Expression and activity up-regulated; regulated by SigmaR1; physically coupled to Orai1[5,91]
KCNQ1/KvLqt1	Colorectal	Tumor suppressor	Identified as a top 10 common insertion site (CIS) gene in a sleeping beauty transposon mutagenesis screen in mice; predicted loss of function in the screen; knockout mouse developed enhanced GI cancer phenotype in *ApcMin* model; expression down-regulated in human colorectal cancer, associated with poor prognosis in stage II, III, and IV disease; found to be down-regulated by β- catenin, which promotes EMT; in turn, KCNQ1 physically interacts with β-catenin, sequestering β-catenin at the plasma membrane[33-38,45]
	Pancreatic	Not determined	Identified as a common insertion site (CIS) gene in two sleeping beauty transposon mutagenesis screens in mice[39,40]
	Gastric	Tumor suppressor	Identified as a CIS gene in a *Sleeping Beauty* transposon mutagenesis screen; predicted loss of function; knockout mouse susceptible to chronic gastritis, hyperplasia and metaplasia; atrial natriuretic peptide reduced proliferation of gastric cancer cells by upregulating KCNQ1[31,32,42,43]
	Hepatocellular	Tumor suppressor	Expression down-regulated by promoter hypermethylation; associated with poor patient prognosis; KCNQ1 regulated EMT; KCNQ1 regulates β-catenin physical interactions at the plasma membrane[41,44]
KCNQ1OT1	Colorectal	Oncogene	Expression up-regulated; promotes Wnt/β-catenin signaling and migration, poor patient prognosis[49,52]
	Esophageal	Oncogene	Expression up-regulated; promotion of metastasis; poor patient prognosis[50]
	Hepatocellular	Oncogene	Expression up-regulated; competes with endogenous miR-504; promotes cell proliferation, associated with TNM stage and poor survival[51]

CRC: Colorectal cancer; GC: Gastric cancer; CIS: Common insertion site; EMT: Epithelial to mesenchymal transition; GI: Gastrointestinal.

liver disease[117,118]. CFTR dysfunction in the exocrine pancreas results in ion transport defects, β-islet cell-related disorders such as dysregulation of insulin release, obstruction of the pancreatic duct, chronic inflammation and cancer initiation[99,119]. CF patients also demonstrate defects in the male reproductive system, chronic sinusitis, and kidney stones. Older CF patients have dysregulation of glucose metabolism and sleep disorders caused by disruption of circadian rhythms[99]. CFTR is also highly expressed in the intestinal epithelium. Loss of CFTR in the intestine causes intestinal obstruction in the ileum and proximal colon known as meconium ileus in infants and distal intestinal obstruction in older patients. CF results in impaired absorption of nutrients due to pancreatic enzyme deficiency and possibly defects in lipid absorption[120]. CF patients are also prone to celiac disease, an intestinal disorder caused by gluten-mediated triggering of T_H1 immune and antibody responses[102]. These and other clinical manifestations of CF demonstrate the profound importance

of CFTR activity in many tissues.

CFTR is a tumor suppressor in CRC

The lifespan of individuals with CF has increased dramatically so that the average life expectancy of a person born with CF today is approximately 44 years[121]. As individuals with CF live longer it has become apparent that they are at increased risk for developing some but not all cancers. Initial evidence linking CF to cancer risk came from a 20-year epidemiological study that compared incidence of cancers in individuals in the United States Cystic Fibrosis registry to the predicted age adjusted risk in the general population to determine standardized incidence ratios. This study reported that the overall risk of cancer was not increased. However, the risk of all types of GI cancers was increased and in particular the risk of CRC, the most common GI cancer, was increased by 6-fold[122]. A recent meta-analysis of population-based studies is consistent with this result[123]. In support of the clinical significance of this finding, endoscopic screening studies of adult CF patients found that polyps in individuals with CF appeared earlier, and were larger and more aggressive than those in the non-CF population[124,125]. As a result of these studies the guidelines for endoscopic screening of CF patients have been modified and CF has been declared a hereditary colon cancer syndrome by the Cystic Fibrosis Foundation[126].

Mouse genetic studies

Mouse genetic studies demonstrated the functional significance of Cftr deficiency. SB transposon-mediated genetic screens initially identified *Cftr* as a candidate cancer-causing gene. *Cftr* was identified in the top 10% to 50% of candidate genes in three SB screens to identify CRC driver genes in *Apc* wildtype[33], *Apc*-deficient[34], and *TGF beta*-deficient[36] backgrounds[127]. Follow up studies evaluated mice carrying a targeted intestinal specific deletion of *Cftr* (*Cftr*$^{-/-}$) for intestinal tumorigenesis. In the tumor sensitized *Apc*^{Min} background *Cftr*$^{-/-}$ mice developed significantly more adenomas than *Apc*^{Min}*Cftr* wildtype mice. Further, *Apc*^{Min}*Cftr*$^{-/-}$ but not *Apc*^{Min}*Cftr* wildtype mice developed invasive lesions. Most striking, *Cftr*-deficiency alone was sufficient to cause adenomas in > 60% of mice after a one year interval[128].

CFTR deficiency in the general population

CFTR deficiency is linked to CRC in the general population as well. In a study of 90 Stage II CRC patients stratified by tumor CFTR expression, disease-free survival at 3 years in the 25% of patients with lowest CFTR expression was 30% lower than those with higher expression[128]. In a second cohort, CFTR mRNA and protein expression was lower in tumor *vs* normal tissue and CFTR mRNA expression was lower in metastatic *vs* non-metastatic tumors. In this study, CFTR-depleted CRC cell lines showed enhanced oncogenic characteristics including increased colony formation, migration and invasion[129]. RAS, PKC and IFNα have been reported to be involved in the down-regulation of CFTR in the colon[119]. It is also possible that low CFTR expression in sporadic tumors is caused by epigenetic silencing of the CFTR promoter as has been reported in other cancers such as lung and bladder[130-133].

CFTR and the stem cell compartment

The single cell layer of the intestinal epithelium is replaced approximately every 5 days. Stem cells at the base of the crypt drive this renewal[114]. These stem cells and progenitor cells that acquire stem-like characteristics are thought to be the CRC initiating cells[134]. In the colon, CFTR expression is highest at the base of the crypt[112] and expression has been reported in the stem cell itself[93]. CFTR has also been reported to be involved in intestinal lineage differentiation with its knockdown causing proliferation, migration and expression of EMT genes[135]. Thus, CFTR is in a position to influence the renewal process of the intestinal epithelium and CFTR deficiency may directly influence cancer initiating cells. Intestinal crypts can be cultured *in vitro* as 3D organoid cultures. Organoid cultures recapitulate the intestinal luminal epithelial structure as well as the renewal and differentiation process[136]. CFTR is expressed in these cultures and maintains its ion channel function[137,138]. Thus, organoid cultures have been developed as surrogates to test the effectiveness of CF therapeutics as treatments for specific mutations[139-141]. In addition, organoid cultures derived from oncogenic or pre-oncogenic tissues maintain the oncogenic characteristics of these tissues[142,143]. Accordingly, organoid cultures have been used to determine the oncogenic characteristics of CFTR deficiency. Than *et al*[128] determined that Cftr-deficient colon organoids demonstrate increased clonogenicity. Analysis of small intestinal organoids by Strubberg *et al*[93] demonstrated increased proliferation of Cftr-deficient organoids and localization of Cftr to the LGR5 stem cell. These findings support a role for Cftr deficiency in the environment of the cancer initiating cell or the stem cell itself.

Wnt/β-catenin signaling

The Wnt/β-catenin signaling pathway is dysregulated in more than 85% of human CRC. Dysregulation contributes to initial adenoma formation and to maintenance of invasive tumors[144]. In the intestine CFTR deficiency is primarily associated with enhanced Wnt/β-catenin signaling. Than *et al*[128] identified nuclear localization of β-catenin, indicative of activation, as well as increased expression of Wnt/β-catenin target genes, in intestinal tumors deficient only for Cftr. Strubberg *et al*[93] identified increased active β-catenin in Cftr KO crypts and organoids which correlated with increased proliferation. Mechanistically, Cftr KO or transient inhibition of channel activity by CFTR(inh)-172 resulted in increased intracellular pH in LGR5+ stem cells. Increased pH in turn promoted association of Dvl2, a member of the Wnt/β-catenin signaling pathway, with PM phospholipids, which positioned it to enhance Wnt/β-catenin signaling[93] (Figure 1A). In contrast, Chan and colleagues reported that intestinal Cftr deficiency in mice carrying the F508del mutation and knockdown of CFTR in the Caco-2 CRC cell line results in diminished β-catenin signaling. Mechanistically, loss of CFTR destabilizes β-catenin membrane localization allowing it to be degraded leading to oncogenic phenotypes *via* activation of NF-κB[145] (Figure 1B). The differences between these two studies may reflect tissue specificity. This study analyzed Wnt/β-catenin signaling in total intestinal tissue rather than the crypt and so may reflect a different role for CFTR outside the stem cell compartment. Further, the effect of CFTR deficiency on Wnt/β-catenin activity varied by tissue in several other studies by this group[100]. In addition, Wnt/β-catenin signaling may also play different roles at different stages of CRC. Although it is generally accepted that dysregulated Wnt/β-catenin signaling is a driving force for initiation of CRC and progression[144] there is evidence that low levels of expression of Wnt/β-catenin targets are associated with poor prognosis in CRC[146]. Further, inhibition of Wnt/β-catenin has been shown to be necessary for survival of latent metastatic cells[147].

Additional mechanisms of tumor suppression

Extensive evidence that CFTR deficiency plays a role in many types of cancers suggests that tumor suppression by CFTR goes beyond regulation of Wnt/β-catenin signaling. The mechanisms of tumor suppression by CFTR are not well understood. However, the roles of CFTR in normal issue and the consequences of CF suggest plausible mechanisms.

Intestinal barrier integrity

CFTR plays a pervasive role in intestinal homeostasis through its influence on several inter-related processes: Epithelial barrier maintenance, microbiota composition and immune homeostasis. Breakdown of these processes is responsible for some of the clinical manifestations of CF and is also potentially oncogenic (Figure 2).

CFTR deficiency disrupts protective physical barriers

The intestinal lumen is home to trillions of bacteria. These bacteria provide essential nutrients and signals needed for intestinal epithelial and immune cell homeostasis. However, direct contact with the intestinal epithelium must be prevented. The colon epithelium is protected from direct contact by protective mucus layers and by tight junctions between the lateral surfaces of the single layer of epithelial cells lining the lumen. The apical, luminal surface of the epithelium is protected by a dense mucus layer impenetrable to bacteria that is generated by constitutive secretion of Muc2 from luminal goblet cells. This dense layer is partially enzymatically digested to generate a looser outer layer inhabited by commensal bacteria[148]. Muc2 is secreted in a condensed conformation that expands to form a fully functional mucus layer in the presence of extracellular $HCO3^-$ and H_2O. CFTR ion channel activity is required for direct export of bicarbonate and also for indirect support of export of bicarbonate and water through other channels[149]. Loss of CFTR ion channel activity results in accumulation of dehydrated mucus in the distal small intestine and proximal colon, which causes intestinal obstruction seen in CF patients in the form of meconium ileus and distal intestinal obstruction[120]. In addition, severely dilated crypts are seen in the CFTR-deficient intestine due to accumulation of mucus in goblet cells which may reflect defects in secretion[150,151]. The CFTR-deficient mucus layer appears to allow dysregulated bacterial contact. Bacterial colonies are reported in crypts with accumulated mucus[150]. Further, comparison of gene expression in the Cftr-deficient mouse intestine to changes in Muc2-deficient intestine using gene set enrichment analysis (GSEA) showed enrichment in inflammatory gene expression changes[128] suggesting that Cftr deficiency, like Muc2 deficiency, allows illicit bacterial contact. The basolateral epithelial surface and underlying lamina propria, including resident immune cells, are protected from bacterial contact by tight junctions and CFTR participates in maintenance of these junctions. In Cftr-deficient mice the small

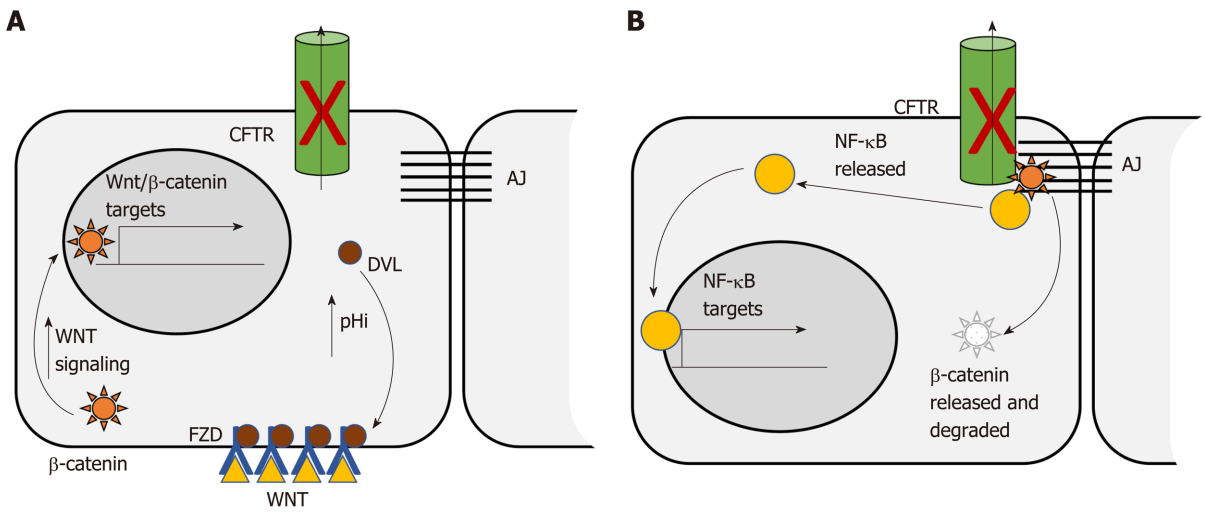

Figure 1 Two models for the effect of CFTR deficiency on Wnt/β-catenin signaling. A: CFTR deficiency promotes Wnt/β-catenin signaling. CFTR deficiency causes increased intracellular pH. Increased pH promotes association with Dishevelled (DVL) at the membrane and with the Wnt receptor Frizzled (FZD). DVL association with FZD enhances Wnt/β-catenin signaling leading to increased nuclear localization of β-catenin. Nuclear β-catenin promotes transcription of genes involved in proliferation, survival and stemness[93]; B: CFTR deficiency inhibits Wnt/β-catenin signaling. CFTR deficiency releases membrane associated β-catenin to the cytosol where it is degraded thus decreasing Wnt/β-catenin activity. Loss of β-catenin releases NF-κB which translocates to the nucleus where it promotes transcription of inflammatory targets[145]. FZD: Frizzled; DVL: Dishevelled; AJ: Adherens junctions.

intestine displays increased intestinal permeability and disruption of tight junctions[152]. Several studies link CFTR interactions mediated by its C-terminal PDZ-binding domain to integrity of tight junctions[152]. Studies in cultured airway epithelial cells show that interaction between the CFTR-PDZ binding (CFTR PDZBD) domain and the PDZ domain of NHERF1 (SLC9A3R1) maintains actin cytoskeletal organization and tight junctions[153]. CFTR deficiency may also maintain tight junctions by *via* direct interaction with ZO-1 an essential component of these junctions[8].

CFTR deficiency causes dysbiosis

As described above, CFTR deficiency as seen in CF patients and animal models creates an altered luminal environment. This environment, as well as a high fat/high calorie diet maintained to overcome nutritional deficits, and CF therapies such as frequent antibiotic exposure, contributes to bacterial dysbiosis[154,155]. In early studies dysbiosis was reported as small intestinal bacterial overgrowth in a CF mouse model[156]. Analysis of microbial 16S DNA in small intestine flushed luminal contents by qRT-PCR demonstrated an estimated 40X increase in bacterial load with decreased diversity[157]. In a second study using phylogenetic microarray analysis of flushed small intestinal contents there was a marked decrease in bacterial community richness, evenness and diversity, a decrease in the relative abundance of protective species such as *Acinetobacter Iwoffii* and *Lactobacilliales* members, but an increase in *Mycobacteria* species and *Bacteriodes fragilis* associated with GI infection and immunomodulation[156]. More recently, microbial population composition of CF *vs* non-CF individuals has been determined using targeted and metagenomic analysis of 16s rRNA DNA from fecal DNA[94,95,155,158]. Further, CF patients have been reported to be susceptible to Crohn's Disease with specific *CFTR* mutations influencing microbial dysbiosis with increased intestinal permeability[159]. In each of these studies CF microbiota demonstrated greatly reduced diversity and significant differences from non-CF controls indicating that the altered luminal environment not only creates increased bacterial access but also changes bacterial composition[95,103,156]. Importantly, alterations to the intestinal microbiome are increasingly associated with CRC[103,160].

CFTR deficiency is associated with immune infiltration

Disruption of the mucus layers and of tight junctions allows bacterial access to immune cells and immune infiltration of the epithelial layer. Although immune infiltration and subsequent inflammation can cause overt damage, this effect has only been reported in one study in which a capsule endoscopy study of CF patients revealed duodenal lesions[161]. More commonly, immune infiltration has been detected under *CFTR*-deficient conditions but has not been accompanied by obvious morphological and histological damage[162]. In human studies CF patients exhibited evidence of immune infiltration in studies evaluating whole gut lavage[163], fecal

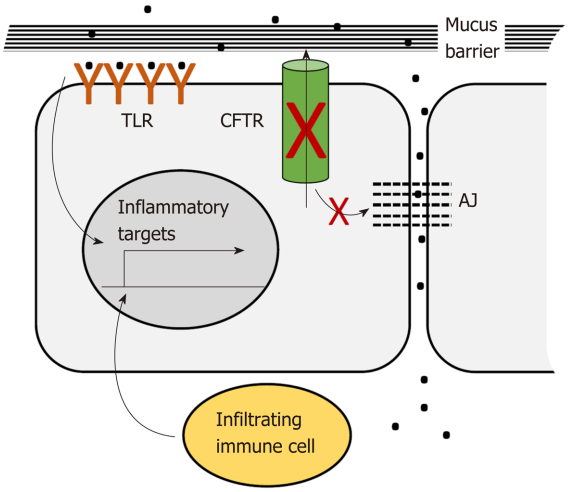

Figure 2 CFTR deficiency disrupts epithelial barrier integrity. CFTR deficiency disrupts the mucin barrier and adherens junctions. This allows bacterial contact with the apical and basal surfaces of the epithelial layer. Contact with the apical layer stimulates inflammatory signaling *via* toll-like receptors. Contact with the basal layer leads to immune cell infiltration which results in additional inflammatory signaling. AJ: Adherens junctions; TLR: Toll-like receptors.

calprotectin[158,161,164] and rectal nitric oxide[165]. Further, microarray analysis of the small intestine in an early study by Norkina *et al*[162] identified upregulation of genes expressed by granulocytes which was supported by microscopy that identified increased mast cells and neutrophils throughout the length of the intestine. Similarly, Chan and colleagues reported immunocytochemical evidence of an increased presence of macrophage and neutrophils in the small intestine of F508del mice[145].

Potential oncogenic changes

Loss of physical barriers in the GI tract allows dysregulated access of bacteria and infiltration of immune cells both of which contribute to inflammatory signaling[166-169]. These contacts have the potential to activate pro-inflammatory signaling in the intestinal epithelium both directly by interaction between bacteria and epithelial toll-like receptors[170] and indirectly *via* activation of lymphocyte cytokine signaling. For example, Fiorotto *et al*[171] demonstrated that in primary biliary epithelial cells CFTR binds Src inhibitors to position them for interaction with Src. Loss of CFTR leads to mislocalization of these inhibitors and activation of Src. In the presence of bacterial products this leads to NF-κB signaling which ultimately disrupts tight and adherens junctions. Proinflammatory signaling by intestinal epithelial cells is associated with dysregulated proliferation and expansion of the stem cell compartment through reversion of progenitor cells to stem cells[172]. Loss of barrier integrity affects other processes as well. Loss of tight junctions facilitates increased migration and invasion[173]. Dysbiosis may result in appearance of bacterial species associated with CRC. Phylogenetic microarray analysis of *Cftr*-deficient mice detected increased representation of *Bacteroides fragilis*[156], a species directly linked to CRC by virtue of its activation of Stat3 signaling through a Th17 immune response[174].

In vitro models of pro-inflammatory signaling

CFTR deficiency has been linked to NF-κB activation in *in vitro* models. Chan and colleagues have carried out a series of studies in cell lines derived from several cancers, that delineate a pathway in which CFTR deficiency leads to activation of NF-κB, transcription of UPA, and enhanced migration and invasion[175-177]. Other groups have shown increased basal levels of pro-inflammatory cytokines and other factors and NF-κB pathway members in *CFTR*-deficient Caco-2, HT-29 and HEK293 cells and increased response to inflammatory stimuli, including an increase in TNF-α, IL-6, IL-1β-induced secretion of IL-8, COX-2 and PGE_2, plus increased activities of ERK1/2, MAPK, IκBα and NF-κB[92,96,178]. Santa-Coloma and colleagues report activation of NF-κB as a result of autocrine signaling by IL-1β[179]. In addition, the Hedgehog pathway has recently been reported to be inhibited under CFTR-deficient conditions which is predicted to activate inflammatory signaling[180].

Stress responses

CFTR deficiency has been reported to both increase oxidative stress *via* impaired

mitochondrial activity[96,97] and to reduce stress by cellular retention of the antioxidant GSH[181]. In Caco-2/15 cells CFTR knockdown caused an increase in lipid peroxidation levels accompanied by a decrease in oxidant defenses such as glutathione peroxidase and catalase[182]. In addition, loss of CFTR has been reported to disable autophagy *via* activation of Transglutaminase-2 whose cross-linking activity causes essential autophagy proteins to be sequestered in aggresomes[183]. Finally, HIF-1-mediated repression of CFTR in intestinal epithelium resulted in a reduction in CFTR mRNA, protein and activity in hypoxic epithelium[184]. The impact of these activities on oncogenesis is not yet known.

Relationship to CRC

CFTR deficiency results in dysregulation of many processes that may be oncogenic. However, the relative contributions of these processes to the development of CRC *in vivo* are not yet clear. In the future it will be important to study CFTR in the environment in which it most likely contributes to development of CRC, the intestinal stem cell compartment. In the colon, CFTR is most highly expressed in the base of the crypt which comprises the stem cell compartment and the likely site of origin of CRC. Because the crypt is the environment for the intestinal stem cell it has unique protective mechanisms. The crypt maintains a unique microbiota with reduced number and diversity compared to the lumen[185]. Limited contact between bacterial products and crypt epithelial cells contributes to this protected environment. For example, goblet cells at the neck of the crypt orchestrate the release of mucin in response to contact with bacterial products[186]. The crypt also has unique mechanisms to protect from cellular stress. In the stem cell, interaction between the cytosolic innate immune sensor NOD2 and the bacterial peptidoglycan motif MMP is necessary for stem cell survival in the face of oxidative stress[187]. Finally, cells in this compartment are uniquely susceptible to the effects of inflammatory signaling. In a mouse genetic study NF-κB was shown to synergize with Wnt/β-catenin in intestinal crypt cells to promote conversion of progenitor cells to stem-like cells with tumor initiating capacity[172]. These considerations highlight the importance of studying potential oncogenic phenotypes of CFTR deficiency in the crypt and crypt-derived organoid models.

CFTR and other cancers

Given the widespread distribution of CFTR and its impact on cellular homeostasis it is not surprising that dysregulation of CFTR is implicated in many cancers. CFTR overexpression has been correlated with cancer in individual studies, in particular, gastric and ovarian cancers[101,188-192]. However, in the vast majority of studies CFTR deficiency is associated with cancer occurrence, and in most of these studies, cancers with *CFTR* mutations or down-regulation of expression in tumors are more likely to exhibit rapid expansion, EMT, decreased apoptosis, increased metabolic potential, and increased patient morbidity and mortality. Decreased CFTR expression and/or inactivating mutations are associated with non-small cell lung cancer[130,175,193], glioblastoma[194], bladder[131-133], EC[195,196], PC[197,198], nasopharyngeal[199], prostate[176], and breast[177] cancers. In most of these cancers CFTR deficiency was associated with increased tumor stage and poor survival. Germline *CFTR* mutations, as seen in CF, have been linked to increased risk of PC among younger patients[197,198]. Germline mutations may be an important risk factor in the general population as an estimated 3% of the United States population are heterozygote carriers for deleterious CF-causing *CFTR* mutations[200] and therefore potentially at risk due to loss of heterozygosity or haploinsufficiency. However, in most studies CFTR deficiency was associated with differentially decreased expression but not specifically with germline defects. Although germline mutations were not examined in these cases it is unlikely that decreased expression was caused primarily by CF-causing germline mutations because F508del, which makes up approximately 70% of CF alleles, causes a 90% decrease in protein levels but only a modest decrease in mRNA. Known causes of decreased CFTR expression include hypermethylation as seen in lung and bladder cancer studies[130-133] and somatic mutations seen in a NSCLC study[130]. In addition, cigarette smoke (CS) has recently been shown to down-regulate CFTR expression and CFTR down-regulation has been linked to the etiology of COPD[201]. However, a recent study also linked CS-mediated down-regulation of CFTR to exacerbation of oncogenic phenotypes in the A549 lung cancer cell line[202].

CFTR therapies and translational and clinical cancer implications

In the era of precision medicine new CF modulator therapies targeted to specific *CFTR* mutations have entered the clinic. These include potentiator drugs that increase anion flow through CFTR channels already present on the PM and corrector drugs that promote correct folding of mutant proteins. Three modulators, ivacaftor,

tezacaftor, and lumacaftor, have been approved for treatment of CF[203]. Ivacaftor is a potentiator approved for treatment of > 25 CF-causing mutations including gating, residual function and splice mutations. Tezacaftor and lumacaftor are correctors designed to improve the function of the F508delta mutation which makes up approximately 70% of CF alleles. As discussed by Bodewes *et al*[204] these drugs are finally targeting CF-related GI diseases, with potential use in cancer therapy. Some examples include treatment of pancreatitis with Ivacaftor[204], drug treatment of CF patients that improved proximal small intestine pH as a regulator of bicarbonate secretion, improvement in cell motility and clinical outcomes in patients with *CFTR* G551D mutations[104], improvement in bicarbonate permeability following Lumacaftor-rescued F508del mutations[205], improvement in gut microbiota and intestinal inflammation following treatment with Ivacaftor, including an increase in *Akkermansia* and a decrease in *Enterobacteriaceae*, and a significant reduction in inflammation in patients treated with Ivacaftor[206]. Additional modulators and combinations of modulators are under development such as the combination of Ivacaftor with 5-Nitro-2-(3-Phenylpropylamino) Benzoate, that is reported to act synergistically in activation of G551D mutant *CFTR*[207]. These mutation-specific drugs are potentially applicable to the 3% of Caucasians who are CF carriers, having one CF-causing *CFTR* mutation. These individuals are potentially at risk for developing CRC due to haploinsufficiency or loss of heterozygosity. In addition to these Food and Drug Administration (FDA)-approved therapies, another category of modulator known as amplifiers, represented by PTI-428, is in clinical trials. This drug is designed to enhance translation of CFTR mRNA to increase protein production and facilitate the activity of corrector drugs[99]. Thus, this drug has the potential to improve CFTR activity in sporadic CFTR-low CRC. In addition, testing strategies used for screening and diagnosis of CF may be applicable to early detection and even prevention of CRC. Currently genetic testing for CF-causing mutations is recommended for all pregnant couples. If carrier status proves to be a risk factor for CRC, then this genetic testing may be adaptable for early detection of CRC as well. A significant technical advance to test new CF therapies has been the development of patient-derived colorectal organoids. An example is the work of Dekkers *et al*[208] who have demonstrated proof of this idea in rectal organoids derived from CF patients to test a range of CF drugs, including Ivacaftor and Lumacaftor. While these new targeted CF therapies have yet to enter the cancer clinic, they may soon do so, along with the repurposing of other ion channel activators and blockers as more becomes known about the precise contribution of ion channels to cancer development.

Ca^{2+} activated Cl^- channels

Ca^{2+} activated Cl^- channels (CLCAs) are a family of secreted self-cleaving proteins that activate Ca^{2+}-dependent Cl^- channels. CLCAs are involved in Cl^- conductance across epithelial cells and therefore influence epithelial fluid secretion, cell-cell adhesion, apoptosis, cell cycle control, tumorigenesis and metastasis, mucus production and cell signaling[209,210]. There are four CLCAs (1-4) in humans and two of these family members (CLCA1,2) are implicated in GI cancers, almost invariably as tumor suppressors whose down-regulation is associated with cancer progression and poor patient prognosis. CLCA1 is down-regulated in CRC and this is associated with poor patient prognosis[4,211,212]. CLCA1 has also been reported to suppress CRC aggressiveness *via* inhibition of the Wnt/β-catenin signaling pathway[213]. CLCA1 is also reported to be a prognostic biomarker for PC, with lower CLCA1 expression correlated with shorter disease-free survival[214]. CLCA2 is also down-regulated in CRC[4,212].

Cl^- intracellular channels

Cl^- intracellular channels (CLICs) are novel, auto-inserting, self-assembling intracellular anion channels involved in a wide variety of fundamental cellular events including regulated secretion, cell adhesion, cell cycle and apoptosis. However, the actions of CLICs remain to be fully explained. They are a class of intracellular anion channels that do not resemble classical ion channel proteins. CLICs can exist as both soluble globular proteins and integral membrane proteins with ion channel function. Human CLIC1 adopts at least two stable soluble structures, with redox status controlling the transition between them. CLIC proteins are characterized by the presence of a 240 residue CLIC module whose structure belongs to the glutathione S-transferase fold superfamily[215]. Three CLICs appear to be functional in humans (CLIC1,3,4) with two CLIC proteins (CLIC1 and CLIC4) that appear to be essential molecular components of anion channels, with CLIC1 capable of forming anion channels in planar lipid bilayers in the absence of other cellular proteins. However, these putative ion channel proteins are controversial because they exist in both soluble and membrane forms, with at least one transmembrane domain[209,215]. All three

CLICs are involved in GI cancers, with all implicated as oncogenes. CLIC1 is over-expressed in CRC[216-219]; in PC[65,217], where it was reported to be upregulated in 69% of tumors and associated with poor patient prognosis; and in GC[65,218,220-223], where it was found upregulated in 68% of tumors, correlated with lymph node metastasis, lymphatic invasion, perineural invasion and poor patient prognosis. It was also reported to promote GC progression by regulating ROS-mediated MAPK/AKT signaling. CLIC1 was also reported to be upregulated in HCC[65,224] and gall bladder cancer[225,226]. CLIC3 is upregulated in PC where it was reported to promote integrin recycling from late endosomes to drive PC progression[65,227]. CLIC3 is also a secreted protein that is reported to drive cancer progression through its glutathione-dependent oxireductase activity. In particular CLIC3 was identified as part of the cancer associated-fibroblast secretome where it promotes the invasive behavior of endothelial cells to promote angiogenesis and invasion. CLIC3 is also secreted by cancer cells[228], and CLIC3 is described as a pH sensor, important as changes in cellular pH influence cell proliferation and the balance between cell survival and cell death[229]. CLIC4 is upregulated in CRC where it was found to be a direct response gene for C-MYC and TP53, with its overexpression associated with poor 5-year patient survival[218,230]. CLIC4 is also upregulated in PC with its expression associated with tumor grade, invasion and poor patient survival[231]. CLIC4 was also found to be expressed in mitochondria where it regulates pH and cell volume.

ANO1

ANO1 is also referred to as Anoctamin-1, DOG1 and TMEM16A. ANO1 is a Ca^{2+}-activated Cl^- channel that mediates receptor-activated Cl^- currents that are involved in a range of physiological processes. ANO1 is activated by intracellular Ca^{2+}. ANO1 has 8 putative transmembrane domains and demonstrates no similarity to other ion channels. It is expressed in a variety of secretory epithelia, including the gut. ANO1 activity is implicated in regulating CFTR Cl^- channel activity. In all of the GI cancers studied, ANO1 expression and activity has been reported to upregulated, thus ANO1 can be described as an oncogene in the GI tract. In CRC, ANO1 expression is upregulated, associated with EMT and poor patient prognosis[232-236]. In PC, ANO1 expression is important for cell migration[234,237]. In EC, ANO1 is a biomarker of EC progression[234,238]. In GC, its expression is upregulated[234,239], and in GI stromal tumors, ANO1 expression is used as a diagnostic biomarker, and it is associated with negative regulation of IGFBP5[240-242]. See Table 2 for a full listing of chloride ion channels in GI cancers.

CALCIUM CHANNELS

Ca^+ is a ubiquitous second messenger, serving as a signaling molecule for a variety of cellular processes such as control of the cell cycle, apoptosis, and migration. Ca^+ concentrations are tightly regulated within the cell, with basal cytoplasmic levels being many orders of magnitude less than in the extracellular space. This control is essential for cellular homeostasis[253]. In addition to extracellular Ca^{2+}, the endoplasmic reticulum (ER) and mitochondria are also significant stores of Ca^{2+}. Selective distribution and activation of Ca^{2+} channels at any of these sources allows for Ca^{2+} micro-domains and adds another level of specificity to Ca^{2+} signaling[6]. Because it is involved in such a broad array of processes, and signaling molecules are often sensitive to very minute changes in Ca^{2+}, it is easy to see how perturbations in Ca^{2+} handling could lead to significant physiological outcomes.

Ca^{2+} signaling is typically initiated through the ligand activation of various receptors that activate phospholipase C[254]. This leads to the production of inositol triphosphate, which diffuses to the ER, where it binds to and opens its receptor and Ca^{2+} channel. Upon its release into the intracellular space, Ca^{2+} binds to calmodulin and a variety of other Ca^{2+}-activated proteins to elicit a wide variety of responses. Over time, these intracellular stores would be depleted if not for being replenished by extracellular Ca^{2+}. The release and depletion of ER Ca^{2+} triggers the process of store-operated calcium entry (SOCE), in which PM Ca^{2+} channels are opened in order to allow entry of extracellular Ca^{2+}[254]. One of the proteins responsible for sensing the depletion of ER stores, and initiating SOCE, is stromal interaction protein 1 (STIM1)[254]. In CRC, STIM1 over-expression has been associated with increased tumor size, depth of invasion, lymph node metastasis, and increased serum levels of carcinoembryonic antigen[15,255,256]. When injected into immunocompromised mice, CRC cells expressing a higher level of STIM1 had a much higher incidence of lung and liver metastasis than those expressing lower levels[257].

The PM Ca^{2+} channels opened by STIM1 activation include ORAI1 as well as

Table 2 Chloride channels

Gene name (s)	Cancer	Role	Functional activity
CLCA1/Chloride channel accessory 1	Colorectal	Tumor suppressor	Expression down-regulated; down regulated in primary tumors and CRC cells; acts via inhibition of Wnt/β-catenin signaling; there is one report that high expression associated with non-response to chemo radiation therapy in rectal cancer[4,211-213]
	Pancreatic	Tumor suppressor	Expression down-regulated; low expression associated with poor patient prognosis[214]
CLCA2/Chloride channel accessory 2	Colorectal	Tumor suppressor	Expression down-regulated[4,212]
CLIC1/Chloride intracellular channel 1	Colorectal	Oncogene	Expression up-regulated; overexpressed by MS analysis of human CRCs; expressed on nuclear and plasma membranes[216-219]
	Pancreatic	Oncogene	Expression up-regulated; over expression associated with poor patient prognosis, tumor grade and size; overexpression in 69% of tumors; knockdown of PC cells reduced cell proliferation and anchorage-independent growth on soft agar, and cell migration[65,217-219]
	Gastric	Oncogene	Expression up-regulated; overexpression associated with poor patient prognosis; upregulated in 68% of gastric cancer, correlates with lymph node metastasis, lymphatic invasion, perineural invasion and pathological staging; induced proliferation, apoptosis, invasion and migration of GC cells in culture; promotes progression by regulating MAPK/AKT pathway; regulates migration and invasion via ROS-mediated P38 MAPK pathway[65,220-223]
	Hepatocellular	Oncogene	Expression up-regulated[65,224]
	Gall bladder	Oncogene	Expression up-regulated; knockdown in GBC cells reduced proliferation, migration and invasion of cells; associated with metastasis, based on proteomic analysis[284-286]
CLIC3/Chloride intracellular channel 3	Pancreatic	Oncogene	Expression up-regulated; CLIC3 and Rab25 collaborate to promote integrin recycling from late endosomes/lysosomes to drive PaC progression[65,227]
CLIC4/Chloride intracellular 4	Colorectal	Oncogene	Expression up-regulated; associated with poor 5-yr patient survival; CLIC4 regulated by TP53 and TNF-alpha and is a direct response gene for C-MYC and TP53[218,230]
	Pancreatic	Oncogene	Expression up-regulated; associated with tumor grade, lymph node metastasis, tumor invasion and poor patient survival; expressed in mitochondria and regulates pH and cell volume[231]

CFTR/Cystic fibrosis transmembrane conductance regulator	Colorectal	Tumor suppressor	Expression down-regulated; CF patients at significant risk of early aggressive colorectal tumors based on colonoscopy screening and other clinical findings; CFTR down-regulated in sporadic CRC, associated with worse prognosis; CFTR was a CIS gene in four sleeping beauty transposon mutagenesis screens in mice, both CRC and GC; > 60% conditional CFTR KO mice develop intestinal tumors and crossing to *ApcMin* model causes an enhanced tumor phenotype and the development of adenocarcinomas; enhanced organoid outgrowth; CFTR deficiency causes an invasive phenotype in CRC cells; loss of CFTR causes upregulation of NF-κB; CFTR modulates Wnt/β-catenin signaling and stem cell proliferation; enhanced risk of CRC in CF patients following lung trans-plants[33-34,36,93,103,119,123-126,128,243-247]
	Pancreatic	Tumor suppressor	Expression down-regulated; increased risk of PC in carriers of 4 specific CFTR mutations[101,118,122,123,197,198,248-250]
	Small intestine	Tumor suppressor	Expression down-regulated[122-123,243]
	Gastric	Oncogene	Expression up-regulated in late stage[101,191,192]
	Esophageal	Tumor suppressor	Expression down-regulated; silencing of CFTR caused upregulation of NFKB; CFTR inhibitors caused enhanced growth of EC cell mouse xenografts; enhanced risk of EC in CF patients following lung transplants; CFTR heterozygous carriers at enhanced risk of EC[195,196,243,251]
	Hepatocellular	Tumor suppressor	Expression down-regulated by promoter hypermethylation[89,252]
ANO1/anoctamin1/TMEM16A/DOG1	Colorectal	Oncogene	Expression up-regulated; negatively regulated by miR-144 miR-9, and miR-132; associated with EMT and poor patient prognosis[232-236]
	Pancreatic	Oncogene	Expression up-regulated; important for cell migration[234,237]
	Esophageal	Oncogene	Expression up-regulated; biomarker for EC progression[234,238]
	Gastric	Oncogene	Expression up-regulated[234,239]
	GI stromal (GIST)	Oncogene	Expression up-regulated; used as a diagnostic biomarker; associated with negative regulation of IGFBP5[240-242]

CRC: Colorectal cancer; PC: Pancreatic cancer; GC: Gastric cancer; GBC: Gall bladder cancer; EMT: Epithelial to mesenchymal transition; EC: Esophageal cancer; GIST: Gastrointestinal stromal tumors; CF: Cystic fibrosis.

members of the TRP superfamily of cation channels. In addition to replenishing intracellular stores, these PM channels also contribute to Ca^{2+} signaling in response to intracellular or extracellular cues. Different members of the TRP superfamily are activated by physical changes such as mechanical stress, osmotic pressure, or temperature; or chemical changes such as pH, growth factors/cytokines, pO2, or ROS[258]. With all of these being central factors in a tumor microenvironment, how a cell alters its response to these conditions could ultimately influence the death or survival of a potentially cancerous cell.

Transient receptor potential family

Various members of the transient receptor potential (TRP) superfamily have been implicated in GI cancers, in particular the TRPM (m = melastatin) and TRPC (c = canonical) sub-families. The cold-activated TRPM8, well known for its role in androgen-dependent prostate carcinoma, is also over-expressed and necessary for the

proliferation and migration of PC cells[259-262]. TRPM7 also plays a role in PC, but by increasing motility and thus the potential for metastasis, with its depletion causing a decrease in invasiveness through the HSP90α/uPA/MMP-2 proteolytic axis and targeted senescence, while results vary as to its role in proliferation[5,258-261,263-267]. TRPM7 has also been implicated as an oncogene in CRC[268] and GC[269-271]. Though a specific mechanism has not been proposed, TRPM2 over-expression has also been shown to reduce PC as well as GC patient survival by increasing invasiveness and proliferation[272,273]. Expression of TRPC1 is reported to be upregulated in CRC[5,256,274], where it promotes metastasis, EC[275], and GC[68]. TRPC6 expression is upregulated in EC[276,277] where it was found to be necessary for Ca^{2+} increase to promote G2 progression, and association with tumor stage and poor patient prognosis, GC[5,278], and HCC[4].

ORAI1

Through its contribution to hyperactivity of intracellular Ca^{2+} oscillations, ORAI1-high cells had increased activation of ERK, AKT, and myocyte enhancer factor 2D, indicating a possible mechanism explaining their increased proliferative and invasive phenotype[275]. ORAI1 expression is also upregulated in CRC[5], where it is activated by STIM1; PC[92,279], where it mediates SOCE and promotes apoptotic resistance in PC cells; GC[68], where it is associated with promoting metastasis; and in EC, where ORAI1 over-expression had a negative effect on patient prognosis.

Other oncogenic Ca^{2+} channels include SIGMAR1 in CRC[280-282], L-TYPE CACNA1C in CRC[283], T-type CACNA1I in GC[284], where its upregulation is associated with poor patient survival, and T-type CACNA1H in GC[284], where it is also associated with poor patient survival.

Tumor suppressor Ca^{2+} channels

While most Ca^{2+} channels appear to function in an oncogenic fashion when dysregulated there are several exceptions. TRPM6 has been reported to be down-regulated in CRC (16 of 20 tumors studied) with high expression associated with better patient survival[285]. STIM2 is reported to be down--regulated in CRC leading to apoptosis resistance[15,256,274]. Expression of the T-type channel CACNA1G is reported to be down-regulated by promoter hypermethylation in CRC[286], PAC[287], and GC[288], with high expression associated with improved overall survival. CACNA2D3 expression is down-regulated by promoter hypermethylation, associated with worse patient prognosis[91,289] and expression of CACNB2 is also reported to be down-regulated by promoter hypermethylation[90]. See Table 3 for a full listing of the role of calcium ion channels in GI cancers.

SODIUM CHANNELS

Voltage-gated sodium channels (VGSCs) are classically associated with the initiation and conduction of action potentials in electrically excitable cells such as neurons and muscle cells. They are typically heteromeric complexes composed of one of 9 pore-forming α-subunits and one of 5 regulatory β-subunits, though the α-subunit can usually be a functional channel by itself. These channels have recently been discovered in non-excitable cell types such as glia, fibroblasts, immune cells, and cancer cells, where their function is less understood[290]. In the developing central nervous system, VGSCs regulate the migration and pathfinding of neurite outgrowth[291-293]. Similarly, in the vast majority of cancers where these channels have been studied, their major influence has been to increase the motility and invasiveness of cancer cells.

The VGSC $Na_V1.5$ is abundantly expressed in human CRC cells SW620, SW480, and HT29, as well as being highly expressed in tumor biopsies relative to adjacent normal tissue[294]. Through pharmacological inhibition, siRNA knockdown, and controlling for Ca^{2+} influence, House *et al*[294] demonstrated that conductance through $Na_V1.5$ contributes significantly to CRC cell invasiveness and cancer progression. This group later demonstrated this contribution mechanistically through correlating $Na_V1.5$ activity to RAP-1 mediated MAPK activation and changes in gene expression through the PKA/ERK/c-Jun/ELK-1/ETS-1 signaling pathway[295]. While this group demonstrated the metastatic contribution of $Na_V1.5$, Peng *et al*[296] have recently shown $Na_v1.5$ expression to be predictive of clinical outcomes in non-metastatic CRC, with the potential involvement of estrogen receptor (ER)-β, implicating multiple roles for the same channel in different stages of cancer progression.

In GC, high expression of the VGSC $Na_v1.7$ has similarly been shown to correlate with higher recurrence and reduced survival[297]. The detailed mechanism proposed by Xia *et al*[297] involves increased expression of the oncoprotein metastasis-associated in

Table 3 Calcium channels

Gene name (s)	Cancer	Role	Functional activity
TRPC1	Colorectal	Oncogene	Expression up-regulated; promotes metastasis[5,256,274]
	Esophageal	Oncogene	Expression up-regulated[275]
	Gastric	Oncogene	Expression up-regulated[276]
	Hepatocellular	Oncogene	Expression up-regulated[249]
TRPC6	Esophageal	Oncogene	Expression up-regulated; necessary for Ca^{2+} increase to promote G2 progression; associated with tumor stage and poor prognosis[276,277]
	Gastric	Oncogene	Expression up-regulated[5,278]
	Hepatocellular	Oncogene	Expression up-regulated[4]
TRPM2	Colorectal	Oncogene	Expression up-regulated[270]
	Pancreatic	Oncogene	Expression up-regulated; enhanced proliferation, invasion & metastasis[272,273]
	Gastric	Oncogene	Expression up-regulated; inhibition reduced proliferation of gastric cancer cells, increased autophagy and sensitized cells to paxlitaxel and doxorubicin[68,272,273]
TRPM6	Colorectal	Tumor suppressor	Expression down-regulated in 16/20 (80%) of primary tumors; high expression associated with better patient survival[285]
TRPM7	Colorectal	Not determined	Genetic polymorphism associated with enhanced risk of adenomas, linked to high Ca^{2+}:Mg^{2+} ratio in diet[268]
	Pancreatic	Oncogene	Expression up-regulated; increased tumor growth, invasiveness and metastasis; targeted silencing induced replicative senescence[5,258-261,263-267]
	Gastric	Oncogene	Highly expressed in gastric cancer cell lines; required for GC survival linked to Mg; suppression induces cell death in culture[4,269-271]
TRPM8	Pancreatic	Oncogene	Expression up-regulated; regulates proliferation and migration; silencing in cell lines induces replicative senescence [259-262]
L-type/a1c subunit/*CACNA1C*	Colorectal	Oncogene	Expression up-regulated[283]
Sig1R/SIGMAR1	Colorectal	Oncogene	Expression up-regulated in CRC cell lines and primary CRC tumors[280-282]
Stim1/Stromal interaction protein 1	Colorectal	Oncogene	Expression up-regulated; increased CRC cell motility; STIM1 overexpression enhanced lung and liver metastases in mouse xenograft models; also associated with poor prognosis in CRC patients[15,255,256]
	Pancreatic	Oncogene	Expression up-regulated; promotes invasion and metastasis; STIM1 and Orai1 are the molecular components of SOCE[14]
Stim2/Stromal interaction protein 2	Colorectal	Tumor suppressor	Expression down-regulated; depletion causes apoptosis resistance[15,256,274]
Orai1/CRAMC1	Colorectal	Oncogene	Expression up-regulated; activated by STIM1[5]
	Pancreatic	Oncogene	Expression up-regulated; mediate SOCE and promote apoptotic resistance in pancreatic cancer cells[91,279]
	Esophageal	Oncogene	Expression up-regulated; promotes tumor-promoting Ca^{2+} oscillations in EC[275]

	Gastric	Oncogene	Expression up-regulated; promotes metastasis[68]
T-type *CACNA1G*/CaV3.1	Colorectal	Tumor suppressor	expression down-regulated by promoter hypermethylation[284,286]
	Pancreatic	Tumor suppressor	Expression down-regulated by promoter hypermethylation[284,287]
	Gastric	Tumor suppressor	Expression down-regulated by promoter hypermethylation; high expression associated with improved overall survival[284,288]
T-type *CACNA1I*/CaV3.3	Gastric	Oncogene	High expression associated with poor survival[284]
T-type *CACNA1H*/CaV3.2	Gastric	Oncogene	High expression associated with poor survival[284]
CACNA2D3	Gastric	Tumor suppressor	Expression down-regulated by promoter hypermethylation, associated with worse prognosis[91,289]
CACNB2	Esophageal	Tumor suppressor	Expression down-regulated by promoter hypermethylation[90]

GC: Gastric cancer; CRC: Colorectal cancer; EC: Esophageal cancer; SOCE: Store-operated calcium entry; STIM1: Stromal interaction protein 1.

colon cancer-1 (MACC1) by $Na_v1.7$ activation in a p38/NFkB-dependent manner. Increased MACC1 expression subsequently leads to HGF/c-Met/c-Jun-dependent activation of another oncoprotein, the Na^+/H^+ exchanger-1 (NHE1) which, through its involvement in maintaining intracellular and extracellular pH, has been shown elevated in a variety of cancers[298-301]. Reports involving other Na_v channels in GI cancer are fewer than for $Na_v1.5$ and $Na_v1.7$. $Na_v1.1$ has been reported to be upregulated in CRC[302], associated with a shorter time to cancer recurrence in stage II and III disease. In contrast, there is one report of $Na_v1.6$ being down-regulated in CRC[303].

The mechanisms through which VGSC α and β subunits contribute to cancer progression typically differs according to their physiological functions: α subunits, through the conduction of Na^+ currents, and β subunits through the regulation of adhesion interactions, though contributions of any β subunit have yet to be shown in a GI cancer. The reason that only α subunits have been associated with GI cancers may owe in part to these differences in function relative to the demands of GI epithelial physiology. This specificity may also prove advantageous in pharmacologically targeting these subsets of cancers, as drugs targeting α subunit function have proven effective in reducing metastasis in murine xenograft breast cancer models[304,305]. See Table 4 for a full listing of sodium ion channels in GI cancer.

ZINC TRANSPORTERS

Zinc is a key trace element in the human body. Approximately 10% of human genes have the potential for zinc binding and a large number of human proteins (as many as 3000), including transcription factors, a variety of receptors, kinases, ligases and other enzymes, require zinc for their catalytic activity or tertiary structure[306,307]. Zinc exists in cells as Zn^{2+} ions that are maintained in this valence state under all biologically relevant redox potentials and pH conditions in a cell. Zinc mediates a wide range of cellular processes that are important for maintenance of tissue homeostasis[306,308,309]. Therefore, alterations in cellular zinc levels, such as zinc deficiency, can disrupt cellular function[306,308]. Furthermore, zinc is toxic to cells, thus zinc levels are tightly regulated within cells. As zinc cannot freely pass across cellular membranes a variety of Zn^{2+}-permeable channels and transporters regulate Zn^{2+} entry and exit from cells[306]. Zinc transporters include both influx and efflux transporters[306,310,311]. Zinc also enters cells *via* other ion channels, such as Zn^{2+} activation of several of the Zn^{2+}-permeable voltage-gated Ca^{2+} ion channels[309]. Examples include the store-operated Ca^{2+} entry (SOCE) channels and the TRP family of Ca^{2+} channels (discussed earlier in this review).

Here, the review will focus on the functions of the two major families of Zn^{2+} transporters: ZnT/SLC30A and ZIP/SLC39A. Members of both of these families of proteins are reported to be dysregulated in human cancers, including GI cancers. There are 14 ZIP proteins (ZIP1-ZIP14) and 10 ZnT proteins (ZnT1-ZnT10) in the

Table 4 Sodium channels

Gene name (s)	Cancer	Role	Functional activity
SCN1A/Nav1.1	Colorectal	Oncogene	Time to reoccurrence of stage II and III CRC is shorter in patients carrying Nav1.1 variants[302]
SCN5A/Nav1.5	Colorectal	Oncogene	Expression up-regulated; mediates invasion via MAPK signaling; key regulator of a transcriptional network that includes Wnt/β-catenin signaling; associated with poor patient prognosis; linked to upregulation of ER-β[5,294-296]
SCN8A/Nav1.6	Colorectal	Tumor suppressor	Expression down-regulated in CRC tumor tissues compared with control[303]
SCN9A/Nav1.7	Gastric	Oncogene	Expression up-regulated; mechanistically related to upregulation of MACC1 and NHE1[297]

CRC: Colorectal cancer.

human body with differential tissue-specific expression[310,311].

ZIPs

These proteins are encoded by the solute carrier family 39A genes (SLC39A1-SLC39A14). ZIPs mainly participate in the uptake of Zn into the cytoplasm from the extracellular space or from intracellular compartments such as the ER, Golgi or mitochondria. In the GI tract ZIPs 1, 3, 4, 5, 6, 7, 8, 9, 10, 11 and 14 are reported to be normally expressed in the intestines; ZIPs 1, 3, 4, 5, 6, 7, 8, 9, 10, and 14 in the pancreas; ZIPs 1, 2, 3, 5, 6, 7, 8, 9, and 14 in the liver; and ZIPs 1, 3, 4, 5, 6, 7, 8, 9 10, and 11 in stomach[310,312,313].

ZnTs

These proteins are encoded by the solute carrier family 30A genes (SLC30A1-SLC30A10). ZnTs participate in the efflux of Zn from the cytoplasm into the extracellular space or to sequester Zn in intracellular compartments. In the GI tract, ZnT1 expression is normally expressed ubiquitously; ZnTs 2, 3, 5, 6, 7 and 10 are normally expressed in the intestines; ZnTs 2, 3, 5, 6, 7 and 8 in pancreas; and ZnT 5, 6, 7 and 10 in liver and ZnT 6, and 7 in stomach[310,312,313].

Zinc and zinc transporters in the GI tract

It is well known that dysregulation of zinc homeostasis results in GI dysfunction[311,312,314,315]. Examples include zinc deficiency resulting in diarrhea[311], and inflammatory bowel disease[316], clinical problems that are ameliorated by zinc supplementation[311,316,317]. This effect of zinc supplementation may be mediated by a zinc sensing receptor molecule, GPR39, that is similarly shown to be protective in the colon[318]. Zinc is also reported to enhance tight junctions and intestinal mucosal barrier functions in general[319,320]. Maintenance of zinc homeostasis is primarily mediated by zinc transporters that act to absorb diet-derived zinc for distribution to the peripheral tissues[321]. But excess zinc is toxic to both intestinal epithelial cells[308,315] and also peripheral tissue cells, thus zinc transporters maintain cytosolic zinc levels *via* both influx and efflux of zinc ions[306]. The physiological role of zinc and zinc transporters have been best studied in the intestinal tract, where zinc is required for intestinal epithelial homeostasis, a process mediated by several zinc transporters. In intestinal absorptive enterocytes, ZIP4 and ZnT1 regulate zinc absorption, while ZIP5 and ZnT5B regulate zinc excretion. In intestinal Paneth cells, ZnT2 and ZIP4 are required for zinc accumulation in Paneth cell granules and are important for Paneth cell maintenance overall. Of potential importance to intestinal cancer, ZIP4 and ZIP7 are expressed in the intestinal crypt stem cell compartment where they contribute to Lgr5+ stem cell maintenance and help maintain transit amplifying cell proliferation[312].

Zinc transporters and GI cancer

The role of zinc transporters in human cancer have been best studied in prostate and breast cancer[308,322]. In the prostate gland ZIPs, especially ZIP1, are considered tumor

suppressors[323]. In breast cancer, increased levels of zinc correspond to upregulation of several ZIPs (and a few ZnTs as well), thus zinc transporters as a group are considered as oncogenes in breast cancer[324]. In the GI tract, the majority of reports show upregulation of both ZIPs and ZnTs in all major GI organ cancers (Table 5), although for some cancers there is clearly conflicting evidence. The strongest evidence for dysregulation of zinc transporters in GI tract cancers has been for PC, where zinc transporter upregulation has been associated with enhanced cancer cell migration and worse patient prognosis. This is especially true for ZIP4. In one study increased mRNA expression of ZIP4 was observed in 16 of 17 pancreatic adenocarcinoma samples[325], a finding that was supported in ZIP4-expressing xenografts in mice that yielded larger tumors[326]. These findings were confirmed by several other groups[327-329], such as Xu *et al*[329] who found that ZIP4 was upregulated in a set of 23 PCs and that ZIP4 expression could be used as a diagnostic and prognostic marker. While the case of ZIP4 as an oncogene is very clear, for other zinc transporters the data is sometimes contradictory, with some reports of upregulation and others of down-regulation of non-ZIP4 zinc transporters[313,330]. Studies employing zinc-sensitive histochemical staining have reported a general reduction in zinc levels in PC samples[331,332], a report that is difficult to reconcile with upregulation of zinc transporters, although it has also been noted that increases in zinc transporter mRNAs do not always correspond to increased protein expression and activity as many zinc transporters, including ZIP4, are regulated by posttranslational mechanisms[322]. Further, some studies have shown down-regulation of virtually all zinc transporters in PC, with the exception of ZIP4[313]. Another case for zinc transporters acting as an oncogene or tumor suppressor is in EC, where an inherited genetic variant in the 5′ untranslated region of ZIP6 results in constitutive expression of ZIP6, enhancing the malignancy of ESCC cells[333]. In ESCC cell lines upregulation of ZIP6 enhances proliferation, migration, and invasion of cancer cells, while down-regulation of ZIP6 suppresses these effects[333]. Similarly, Jin *et al*[334] reported that knockdown of ZIP5 significantly inhibited human ESCC cell progression and Kumar *et al*[335] reported upregulation of ZIP7 in ESCC. In contrast, other studies have reported that zinc deficiency contributes to progression of EC. Choi *et al*[336] reported that zinc supplementation inhibited proliferation of ESCC cell lines, a process mediated by inhibition of Orai-mediated SOCE and subsequent Ca^{2+} oscillations. For other GI cancers such as CRC and GC, there is less evidence but those reports point to an oncogenic role for zinc transporters[337-339], despite zinc's known protective effects against colonic inflammation.

Mechanisms in cancer cells

The mechanisms underlying the effects of dysregulation of zinc homeostasis and zinc transporter expression in cancer are unclear, and are almost certainly tissue and cancer specific as seen in the evidence that zinc transporters appear to clearly act as tumor suppressors in the prostate gland but as oncogenes in breast cancer. Less is known in GI tract cancers, and that data is controversial (see conflicting PC and EC studies above) but some potential mechanisms of action are listed below.

Zinc signaling within cells: Changes in intracellular concentration can lead to it acting as a second messenger for external signals, including activation of mitogen-activated protein kinase (MAPK), extra-cellular signal-related kinase (ERK) and the c-Jun N-terminal kinase (JNK) pathways that can act to phosphorylate proteins involved in regulating cell proliferation, differentiation and apoptosis[306,322]. This has been best studied in breast cancer where ZIP-mediated signaling pathways promote cell proliferation and metastasis in luminal and tamoxifen-resistant breast cancer cells[322]. Overall, zinc is recognized as an important signaling molecule in normal cell functions as well as pathologies such as cancer. How zinc signaling is conveyed from zinc transporters to downstream signaling pathways is still largely unclear.

Regulation of the intestinal stem cell compartment: In normal tissue several zinc transporters are involved in maintenance of Lgr5+ stem cells and Paneth cells, which themselves are important for stem cell maintenance[312]. Further evidence for this mechanism is the role of ZIP7 in resolving ER stress in the intestinal stem cell compartment[340]. Mice lacking ZIP7 in intestinal epithelium triggered ER stress that led to loss of intestinal stem cells and proliferative progenitor cells.

Requirement for zinc in rapidly proliferating cells: Zinc is required for protein structure, catalytic activity, such as for zinc finger transcription factors.

Action of increased cellular zinc on other ion channels: TRP Ca^{2+} and K^{+} channels are sensitive to zinc activation[308].

Table 5 Zinc transporters

Gene name	Cancer	Role	Functional activity
ZnT1/SLC30A1	Pancreatic	Tumor suppressor	Decreased mRNA expression in tumors[313]
ZnT2/SLC30A2	Pancreatic	Tumor suppressor	Decreased mRNA expression in tumors[313]
ZnT3/SLC30A3	Pancreatic	Tumor suppressor	Decreased mRNA expression in tumors[313]
ZnT4/SLC30A4	Pancreatic	Tumor suppressor	Decreased mRNA expression in tumors[313]
ZnT5/SLC30A5	Colorectal	Oncogene	Increased mRNA expression in tumors[337]
ZnT6/SLC30A6	Colorectal	Oncogene	Increased mRNA expression in tumors[337]
ZnT7/SLC30A7	Esophageal colorectal	Oncogene oncogene	Increased mRNA expression in tumors[308,335] Increased mRNA expression in tumors[337]
ZnT8/SLC30A8	Pancreatic	Tumor suppressor	Decreased mRNA expression in tumors[313]
ZnT9/SLC30A9	Pancreatic	Tumor suppressor	Decreased mRNA expression in tumors[313]
ZIP1/SLC39A1	Gastric	Oncogene	Increased mRNA expression in tumors, worse patient prognosis[338]
	Pancreatic	Tumor suppressor	Down regulated mRNA expression in tumors[313]
ZIP2/SLC39A2	Gastric	Oncogene	Increased mRNA expression in tumors, worse patient prognosis[338]
	Pancreatic	Tumor suppressor	mRNA expression down-regulated in tumors[313]
ZIP3/SLC39A3	Pancreatic	Tumor suppressor	Decreased expression in adenocarcinoma[308,313,331,343]
		Oncogene	Medium to high mRNA expression in multiple human PC cell lines[313]
ZIP4/SLC39A4	Hepatocellular	Oncogene	Increased mRNA and protein expression, repressed apoptosis, enhanced cell cycle and migration[308,325,344-346]
	Pancreatic	Oncogene	Increased expression in PDAC and PC cell lines, link to CREB-miR-373 axis, promotes cancer xenograft growth in nude mice[313,325-327]
	Gastric	Oncogene	Increased mRNA expression in tumors, worse patient prognosis[338]
ZIP5/SLC39A5	Esophageal	Oncogene	Increased expression in ESCC, knockdown in cell lines inhibited migration and invasion[308,334]
	Gastric	Oncogene	Increased mRNA expression in tumors, worse patient prognosis[338]
	Pancreatic	Tumor suppressor	Decreased mRNA expression in tumors[313]
ZIP6/LIV-1/SLC39A6	Pancreatic	Oncogene	Increased expression in tumors and cell lines[313,330]
		Tumor suppressor	Decreased mRNA expression in tumors[313]
	Hepatocellular	Oncogene	Increased mRNA and protein expression[308,347]
	Esophageal	Oncogene	Increased expression in ESCC[308,333]
	Colorectal	Oncogene	Increased mRNA expression in tumors[337]
	Gastric	Oncogene	Increased mRNA expression in tumors, worse patient prognosis[338]
ZIP7/SLC39A7	Pancreatic	Tumor suppressor	Decreased mRNA expression in tumors[313]
		Oncogene	Medium to high mRNA expression in multiple human cell lines[313]

	Colorectal	Oncogene	Increased mRNA expression in tumors and CRC cell lines, knockdown inhibits cell growth and induces apoptosis in cell lines[339]
	Gastric	Undetermined	Increased mRNA expression, but better patient prognosis[338]
ZIP8/SLC39A8	Gastric	Oncogene	Increased mRNA expression in tumors, worse patient prognosis[338]
	Pancreatic	Tumor suppressor	Decreased mRNA expression in tumors[313]
		Oncogene	Medium to high mRNA expression in multiple human cell lines[313]
ZIP9/SLC39A9	Colorectal	Oncogene	Increased mRNA expression in tumors[337]
	Gastric	Oncogene	Increased mRNA expression in tumors, worse patient prognosis[338]
	Pancreatic	Tumor suppressor	Decreased mRNA expression in tumors[313]
		Oncogene	Medium to high mRNA expression in multiple human cell lines[313]
ZIP10/SLC39A10	Colorectal	Oncogene	Increased mRNA expression in tumors[337]
	Pancreatic	Tumor suppressor	Decreased mRNA expression in tumors[313]
		Oncogene	Medium to high mRNA expression in multiple human cell lines[313]
ZIP11/SLC39A11	Colorectal	Oncogene	Increased mRNA expression in tumors[337]
	Gastric	Undetermined	Increased mRNA expression in tumors, better patient prognosis[338]
	Pancreatic	Tumor suppressor	Decreased mRNA expression in tumors[313]
		Oncogene	Medium to high mRNA expression in multiple human cell lines[313]
ZIP12/SLC39A12	Gastric	Oncogene	Increased mRNA expression in tumors, worse patient prognosis[338]
	Pancreatic	Tumor suppressor	Decreased mRNA expression in tumors[313]
ZIP13/SLC39A13	Gastric	Oncogene	Increased mRNA expression in tumors, worse patient prognosis[338]
	Pancreatic	Tumor suppressor	Decreased mRNA expression in tumors[313]
		Oncogene	Medium to high mRNA expression in multiple human cell lines[313]
ZIP14/SLC39A14	Hepatocellular	Tumor suppressor	Decreased expression in hepatoma tissues[308,348]
	Gastric	Undetermined	Increased mRNA expression in tumors, but better patient prognosis[338]
		Tumor suppressor	Decreased mRNA expression in tumors[313]
	Pancreatic	Oncogene	Medium to high mRNA expression in multiple human cell lines[313]

PDAC: Pancreatic ductal adenocarcinoma; ESCC: Esophageal squamous cell carcinoma; CRC: Colorectal cancer; PC: Pancreatic cancer.

Zinc deficiency: Zinc deficiency leading to disruption of intestinal epithelial barrier function and enhanced colonic inflammation as zinc supplementation has been shown to improve inflammatory bowel disease-related phenotypes in animal models.

Therapeutic opportunities

In contrast with other ion channels discussed in this review drugs targeting various zinc transporters have been slow in development. Myers[341] and Bin *et al*[342] discuss some of the challenges to drug development represented by zinc transporter structure. One of these, ZnT8, which is especially associated with type-2 diabetes is the subject of current drug development research. Potentially, knowledge gained from

targeting ZnT8 could lead to targeting of other transporters such as ZIP4 in PC. One therapeutic area that is immediately available is zinc supplementation, also discussed by Myers and Bin *et al*[342]. For conditions, including cancers, that demonstrate zinc deficiency, zinc supplementation may have immediate therapeutic value. This has been demonstrated for inflammatory bowel disease and may have utility for zinc-deficient cancers. See Table 5 for a full listing of zinc transporters and their role in GI cancers.

CONCLUSION

Ion channels play an essential role in the GI tract, mediating a range of cellular and tissue processes. They are also commonly dysregulated in major GI malignancies such as CRC, HCC, PC, GC and EC. In these cancers, ion channels modulate many of the well-known hallmarks of cancer, with increasing evidence that ion channels are important for the regulation of tissue and cancer stem cells. The influence of ion channels on cancer cell processes has led to cancer being described as a channelopathy. For a summary of mechanisms of selected ion channels in GI cancer see Figure 3. Notably, ion channels represent potentially important clinical targets for several reasons.

First, ion channels and transporters are predominantly found in the PM of the lumens of GI tract organs thus the majority of ion channels should be accessible to therapeutic drugs.

Second, the structures of all of the major families of ion channels found in the GI tract are known and their functions have been well-characterized, primarily due to studies prompted by dysregulation of these ion channels outside the GI tract, *e.g.*, CFTR in CF lung pathology, thus they should be readily amenable to new drug design and preclinical and clinical testing.

Third, drugs are currently used to target several ion channels for disorders outside the GI tract *e.g.*, retigabine for KCNQ-deficiency and many other examples, thus these drugs can be repurposed for clinical use in the GI tract. Current examples of drug repurposing in the GI tract include CFTR potentiators, activators, correctors, and amplifiers such as for patients with specific CFTR mutations. These include Ivacaftor (potentiator for > 25 CF-causing mutations, including G551D), and Tezacaftor and Lumacaftor (correctors for patients with F508delta mutations which account for aaproximately 70% of CF patients). These drugs have been shown to be effective in ameliorating lung pathology in CF patients and are now being used to treat several GI pathologies in CF patients. For example, Ivacaftor is being used to treat pancreatitis, and intestinal inflammation (including producing an improvement in gut microbiota) and Lumacaftor has been shown to improve intestinal bicarbonate permeability. As CF patients are highly susceptible to the development of CRC, these drugs may be readily repurposed for prevention and treatment of CRC, likely in combination with other standard therapy. Data generated by the repurposing efforts underway for non-cancer therapy will provide support for the next step into the cancer clinic.

Fourth, further research into the mechanisms of action of various ion channels, including the rapidly growing utility of bioinformatics analysis, can lead to greater drug repurposing strategies. For example, through a bioinformatics approach, tricyclic antidepressants were recently and rapidly repurposed by the FDA for use in treatment of small cell lung carcinoma[349]. In other cases, research into mechanisms of drug action reveals ion channels as novel mediators. For example, it has long been known that daily aspirin is protective against CRC, but only recently has it been determined that the mechanism of this effect is likely through the remodeling of the SOCE Ca^{2+} channel[256]. Further understanding of the canonical and non-canonical roles that ion channels play in cancer development can lead to further repurposing of drugs to treat specific GI cancers[350]. This is especially true as precision cancer medicine embraces combinatorial treatment modalities.

Fifth, ion channels and transporters can provide novel cancer biomarkers with diagnostic and prognostic implications, *e.g.*, KCNQ1 in later stage CRC, where patients who maintain high expression of KCNQ1 show better disease free survival at stage II and III CRC[38], and a 23-month survival advantage for stage IV CRC patients following hepatic resection[37]; CFTR in CF-related CRC[122-126]; hERG1 in several GI cancers[59-74]. and zinc transporters such as ZIP4 in PC[325-329]. For example in CRC, hERG1 is not expressed in normal colon mucosa but is upregulated in adenocarcinoma, with its highest expression in CRC metastasis. It is noted that stereoselective hERG1 channel blockers for treatment of cardiac arrhythmias have been used in the clinic for several decades.

Sixth, there is research data linking several ion channels to regulation of the stem

Chloride channels

CFTR-tumor suppressor
Promotes H_2O and ion homeostasis in GI epithelia[107,108].
Deficiency is associated with dysregulation of Wnt/β-catenin and inflammatory signaling[92,93,96,128,161-175,178-180].

CLCA1-tumor suppressor
Expression is associated with decreased nuclear β-catenin and decreased expression of EMT markers[211-213].
Loss is associated with oncogenic phenotypes including increased tumorigenesis and metastasis *in vivo*[214].

Potassium channels

KCNQ1- tumor suppressor
Provides electrical driving force for export of Cl-[23,24].
Sequesters β-catenin at adherens junctions[45].
Loss inhibits activity of CFTR and allows release of β-catenin to the cytosol[44].

KCNH2/hERG1-oncogene
Interaction with integrin promotes PI3Kinase-AKT-HIF1-VEGFa signaling[72].
Upregulation is associated with increased angiogenesis[71-74].

Zinc transporters

SLC39A7-oncogene
Member of the SLC39A/ZIP family[310].
This family and the SLC30A/ZnT family control uptake and efflux of Zn^{2+} to maintain balance between activity of Zn dependent enzymes and toxicity[310,312].
In GI cancers upregulation of these transporters is associated with cancer[324-329,333-339].
SLC39A7 supports intestinal epithelial stem cells by limiting ER stress[340].

Sodium channels

SCCN5/Na_v1.5- oncogene.
Member of the VGSC family that promotes migration in normal cells and invasiveness in GI cancers[291-294].
Na_v1.5 promotes PKA-ERK-c-Jun-ELK-1-ETS-1 signaling leading to transcription of EMT and invasion genes[295].

Calcium channels

ORAI1 and STIM1- oncogenes
Required for maintenance of calcium homeostasis *via* SOCE[254].
Upregulation promotes changes in intracellular distribution of Ca^{2+}, and dysregulation of Ca^{2+} mediated signaling leading to proliferation, migration and invasion[255-257].

TRPM7-oncogene
Member of TRP family that regulates local intracellular concentrations of Ca^{2+} and modulate Ca^{2+} dependent signaling[258].
Upregulation of TRPM7 promotes an invasive Hsp90α-uPA-MMP-2 pathway[264].

Figure 3 Oncogenic mechanisms of selected ion channels. Because ion channels influence the basic biochemical environment of the cell as well as complex interactions with other proteins, they have profound and pleiotropic effects on cell function. As a result, it is often difficult to determine specific mechanisms for oncogenic phenotypes. However, progress has been made in defining mechanisms in some cases. This figure shows examples from each category of channels with accompanying pathways linking dysregulation of channel function to tumorigenesis. For additional information and references please see text and Tables 1-5. GI: Gastrointestinal; EMT: Epithelial to mesenchymal transition; TRP: Transient receptor potential; SOCE: Store-operated calcium entry; VGSC: Voltage-gated sodium channels; STIM1: Stromal interaction protein 1.

cell compartment in GI tract organs. This is especially true for the intestinal tract. For example, both CFTR[93,112] and KCNQ1 (R.Cormier and P.Scott, unpublished studies) are expressed in the stem cell compartment of the colon, where they have been shown to influence stem call capacity in mouse organoids models[37,128], as well as regulating the expression of stem cell related genes, again in transgenic mouse models[37,128]. As the intestinal stem cell is thought to be the precursor for the intestinal cancer stem cell, better understanding of the underlying mechanisms of how ion channels such as CFTR and KCNQ1 may regulate stem cell function will be very important. A key tool in this research and a technical advance in therapeutic development has been the creation of patient-derived tissue and cancer organoid surrogate models[136], *e.g.*, that have been used to test the function of CFTR[137,138], as well as the efficacy of CFTR modulator drugs in CF patient-derived rectal organoids[208]. The same strategy of biobanking of patient-derived organoids is currently used to test patient-specific CRC treatment protocols that can include ion channel modulator drugs.

Seventh, as patient genomic sequencing efforts increase, more has become known about specific germline genomic variants in ion channel genes and potential susceptibility to GI tract cancers. For example, CF patients who are homozygous for CFTR mutations are at a significantly heightened risk for developing early onset CRC. What is the risk for CRC for heterozygous carriers of CFTR mutations, a group that represents more than 10 million individuals in the United States alone? CRC patients whose cancers show reduced expression of CFTR demonstrate worse CRC disease prognosis[128]. Current ongoing studies of CRC risk in CFTR carriers may help inform on the lifetime risk of CRC in these patients, potentially leading to earlier screening and chemoprevention.

A final comment here, as discussed by Humphries *et al*[351], is that while there exists great promise in targeting ion channels in human diseases there remains significant challenges, especially related to target specificity and off-target toxicity, before development of ion channel targeting can lead to more widespread effective personalized cancer treatment.

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MINIREVIEWS

Targeted therapies in metastatic gastric cancer: Current knowledge and future perspectives

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Abstract

Gastric cancer (GC) represents a leading cause of cancer related morbidity and mortality worldwide accounting for more than 1 million of newly diagnosed cases and thousands of deaths every year. In the last decade, the development of targeted therapies and the optimization of already available chemotherapeutic drugs has expanded the available treatment options for advanced GC and granted better survival expectations to the patients. At the same time, global efforts have been undertaken to investigate in detail the genomic and epigenomic heterogeneity of this disease, resulting in the identification of new specific and sensitive predictive and prognostic biomarkers and in innovative molecular classifications based on gene expression profiling. Nonetheless, several randomized studies aimed at exploring new innovative agents, such as immune checkpoint inhibitors, failed to demonstrate clinically meaningful survival advantages. Therefore, it is essential to further improve the molecular characterization of GC subgroups in order to provide researchers and medical oncologists with new tools for patients' selection and stratification in future clinical development programs and subsequent trials. The aim of the present manuscript is to provide a global overview of the recent molecular classifications from The Cancer Genome Atlas and the Asian Cancer Research Group and to present key promising developments in the field of immunotherapy and targeted therapies in metastatic GC.

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Core tip: Gastric cancer (GC) still represents a leading cause of cancer related morbidity and mortality worldwide accounting for more than 1 million of newly diagnosed cases and thousands of deaths every year. In the last decade, global efforts have been undertaken to investigate in detail the genomic and epigenomic heterogeneity of this disease, resulting in innovative molecular classifications of GC based on gene expression profiling and in the identification of new specific and sensitive predictive and prognostic biomarkers. At the same time, the development of targeted therapies has expanded the treatment scenario for advanced GC. The aim of the present manuscript is to provide a detailed and comprehensive overview of the recent molecular classifications from The Cancer Genome Atlas and the Asian Cancer Research Group and to present key promising developments in the field of immunotherapy and targeted therapies in metastatic GC.

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INTRODUCTION

Gastric Cancer (GC) is the third leading cause of cancer death and the fifth most common malignancy worldwide for both sex, accounting for over 1 million new diagnoses and almost 800000 patients deaths in 2018[1]. Over 70% of GCs occur in low/middle income countries with the highest rates in Eastern Asia, Eastern Europe and South America and the lowest rates in North America and Western European countries[2,3].

Since the middle of the 20th century a progressive decline of distal GC incidence has been observed, possibly due to recognition and eradication of certain risk factors such as *Helicobacter pylori* (*H. pylori*), the introduction of refrigerators and an increased use of fresh food[4,5]. Conversely, the rate of gastroesophageal junction cancer (GEJC) is increasing in Western countries, which probably reflects the increase of gastroesophageal reflux disease and visceral obesity[6,7]. Considering the rising of worldwide population, the absolute number of new diagnoses per year is increasing, so that GC still remains an important cause of cancer-related mortality and a main global health-care problem.

Surgery is the only potentially curative treatment while neoadjuvant and adjuvant therapies should be integrated with surgery in locally advanced disease. Despite these multimodal treatments the 5 years overall survival is less than 30%, and in metastatic setting the prognosis remains poor with a median overall survival (OS) of 1 year[8].

The last decade has been characterized by a better understanding of molecular mechanisms of pathogenesis and biology of GC with the definition of new genomic classifications and identification of prognostic and predictive biomarkers potentially predictive of response to innovative target agents. Despite this, up to now, few target-directed options have been approved for metastatic GC. The anti-human epidermal growth factor receptor-2 (HER2) drug trastuzumab has been the first target agent approved for HER2 high expressing advanced GCs and GEJCs, while the antiangiogenic drug ramucirumab has received approval in the second-line setting as a monotherapy or in combination with paclitaxel. More recently, anti-PD1 agents such as nivolumab and pembrolizumab have been approved for patient with heavily pretreated advanced GC in some Asian countries and North America, respectively.

More than 90 percent of GCs are adenocarcinomas. According to Lauren's criteria, gastric adenocarcinomas are divided into intestinal (54%), diffuse (32%), and indeterminate type (15%), which present distinct epidemiology, histological appearance, cell differentiation and molecular pathogenesis[9,10]. Intestinal carcinomas are more likely to be sporadic than inherited and causally related to *H. pylori* infection

and the Correa's phenotypic multistep cascade (*i.e.*, longstanding gastritis, atrophic gastritis, dysplasia and adenocarcinoma)[11]. Histologically, diffuse-type adenocarcinomas are poorly differentiated and composed by discohesive cells usually characterized by with a dysregulation in the expression of E-cadherin, a key cell surface and connection protein[12]. Both Lauren classification and the World Health Organization (WHO) 2010 classification[13], although widely used in the clinical practice, remain insufficient to guide precision treatments for the individual patient and GC histotype is not a parameter considered in the treatment decision process.

GC MOLECULAR CLASSIFICATION

The Cancer Genome Atlas research group

In 2014, the The Cancer Genome Atlas (TCGA) network proposed a comprehensive molecular analyses of 295 primary GC based on array-based somatic copy number analyses, whole exome and genome sequencing, messenger RNA and microRNA sequencing, and reverse-phase protein array profiling. As a result, four GC subgroups were identified: Epstein-Barr (EBV) positive tumors, tumors with microsatellite instability (MSI), genomically stable tumours (GS) and tumors with chromosomal instability (CIN)[14].

EBV activation was found in about 9% of tumor samples. All EBV positive tumours were associated to extreme DNA hypermethylation with high levels of CIMP (*i.e.*, CpG island methylation) of *CDKN2A (p16 NK4A)* promoter but not of *MutL homolog 1* (*MLH1*), characteristic of MSI associated CIMP. As previously reported, phoshatidylinositol-4-5-bisphosphate 3-kinase catalytic subunit alpha (*PIK3CA*) mutations were detected in 80% of EBV-positive GC[15,16]. Moreover, the finding of an overexpression of programmed death ligands 1 and 2 (PD-L1 and PD-L2) and of a significant immune cell presence supported the rationale to evaluate checkpoint inhibitors in this GC subgroup.

The MSI group (22%) was characterized by loss of mismatch repair functions which lead to alterations in length of repetitive regions in DNA known as microsatellites. The hypermethylation of *MLH1* promoter region was the most representative mismatch repair defect in patient with MSI sporadic GCs. Alterations of *PIK3CA*, *ERBB3* and *ERB22* were found and in contrast to MSI colorectal cancers, *BRAF* mutations have never been described in MSI-GCs. MSI GCs can be part of the spectrum of inherited malignancies such as Lynch syndrome and nonpolyposis colorectal cancer syndrome which are associated to inherited germline mismatch repair defects[17]. Although colorectal and endometrial cancers are the most common cancer associated to these syndromes, other extracolic tumours including GC, can occur[18]. MSI GCs are mainly associated with intestinal histotype, are localized in the antrum, with less frequent lymph-node involvement, occur mainly in elderly age and have a more favourable prognosis[19,20].

GS tumors (20%) are characterized by low copy number alterations and a low mutation rate. *ARID1*, *RHOA* and *CDH1* mutations are the principal somatic genomic alterations observed in this class. An interchromosomal translocation between *CLDN18* and *ARHGAP26*, mutually exclusive from RHOA mutations, was found in the 15% of GS GCs. These tumors usually occur in younger patients (median age 59), and are correlated with diffuse histology and distal localization. GS subtype was associated with the worst OS and prognosis among the four TCGA subtypes.

The fourth TCGA group are GCs with CIN (50%) characterized by DNA aneuploidy, highly variable chromosomal copy numbers, and mutations of the tumor suppressor *TP53*, which is responsible for chromosomal instability. Frequent genomic amplifications of receptor tyrosine kinases (RTKs)/RAS pathway were found, including *epidermal growth factor receptor* (*EGFR*), *ERBB2*, *ERBB3*, MET proto-oncogene (*MET*), *fibroblast growth factor receptor 2* (*FGFR2*), *vascular endothelial growth factor A* (*VEGFA*), and *KRAS*. Most of CIN GCs are classified as intestinal-type, frequently located at the gastroephageal junction/cardia[21,22].

The Asian Cancer Research Group

In 2015 the Asian Cancer Research Group (ACRG) proposed a different molecular classification based on gene expression profiling, genome-wide copy number microarrays and targeted gene sequencing of 300 primary tumors with the definition of four molecular subtypes: Microsatellite unstable type, epithelial to mesenchymal-like type (MSS/EMT), MSS/TP53 and MSS/TP53 negative subtypes[23]. Each of these molecular subtypes was correlated to distinct clinical phenotypes and genomic alterations.

MSI GCs occurred preferentially in the antrum, with intestinal histology (more than

60%) and half of them were diagnosed at an early stage disease. MSI tumors were characterized by loss of expression of *MLH1* and an elevated DNA methylation signature. The MSI subtype was associated with the presence of hypermutation, with mutations of *ARID1A* (44.2%), the *PI3K-PTEN-mTOR* pathway (42%), *KRAS* (23.3%) and *ALK* (16.3%).

The MSS/EMT subtype was observed at significantly younger age, with diffuse histology at stage III/IV and showed *CDH1* loss of expression. The EMT subtype presented a lower number of mutation events when compared to the other MSS groups. The MSS/EMT had the worst prognosis, while the MSI subtype showed the best prognosis of the four. The authors observed that the MSS/EMT group presented a higher percentage of recurrence *vs* the MSI group (63% *vs* 23%). The MSS/EMT GC subtype was associated to a higher frequency of peritoneal metastases compared to all other subtypes, while a higher percentage of liver-limited metastasis in the MSI and MSS/TP53 subtypes was found.

MSS/TP53 positive and MSS/TP53 negative showed an intermediate prognosis and also an intermediate chance of recurrence. EBV infection was more frequently associated to MSS/TP53 positive group. MSS/TP53 negative subtype exhibited the highest prevalence of *TP53* mutations (60%) and a low frequency of other mutations, as well as recurrent focal amplification of *ERBB2*, *EGFR*, *CCNE1*, *CCND1*, *MDM2*, *ROBO2*, *GATA6* whereas the MSS/TP53 positive subtype showed a relative higher (compared to MSS/TP53 negative) of mutations in *APC*, *ARID1A*, *KRAS*, *PIK3CA* and *SMAD4*.

Comparison between the TCGA and ACRG classification

Comparisons between TCGA and ACRG genomic subtypes showed similarities and differences (Figure 1). Both molecular classifications revealed MSI positive tumors and TCGA GS, EBV and CIN subtypes are likely to be approximated to ACRG MSS/EMT, MSS/TP53 positive and MSS/TP53 negative subtypes, respectively. Tumors classified as the GS subtype in the TCGA set were present across all ACRG subtypes in the ACRG data set, while tumors classified as the TCGA CIN subtype were present across all ACRG subtypes in the TCGA data set. A substantially lower percentage of Lauren's diffuse subtype cases were found in the TCGA cohort compared to ACRG database (24% *vs* 45% respectively) with the majority (57%) of Lauren's diffuse-sub-type cases present in the TCGA GS group but only 27% cases present in the ACRG MSS/EMT subtype. Additionally, *CDH1* and *RHOA* mutations, which were mutated in TCGA GS, were infrequent in the ACRG MSS/EMT subtypes. These differences suggest that TCGA GS type is not equivalent to the ACRG MSS/EMT subtype.

Collectively, these findings confirm that the TCGA and ACRG classification systems are related but distinct in terms of demographics, molecular mechanisms, driver genes and prognosis. Although these novel classifications have provided a deeper understanding of GC biology, some limitations can be observed. First, these analyses are based on complex molecular technologies and could not be replied in standard laboratories. Furthermore, a prospective validation on large scale including patients of different age and ethnicity is needed. Finally TCGA and ACRG classifications are the result of comprehensive molecular analysis on malignant GC epithelial cells that don't consider the impact of peritumoral stromal cells. Of note, novel stromal gene signatures have been analyzed in comparison to the dominant cancer phenotypes identified by TCGA and ACRG, revealing distinct stromal phenotypes[24,25].

CURRENT STANDARD TREATMENTS IN METASTATIC GC

Chemotherapy remains the backbone of therapy in patients with metastatic GC and good performance status. Available data from randomized clinical trials showed a statistically significant benefit of palliative chemotherapy, compared with best supportive care (BSC), in terms of symptom control, improvement of quality of life (QoL) and overall survival (OS).

In the first line setting a variety of cytotoxic drugs, including platinum compounds, fluoropyrimidines, anthracyclines, taxanes, and irinotecan, have shown activity in GC. Currently, a combination including a platinum compound (cisplatin or oxaliplatin) plus a fluoropyrimidine (5-FU,capecitabine, or S-1) agent is one of the most widely used doublet on the basis of a balanced benefit-to risk ratio[26].

Currently, trastuzumab is the only molecularly targeted drug accepted in first-line therapy, in combination with cisplatin and a fluoropyrimidine, for the treatment of patients with metastatic HER2-overexpressing GC or GEJC who have not received

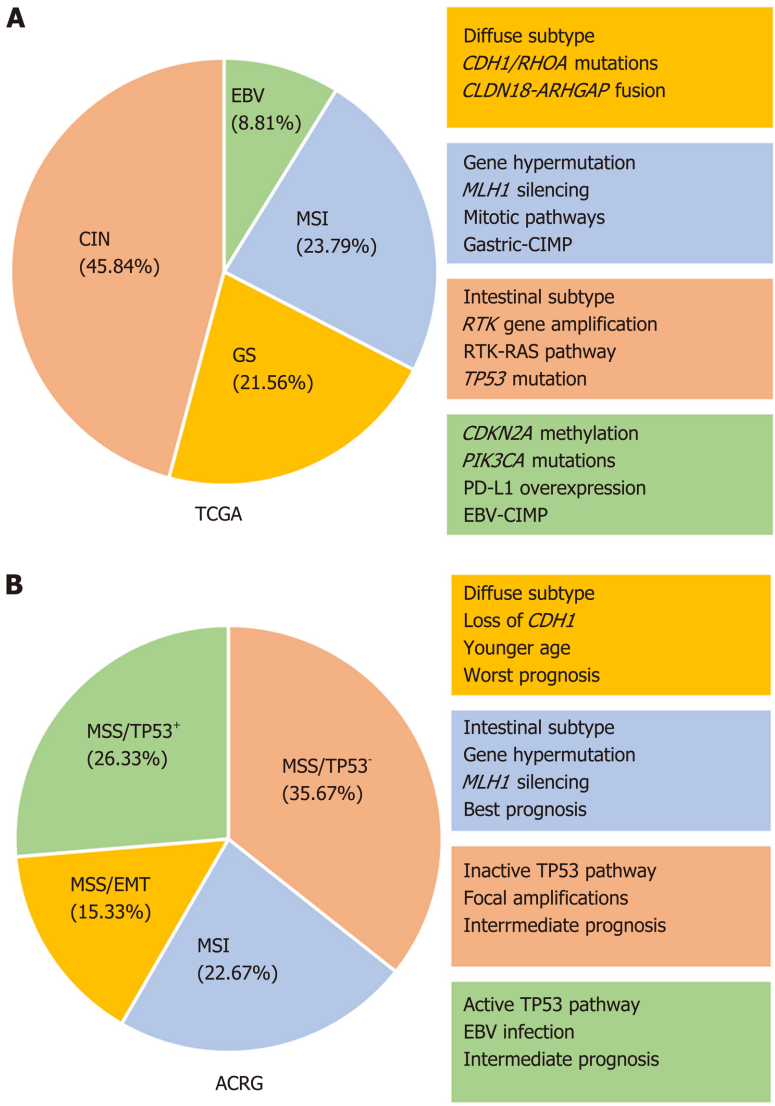

Figure 1 The cancer genome atlas and the Asian cancer research group molecular classification of gastric cancer. EBV: Epstein-Barr; CIN: Chromosomal instability; MSI: Microsatellite instability; GS: Genomically stable tumours; MSS/EMT: Microsatellite unstable type, epithelial to mesenchymal-like type.

anti-cancer treatment for their metastatic disease. As a result, all cases of advanced GC should be characterized for HER2 status. HER2 is a member of the epidermal growth factor receptors (EGFRs) family and is involved in transmembrane signaling, and overexpression/activation leads to increased cell proliferation, growth and survival[27]. HER2 overexpression or/and amplification is found in approximately 20% of metastatic GC, although there are differences depending on tumor subtype, is more common in intestinal GC than diffuse GC, and more common in GEJC than distal GC[28].

The phase III, open-label, randomised controlled ToGA trial (Trastuzumab for GC) compared in a population of 594 previously untreated patients standard chemotherapy (six courses of cisplatin plus either infusional fluorouracil or capecitabine) with and without trastuzumab until disease progression. All end points were improved with the addition of trastuzumab to chemotherapy, including objective response rate (ORR) (47.3% *vs* 34.5%), PFS (6.7 *vs* 5.5 mo; HR: 0.71; 95%IC: 0.59-0.85; $P < 0.0002$), and at a median follow-up of 17 to 19 mo, median OS was significantly better with trastuzumab (13.8 *vs* 11.1 mo) (HR: 0.74; 95%CI: 0.60-0.91; $P = 0.0046$). The inclusion criteria of the ToGA trial were a 3+ HER2 immunohistochemical (IHC) overexpression or the presence of *HER2* gene amplification as assessed by fluorescent in situ hybridization (FISH), irrespective of IHC score[29].

Despite the demonstrated efficacy of numerous chemotherapy options, only 40% of patients who progressed to first-line chemotherapy are susceptible to a second-line chemotherapy on progression[30]. In this setting, ramucirumab, a fully human monoclonal antibody VEGFR-2 antagonist, is the only molecular-targeted drug with a

confirmed, although modest, survival benefit.

The activity of ramucirumab, in second-line treatment of GC was investigated by the phase III REGARD trial (Ramucirumab monotherapy for previously treated advanced gastric or gastro-oesophageal junction adenocarcinoma), a randomized, double-blind, placebo-controlled study. In the REGARD trial 355 patients with previously treated advanced GC or GEJC adenocarcinomas were randomized to best supportive care plus either ramucirumab or placebo. Median OS was 5.2 mo in the ramucirumab arm and 3.8 mo in the placebo arm (HR: 0.78; 95%CI: 0.603-0.998; P = 0.047)[31]. However, the RAINBOW trial (Ramucirumab plus paclitaxel *vs* placebo plus paclitaxel in patients with previously treated advanced gastric or gastro-oesophageal junction adenocarcinoma) was the landmark study that demonstrated the benefit of ramucirumab in second line setting in combination with chemotherapy, which compared weekly paclitaxel (80 mg/m^2 on days 1, 8, and 15 of each 28-day cycle) plus ramucirumab (8 mg/kg IV every two wk) or placebo in 665 patients. Median OS and PFS were significantly longer in patients treated with ramicurumab than in the placebo-plus-paclitaxel group (median OS: 9.6 *vs* 7.4 mo, HR: 0.81, 95%CI: 0.678-0.962, P = 0.017 and median PFS 4.4 *vs* 2.9 mo, HR: 0.635, 95%CI: 0.539-0.752, $P \leq 0.001$, respectively)[32].

Largely based on this trial results, ramucirumab plus paclitaxel is currently the preferred choice for second-line therapy. More recently, the phase III TAGS study (Trifluridine/tipiracil *vs* placebo in patients with heavily pretreated metastatic GC) proved that trifluridine/tipiracil is an effective treatment option for patients with heavily pretreated metastatic GC. The study demonstrated a 31% reduction in risk of death and a 2.1-month improvement in median OS in treated patients[33].

HER2: PRIMARY AND ACQUIRED RESISTANCE

The anti-HER2 monoclonal antibody trastuzumab plus standard chemotherapy have significantly improved response rate and survival outcomes in primary GC and GEJC displaying HER2 overexpression/amplification. Unfortunately, about 50% of patients did not respond to the combination treatment suggesting the existence of a primary resistance[29]. At same time, acquired resistance usually limits the duration of response to this treatment.

Genomic alterations of the RTK pathway such as *EGFR*, *FGFR2*, *MET*, and *KRAS* amplification may be responsible for primary resistance to HER2-targeting drugs[14]. Recently, amplifications of cell-cycle–related genes such as *CCNE1* and *CDK6*, *PI3K* mutations, and amplification of *MET* have shown to confer resistance to anti-HER2 agents in vitro *HER2*–amplified cell-line models[34]. Although uncommon, other rare alterations in RTK pathways such as *ALK*, *ROS1*, *NTRK1/2/3* and *RET* fusion could be correlated with primary resistance to trastuzumab[35-37]. To confirm these data, a recent study investigated a panel of genomic alterations including mutations in the *EGFR / MET / KRAS / PI3K / PTEN* genes and amplifications in *EGFR / MET / KRAS* in 37 patients treated with trastuzumab (17 responders and 20 patients with primary resistance). Interestingly, panel alterations were significantly more frequent in resistant (11 of 20, 55%) as compared with sensitive patients and in HER2 IHC 2+ than 3+ tumors. Patients with no alteration had a significantly longer median PFS and OS[38].

Mechanisms of acquired resistance to anti-HER2 treatment in GC are largely unknown. Pietrantonio *et al*[39] have analyzed 22 matched tumor samples taken at baseline and post-progression in patients receiving chemotherapy and trastuzumab for advanced HER2 IHC 3+ or 2+ GC. Loss of HER2 was identified as a mechanism of resistance in 32% of samples and the probability of loss of HER2 positivity was significantly higher in patients with baseline IHC score 2+ *vs* 3+. Similarly, loss of HER2 and frequent secondary alterations in the RTK/RAS/PI3K pathway in HER2 positive adenocarcinoma have been observed in patients treated with trastuzumab[40].

In a recent study evaluating capecitabine and oxaliplatin as first line neoadjuvant therapy in patients with previously untreated, HER2-positive GC, the analysis of circulating free DNA (cfDNA) at disease progression demonstrated the emergences of genomic aberrations such as *MYC*, *EGFR*, *FGFR2* and *MET* amplifications[41]. However, none of these biomarkers is evaluated in the current clinical practice.

Other anti-HER2 strategies have failed to demonstrate a clinical benefit in second line HER2 positive GC. In the GATSBY trial (Trastuzumab emtansine *vs* taxane use for previously treated HER2-positive locally advanced or metastatic gastric or gastro-oesophageal junction adenocarcinoma) the antibody-drug conjugate consisting of the monoclonal antibody Trastuzumab linked to microtubule inhibitor emtansine (T-DM1) compared to taxans, failed to prolong survival in patients with previously

treated HER2-positive advanced GC[42]. In the phase III randomized TyTAN trial (Lapatinib plus paclitaxel *vs* paclitaxel alone in the second-line treatment of HER2-amplified advanced GC in Asian populations) comparing the efficacy and safety of the tyrosine kinase inhibitor of EGFR and HER2 Lapatinib plus paclitaxel with paclitaxel alone, the combination treatment demonstrated activity in the second-line but did not significantly improve OS in the intent-to-treat population (ITT)[43]. Moreover, in a recent trial comparing weekly paclitaxel alone with weekly paclitaxel plus Trastuzumab in patients with HER2-positive advanced GC/GEJC progressing during Trastuzumab-containing therapy, Trastuzumab beyond progression strategy did not improve PFS[44].

Results from ongoing phase III (NCT01774786) and phase II (NCT01522768) clinical trials of Pertuzumab and Afatinib respectively, will hopefully contribute to the unmet need in this setting of patients whose therapeutic options still remain limited (Table 1).

RTK/RAS - TARGET THERAPIES

CIN tumors are frequently characterized by the presence of activation of the RTK/RAS pathway and *EGFR*, *HER2*, *HER3*, *JAK2*, *MET*, *FGFR2*, *PIK3CA* and *KRAS/NRAS* amplification[14]. Other works have reported that at least one third of GC patients present alterations of the RTK/RAS pathway and may be potentially treatable by directed therapies[45]. Unfortunately, most of phase II and III trials evaluating RTK/RAS target therapies failed to demonstrate activity in metastatic GC.

The *EGFR* gene is amplified in the 5% and EGFR is overexpressed in more than 30% of GC[14,46]. Both anti-EGFR drug cetuximab and panitumumab have been tested in two phase III trial. In the EXPAND trial (Capecitabine and cisplatin with or without cetuximab for patients with previously untreated advanced GC), the addition of the chimeric monoclonal antibody cetuximab to capecitabine-cisplatin provided no additional benefit in terms of PFS to chemotherapy alone in the first-line treatment of advanced GC (HR: 1.09; 95%CI: 0.92-1.29; P = 0.32)[47]. The REAL 3 trial (Epirubicin, oxaliplatin, and capecitabine with or without panitumumab for patients with previously untreated advanced oesophagogastric cancer), with 553 patients randomized to receive epirubicin, oxaliplatin, and capecitabine (EOC) plus the human monoclonal antibody panitumumab or EOC alone, failed to show a benefit in OS of the combination therapy compared with the only chemotherapy (HR: 1.37; 95%CI: 1.07-1.76; P = 0.013)[48]. However, none of these studies have selected patients on the basis of *EGFR* overexpression/amplification. In metastatic colorectal cancer, *RAS* mutations are a negative predictive biomarkers of response to anti-EGFR therapy but can be detected only in about 3% of GC and GEJC.

Several works have reported that EGFR expression, *EGFR* gene copy number, or expression of other EGFR ligands such as epiregulin and amphiregulin, might be potential markers for efficacy of anti-EGFR target therapies[49-51]. However, in the EXPAND trial, no substantial differences between the treatment groups for PFS or OS according to EGFR immunohistochemistry score was noted[47]. Results from a phase III trial comparing the efficacy of nimotuzumab, a recombinant humanized anti-EGFR antibody, and irinotecan on irinotecan alone in patients with EGFR overexpressed advanced GC/GEJC are expected (ENRICH study, NCT01813253, Table 1).

The tyrosine kinase receptor c-MET and its own ligand, hepatocyte growth factor (HGF), have been investigated as potential target in advanced GC. In GC, alteration of the MET/HGF pathway is related to a more aggressive disease and poor prognosis, with MET activation stimulating tumor invasiveness[52,53]. Onartuzumab, a monovalent monoclonal antibody binding with the extracellular of MET, has been tested in a phase III trial of 562 patients randomized to receive onartuzumab plus FOLFOX6 *vs* placebo plus mFOLFOX6 in patients with metastatic HER2-negative and MET-positive GEC. However, the addition of onartuzumab to mFOLFOX6 did not attain significant differences in OS or PFS compared with placebo plus mFOLFOX6 in ITT (OS HR: 0.82; 95%CI: 0.59-1.15; P = 0.24; PFS HR: 0.90; 95%CI: 0.71-1.16; P = 0.43) or MET 2+/3+ populations (OS HR: 0.64; 95%CI: 0.40-1.03; P = 0.06; PFS HR: 0.79; 95%CI: 0.54-1.15; P = 0.22)[54]. The RILOMET phase III trial (Rilotumumab plus epirubicin, cisplatin, and capecitabine as first-line therapy in advanced MET-positive gastric or gastro-oesophageal junction cancer), evaluating the fully human monoclonal antibody anti-MET Rilotumumab plus epirubicin, cisplatin, and capecitabine or placebo plus epirubicin, cisplatin, and capecitabine as first line in advanced GC, was ceased subsequently the finding by an independent data monitoring committee of a higher number of deaths in the rilotumumab group compared with the placebo group[55].

Table 1 Ongoing phase II/III target trials in advanced gastric cancer

Study	Line	Control arm	Experimental arm	Target	NCT number
JACOB	1st	Placebo + Trastuzumab + Chemotherapy	Pertuzumab + Trastuzumab + Chemotherapy	HER2	NCT01774786
ID NUMBER:11-166	2nd	-	Afatinib + Paclitaxel	HER2	NCT01522768
NIEGA	2nd	-	Irinotecan + Nimotuzumab	EGFR	NCT03400592
ENRICH	2nd	Irinotecan	Irinotecan + Nimotuzumab	EGFR	NCT01813253
CheckMate-649	1st	Oxaliplatin + Fluoropyrimidine	- Nivolumab + Oxaliplatin + Fluoropyrimidine - Ipilimumab + Nivolumab	PD-1, CTLA-4	NCT02872116
ATTRACTION-4	1st	Placebo + Oxaliplatin + S-1/Capecitabine	Oxaliplatin + S-1/Capecitabine + Nivolumab	PD-1	NCT02746796
JAVELIN Gastric 100	1st	Maintenance 1st line	Avelumab	PD-L1	NCT02625610
KEYNOTE-062	1st	Platin/fluoropyrimidine	- Pembrolizumab - Pembrolizumab + Platin/fluoropyrimidine	PD-1	NCT02494583
SPOTLIGHT	1st	Oxaliplatin + Fluoropyrimidine	Zolbetuximab + Oxaliplatin + Fluoropyrimidine	CLDN18.2	NCT03504397
ILUSTRO	1st/3rd	-	- Zolbetuximab monotherapy, 3rd line - Zolbetuximab + FOLFOX, 1st line	CLDN18.2	NCT03505320
GLOW	1st	Oxaliplatin + Capecitabine	Zolbetuximab + Oxaliplatin + Capecitabine	CLDN18.2	NCT03653507
ANGEL	3rd	BSC	Apatinib	VEGFR-2	NCT03042611
INTEGRATE II	3rd	Placebo	Regorafenib	VEGFR1-3, FGFR, PDGFR-β RAF, RET and KIT	NCT02773524

JACOB: A Study of Pertuzumab in Combination With Trastuzumab and Chemotherapy in Participants With Human Epidermal Growth Factor Receptor 2 Positive Metastatic Gastroesophageal Junction or Gastric Cancer; ID NUMBER:11-1669: Afatinib and Paclitaxel in Patients With Advanced HER2-Positive Trastuzumab-Refractory Advanced Esophagogastric Cancer; NIEGA: Study of Nimotuzumab and Irinotecan as Second Line With Recurrent or Metastatic Gastric Adenocarcinoma; ENRICH: Study of Nimotuzumab and Irinotecan as Second Line With Advanced or Recurrect Gastric and Gastroesophageal Junction Cancer; CheckMate649: Efficacy Study of Nivolumab Plus Ipilimumab or Nivolumab Plus Chemotherapy Against Chemotherapy in Stomach Cancer or Stomach/Esophagus Junction Cancer; ATTRACTION-4: Study of ONO-4538 in Gastric Cancer; JAVELIN: Gastric 100Avelumab in First-Line Maintenance Gastric Cancer; KEYNOTE-062: Study of Pembrolizumab as First-Line Monotherapy and Combination Therapy for Treatment of Advanced Gastric or Gastroesophageal Junction Adenocarcinoma; SPOTLIGHT: A Phase 3 Efficacy, Safety and Tolerability Study of Zolbetuximab Plus mFOLFOX6 Chemotherapy Compared to Placebo Plus mFOLFOX6 as Treatment for Gastric and Gastroesophageal Junction Cancer; ILUSTRO: A Study to Assess the Antitumor Activity, Safety, Pharmacokinetics and Biomarkers of Zolbetuximab in Participants With Claudin 18.2 Positive, Metastatic or Advanced Unresectable Gastric and Gastroesophageal Junction Adenocarcinoma; GLOW: A Study of Zolbetuximab Plus CAPOX Compared With Placebo Plus CAPOX as First-line Treatment of Subjects With Claudin 18.2-Positive, HER2-Negative, Locally Advanced Unresectable or Metastatic Gastric or Gastroesophageal Junction Adenocarcinoma; ANGEL: Efficacy and Safety Trial of Apatinib Plus Best Supportive Care Compared to Placebo Plus Best Supportive Care in Patients With Gastric Cancer; INTEGRATEII: A Study of Regorafenib in Refractory Advanced Gastro-Oesophageal Cancer, Best supportive care; HER2: Human epidermal growth factor receptor 2; EGFR: Epidermal growth factor receptor; PD-L1: Programmed death ligand 1; PD-1: Programmed cell death protein 1; CTLA-4: Cytotoxic T-lymphocyte-associated antigen 4; CLDN18.2: Claudine-18.2; VEGFR1-3: Vascular endothelial growth factor receptor 1-3; FGFR: Fibroblastic growth factor receptor; PDGFR-β: Platelet-derived growth factor receptor beta; RAF: Serine/threonine-specific protein kinases RAF; RET: Rearranged during transfection; KIT: Tyrosine-protein kinase Kit.

Approximately 5%-10% of GCs present an *fibroblast growth factor receptor-2* (*FGFR2*) gene amplification, which appears to confer poor prognosis[56-58]. The selective FGFR-1, 2, 3 tyrosine kinase inhibitor AZD4547 showed potent activity in preclinical models[59]. The randomized phase II SHINE study (Efficacy and Safety of AZD4547 *vs* Paclitaxel in Patients With Advanced Gastric or Gastro-oesophageal Cancer) comparing

AZD4547 *vs* paclitaxel as second-line treatment in patients with advanced GC displaying *FGFR2* polysomy or gene amplification did not demonstrated a PFS improvement in the experimental arm (1.8mo with AZD4547 *vs* 3.5mo with paclitaxel; HR: 1.57; 80%CI: 1.12-2.21; P = 0.9581)[60].

IMMUNOTHERAPY

GCs/GEJCs are associated with immune system evasion and overexpression of immune checkpoint proteins including the programmed death ligand 1 (PD-L1) and programmed death ligand 2 (PD-L2) expressed on the surface of tumor and immune cells. An high expression of PD-L1 has been reported in both Western and Asian GC/GEJC cohorts and has been associated with an elevated tumor mutational burden (TMB) and specific subtypes of adenocarcinomas[61,62]. The binding of PD1, a protein of CD28 family expressed on T cells functioning as a negative costimulatory receptor, and its ligands-PD-L1 and PD-L2, can inhibit cytotoxic T-cell responses, allowing tumor cells to evade immune surveillance. Checkpoint inhibitors such as antibodies anti PD1 (pembrolizumab, nivolumab) and PD-L1 (atezolizumab, avelumab, durvalumab) and inhibitors of cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4, like ipilimumab) have demonstrated to antagonize this immune tolerance, which results in an enhanced antitumor effect. In the last years, checkpoint inhibitors have shown activity in several solid tumors and have received approval for a number of clinical indications including advanced melanoma, renal cell carcinoma and non-small lung cancer (NSCLC)[63].

Since their introduction in the treatment scenario, lots of efforts have been undertaken to establish predictive biomarkers of response to these novel agents. Combined data from disease-specific trials with the humanized IgG4 monoclonal antibody pembrolizumab, demonstrated that tumors with a large number of somatic mutations due to high microsatellite instability (MSI-H) or mismatch repair deficiency (dMMR) are susceptible and can benefit to immune checkpoint blockade. On these findings, in 2017 FDA decided to accelerate the approval to Pembrolizumab for patients with unresectable or metastatic solid tumours with positive dMMR or MSI-H biomarkers[64]. Other studies have shown that PD-L1 expression on the membranes of tumor cells or tumor-infiltrating immune lymphocytes (TILs) was associated with a better OS in certain types of tumours treated with checkpoint inhibitors. However, there is currently no consensus on the role of PD-L1 expression as prognostic and predictive biomarker in advanced GC[65].

In GC, checkpoint inhibitors have been firstly assessed in the salvage setting showing a rather wide range of response rate (11.6%-22%)[66,67]. In the phase III Asian ATTRACTION-2 trial (Nivolumab in patients with advanced gastric or gastro-oesophageal junction cancer refractory to, or intolerant of, at least two previous chemotherapy regimens) 493 patients with refractory GC to two or previous chemotherapy regimens were randomized to receive nivolumab (n = 330) or placebo (n = 163) resulting in a median OS of 5.26 mo (95%CI: 4.60-6.37) in the nivolumab group and 4.14 mo (3.42-4.86) in the placebo group (HR: 0.63; 95%CI: 0.51-0.78; P < 0.0001). The OS rates of nivolumab and placebo were 27.3% and 11.6% at 12 mo, and 10.6% and 3.2% at 24 mo, respectively. Based on these results, nivolumab was granted accelerated approval in Japan, South Korea and Taiwan for the treatment of advanced GC progressed to standard chemotherapy[68].

Moreover, Pembrolizumab has recently received accelerated approval by FDA considering the promising results of the KEYNOTE-059 trial (Safety and Efficacy of Pembrolizumab Monotherapy in Patients with Previously Treated Advanced Gastric and Gastroesophageal Junction Cancer). In this phase II, single arm study, 259 patients with advanced GC or GEJC whose disease progressed to two or more lines of therapy, received pembrolizumab every 3 wk achieving an objective response rate (ORR) of 11,6 % (95%CI: 8.0%-16.1%; n = 30/259) with complete response in 2.3% (95%CI: 0.9%-5%; n = 6/259) and manageable safety. Interestingly, patients with PD-L1 positive tumors (PD-L1 combined positive score ≥ 1) had an ORR of 22.7% (95%CI: 13.8-33.8) and patients PD-L1-negative had an ORR of only 8.6% (95%CI: 2.9-19.0). Excluding MSI-H tumors (ORR of 57%, 4 of 7 patients) from that group, the percentage of response to pembrolizumab decreased to 13.3% in PD-L1 positive microsatellite stable (MSS) (or MSI status not available) patients, and 9% (15 of 167) of MSS patients independently of PD-L1 status responded, confirming the importance of the microsatellite status as marker of response to checkpoint inhibitors[66].

Despite the initial enthusiasm, some randomized phase III trial reported negative outcomes with checkpoint inhibitors when compared to chemotherapy. The KEYNOTE-061 phase III trial (Pembrolizumab *vs* paclitaxel for previously treated,

advanced gastric or gastro-oesophageal junction cancer) comparing pembrolizumab *vs* chemotherapy with weekly paclitaxel as second line treatment in patients with GC or GEJC with PD-L1 positivity in at least 1 % of tumor cells, failed to improve OS and PFS[69]. Similarly, the randomized, phase III Javelin Gastric 300 trial comparing the anti-PD-L1 IgG1 monoclonal antibody Avelumab *vs* chemotherapy as third line therapy in 379 patients with advanced GC/GEJC, did not meet its primary endpoint of improving OS or the secondary end points of PFS[70].

In the TCGA study an high level of intra- or peritumoral immune cell infiltration and frequent amplification of the *CD274* gene (which encodes PD-L1) and the *PDCD1LG2* gene (which encodes PD-L2) in the EBV-positive subgroup GC was found[14]. Furthermore, subsequent studies confirmed remarkable PD-L1 expression both in cancer and immune cells in EBV positive GCs[71]. Consistent with these findings, a prospective phase II Korean clinical trial of pembrolizumab with whole exome and RNA sequencing in pre and post biopsy specimens was performed to better define those patient who may benefit from pembrolizumab. Among 61 patients with advanced GC that received pembrolizumab as a second or greater line of treatment, those with MSI-H and EBV positive tumours, which are mutually exclusive, showed dramatic responses to pembrolizumab with ORR of 85.7% (6/7) in the MSI-H group and of 100% (6/6) EBV positive GCs. In addition for the 55 patients for whom PD-L1 combined positive score positivity (cut off value ≥ 1) was available, ORR was significantly higher for PD-L1 positive (n = 28) tumors when compared to PD-L1 negative (n = 27) GCs (50.0% *vs* 0.0%, P < 0.001)[72]. Although this study have provided the first clinical evidence of high activity of pembrolizumab EBV positive GCs, the percentage of EBV positive or MSI-H GCs was higher in this patient cohort compared to Western cohorts. This can be explained at least in part with the different regional risk of acquiring EBV associated GCs that ranges from 1.3%-30.9% (average of 10% worldwide) with the highest risk in Far East Asia, which also presents the highest incidence of GCs[73].

In order to optimize treatment strategies with checkpoint inhibitors, a number of ongoing trials are evaluating these agents in the first line setting (NCT02872116, NCT02746796, NCT02625610, NCT02494583, Table 1). Novel predictive biomarker are needed to select patient subgroups most likely to benefit from checkpoint inhibitors. Recently, Sundar *et al*. reported that epigenomic promoter alterations occur in a substantial proportion of metastatic GCs and cause reduced expression immunogenic peptides, which allow immune evasion and remarkable resistance to anti-PD1 immune checkpoint inhibition[74].

CLAUDIN 18.2

Claudins are main components of tight junctions in epithelial cellular sheets. Distinct claudins isoforms have been identified in different organs and their altered function has been discovered to be associated to the cancerogenesis of respective tissues[75,76]. Claudin 1-5, 7-12,16 and 18 proteins are expressed in healthy gastric tissue[77]. In particular the isoform 2 of the tight junction molecule Claudine-18 (CLDN18.2) is strictly confined to differentiated gastric epithelial cells where controls the paracellular permeability to Na^+ and H^+. In a significant percentage of primary GCs and metastases, the cell polarity perturbations lead to exposure of CLDN18.2 on the surface of GC cells so that it is available for monoclonal antibody binding[78]. CLDN18.2 is not exclusive of GC and is broadly expressed in various cancer types including biliary duct, pancreatic, ovarian cancer and NSCLC. A recent work have analyzed 286 GC/GEJC tissue samples from North America, Asia and Europe, demonstrating that 30% of samples (n = 86/286) presented high expression CLDN18.2 (moderate-to-strong CLDN18.2 membrane staining in ≥ 75% of tumor cells) with limited overlap with HER2[79]. These biological findings suggested that CLDN18.2 could be targetable and led to the further development of monoclonal antibodies against this protein. Zolbetuximab (IMAB362) is an anti-CLDN18.2 antibody that binds GC cell lines with high relative affinity and selectivity, then mediates a lysis of CLDN18.2-positive cells through antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC). In the phase II FAST trial a total of 161 patients were randomized to receive zolbetuximab plus epirubicine and oxaliplatin (EOX) or EOX alone. Median PFS was significantly higher with zolbetuximab + EOX (7.5 mo) *vs* EOX alone (5.3 mo; P < 0.0005; HR: 0.44, 95%CI: 0.29-0.67) and median OS (13 *vs* 8.4 mo; P = 0.0008; HR: 0.56, 95%CI: 0.40-0.79) and ORR (39 *vs* 25%; P = 0.022) were also demonstrated to be longer with zolbetuximab + EOX *vs* EOX alone with an increased efficacy in patients with high CLDN18.2 expression[80]. Consistent with these results, several trials are investigating zolbetuximab in different

setting (NCT03504397, NCT03504397, NCT03653507, Table 1).

ANGIOGENIC AND STROMAL INHIBITORS

Based on the positive results of the REGARD and RAINBOW trial, other agents were assessed for angiogenic inhibition in GC. The VEGFR-2 tyrosine kinase inhibitor apatinib was tested in a phase II trial of patients with advanced GC refractory to two or more lines of prior chemotherapy, showing compared to placebo, prolonged OS (6.5 mo; 95%CI: 4.8 -7.6 *vs* 4.7 mo; 95%CI: 3.6-5.4; P = 0.0149; HR: 0.709; 95%CI: 0.537-0.937; P = 0.0156) and PFS (2.6 mo; 95%CI: 2.0-2.9 *vs* 1.8 mo; 95%CI: 1.4-1.9; P < 0.001; HR: 0.444; 95%CI: 0.331-0.595; P < 0.001)[81]. The ongoing ANGEL phase III trial (Efficacy and Safety Trial of Apatinib Plus Best Supportive Care Compared to Placebo Plus Best Supportive Care in Patients With Gastric Cancer) is evaluating the clinical benefit and safety of apatinib plus Best Supportive Care (BSC) in comparison to placebo plus BSC in patients who failed to at least two prior lines of standard chemotherapies (NCT03042611, Table 1). Other phase III trials are assessing the efficacy of apatinib as maintenance treatment after first line induction therapy (NCT03598348, NCT02510469, NCT02509806). Regorafenib is an oral multi-kinase inhibitor which targets angiogenic (VEGFR-1 and -2, tie-2), stromal (PDGF-β) and oncogenic RTK, largely used in metastatic colorectal cancer and GIST. In the INTEGRATE phase II study (Regorafenib for the treatment of advanced GC) patients with previously treated GC had statistically significantly improved PFS when treated with regorafenib compared to placebo [2.6 *vs* 0.9 mo (HR: 0.40; 95%CI: 0.28-0.59; stratified log-rank: P < 0.001)][82]. Consistent with these results, regorafenib is currently evaluated in the INTEGRATE II phase III trial (NCT02773524, Table 1). Bevacizumab is a recombinant humanized monoclonal antibody that blocks angiogenesis by inhibiting VEGF-A. The AVAGAST and AVATAR trials, comparing the VEGF-antibody bevacizumab plus cisplatin/capecitabine to chemotherapy alone in different populations, failed to show significant benefit in OS[83,84]. Subgroup analysis of the AVAGAST trial showed that non-Asian patients were more likely to benefit from an anti-angiogenic therapy than Asian patients, although in the overall study population, this effect was not observed. Despite the encouraging results in the second line setting, in the recent phase III trial RAINFALL (Ramucirumab with cisplatin and fluoropyrimidine as first-line therapy in patients with metastatic gastric or junctional adenocarcinoma) that randomized patients to receive ramucirumab plus fluoropyrimidine and cisplatin or placebo plus fluoropyrimidine and cisplatin as first-line treatment, the addiction of ramucirumab to chemotherapy did not demonstrated a statistical significant advantage in PFS (HR: 0.961, 95%CI: 0.768-1.203, P = 0.74) and OS [HR: 0.962, 95%CI: 0.801-1.156, P = 0.6757; median OS 11.2 mo (9.9-11.9) in the ramucirumab group *vs* 10.7 mo (9.5-11.9) in the placebo group][85]. Other studies have investigated innovative approach to target the tumor microenvironment. A phase I/Ib study found that the addition of andecaliximab, a monoclonal antibody that inhibits matrix metalloproteinase 9, to FOLFOX showed activity in GC and GEJC. Unfortunately the phase III GAMMA-1 trial (A phase III, randomized, double-blind, placebo-controlled study to evaluate the efficacy and safety of andecaliximab combined with mFOLFOX6 as first-line treatment in patients with advanced gastric or gastroesophageal junction adenocarcinoma) comparing FOLFOX6 plus andecaliximab or mFOLFOX6 plus placebo showed a median OS of 12.5 *vs* 11.8 mo in the andecaliximab *vs* placebo treatment groups, respectively (HR: 0.93, two-sided: P = 0.56) and a median PFS of 7.5 mo in the andecaliximab group *vs* 7.1 mo in the placebo group (HR: 0.94, two-sided: P = 0.10)[86].

CONCLUSION

Recent high-throughput molecular profiling studies have provided a deeper understanding of the multiple genomic and epigenetic landscape of this complex and heterogeneous disease. New gene mutations, chromosomal aberrations, transcriptional and epigenetic alterations have been described with potentially implications for the development of personalized treatment options. However, at present, few target therapies are still available for metastatic GC.

Researches are focusing on the comprehension of primary and acquired mechanisms of resistance to anti-HER2 drugs. Moreover the targeting of other RTKs such as EGFR, MET or FGFR by TKIs or monoclonal antibodies failed to demonstrate a clinical benefit in GC. However, an appropriate molecular selection have not been conducted in many target driven clinical trials and retrospective analyses of these

studies have provided a potential benefit from RTK-inhibitors in molecularly selected subgroups.

It has to be noted that an excessive GC tumor heterogeneity and evolution complicates the efficacy of target strategies. Recent studies showed a significant discrepancy in genomic alterations within the primary tumor and between the primary tumor and disseminated disease and the potential use of plasma-based circulating-tumor DNA (cfDNA) to enhance selection of therapy in GC[41,87].

Based on the promising results of clinical trials of patients with pretreated advanced GC, pembrolizumab and nivolumab were granted accelerated approval in in the United States and in some Asian countries respectively. In contrast, none of the current checkpoint inhibitors have been approved by the European Medicines Agency (EMA). As demonstrated in other solid tumors, GC with MSI-H or dMMR is more likely to respond to checkpoint inhibitors. EBV positive GCs seem to benefit significantly from these drugs, while the role of PD-L1 expression as prognostic and predictive biomarker of response to checkpoint inhibitors has not confirmed in all the studies. In addition, epigenomic promoter alterations have been recently described as a novel potential mechanism of resistance to checkpoint inhibitors in a substantial proportion of GC. The anti-CLDN18.2 antibody zolbetuximab has shown promising results and it is currently investigated in different ongoing trials. As regard angiogenesis, in addition to ramucirumab, other antiangiogenic agents including apatinib and regorafenib are currently under investigation.

In conclusion, remarkable advances in the molecular characterization of GC have expanded our knowledge and paved the way to novel treatment options that will hopefully improve the survival outcomes of patients with metastatic GC.

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ORIGINAL ARTICLE

Basic Study

lncRNA-SNHG15 accelerates the development of hepatocellular carcinoma by targeting miR-490-3p/ histone deacetylase 2 axis

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Author contributions: Dai W and Dai JL performed the majority of experiments and analyzed the data; Ye MS performed the molecular investigations; Tang MH designed and coordinated the research; Fang S wrote the paper.

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Abstract

BACKGROUND

Hepatocellular carcinoma (HCC) has become a great threat for people's health. Many long noncoding RNAs are involved in the pathogenesis of HCC. SNHG15, as a tissue specific long noncoding RNAs, has been studied in many human cancers, except HCC.

AIM

To explore the regulatory mechanism of SNHG15 in HCC.

METHODS

In the present research, 101 HCC patient samples, two HCC cell lines and one normal liver cell line were used. RT-qPCR and Western blot analysis were applied to detect SNHG15, miR-490-3p and histone deacetylase 2 (HDAC2) expression. The regulatory mechanism of SNHG15 was investigated using CCK-8, Transwell and luciferase reporter assays.

RESULTS

Our research showed that up-regulation of SNHG15 was found in HCC and was related to aggressive behaviors in HCC patients. Moreover, knockdown of SNHG15 restrained HCC cell proliferation, migration and invasion. In addition,

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SNHG15 served as a molecular sponge for miR-490-3p. Further, miR-490-3p directly targets HDAC2. HDAC2 was involved in HCC progression by interacting with the SNHG15/miR-490-3p axis.

CONCLUSION

In conclusion, long noncoding RNA SNHG15 promotes HCC progression by mediating the miR-490-3p/HDAC2 axis in HCC.

Key words: SNHG15; miR-490-3p; Hepatocellular carcinoma; Histone deacetylase 2; Pathogenesis; Regulatory mechanism

Core tip: Long noncoding RNA (lncRNA)-SNHG15 is up-regulated in hepatocellular carcinoma (HCC) tissue and cell lines. HCC patients with up-regulated lncRNA-SNHG15 level were more likely to have larger tumor sizes and advanced clinical stage. lncRNA-SNHG15 promotes cell proliferation, migration and invasion in HCC cell lines. The miR-490-3p/histone deacetylase 2 axis was determined to be the target regulated by lncRNA-SNHG15 in HCC, and the regulatory mechanism of lncRNA-SNHG15 has been preliminarily illuminated.

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INTRODUCTION

Hepatocellular carcinoma (HCC) is a high-risk, highly harmful malignant tumor. Recently, HCC has jumped to the top three cancers in the Asia-Pacific region including China, posing a threat to human health[1]. Moreover, the pathogenesis of HCC is not fully understood. It has been reported that environmental and dietary factors affect the occurrence of HCC[2]. At present, surgery is still the first choice for the treatment of HCC. However, the recurrence rate of HCC is still high, and the 5-year survival rate is generally below 50%[3]. Thus, exploring the pathogenesis of HCC to screen for effective diagnostic markers and therapeutic targets is very urgent for HCC patients.

Long non-coding RNAs (lncRNAs) are involved in the occurrence of human diseases and cancers[4]. Recently, the presence of increased numbers of lncRNAs have been demonstrated to be involved in HCC progression. For instance, lncRNA HOXD-AS1 was overexpressed in HCC and accelerated cell proliferation and cell cycle progression through the MEK/ERK pathway[5]. On the contrary, lncRNA TPTEP1 was down-regulated in HCC and restrained HCC progression by suppressing STAT3 phosphorylation[6].

The specific functions of lncRNA SNHG15 in human cancers have caught our attention. It had been reported that SNHG15 was down-regulated and served as a tumor suppressor in thyroid cancer[7]. However, SNHG15 was up-regulated in prostate cancer and acted as a tumor promoter by mediating miR-338-3p[8]. These results indicate that expression of SNHG15 is tissue specific. In particular, SNHG15 expression was increased in HCC and predicted poor survival in HCC patients[9]. However, the specific role of SNHG15 is still unclear and needs to be elucidated.

In addition, it is well-known that lncRNAs exert an effect in human diseases by inhibiting the expression and biological functions of miRNAs[10]. SNHG15 was found to enhance colorectal cancer cell viability through down-regulation of miR-338-3p[11]. In this study, miR-490-3p was predicted to have binding sites with SNHG15. Moreover, miR-490-3p sponging by CircSLC3A2 was reported to regulate HCC progression[12]. It had been shown that miR-490-3p expression was reduced in HCC and restrained autophagy[13]. However, whether SNHG15 regulates HCC progression *via* regulating miR-490-3p remains unknown.

Besides that, histone deacetylase 2 (HDAC2) was predicted as a target of miR-490-

3p in this study. Moreover, HDAC2 expression had been reported to be increased in HCC tissues, which was related to adverse prognosis[14]. Additionally, miR-145 was proposed to act as a tumor inhibitor *via* binding to HDAC2 in liver cancer[15]. However, the interaction between miR-490-3p and HDAC2 has not been reported in previous studies. Therefore, their relationship in HCC was investigated in our study. Further, the regulatory mechanism of lncRNA SNHG15 with the miR-490-3p/HDAC2 axis was elucidated in HCC progression. Our results will contribute to better understand the role of lncRNA SNHG15 in HCC progression.

MATERIALS AND METHODS

Clinical tissues

One hundred and one HCC patients in the Affiliated Hospital of Guangdong Medical University, the second Affiliated Hospital of Guangdong Medical University and the Seventh Affiliated Hospital of Sun Yat-sen University participated in this research. The clinical features of the patients were shown in Table 1. Among them, 33 randomly selected HCC tissues and paired adjacent non-neoplastic liver tissues were applied for further experiments. Before the experiment, written informed consent was collected from all HCC patients. Moreover, patients with HCC did not receive any treatment except for surgery. The permission of this study was obtained from the Institutional Ethics Committee of the Affiliated Hospital of Guangdong Medical University, the second Affiliated Hospital of Guangdong Medical University and the Seventh Affiliated Hospital of Sun Yat-sen University.

Cell culture

HCC cell lines HuH-1, HuH-7 and normal human liver cells L-O2 were obtained from the Japanese Collection of Research Bioresources Cell Bank (JCRB, Shanghai, China). The growth conditions of these cells are 5% CO_2, 37 °C and culture solution (90% DMEM + 10% fetal bovine serum).

Cell transfection

SNHG15 siRNA (si-SNHG15), pcDNA3.1-SNHG15 vectors, HDAC2 siRNA (si-HDAC2) and miR-490-3p mimics or inhibitor were purchased from Genepharma (Shanghai, China). Next, the siRNAs, vectors, mimics or inhibitors (20 nM) were transfected into HuH-1 or L-O2 cells using Lipofectamine 2000 (Invitrogen). Untreated cells were used as the controls.

RT-qPCR

Total RNA isolation was performed with TRIzol reagent (Sigma, United States). The cDNA was reverse transcribed using microRNA reverse transcription kit (TAKARA, Dalian, China). RT-qPCR assay was performing using SYBR Green Master Mix II (TAKARA). U6 or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal reference for RNA or protein. Relative expression of SNHG15, miR-490-3p and HDAC2 were detected with the $2^{-\Delta\Delta ct}$ method.

Western blot analysis

Protein samples were lysed using RIPA buffer (Beyotime, Shanghai, China). Next, 10% SDS-PAGE was used to separate protein. Protein samples were transferred to PVDF membranes and blocked with 5% nonfat milk. Next, protein samples were incubated with HDAC2 and GAPDH primary antibodies (Abcam, Shanghai, China) overnight at 4 °C. After washing, secondary antibodies (Abcam, United States) were incubated with the protein samples for 1 h. Finally, ECL kit (Beyotime) was used to assess protein bands.

CCK-8 assay

Transfected HuH-1 cells were incubated for 24 h (at 37 °C, 5% CO_2) in 96-well plates. Next, HuH-1 cells (3×10^3/well) were incubated in DMEM medium for 24, 48, 72 and 96 h. CCK-8 (Dojindo, Kumamoto, Japan) was added and incubated with the cells for 4 h. Finally, the optical density at 450 nm was observed by a microplate reader (Molecular Devices) to assess cell viability.

Transwell assay

First, cell invasion was detected in the upper chamber with Matrigel (BD Biosciences, United States). After 30 min, Transwell upper chamber was added with HuH-1 or L-O2 cell suspension (5×10^3 cells/well). Next, lower chamber was added with DMEM medium (10% fetal bovine serum). After 24 h, 0.1% crystal violet was applied to stain the moved cells. For cell migration, 5×10^3 transfected cells were placed in the upper

Table 1 Relationship between lncRNA-SNHG15 expression and the clinic-pathological characteristics in hepatocellular carcinoma patients

Characteristics	Cases	lncRNA-SNHG15		*P* value
		High	Low	
Age in yr				0.56
≥ 65	40	26	14	
< 65	61	44	17	
Gender				0.86
Male	68	48	20	
Female	33	22	11	
Tumor size in cm				0.01[a]
< 5 cm	72	51	21	
≥ 5 cm	29	19	10	
Edmondson-Steiner grading				0.01[a]
I + II	75	53	22	
III + IV	26	17	9	
TNM stage				0.02[a]
I-II	80	55	25	
III-IV	21	15	6	

Statistical analyses were performed by the χ^2 test.
[a]$P < 0.05$ was considered significant. lncRNA-SNHG15: Long non-coding RNA-Small nucleolar RNA host gene 15.

chambers without Matrigel. Observation and photographing were performed by a light microscope.

Dual luciferase reporter assay

Reporter plasmids of SNHG15 (wt-SNHG15 and mut-SNHG15) and HDAC2 (wt-HDAC2 and mut-HDAC2) were cloned into empty pGL3 vectors (GenePharma, Shanghai, China. Then, the above reporter vectors were transfected into HuH-1 cells with miR-490-3p mimics. After 48 h, luciferase activities were examined by dual-luciferase reporter assay system (Promega, United States). HuH-1 cells with empty pGL3 vectors were used as the control.

Statistical analysis

Data were analyzed by SPSS 17.0 or Graphpad Prism 6, which are shown as mean ± standard deviation. One-way ANOVA and Student's *t*-test were employed to compare differences among multiple groups. Chi-squared test was used to analyze the association between SNHG15 and clinical features in HCC patients. $P < 0.05$ indicated significant difference.

RESULTS

Dysregulation of SNHG15 in HCC

First, a difference in SNHG15 expression was detected in HCC tissues. We found higher SNHG15 expression in HCC tissues than normal tissues (Figure 1A). Similarly, up-regulation of SNHG15 was found in HuH-1 and HuH-7 cells compared to L-O2 cells (Figure 1B). The expression of SNHG15 in HuH-1 cells is higher than HuH-7 cells. Therefore, HuH-1 cells were used for further experiments. In addition, the association between HCC clinical features and abnormal expression of SNHG15 was analyzed. Tumor size, TNM stage and degrees of differentiation in HCC under Edmondson-Steiner grading system were associated with abnormal SNHG15 expression in HCC patients (Table 1). These results suggested that SNHG15 may affect tumorigenesis of HCC.

Next, SNHG15 siRNA or SNHG15 overexpression vector was transfected into HuH-1 cells to explore its role in HCC. The transfection efficiency was confirmed by RT-qPCR (Figure 1C). CCK-8 assay suggested that knockdown of SNHG15 suppressed proliferation of HuH-1 cells, while up-regulation of SNHG15 accelerated cell proliferation in HuH-1 cells (Figure 1D). In addition, the Transwell assay showed

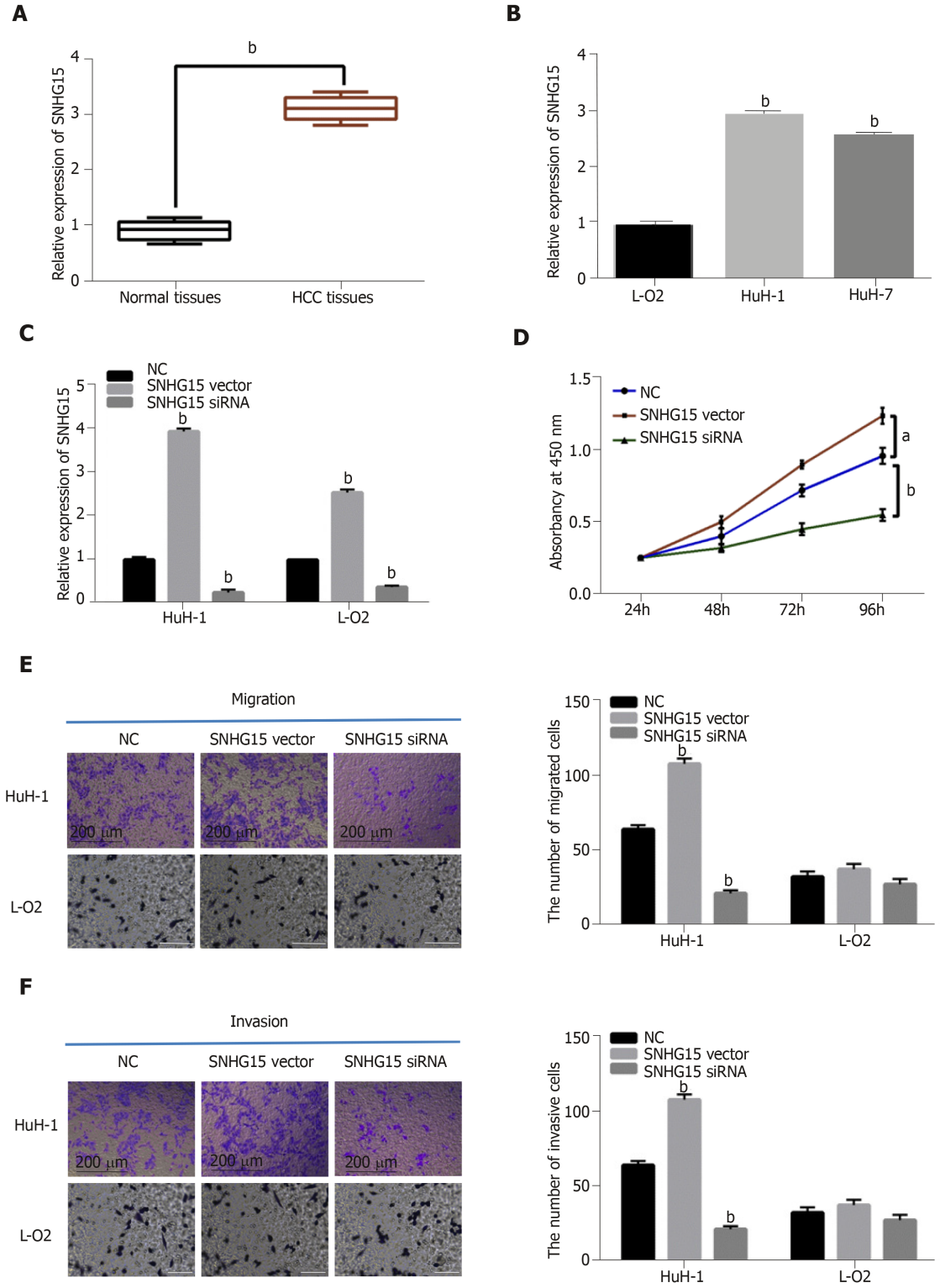

Figure 1 The dysregulation of lncRNA-SNHG15 in hepatocellular carcinoma. A: SNHG15 expression in 33 randomly selected hepatocellular carcinoma tissues and normal tissues; B: SNHG15 expression in HuH-1, HuH-7 and L-O2 cell lines; C: lncRNA-SNHG15 expression in HuH-1 and L-O2 cells containing SNHG15 siRNA or vector; D: Cell proliferation in HuH-1 and L-O2 cells with SNHG15 siRNA or vector; E: Cell migration in HuH-1 cells with SNHG15 siRNA or vector; F: Cell invasion in HuH-1 cells with SNHG15 siRNA or vector. Untreated cells were used as control (NC). $^{a}P < 0.05$, $^{b}P < 0.01$. SNHG15: Small nucleolar RNA host gene 15; HuH-1: Hepatocellular carcinoma cell line; HuH-7: Hepatocellular carcinoma cell line; L-O2: Normal human liver cell lin3; siRNA: Small interfering RNA.

that cell migration was also promoted by up-regulation of SNHG15 and restrained by knockdown of SNHG15 (Figure 1E). We also found that SNHG15 siRNA decreased its expression in L-O2 cells, whereas SNHG15 overexpression vector enhanced its expression in L-O2 cells (Figure 1C). However, up-regulation or down-regulation of SNHG15 had little effect on cell migration and invasion in normal human liver cells L-O2 (Figure 1E and F). These findings indicated the potential carcinogenesis of

SNHG15 in HCC.

Direct interaction of SNHG15 and miR-490-3p in HCC

To explore the regulatory mechanism of SNHG15, its target miRNA was searched in starBASEv2.0 database (http://starbase.sysu.edu). It predicted that SNHG15 has potential binding sites with miR-490-3p (Figure 2A). Luciferase reporter assay showed that miR-490-3p mimics decreased SNHG15-wt luciferase activity, but not SNHG15-mut (Figure 2B). Next, lower miR-490-3p expression was identified in HCC tissues compared to normal tissues (Figure 2C). Furthermore, SNHG15 was negatively associated with miR-490-3p expression in HCC tissues (Figure 2D). In HuH-1 cells, knockdown of SNHG15 enhanced miR-490-3p expression, while up-regulation of SNHG15 reduced miR-490-3p expression (Figure 2E). Interestingly, down-regulation or overexpression of miR-490-3p could also inversely regulate SNHG15 expression in HuH-1 cells (Figure 2F). To further explain their interaction, the SNHG15 vector was transfected into HuH-1 cells containing miR-490-3p mimics. Moreover, the increased miR-490-3p expression mediated by its mimics was weakened by up-regulation of SNHG15 (Figure 2G). Functionally, miR-490-3p induced inhibition of cell proliferation was also restored by SNHG15 up-regulation (Figure 2H). Similarly, up-regulation of SNHG15 also weakened the suppressive effect of miR-490-3p on migration and invasion of HCC cells (Figure 2I and 2J). Based on the results, we hypothesized that SNHG15 may accelerate HCC progression *via* molecular sponging of miR-490-3p.

miR-490-3p directly targets HDAC2

TargetScan (http://www.targetscan.org) predicted that miR-490-3p has a binding site to HDAC2 (Figure 3A). Dual-luciferase reporter assay indicated that miR-490-3p mimics reduced HDAC2-wt luciferase activity, but not HDAC2-mut (Figure 3B). Moreover, miR-490-3p mimics were found to decrease HDAC2 expression, while a miR-490-3p inhibitor up-regulated HDAC2 in HuH-1 cells (Figure 3C and D). These studies indicated that miR-490-3p directly targets HDAC2. In addition, the dysregulation of HDAC2 was identified in HCC tissues. Furthermore, HDAC2 was up-regulated in HCC tissues compared with normal tissues (Figure 3E), and miR-490-3p had an inverse correlation with HDAC2 expression in HCC tissues (Figure 3F). On the contrary, a positive correlation between SNHG15 and HDAC2 expression was identified in HCC tissues (Figure 3G). According to these results, we suspected that HDAC2 may be involved in HCC progression by affecting the SNHG15/miR-490-3p axis.

HDAC2 regulated HCC progression through mediating SNHG15/miR-490-3p

Finally, HuH-1 cells containing HDAC2 siRNA were transfected with a SNHG15 vector or a miR-490-3p inhibitor to further explain their interaction. First, we found that mRNA and protein expression of HDAC2 was down-regulated by HDAC2 siRNA. However, the decreased expression of HDAC2 was reversed by the miR-490-3p inhibitor or SNHG15 vector (Figure 4A and B). Functionally, the inhibition of cell proliferation induced by HDAC2 siRNA was restored by miR-490-3p down-regulation or SNHG15 up-regulation (Figure 4C). Similarly, the reverse effects of the miR-490-3p inhibitor or SNHG15 vector on migration and invasion were also identified in HuH-1 cells with HDAC2 siRNA (Figure 4D and 4E). These results showed that the SNHG15/miR-490-3p axis exerts an effect in the development of HCC progression by interacting with HDAC2.

DISCUSSION

As potential therapeutic targets, many lncRNAs have been found to regulate HCC progression. For example, lncRNA-MNX1-AS1 was found to accelerate HCC development *via* regulating the miR-218-5p/COMMD8 axis[16]. In our research, lncRNA SNHG15 also served as an oncogene in HCC. In particular, up-regulation of SNHG15 was identified in HCC, which was related to adverse clinical outcomes in HCC patients. Functionally, knockdown of SNHG15 decreased migration, invasion and proliferation of HCC cells. At the same time, SNHG15 was found to accelerate HCC progression by targeting the miR-490-3p/HDAC2 axis, indicating that SNHG15 may be a therapeutic target for HCC patients.

Consistent with our results, up-regulation of SNHG15 was also detected in colorectal carcinoma and lung cancer[17,18]. Functionally, up-regulated expression of SNHG15 accelerated proliferation and invasion of gastric cancer cells[19]. In addition, Kong *et al*[20] reported that SNHG15 facilitated human breast cancer cell migration by sponging miR-211-3p. The same effect of SNHG15 was also identified in HCC, which was consistent with previous studies. Besides that, Zhang *et al*[9] demonstrated that

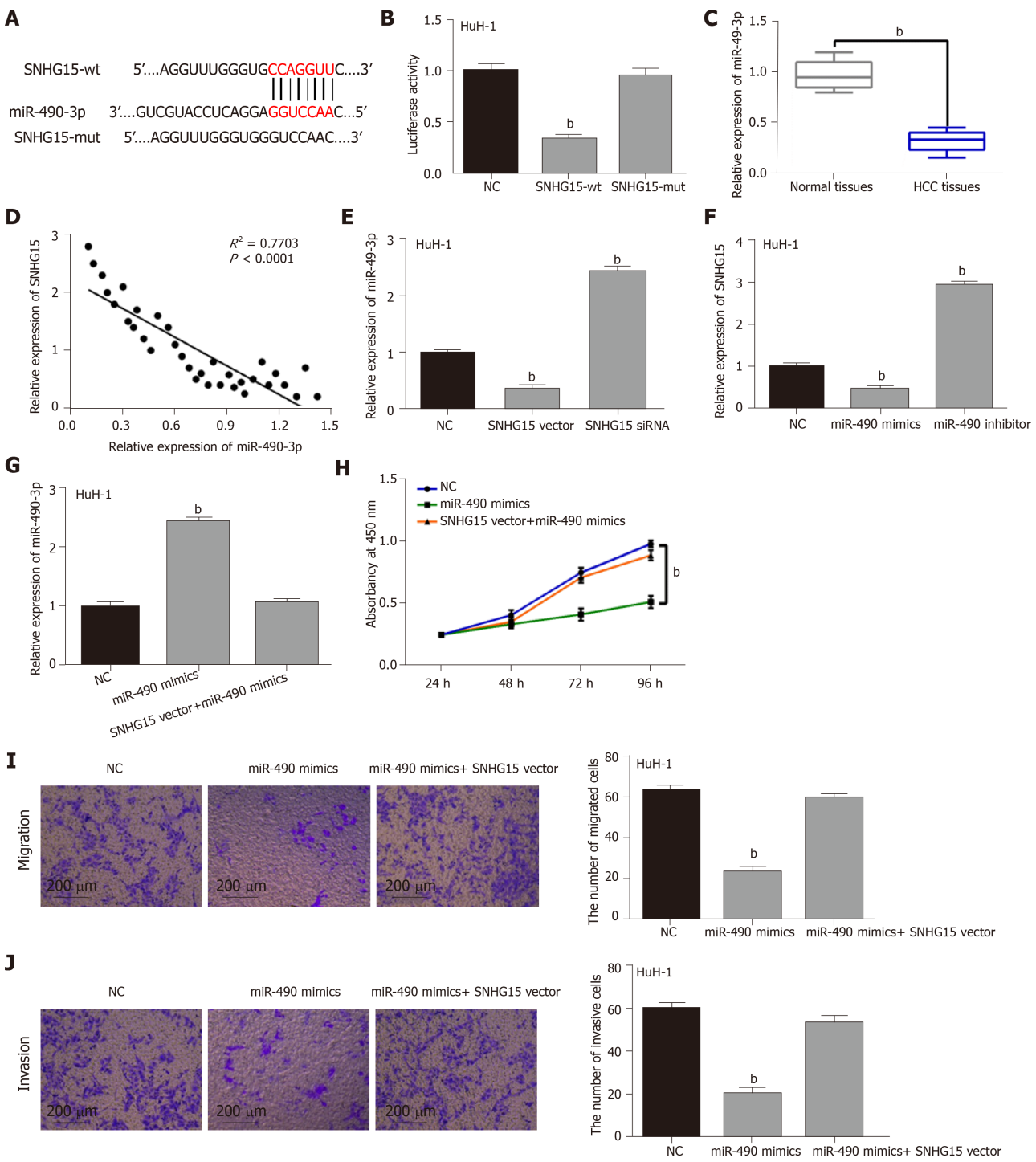

Figure 2 Direct interaction of lncRNA-SNHG15 and miR-490-3p. A: The binding sites between lncRNA-SNHG15 with miR-490-3p; B: Luciferase reporter assay; C: miR-490-3p expression in 33 randomly selected HCC tissues and normal tissues; D: SNHG15 had negative correlation with miR-490-3p expression in 33 randomly selected HCC tissues; E: miR-490-3p expression regulated by SNHG15 siRNA or vector in HuH-1 cells; F: SNHG15 expression in HuH-1 cells containing miR-490-3p mimics or inhibitor; G: miR-490-3p expression in cells with miR-490-3p mimics or miR-490-3p mimics + SNHG15 vector. H: Cell proliferation in HuH-1 cells containing miR-490-3p mimics or miR-490-3p mimics + SNHG15 vector; I: Cell migration in HuH-1 cells containing miR-490-3p mimics or miR-490-3p mimics + SNHG15 vector; J: Cell invasion in HuH-1 cells containing miR-490-3p mimics or miR-490-3p mimics + SNHG15 vector. Untreated cells were used as control (NC). $^{b}P < 0.01$. SNHG15: Small nucleolar RNA host gene 15; miR-490-3p: Hsa-miR-490-3p; HCC: Hepatocellular carcinoma; siRNA: Small interfering RNA; HuH-1: Hepatocellular carcinoma cell line.

abnormal expression of SNHG15 was related to TNM stage and vein invasion in HCC patients. Similarly, tumor size, Edmondson-Steiner grading and TNM stage were also related to SNHG15 expression in our research. Further, SNHG15 was confirmed as a molecular sponge for miR-490-3p in this study, which has not been found in previous studies.

The dysregulation of miR-490-3p has been identified in the tumorigenesis of human

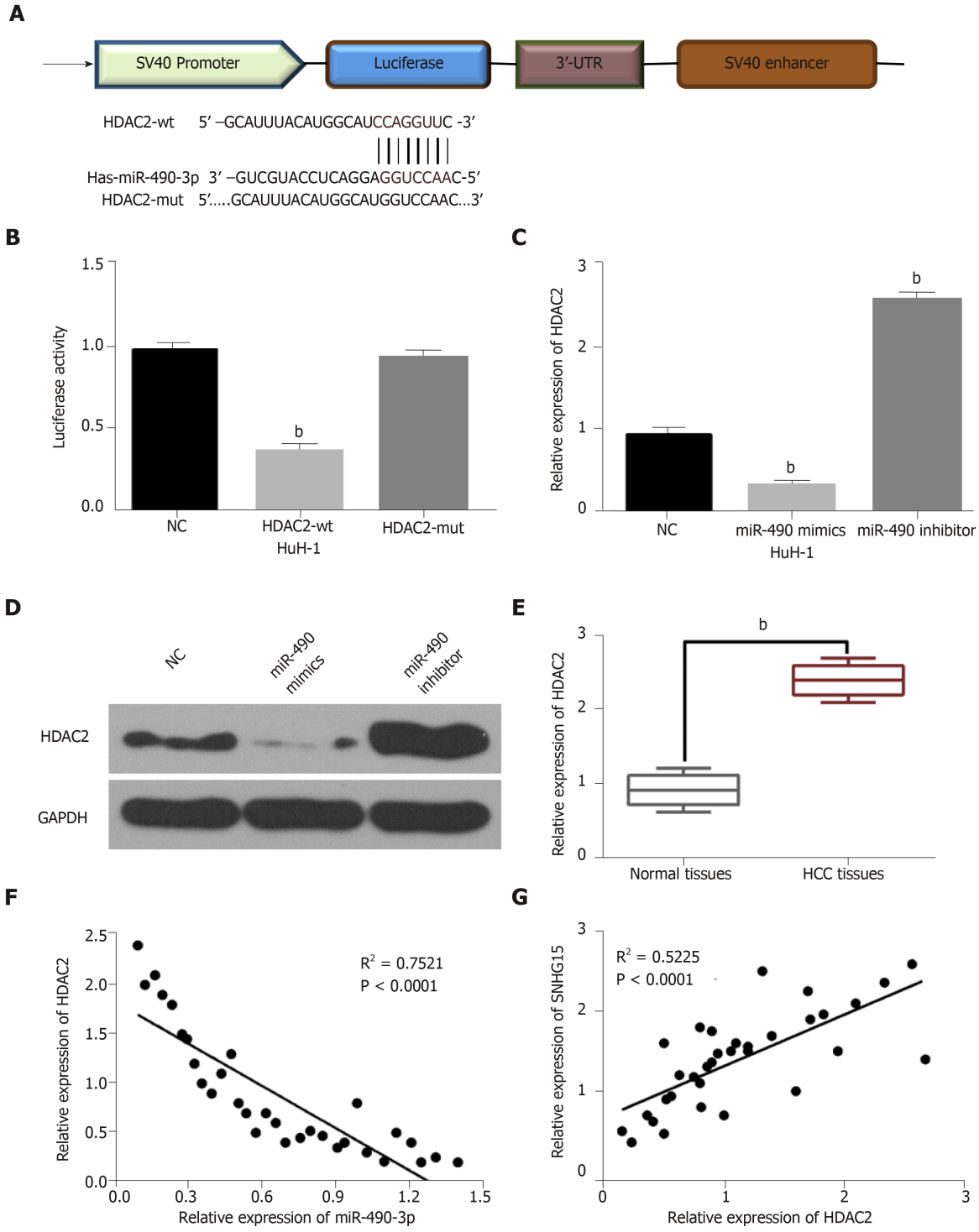

Figure 3 miR-490-3p directly targets HDAC2. A: The binding sites between miR-490-3p and HDAC2; B: Luciferase reporter assay; C: miR-490-3p regulated HDAC2 expression in HuH-1 cells; D: miR-490-3p regulated HDAC2 expression in HuH-1 cells; E: HDAC2 expression in 33 randomly selected hepatocellular carcinoma tissues and normal tissues; F: HDAC2 was negatively correlated with miR-490-3p in 33 randomly selected HCC tissues; G: lncRNA-SNHG15 was positively correlated with HDAC2 in 33 randomly selected HCC tissues. Untreated cells were used as control (NC). $^{b}P < 0.01$. miR-490-3p: Hsa-miR-490-3p; HDAC2: Histone deacetylase 2; HuH-1: Hepatocellular carcinoma cell line; HCC: Hepatocellular carcinoma; SNHG15: Small nucleolar RNA host gene 15.

cancers. Down-regulation of miR-490-3p had been found in ovarian epithelial carcinoma and colorectal cancer[21,22]. In this research, miR-490-3p was also down-regulated in HCC. Furthermore, miR-490-3p overexpression was found to decrease migration, invasion and proliferation of HCC cells. Consistently, the inhibitory role of miR-490-3p had been also identified in lung cancer and colorectal cancer[23,24]. miR-490-3p promoted viability and epithelial to mesenchymal transition of HCC cells[25], which also supports the accuracy of our results. In addition, reciprocal suppression between SNHG15 and miR-490-3p was identified in HCC. Furthermore, SNHG15 up-

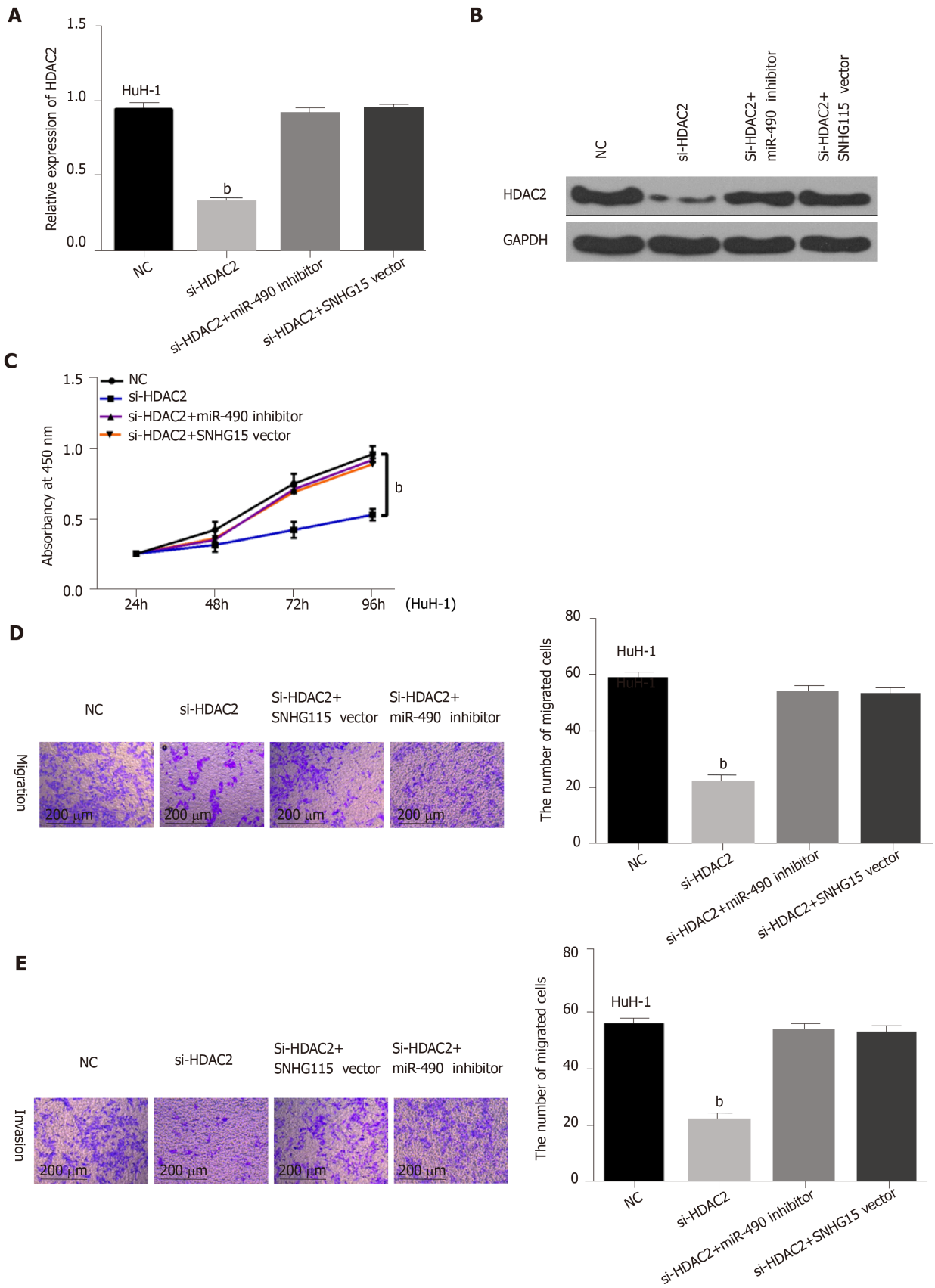

Figure 4 HDAC2 regulated hepatocellular carcinoma progression through mediating SNHG15/miR-490-3p. A: HDAC2 expression in HuH-1 cells containing HDAC2 siRNA, si-HDAC2 + miR-490-3p inhibitor or si-HDAC2 + SNHG15 vector; B: HDAC2 expression in HuH-1 cells containing HDAC2 siRNA, si-HDAC2 + miR-490-3p inhibitor or si-HDAC2 + SNHG15 vector; C: Cell proliferation in HuH-1 cells containing HDAC2 siRNA, si-HDAC2 + miR-490-3p inhibitor or si-HDAC2 + SNHG15 vector; D: Cell migration in HuH-1 cells containing HDAC2 siRNA, si-HDAC2 + miR-490-3p inhibitor or si-HDAC2 + SNHG15 vector; E: Cell invasion in HuH-1 cells containing HDAC2 siRNA, si-HDAC2 + miR-490-3p inhibitor or si-HDAC2 + SNHG15 vector. Untreated cells were set as control (NC). $^{b}P < 0.01$. SNHG15: Small nucleolar RNA host gene 15; miR-490-3p: Hsa-miR-490-3p; HDAC2: Histone deacetylase 2; HuH-1: Human hepatoma cell line; siRNA: Small interfering RNA; si-HDAC2: HDAC2 siRNA.

regulation abolished the suppressive effect of miR-490-3p in HCC progression. These findings imply that SNHG15 accelerated HCC development by sponging miR-490-3p.

We explored the downstream mechanism of miR-490-3p in HCC. We found that miR-490-3p directly targets HDAC2. Moreover, up-regulation of HDAC2 was found in HCC, and a negative association between their expressions was detected in HCC. Meanwhile, a positive correlation between the expression of SNHG15 and HDAC2 was observed in HCC. Previous studies suggested that HDAC2 was up-regulated in breast cancer and colorectal cancer, acting as an oncogene[26,27]. In our research, knockdown of HDAC2 was also found to inhibit HCC progression, serving as a tumor promoter. More importantly, down-regulation of miR-490-3p or up-regulation of SNHG15 was identified to recover the inhibitory effect of HDAC2 silencing in HCC. Taken together, we for the first time demonstrated that HDAC2 regulated by the SNHG15/miR-490-3p axis promoted the tumorigenesis of HCC.

ARTICLE HIGHLIGHTS

Research background

Among all cancers, hepatocellular carcinoma (HCC) related mortality is one of the highest and has seen a dramatic increase in annual global incidence rate. Many recent studies have demonstrated how transcriptional regulation affects HCC. Long non-coding RNAs (lncRNA) play a role in the initiation and progression of HCC, such as maintenance of cell growth, evasion of apoptosis, promotion of invasion and metastasis, stemness maintenance and epithelial to mesenchymal transition.

Research motivation

To discover biomarkers for the diagnosis and treatment of HCC.

Research objectives

To investigate the underlying mechanisms of lncRNA-SNHG15 in HCC.

Research methods

lncRNA-SNHG15 expression was observed by qRT-PCR assay in HCC tissue and cell lines. Clinicopathological characteristics were collected, arranged and combined with expression analysis of HCC to evaluate the functions of lncRNA-SNHG15. Moreover, cell function assays and western blot were performed to explore the functions of lncRNA-SNHG15 and targets regulated by lncRNA-SNHG15 in HCC cell lines.

Research results

We found that lncRNA-SNHG15 was increased in HCC tissues and cell lines and exhibited a significantly positive relationship with tumor sizes, TNM stage and Edmondson-Steiner grading. Cell experiments showed SNHG15 increased the proliferation and invasion capacity of HCC cell lines, and miR-490-3p/histone deacetylase 2 may be the target regulated by lncRNA-SNHG15 in HCC cells.

Research conclusions

Our study demonstrated that lncRNA-SNHG15 can significantly promote cell growth, migration and invasion of HCC. Furthermore, it can work through miR-490-3p/histone deacetylase 2. Therefore, our study provides some molecular mechanism and three new biomarkers for HCC.

Research perspectives

In the future, research may reveal the important role of lncRNA-SNHG15 that enhances the sensitivity of HCC detection and further develop its application in anti-cancer treatments.

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ORIGINAL ARTICLE

Basic Study

Sirtuin 1 alleviates endoplasmic reticulum stress-mediated apoptosis of intestinal epithelial cells in ulcerative colitis

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Author contributions: Zhou XX designed the experiments; Ren MT, Gu ML, Yu MS, and Pan HH performed the experiments and analyzed the data; Ren MT drafted the manuscript; Zhou XX and Gu ML critically revised the manuscript; Ji F and Ding CY offered help during the experiments; all authors have read and approved the final manuscript.

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Abstract

BACKGROUND

Sirtuin 1 (SIRT1) is a nicotinamide adenine dinucleotide (NAD^+)-dependent protein deacetylase that is involved in various diseases, including cancers, metabolic diseases, and inflammation-associated diseases. However, the role of SIRT1 in ulcerative colitis (UC) is still confusing.

AIM

To investigate the role of SIRT1 in intestinal epithelial cells (IECs) in UC and further explore the underlying mechanisms.

METHODS

We developed a coculture model using macrophages and Caco-2 cells. After treatment with the SIRT1 activator SRT1720 or inhibitor nicotinamide (NAM), the expression of occludin and zona occludens 1 (ZO-1) was assessed by Western blot analysis. Annexin V-APC/7-AAD assays were performed to evaluate Caco-2 apoptosis. Dextran sodium sulfate (DSS)-induced colitis mice were exposed to SRT1720 or NAM for 7 d. Transferase-mediated dUTP nick-end labeling (TUNEL) assays were conducted to assess apoptosis in colon tissues. The expression levels of glucose-regulated protein 78 (GRP78), CCAAT/enhancer-binding protein homologous protein (CHOP), caspase-12, caspase-9, and caspase-3 in Caco-2 cells and the colon tissues of treated mice were examined by

potential conflicts of interest exist.

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ARRIVE guidelines statement: The authors have read the ARRIVE guidelines, and the manuscript was prepared and revised according to the ARRIVE guidelines.

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quantitative real-time PCR and Western blot.

RESULTS

SRT1720 treatment increased the protein levels of occludin and ZO-1 and inhibited Caco-2 apoptosis, whereas NAM administration caused the opposite effects. DSS-induced colitis mice treated with SRT1720 had a lower disease activity index ($P < 0.01$), histological score ($P < 0.001$), inflammatory cytokine levels ($P < 0.01$), and apoptotic cell rate ($P < 0.01$), while exposure to NAM caused the opposite effects. Moreover, SIRT1 activation reduced the expression levels of GRP78, CHOP, cleaved caspase-12, cleaved caspase-9, and cleaved caspase-3 in Caco-2 cells and the colon tissues of treated mice.

CONCLUSION

SIRT1 activation reduces apoptosis of IECs *via* the suppression of endoplasmic reticulum stress-mediated apoptosis-associated molecules CHOP and caspase-12. SIRT1 activation may be a potential therapeutic strategy for UC.

Key words: Sirtuin 1; Endoplasmic reticulum stress; Apoptosis; Ulcerative colitis; Intestinal barrier

Core tip: The purpose of this article was to investigate the role of sirtuin 1 (SIRT1) in intestinal epithelial cells (IECs) in ulcerative colitis (UC) in a UC coculture model and in mice with dextran sodium sulfate (DSS)-induced colitis. It was found that SIRT1 activation contributes to enhanced intestinal barrier and reduced apoptosis of IECs via the suppression of endoplasmic reticulum stress-mediated apoptosis-associated molecules CCAAT/enhancer-binding protein homologous protein and caspase-12. SIRT1 activation may be a potential therapeutic strategy for UC.

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INTRODUCTION

Ulcerative colitis (UC), the main subtype of inflammatory bowel disease (IBD), is a chronic relapsing inflammatory disorder of the large intestine. The incidence and prevalence of UC have increased in recent years[1]. The etiology of UC remains obscure and involves a combination of genetics, environment, microbiota, and the immune system[2,3]. It is widely believed that dysregulation of cytokines (*e.g.*, tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1), IL-10, and IL-21), oxidative stress, and abnormal immune responses are key players in the progression of UC[4]. Recent data demonstrate that the intestinal epithelium, which plays a crucial role in the occurrence and persistence of UC, is a highly dynamic tissue rather than a simple physical barrier[5]. Although a large number of therapeutic agents, including 5-ASA drugs, immunosuppressants, steroids, and emerging biological agents, have appeared in the past few years, most patients still experience severe complications or recurrence of the disease, which greatly reduces their quality of life[6]. Therefore, it is imperative to develop effective treatments for UC.

The endoplasmic reticulum (ER) is a principal compartment in eukaryotic cells for protein folding and trafficking. Cellular stresses such as perturbations of Ca^{2+} homeostasis and oxidative stress disrupt ER homeostasis, resulting in the accumulation of unfolded and misfolded proteins in the ER lumen, which initiates the unfolded protein response (UPR)[7]. The UPR reduces protein synthesis, accelerates protein folding, and activates ER-associated degradation to orchestrate the recovery of ER function. However, if ER stress is too severe or persistent, intrinsic apoptotic pathways are eventually triggered, leading to cell death[8,9]. Recently, increasing evidence suggests that ER stress and UPR are involved in the pathogenesis of UC by

regulating apoptosis, autophagy, and inflammatory responses[6,10].

Sirtuin 1 (SIRT1), a member of the mammalian sirtuin family of proteins, is a nicotinamide adenine dinucleotide (NAD^+)-dependent protein deacetylase and plays an essential role in caloric restriction, life span modulation, and cell fate determination[11,12]. Recently, a number of studies have demonstrated that SIRT1 plays a protective role in colitis[13-16]. In particular, a report by Melhem *et al*[14] illustrated that SIRT1 relieves experimental colitis by modulating ER stress and reducing the UPR. However, the mechanism underlying the regulatory effect of SIRT1 on ER stress-mediated apoptosis in colitis is still unclear.

In the present study, we aimed to investigate the role of SIRT1 in the intestinal barrier in a UC coculture model and in mice with dextran sodium sulfate (DSS)-induced colitis. The mechanisms underlying the effect of SIRT1 on ER stress-mediated apoptotic pathways within intestinal epithelial cells (IECs) were further explored.

MATERIALS AND METHODS

Reagents

The SIRT1 activator SRT1720 and inhibitor nicotinamide (NAM) were obtained from Selleck Chemicals (Houston, TX, United States). DSS was purchased from MP Biomedical (Santa Ana, CA, United States). Anti-occludin, anti-zona occludens 1 (ZO-1), and anti-caspase-3 primary antibodies were purchased from Proteintech (Wuhan, China), anti-caspase-12 and anti-caspase-9 antibodies were obtained from LSBio (Seattle, WA, United States), and anti-SIRT1, anti-glucose-regulated protein 78 (GRP78), anti-CCAAT/enhancer-binding protein homologous protein (CHOP), and anti-β-actin antibodies were obtained from Abcam (Cambridge, UK).

Cell culture and coculture

We established an *in vitro* coculture model of Caco-2 and THP-1 cells based on previous studies[17-19].The human colon carcinoma Caco-2 and monocyte THP-1 cell lines were obtained from the American Type Culture Collection (ATCC; Manassas, VA, United States), cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco, Carlsbad, CA, United States) and RPMI-1640 cell culture medium (Gibco), respectively, supplemented with 10% fetal bovine serum (FBS; Gibco), and incubated at 37 °C in a 5% CO_2 atmosphere. To establish the coculture model, Caco-2 cells were cultured in 6-well culture inserts (Transwell inserts; Corning Costar, NY, United States) at a density of 2×10^5 cells/insert for 17-20 d to obtain an integrated monolayer. THP-1 cells were cultured in 6-well plates at a density of 1.5×10^6 cells/well and treated with serum-free RPMI-1640 medium containing 100 ng/mL phorbol-12-myristate-13-acetate (PMA; Sigma-Aldrich, St. Louis, MO, United States) and 0.3% bovine serum albumin (BSA; Sigma-Aldrich) for 48 h. After confirming that THP-1 cells had differentiated into macrophages, the Transwell insert on which Caco-2 cells had been cultured for 17-20 d was placed in the culture well in which human macrophage-like THP-1 cells were cultivated, then lipopolysaccharide (LPS; Sigma-Aldrich) was added to the lower chamber at a final concentration of 10 ng/ml. Ultimately, the two cell lines were cocultured for 24 h. Once the coculture model was established, the SIRT1 activator SRT1720 or inhibitor NAM was added to the upper chamber medium at a final concentration of 10 μM and 5 mM, respectively.

Enzyme-linked immunosorbent assay (ELISA)

The levels of secreted inflammatory cytokines IL-1β and TNF-α in the coculture model as well as in the colon tissues of treated mice were assayed using ELISA kits (Boster, Wuhan, China) according to the manufacturer's instructions. Cell-free supernatants from the upper chamber after coculture for 24 h and colon homogenate supernatants of mice were collected. The absorbance at 450 nm was detected with a microplate reader (Thermo Fisher Scientific, Waltham, MA, United States).

Annexin V-APC/7-AAD assays

Caco-2 apoptosis was evaluated with an Annexin V-APC/7-AAD Apoptosis Detection kit (Keygen Biotech, Nanjing, China). After SRT1720 or NAM treatment for 48 h, Caco-2 cells were harvested with EDTA-free trypsin, washed twice with cold phosphate-buffered saline, and resuspended in 500 μL of 1 × binding buffer. The resuspended cells were incubated with 5 μL of Annexin V-APC and 5 μL of 7-AAD for 5 min in the dark prior to being analyzed with a CytoFLEX flow cytometer (Beckman Coulter, CA, United States).

Animals

Twenty-four female C57BL/6 mice (6-8 wk old, weighing 18-22 g) were obtained from

SIPPR-BK Lab Animal Co. Ltd. (Shanghai, China) and kept at room temperature (22-23 °C), with a light/dark cycle of 12/12 h, and free access to food and water. All experimental protocols were designed to minimize pain or discomfort to the animals and were approved by the Ethics Committee of the First Affiliated Hospital, College of Medicine, Zhejiang University.

Female mice were randomly divided into four groups of six per group: The control group had free access to drinking water; the UC group was fed 3% DSS (w/v) for 7 consecutive days; and the UC + SRT1720 and UC + NAM groups received 3% DSS (w/v) for 7 consecutive days, followed by treatment with SRT1720 (100 mg/kg·d, intraperitoneal injection) or NAM (500 mg/kg·d, intraperitoneal injection) for another 7 d, respectively. The disease activity index (DAI) was measured daily after successful induction of acute colitis, as previously described[20].

All mice were sacrificed by decapitation. Distal colon samples were harvested for subsequent studies. The histological score (HS) of colon sections stained with hematoxylin and eosin was evaluated as described previously[20].

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

Apoptosis of cells in the colon tissue was assessed using a commercially available TUNEL assay kit (In Situ Cell Death Detection kit; Roche Applied Science, Basel, Switzerland) according to the manufacturer's instructions. In brief, tissue sections were incubated with proteinase K solution at 37 °C for 15 min. Afterwards, the enzyme solution and label solution were mixed (1:9) and added to the samples. The addition of 50 μL of converter-POD for 30 min was performed sequentially. Ten fields per section were assayed randomly in each experiment, and the percentage of positive cells was calculated.

Quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from human macrophage-like THP-1 and Caco-2 cells using TRIzol reagent (TaKaRa, Shiga, Japan) and reverse transcribed using a PrimeScript™ RT reagent kit with gDNA Eraser (TaKaRa). qRT-PCR was performed on an Applied Biosystems 7500 Fast Real-Time PCR System using the SYBER Green Premix Ex Taq kit (TaKaRa) according to the manufacturer's protocol. The primer sequences are listed in Table 1. The relative mRNA expression was analyzed by the 2^{-Ct} method.

Western blot analysis

Total protein from Caco-2 cells and colon segments of mice was isolated with RIPA buffer (Beyotime Biotechnology, Shanghai, China). Protein was quantified by BCA assay, separated by 10% SDS-PAGE, and transferred to polyvinylidene difluoride membranes (Millipore, Billerica, MA, United States). The membranes were blocked with 5% BSA diluted in TBS containing 5% Tween-20 for 2 h at room temperature, and incubated with primary antibodies at 4 °C overnight. Finally, the membranes were treated with ECL reagent and exposed to X-ray film. β-actin was used as an internal control.

Statistical analysis

Data are shown as the mean ± standard deviation (SD). All statistical analyses were performed with GraphPad Prism 7.0 (GraphPad Software, San Diego, United States) using unpaired Student's *t*-test or one-way analysis of variance followed by Tukey's test for multiple comparisons. $P < 0.05$ indicated a statistically significant difference.

RESULTS

Establishment of a coculture model in vitro

Cell-free supernatants from the upper chamber, human macrophage-like THP-1 cells, and Caco-2 cells were collected for the evaluation of IL-1β and TNF-α levels by ELISA or qRT-PCR. In the present study, the levels of secreted IL-1β and TNF-α in the upper chamber supernatants were dramatically increased upon LPS stimulation (Figure 1A and B; $P < 0.001$ *vs* coculture). In addition, the mRNA expression levels of inflammatory cytokines in macrophages and Caco-2 cells were significantly increased compared with the cells cocultured without added LPS (Figure 1C; $P < 0.01$ for IL-1β in Caco-2 *vs* coculture, $P < 0.001$ for IL-1β and TNF-α in macrophages and TNF-α in Caco-2 *vs* coculture). Herein, LPS administration for 24 h significantly increased IL-1β and TNF-α levels in the coculture model, which is in accordance with precious studies[17-19], suggesting that it is a suitable model to mimic acute colitis *in vitro*.

SIRT1 activation enhances tight junctions in Caco-2 monolayers

To assess the integrity of the intestinal barrier, we detected the expression levels of

Table 1 Primer sequences for quantitative real-time polymerase chain reaction

Gene name		Primer sequence
IL-1β	Forward	5′-ATGGCTTATTACAGTGGCA-3′
	Reverse	5′-TGTAGTGGTGGTCGGAGA-3′
TNF-a	Forward	5′-TCAGAGGGCCTGTACCTCAT-3′
	Reverse	5′-GGAAGACCCCTCCCAGATAG-3′
GRP78	Forward	5′-GGAACCATCCCGTGGCATAA-3′
	Reverse	5′-CTTGGTAGGCACCACTGTGT-3′
CHOP	Forward	5′-CACCACTCTTGACCCTGCTTCTC-3′
	Reverse	5′-TGACCACTCTGTTTCCGTTTCC-3′
β-actin	Forward	5′-AGCGAGCATCCCCCAAAGTT-3′
	Reverse	5′-GGGCACGAAGGCTCATCATT-3′

GRP78: Glucose-regulated protein 78; CHOP: CCAAT/enhancer-binding protein homologous protein.

tight junction (TJ) proteins occludin and ZO-1. After coculturing with SRT1720 (10 μM) or NAM (5 mM), Caco-2 cells were collected, and the protein was extracted for Western blot analysis. As expected, compared with the UC group, SRT1720 administration significantly upregulated the expression of the TJ proteins occludin and ZO-1, while NAM treatment suppressed the expression of occludin and ZO-1 (Figure 2). Clearly, our data indicate that drug treatment for 48 h results in the strongest protective and damaging effect on Caco-2 monolayers, respectively. Therefore, we chose 48 h as the time point for drug treatment in subsequent experiments.

SIRT1 inhibits Caco-2 apoptosis

Annexin V-APC/7-AAD staining assays were applied to assess the apoptosis of Caco-2 cells treated with SRT1720 or NAM for 48 h. As shown in Figure 3A, the induction of colitis caused significant damage to the Caco-2 monolayers, with the apoptosis rate (Annexin V-APC+/7-AAD+ quadrant and Annexin V-APC+/7-AAD- quadrant) reaching 10.21%. Though administration of SRT1720 reduced the apoptosis rate of Caco-2 cells to some extent, there were no significant differences between the UC + SRT1720 group and the UC group (Figure 3B). However, NAM treatment led to a significant increase in the rate of apoptosis (Figure 3B; $P < 0.001$ *vs* UC).

SIRT1 negatively regulates ER stress-mediated apoptotic pathways in Caco-2 monolayers

As shown in Figure 4A, SRT1720 administration increased the protein level of SIRT1, while NAM treatment downregulated its expression. To explore the molecular mechanisms underlying the protective role of SIRT1, we detected the mRNA levels of the ER stress chaperone GRP78 and the ER stress-induced apoptosis marker CHOP in Caco-2 cells. Exposure to SRT1720 for 48 h resulted in significantly decreased mRNA expression levels of GRP78 (Figure 4B; $P < 0.001$ *vs* UC) and CHOP (Figure 4C; $P < 0.01$ *vs* UC), whereas NAM treatment increased the expression of GRP78 and CHOP (Figure 4B and C; $P < 0.01$ *vs* UC). Western blot was also performed to verify the protein levels of ER stress- and apoptosis-related molecules. Consistent with the mRNA levels, we found that the level of GRP78 was significantly decreased in the SRT1720-treated group compared with the UC group (Figure 4D). In addition, the levels of CHOP and cleaved caspase-12, which play important roles in ER stress-induced apoptosis, were also decreased after SRT1720 treatment (Figure 4D). Moreover, the expression of downstream molecules, such as caspase-9 and caspase-3, was also suppressed (Figure 4D). In contrast, NAM treatment increased the expression of GRP78 and CHOP and upregulated the levels of the activated forms of caspase-12, caspase-9, and caspase-3 (Figure 4D).

SIRT1 clinically and histologically ameliorates DSS-induced colitis

Symptoms of acute colitis, including weight loss, diarrhea, and rectal bleeding, were observed daily after 7 d of DSS exposure. As expected, the administration of DSS successfully induced colitis, as the DAI dramatically increased in the UC group compared with the control group (Figure 5A; $P < 0.001$ *vs* control). The DAI score was higher in the UC + NAM group than in the UC group (Figure 5A; $P < 0.01$ *vs* UC), and SRT1720 treatment markedly reduced the DAI score (Figure 5A; $P < 0.01$ *vs* UC, $P < 0.001$ *vs* UC). Histologically, integrity loss, goblet cell damage, and inflammatory cell

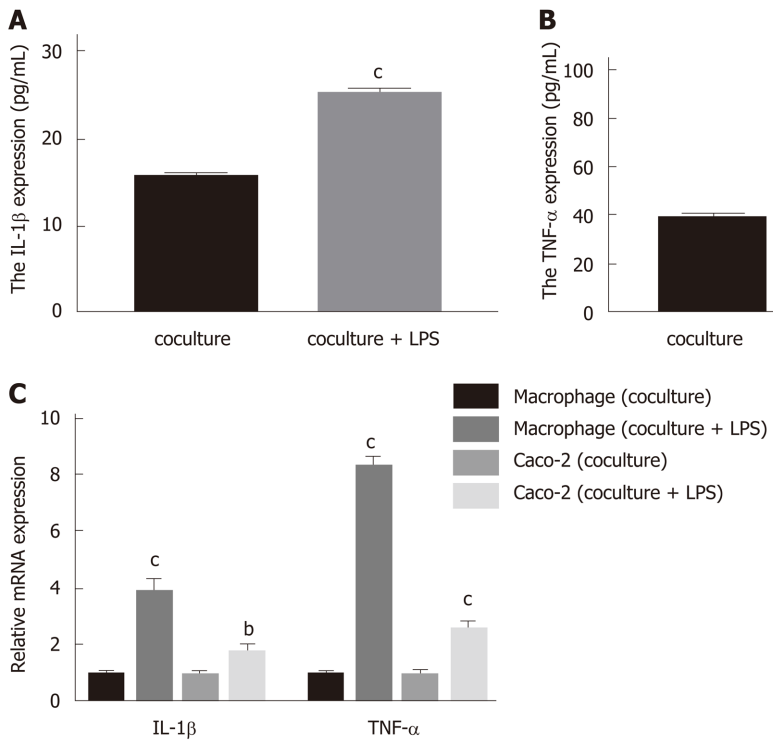

Figure 1 Establishment of a coculture model *in vitro*. A: The levels of secreted IL-1β in the upper chamber supernatants were detected by ELISA; B: The levels of secreted TNF-α in the upper chamber supernatants were detected by ELISA; C: The mRNA expression levels of IL-1β and TNF-α in macrophage-like THP-1 cells and Caco-2 cells were tested by quantitative real-time PCR. Data are presented as the mean ± SD. $^{b}P < 0.01$ *vs* coculture; $^{c}P < 0.001$ *vs* coculture. LPS: Lipopolysaccharide; IL-1β: Interleukin-1β; TNF-α: Tumor necrosis factor-α; ELISA: Enzyme-linked immunosorbent assay; PCR: Polymerase chain reaction.

infiltration were observed in the DSS group compared with the control group (Figure 5B and C; $P < 0.001$ *vs* control). Compared with those in the UC group, the above changes were ameliorated in the SRT1720-treated group and aggravated in the NAM-treated group (Figure 5B). The HS of the UC + SRT1720 group was higher than that of the UC group (Figure 5C; $P < 0.001$ *vs* UC), while the UC + NAM group did not show significantly aggravated colitis (Figure 5C). Taken together, these data show that SIRT1 activation reduces susceptibility to DSS-induced acute colitis both clinically and histologically.

SIRT1 decreases inflammatory cytokine expression in DSS-induced colitis

The expression levels of IL-1β and TNF-α in colon tissues were detected by ELISA to assess the inflammatory response. The data indicated that DSS treatment increased the levels of IL-1β and TNF-α significantly (Figure 6A and B; $P < 0.001$ *vs* control). Reduced expression levels of inflammatory cytokines were observed in the UC + SRT1720 group compared with the UC group (Figure 6A and B; $P < 0.01$ for IL-1β and $P < 0.001$ for TNF-α *vs* UC). In addition, the UC + NAM group showed increased expression levels of IL-1β and TNF-α (Figure 6A and B; $P < 0.05$ *vs* UC).

SIRT1 activation reduces the apoptotic cell rate in DSS-induced colitis

To estimate apoptosis in the colon tissue, TUNEL assays were performed, and positively stained cells were counted. The DSS group presented a dramatically larger number of apoptotic cells than the control group (Figure 7A and B; $P < 0.001$ *vs* control). There were fewer apoptotic cells in the UC + SRT1720 group than in the UC group (Figure 7A and B; $P < 0.01$ *vs* UC). Moreover, NAM administration decreased the percentage of apoptotic cells (Figure 7A and B; $P < 0.001$ *vs* UC).

SIRT1 activation suppresses ER stress-mediated apoptotic pathways during DSS-induced colitis

Protein levels in colon tissues of treated mice were assessed by Western blot. DSS administration caused a lower protein level of SIRT1. Besides, SRT1720 and NAM treatment upregulated and downregulated SIRT1 expression levels, respectively (Figure 8A). As shown in Figure 8B, compared with the control group, the DSS-treated group showed significantly elevated protein levels of GRP78, CHOP, and cleaved caspase-12, suggesting that ER stress and the UPR were activated. Consistent

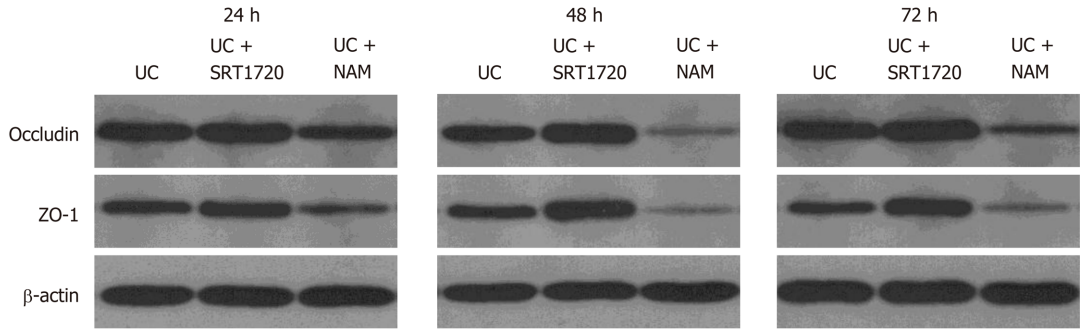

Figure 2 Sirtuin 1 activation enhances tight junctions in Caco-2 monolayers. The protein levels of occludin and ZO-1 in Caco-2 monolayers were examined after administration of the drug for 24 h, 48 h, or 72 h. UC: Ulcerative colitis; NAM: Nicotinamide; ZO-1: Zona occludens 1.

with the *in vitro* results, SRT1720 administration downregulated the protein levels of GRP78, CHOP, cleaved caspase-12, cleaved caspase-9, and cleaved caspase-3 (Figure 8B). Additionally, NAM administration caused the opposite effect (Figure 8B). Altogether, these results show that SIRT1 activation inhibits ER stress-mediated apoptotic pathways in DSS-induced colitis mice.

DISCUSSION

UC is characterized by chronic colonic mucosal inflammation and is a known risk for colorectal cancer. Although novel pharmacological therapies have been emerging recently, the existing treatments for UC are still not satisfactory[21]. Thus, developing effective drugs is essential. As a well-known modulator of lifespan, SIRT1 is involved in various diseases, including cancers, metabolic diseases, and inflammation-associated diseases[22-25]. However, the role of SIRT1 in the intestinal barrier and intestinal inflammation is still obscure. Here, we applied the SIRT1 activator SRT1720 and inhibitor NAM to investigate the potential effects of SIRT1 on the intestinal barrier in a UC coculture model and in mice with DSS-induced colitis. Our results demonstrate that the pharmacological activation of SIRT1 enhances TJ integrity of the intestinal barrier and reduces apoptosis of IECs by downregulating the expression of CHOP and suppressing the activation of caspase-12, which are key molecules in ER stress-mediated apoptotic pathways.

SIRT1 was found to be downregulated in colonic epithelium and lamina propria mononuclear cells (LPMCs) of IBD patients and elevated after successful infliximab treatment, suggesting that SIRT1 is involved in the development of IBD[13,14]. Furthermore, previous studies have identified the protective role of SIRT1 in intestinal inflammation, the molecular mechanisms of which include intestinal microbiota alteration and nuclear factor kappa B (NF-κB) pathway suppression[13,16,26]. In our study, SIRT1 activation significantly alleviated DSS-induced experimental colitis both clinically and histologically; this alleviation of colitis was accompanied by the downregulation of the levels of the inflammatory cytokines IL-1β and TNF-α in colon tissues, which is consistent with previous studies. Nevertheless, further studies are required to illuminate the underlying mechanisms.

The intestinal epithelial barrier isolates the internal milieu from the external environment and plays a crucial role in intestinal homeostasis. Intestinal inflammation is associated with increased permeability of the intestinal mucosa caused by intestinal barrier damage[27]. Occludin and ZO-1 are important TJ proteins and are essential for the maintenance of intestinal mucosal barrier integrity[28]. Occludin interacts directly with claudins and actin and takes part in the regulation of the intestinal barrier[29]. ZO-1 is a peripheral membrane protein that is essential for TJ assembly and maintenance[29]. A recent study revealed that occludin and ZO-1 expression in UC patients was not only significantly decreased compared with that of healthy controls but also positively related to intestinal mucosal healing[30]. Moreover, previous studies have demonstrated that SIRT1 enhances TJs in other physical barriers[31,32]. Herein, we found that SIRT1 activation significantly increased the expression of occludin and ZO-1 in Caco-2 monolayers, suggesting that SIRT1 may exert protective effects on colitis by promoting intestinal barrier integrity.

IECs, which comprise enterocytes, goblet cells, and Paneth cells, have well-developed ER structures for the biosynthesis of large amounts of proteins. Increasing evidence suggests that ER stress and the UPR in IECs are involved in the pathogenesis

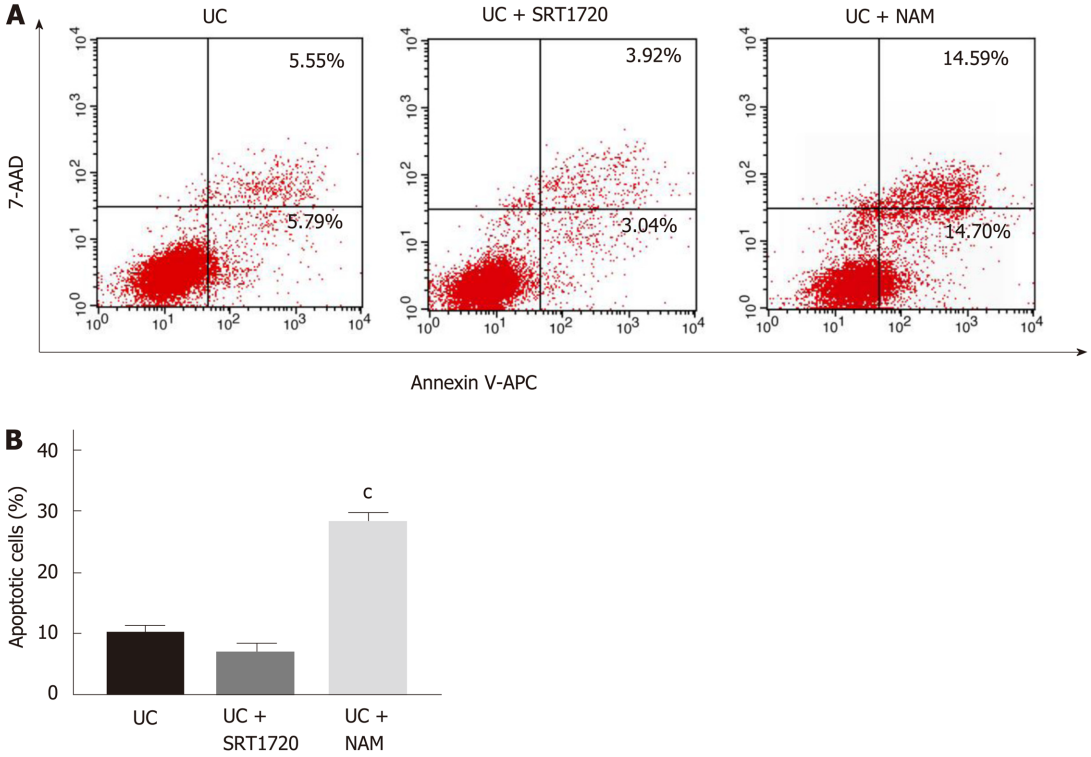

Figure 3 Sirtuin 1 inhibits Caco-2 apoptosis. A: Cells in the upper-right (UR) portion are late apoptotic cells, whereas cells in the lower-left and lower-right (LR) portions are viable and early apoptotic cells, respectively; B: Apoptotic rate (UR + LR) of Caco-2 cells. Each value is presented as the mean ± SD. $^{c}P < 0.001$ *vs* UC. UC: Ulcerative colitis; NAM: Nicotinamide.

of UC[10,33,34]. Unresolved ER stress is a common character of the UC epithelium and results in the activation of the UPR to restore ER homeostasis or the induction of cell apoptosis if ER stress is too severe to be rescued[35]. Additionally, ER stress in IECs is related to intestinal dysbiosis and dysregulated immune response, leading to cell dysfunction, mucosal barrier damage, and intestinal inflammation[36]. In the present study, exposure to DSS increased the expression of the ER stress marker GRP78, which was accompanied by an increased rate of apoptosis in the colon and the activation of apoptosis-related proteases caspase-9 and caspase-3. GRP78 is an ER chaperone that binds to transmembrane ER stress sensors (inositol-requiring enzyme 1α (IRE1α), double-stranded RNA-activated protein kinase-like ER kinase (PERK), and activating transcription factor 6α (ATF6α)) in non-stressed cells[37]. It is released and contributes to the apoptosis of IECs upon ER stress[6]. Moreover, we found that SIRT1 activation reduced apoptosis in Caco-2 monolayers and colon tissues of DSS-induced colitis mice, indicating that SIRT1 may play a protective role in ER stress-induced injury.

To further confirm the protective effect of SIRT1, we detected the protein levels of CHOP and caspase-12. As a downstream transcriptional factor of PERK/eukaryotic translation initiation factor 2α (eIF2α)/activating transcription factor 4 (ATF4), CHOP plays a critical role in ER stress-induced apoptosis through suppression of the antiapoptotic protein Bcl-2 and induction of the proapoptotic molecules Bim, death receptor 5 (DR5), and telomere repeat binding factor 3 (TRB3)[38]. The intestinal epithelium of IBD patients shows higher expression of CHOP compared with normal people[39]. Furthermore, CHOP overexpression increases susceptibility to intestinal inflammation and mucosal tissue injury in mice, whereas knockdown of CHOP alleviates IEC apoptosis[40,41]. Previous studies have revealed that SIRT1 alleviates ER stress-mediated cell apoptosis through the downregulation of the PERK-eIF2α-CHOP axis in the UPR pathway in cardiac cells and chondrocytes[42,43]. Caspase-12 is another marker of ER stress-mediated apoptosis, which is separated from the ER membrane and cleaved into active fragments upon ER stress, resulting in caspase-3 cleavage and apoptosis[44]. Guo *et al*[45] reported that SIRT1 may alleviate ER stress-mediated apoptosis of cardiomyocytes *via* reduced expression levels of CHOP and cleaved caspase-12. Here, we show that SIRT1 activation decreases the expression of CHOP and suppresses the activation of caspase-12 in Caco-2 cells as well as in DSS-induced colitis mice, while treatment with the SIRT1 inhibitor NAM induced the opposite

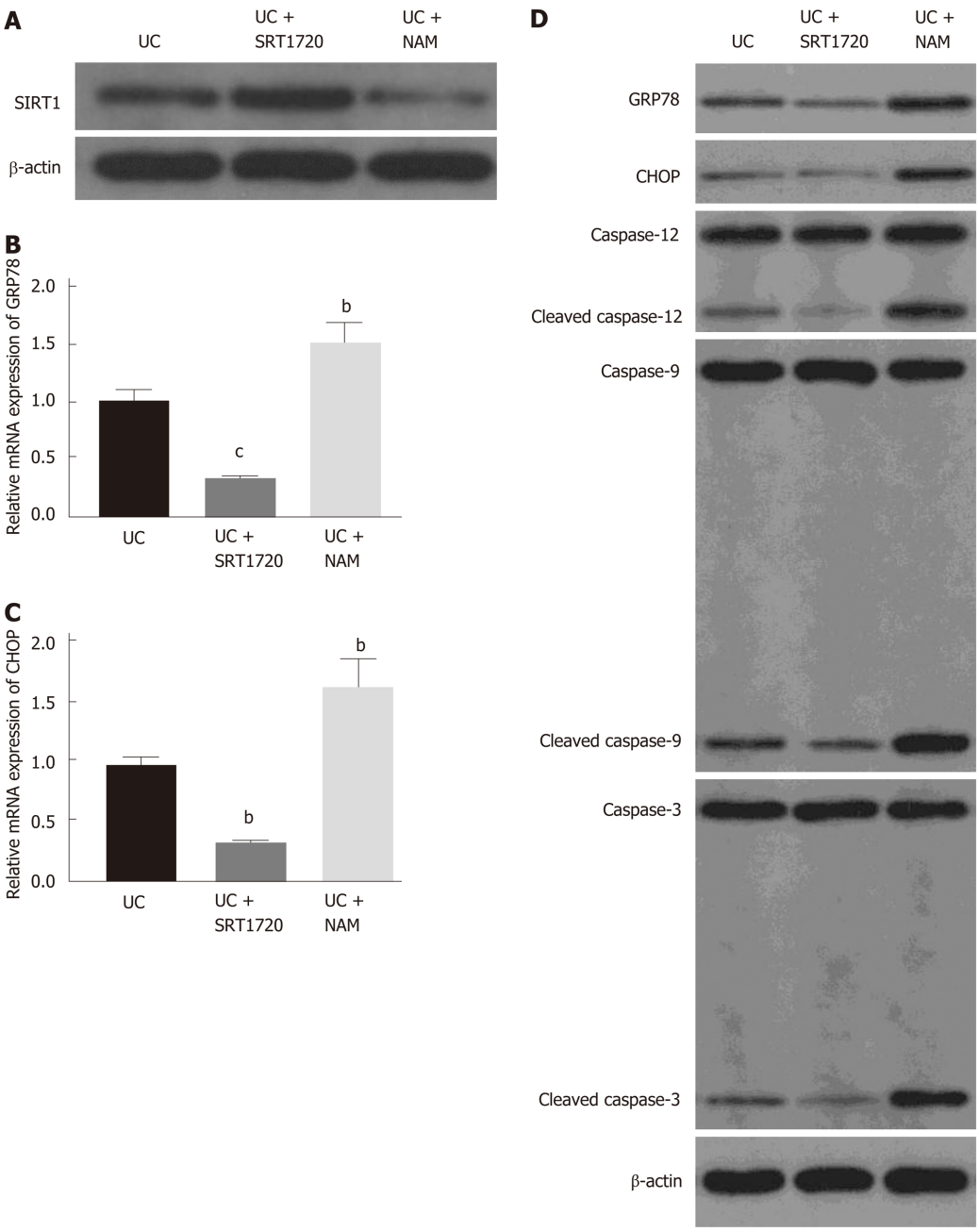

Figure 4 Sirtuin 1 negatively regulates endoplasmic reticulum stress-mediated apoptotic pathways in Caco-2 monolayers. A: The protein levels of SIRT1 in Caco-2 cells were detected by Western blot; B: The mRNA levels of GRP78 in Caco-2 cells were tested by quantitative real-time PCR; C: The mRNA levels of CHOP in Caco-2 cells were tested by quantitative real-time PCR; D: The protein levels of GRP78, CHOP, caspase 12, caspase 9, and caspase 3 were detected by Western blot in Caco-2 cells. Data are presented as the mean ± SD. $^{b}P < 0.01$ *vs* UC; $^{c}P < 0.001$ *vs* UC. UC: Ulcerative colitis; NAM: Nicotinamide; PCR: Polymerase chain reaction; GRP78: Glucose-regulated protein 78; CHOP: CCAAT/enhancer-binding protein homologous protein; SIRT1: Sirtuin 1.

effect, which indicates that SIRT1 protects IECs from ER stress-induced apoptosis by suppressing CHOP, caspase-12, and their downstream signaling cascades.

In conclusion, we discovered that SIRT1 activation contributes to enhanced intestinal barrier integrity and reduced IEC apoptosis *via* the suppression of ER stress-mediated apoptotic proteins such as CHOP and caspase-12. SIRT1 may serve as a novel drug target, and SIRT1 activation is a promising therapeutic strategy for UC.

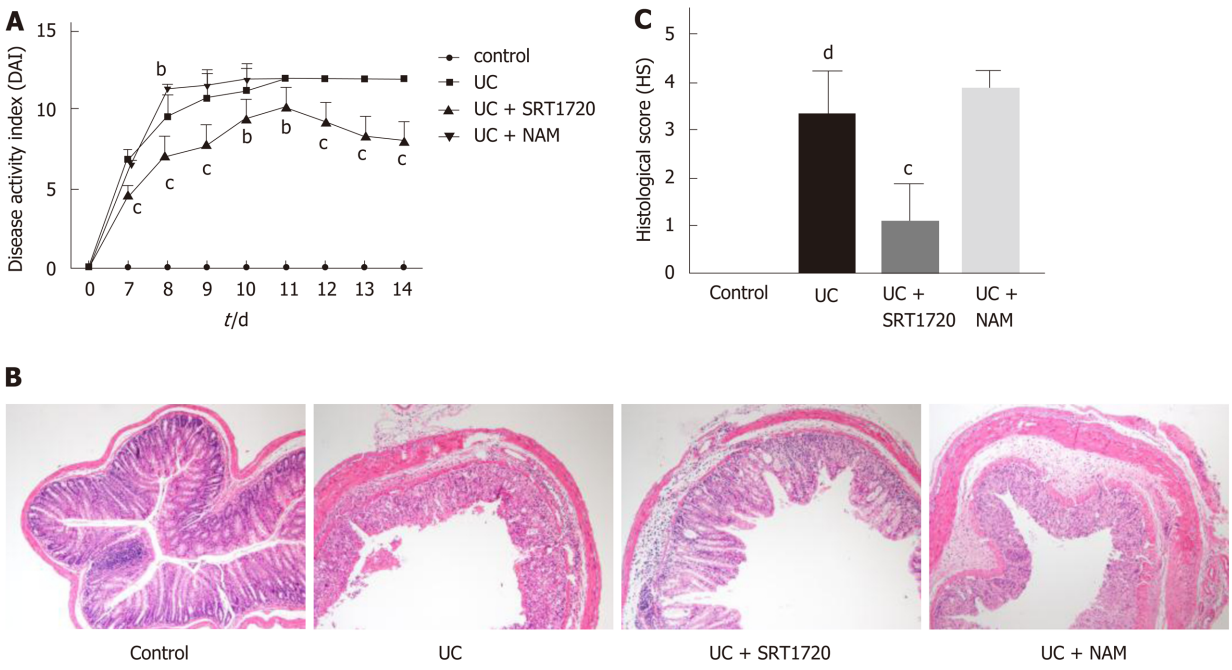

Figure 5 Sirtuin 1 clinically and histologically ameliorates dextran sodium sulfate-induced colitis. A: Disease activity index was calculated; B: Hematoxylin-eosin staining of colon sections was performed (×100); C: Histological scores of stained sections were calculated. Each value is presented as the mean ± SD. $^{b}P < 0.01$ *vs* UC; $^{c}P < 0.001$ *vs* UC; $^{d}P < 0.001$ *vs* control. UC: Ulcerative colitis; NAM: Nicotinamide.

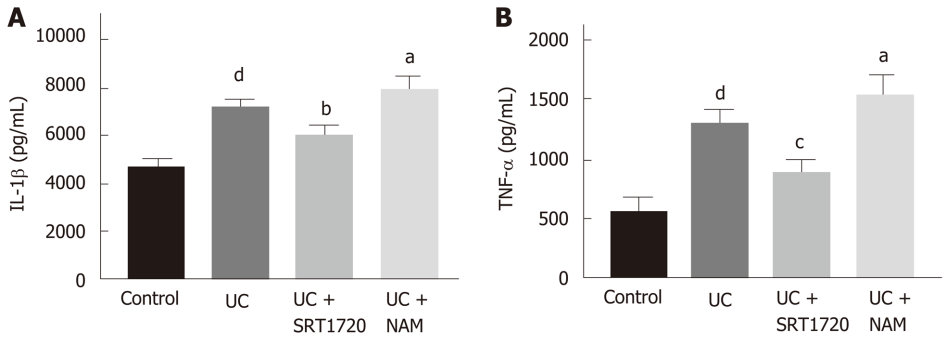

Figure 6 SIRT1 decreases inflammatory cytokine expression in dextran sodium sulfate-induced colitis. The expression levels of IL-1β and TNF-α were detected by ELISA. A: The expression of IL-1β in colon tissues; B: The expression of TNF-α in colon tissues. Data are presented as the mean ± SD. $^{a}P < 0.05$ *vs* UC; $^{b}P < 0.01$ *vs* UC; $^{c}P < 0.001$ *vs* UC; $^{d}P < 0.001$ *vs* control. UC: Ulcerative colitis; NAM: Nicotinamide; IL-1β: interleukin-1β; TNF-α: Tumor necrosis factor-α; ELISA: Enzyme-linked immunosorbent assay.

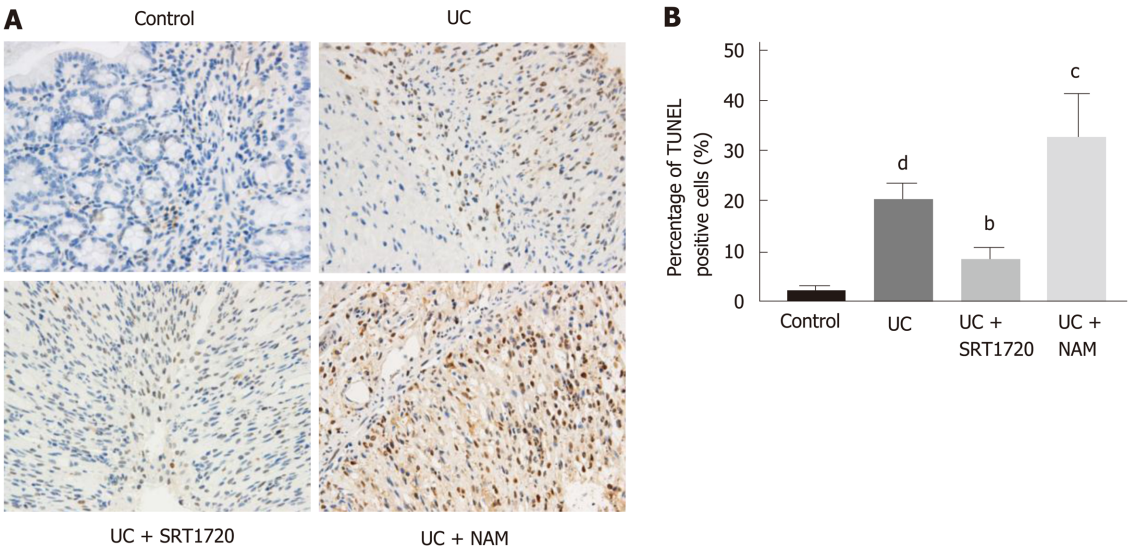

Figure 7 SIRT1 activation reduces apoptotic cell rates in dextran sodium sulfate-induced colitis. A: TUNEL staining of colon tissue was performed; B: The number of TUNEL positive cells per 10000 cells was counted. Each value is presented as the mean ± SD. $^{b}P < 0.01$ *vs* UC; $^{c}P < 0.001$ *vs* UC; $^{d}P < 0.001$ *vs* control. TUNEL: Transferase-mediated dUTP nick-end labeling; UC: Ulcerative colitis; NAM: Nicotinamide.

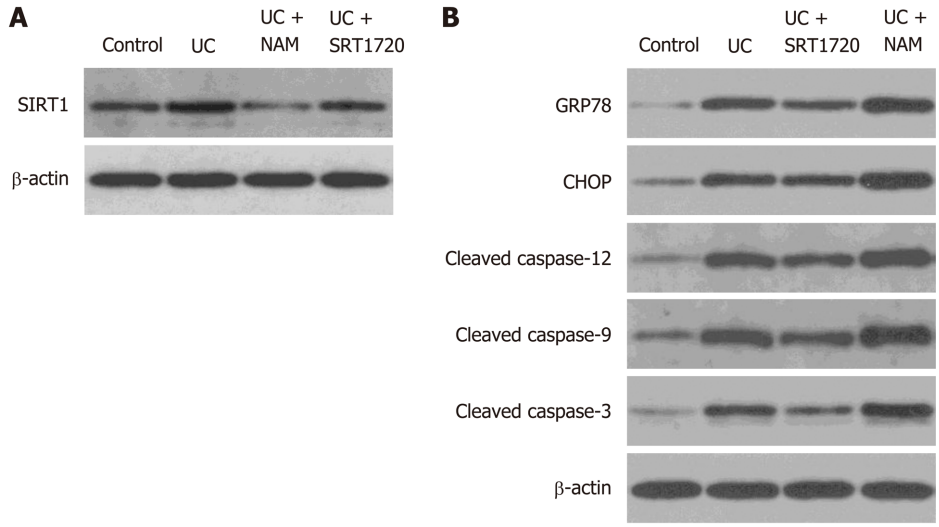

Figure 8 Sirtuin 1 activation suppresses endoplasmic reticulum stress-mediated apoptotic pathways during dextran sodium sulfate-induced colitis. The protein levels of SIRT1, GRP78, CHOP, cleaved caspase-12, cleaved caspase-9, and cleaved caspase-3 in colon tissues were detected by Western blot. UC: Ulcerative colitis; NAM: Nicotinamide; GRP78: Glucose-regulated protein 78; CHOP: CCAAT/enhancer-binding protein homologous protein; SIRT1: Sirtuin 1.

ARTICLE HIGHLIGHTS

Research background

Ulcerative colitis (UC), the main subtype of inflammatory bowel disease (IBD), is a chronic relapsing inflammatory disorder of the large intestine. The incidence and prevalence of UC have increased in recent years. Sirtuin 1 (SIRT1), a member of the mammalian sirtuin family of proteins, is a nicotinamide adenine dinucleotide (NAD^+)-dependent protein deacetylase and plays an essential role in caloric restriction, life span modulation, and cell fate determination. Recently, a number of studies have demonstrated that SIRT1 plays a protective role in colitis.

Research motivation

Although a large number of therapeutic agents, including 5-ASA drugs, immunosuppressants, steroids, and emerging biological agents, have appeared in the past few years, most patients still experience severe complications or recurrence of the disease, which greatly reduces their quality of life.

Research objectives

To investigate the role of SIRT1 in intestinal epithelial cells in UC and further explore the underlying mechanisms.

Research methods

We developed a coculture model using macrophages and Caco-2 cells. After treatment with the SIRT1 activator SRT1720 or inhibitor nicotinamide (NAM), the expression of occludin and zona occludens 1 (ZO-1) was assessed by Western blot. Annexin V-APC/7-AAD assays were performed to evaluate Caco-2 apoptosis. DSS-induced colitis mice was exposed to SRT1720 or NAM for 7 d. Transferase-mediated dUTP nick-end labeling (TUNEL) assays were conducted to assess apoptosis in colon tissues. The expression levels of glucose-regulated protein 78 (GRP78), CCAAT/enhancer-binding protein homologous protein (CHOP), caspase-12, caspase-9, and caspase-3 in Caco-2 cells and the colon tissues of treated mice were examined by quantitative real-time PCR and Western blot.

Research results

SRT1720 treatment increased the protein levels of occludin and ZO-1 and inhibited Caco-2 apoptosis, whereas NAM administration caused the opposite effects. DSS-induced colitis mice treated with SRT1720 had a lower disease activity index ($P < 0.01$), histological score ($P < 0.001$), inflammatory cytokine levels ($P < 0.01$), and apoptotic cell rates ($P < 0.01$), while exposure to NAM caused the opposite effects. Moreover, SIRT1 activation reduced the expression levels of GRP78, CHOP, cleaved caspase-12, cleaved caspase-9, and cleaved caspase-3 in Caco-2 cells and the colon tissues of treated mice.

Research conclusions

SIRT1 activation contributes to enhanced intestinal barrier integrity and reduced apoptosis of intestinal epithelial cells *via* the suppression of endoplasmic reticulum (ER) stress-mediated apoptosis-associated molecules CHOP and caspase-12.

Research perspectives

SIRT1 may serve as a novel drug target, and SIRT1 activation is a promising therapeutic strategy for UC.

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ORIGINAL ARTICLE

Basic Study

Up-regulated Wnt1-inducible signaling pathway protein 1 correlates with poor prognosis and drug resistance by reducing DNA repair in gastric cancer

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Abstract

BACKGROUND

Wnt1-inducible signaling pathway protein 1 (WISP1) is upregulated in several types of human cancer, and has been implicated in cancer progression. However, its clinical implications in gastric cancer (GC) remain unclear.

AIM

To explore the expression pattern and clinical significance of WISP1 in GC.

METHODS

Public data portals, including Oncomine, The Cancer Genome Atlas database, Coexpedia, and Kaplan-Meier plotter, were analyzed for the expression and clinical significance of WISP1 mRNA levels in GC. One hundred and fifty patients who underwent surgery for GC between February 2010 and October 2012 at the Affiliated Hospital of Jiangnan University were selected for validation study. WISP1 levels were measured at both the mRNA and protein levels by RT-qPCR, Western blot analysis, and immunohistochemistry (IHC). In addition, the *in situ* expression of WISP1 in the GC tissues was determined by IHC, and the

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patients were accordingly classified into high- and low-expression groups. The correlation of WISP1 expression status with patient prognosis was then determined by univariate and multivariate Cox regression analyses. WISP1 was knocked down by RNA interference. The 50% inhibitory concentration of oxaliplatin was detected by CellTiter-Blue assay.

RESULTS
WISP1 levels at both the mRNA and protein levels were remarkably upregulated in GC tissues compared to normal tissues. Moreover, IHC revealed that WISP1 expression was associated with T stage and chemotherapy outcome, but not with lymph node metastasis, age, gender, histological grade, or histological type. GC patients with high WISP1 expression showed a poor overall survival. Multivariate survival analysis indicated that WISP1 was an important prognostic factor for GC patients. Mechanistically, knock-down of WISP1 expression enhanced sensitivity to oxaliplatin by reducing DNA repair and enhancing DNA damage.

CONCLUSION
Significantly upregulated WISP1 expression is associated with cancer progression, chemotherapy outcome, and prognosis in GC. Mechanistically, knock-down of WISP1 expression enhances oxaliplatin sensitivity by reducing DNA repair and enhancing DNA damage. WISP1 may be a potential therapeutic target for GC treatment or a potential biomarker for diagnosis and prognosis.

Key words: Wnt1-inducible signaling pathway protein 1; Biomarker; Bioinformatics analysis; Chemotherapy outcome; Gastric cancer

Core tip: The present study for the first time revealed that significantly upregulated Wnt1-inducible signaling pathway protein 1 (WISP1) expression was associated with advanced cancer, drug resistance, and poor prognosis in gastric cancer (GC). WISP1 enhanced oxaliplatin resistance by reducing cell cytotoxicity through enhancing DNA repair. Overall, the findings of the present study suggest that WISP1 is a novel prognostic biomarker for GC and highlight the significance of WISP1 as a promising therapeutic target for GC.

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INTRODUCTION

Gastric cancer (GC) is one of the most common malignancies[1]. In 2018, the incidence and mortality rates of GC ranked fifth and third, respectively, putting a heavy economic burden on the public health system worldwide, especially in East Asian countries[2]. Cancer incidence and mortality have been increasing in China and consequently a major public health problem in the country[3]. Because patients with early-stage GC are often asymptomatic, most patients are usually diagnosed at an advanced stage[4]. Available treatments are mostly unfavorable and inefficient[5]. Early diagnosis of GC and effective medical treatment are crucial for patient survival and therapeutic outcome[6]. Thus, there is an urgent need to better understand the mechanism of GC progression and development, and to identify promising biomarkers for diagnosis and prognosis.

Wnt1-inducible signaling pathway protein 1 (WISP1), highly homologous to CCN1 and CCN2, is a member of the CCN family of secreted, extracellular matrix (ECM)-associated signaling proteins (CCN intercellular signaling protein). This family of proteins regulate diverse cellular functions, including cell proliferation,

differentiation, adhesion, migration, and survival. In recent studies, it was demonstrated that the expression of WISP1 in cancer promotes cell proliferation, and high WISP1 expression correlated with advanced tumors of the brain, breast, colon, and lung. WISP1 reduced p53-mediated apoptosis through activating Akt kinase, and inhibited TNF-induced cell death in cardiomyocytes[7]. In addition, recombinant WISP-1 enhances ECM deposition in human fibroblasts, suggesting that it might play a vital role in matrix remodeling *in vivo*[8]. In GC cells, WISP1 acts as an oncogene by promoting cell proliferation, migration, and invasion[9]. Although WISP1 has been demonstrated in cancer progression, its prognostic value in GC remains unclear.

In this study, we explored whether WISP1 can be a novel biomarker for prognosis or a therapeutic target in GC. First, public databases and websites, including Oncomine and Coexpedia, were used for predicting the expression of WISP1 in GC. In addition, Kaplan-Meier plotter datasets from The Cancer Genome Atlas (TCGA) were used for analyzing the association of overall survival between WISP1 and GC. Subsequently, WISP1 expression was evaluated at both the mRNA and protein levels in 20 freshly frozen GC tissues and 20 non-cancerous GC tissues. Furthermore, immunohistochemistry (IHC) was used to explore the relationship between WISP1 expression and clinicopathologic parameters, including overall survival. WISP1 was knocked down by RNA interference in MKN45 and AGS cell lines. The 50% inhibitory concentration (IC50) was detected by CellTiter-Blue (CTB) assay to evaluate the effect on oxaliplatin resistance.

MATERIALS AND METHODS

Patients and tissue samples

A total of 150 patients who underwent surgery for GC between February 2010 and October 2012 at the Affiliated Hospital of Jiangnan University (Wuxi, China) were selected for this study. Those patients who postoperatively received oxaliplatin-based or cisplatin-based first-line systematic chemotherapy were enrolled in the present study. Tumor assessment was performed after every two cycles of chemotherapy according to the Response Evaluation Criteria in Solid Tumors 1.1 (RECIST 1.1) criteria, and the assessment was classified as complete response (CR), partial response (PR), stable disease (SD), and progressive disease (PD). This study was approved by the Institutional Research Ethics Committee of Affiliated Hospital of Jiangnan University (Wuxi, China). None of the patients selected received chemotherapy or radiotherapy prior to surgery. According to the American Joint Committee on Cancer, the tumor stage classification was determined by three pathologists who were blinded to the data. The tissues dissected from patients were fixed in formalin immediately after harvesting, and embedded in paraffin for further research. Fresh tissue samples obtained were dissected and immediately stored in liquid nitrogen for PCR and Western blot analysis. All participating clinical doctors and patients provided written informed consent prior to the start of the study.

Bioinformatics analysis

The mRNA levels of WISP1 in GC and normal gastric tissues were mined through Oncomine database (www.oncomine.org) and Coexpedia (www.coexpedia.org)[10]. The prognostic value of WISP1 in gastric adenocarcinoma was analyzed through Kaplan-Meier Plotter (kmplot.com/analysis/)[11].

RNA isolation and RT-qPCR

Total RNA was extracted from frozen tissue samples using Trizol reagent (Invitrogen, Carlsbad, CA, United States) according to the manufacturer's guidelines, and reverse transcription was performed using the PrimeScript RT-PCR kit (Takara, Japan)[12]. RT-qPCR was carried out on an ABI 7500 RealTime PCR System (Applied Biosystems, United States) using SYBR Green Master Mix (Takara, Japan), and normalized to levels of β-actin. The primers used in this study are: Forward, 5′-GAA GCAGTCAGCCCTTATG-3′ and reverse, 5′-CTTGGGTGTAGTCCAGAAC-3′ for WISP1; and forward, 5′-CCTGTGGCATCCACGAAACT-3′ and reverse, 5′-GAA GCATTTGCG GTGGACGAT-3′ for β-actin. All reactions were performed at least in triplicate. The $2^{-\Delta\Delta Ct}$ method was used to quantify the relative expression levels of WISP1.

Western blot analysis

Total protein was extracted from GC and paracancerous tissues using RIPA lysis buffer (Pierce, Thermo Scientific, Cramlington, United Kingdom)[13]. Protein concentration was determined with an enhanced bicinchoninic acid assay kit (CWBio, Beijing, China). A total of 40 mg of protein was separated by sodium dodecyl sulfate-

polyacrylamide gel electrophoresis, and transferred to polyvinylidene difluoride membranes (CWBio, Beijing, China). After blocking with 5% non-fat milk for 1 h at room temperature (RT), the membranes were incubated overnight at 4 °C with primary antibodies directed to WISP1 (Abcam, ab178547), XRCC1 (Abcam, ab9147), γH2AX (Abcam, ab2893), and β-actin (Abcam, ab8226). After washing three times with TBST (Tris-buffered saline with Tween 60), the membranes were incubated with horseradish peroxidase-conjugated secondary antibody at 1:5000 dilution for 1.5 h at RT. Protein bands were visualized using an enhanced chemiluminescence system, and exposure of the membranes to X-ray films (Bio-Rad, Hercules, CA, United States). Densitometric analysis was performed using Image Pro-Plus software (Media Cybernetics, United States). Relative protein expression levels were normalized to β-actin.

Immunohistochemistry

Cancerous tissues and adjacent normal tissues were collected from GC patients and subjected to formalin fixation, dehydration, and paraffin embedding. Tissue sections were cut at 5-μm thickness and mounted on glass slides. According to the manufacturer's protocol, IHC staining was performed. In brief, antigen retrieval was performed using a microwave, then the slides were incubated with a primary antibody directed against WISP1 (dilution 1:200, ab178547, Abcam, United States) at 4 °C overnight. Sections were washed in TBST three times for 5 min, and incubated with a rabbit secondary antibody at RT for 1 h. Staining was performed using liquid DAB (3,3′-diaminobenzidine) as the substrate. Three pathologists scored the staining intensity and positive ratio of WISP1 staining in a blinded fashion. By analyzing the percentage of positively stained cells, the sections were graded by five levels: 0 (≤5%), 1 (6%-25%), 2 (26%-50%), 3 (51%-75%), and 4 (>76%). By analyzing the staining intensity, it was graded as negative (0), weak (1), moderate (2), and strong staining (3). A total score, ranging from 0-12, was generated for each case by multiplying the staining intensity score by the score of the extent of the positive cells[14]. The total score of 0-1 was classified as "-", 2-3 as "+", 4-6 as "++", and 6-12 as "+++"[15]. Subsequently, WISP1 expression was divided into two categories: High (scores 4-12) and low (scores 0-3).

RNA interference

Chemically synthesized WISP1 siRNAs (siRNA-1 and siRNA-2) and matched scramble control siRNAs were purchased from RiboBio Company (Guangzhou, China). Their corresponding sequences are: NM_080838.3 (628-646), GGACATCCATACACTCATT and NM_080838.3 (885-903), GGAA TCCCAATGACATCTT. The siRNAs were transiently transfected into MKN45 and AGS cells by using Lipofectamine 3000 reagent (Invitrogen, Carlsbad, CA, United States) according to the manufacturer's instructions. Western blot and qRT-PCR were used to illustrate the protein and mRNA expression levels of WISPI in MKN45 and AGS cells.

CTB assay

CTB assay was used to assess the viability of cells according to the manufacturer's protocol. Briefly, cells at a density of 5000 cells/well were seeded in 96-well plates and cultured in RPMI 1640 medium supplemented with 10% fetal calf serum, 100 U/mL penicillin, and 100 mg/ml streptomycin (Gibco, Grand Island, NY, United States) at 37 °C in a 5% CO_2 incubator for 12 h. Then, the medium was replaced with medium containing oxaliplatin at different concentrations[16]. After treatment with oxaliplatin for 48 h, 10 μL of CTB was added to each well and the plates were incubated for 4 h at 37 °C. Fluorescent signals were recorded on a microplate reader (Thermo Labsystems, Helsinki, Finland).

Statistical analysis

Statistical analyses were performed with R version 3.5.3 software. Differences in WISP1 expression between tumor and normal samples were evaluated using the Student's *t*-test. The associations of WISP1 with clinicopathological features were assessed by the chi-square test or Fisher's exact test when appropriate. The overall survival curve and its significance were determined by Kaplan-Meier survival analysis and log-rank test, respectively. Univariate and multivariate analyses of prognosis were carried out using the Cox proportional hazard regression model. All *P*-values were two-tailed and considered statistically significant when less than 0.05.

RESULTS

WISP1 is up-regulated in GC

Data from the Oncomine database, Gene Expression Omnibus, Coexpedia database, and TCGA were analyzed by the bioinformatics method to determine the differential expression of WISP1 mRNA between GC and normal tissues. As shown in Figure 1, the WISP1 mRNA expression level was remarkably higher in GC tissues compared to normal gastric tissues ($P < 0.05$). Meta-analysis of the 17 datasets from five studies on WISP1 mRNA levels in GC versus normal gastric tissues was performed based on the Oncomine database. Data showed that WISP1 was up-regulated in GC. From the Coexpedia database, WISP1 was not only up-regulated in GC, but also positively associated with clinical stage ($P < 0.05$).

To validate the predictive results, RT-PCR was performed to determine WISP1 mRNA levels in 20 cases of GC and matched normal gastric tissues. Furthermore, Western blot analysis and IHC were performed to determine WISP1 protein levels in 20 cases of GC and normal gastric tissues (Figure 2). Our findings indicated that WISP1 was mainly located in the cytoplasm of GC cells. The highly positive rate of WISP1 staining in GC was 85% (17/20). When compared to matched normal tissues, the expression level of WISP1 protein in GC was dramatically higher ($P < 0.001$).

WISP1 is associated with T stage and chemotherapy outcome

To determine the protein expression of WISP1 in GC, IHC was used on 150 cases of GC samples. Subsequently, to verify the predictive results on the protein level, immunohistochemical staining was performed and the results were statistically analyzed. Our findings showed that positive staining for WISP1 was predominantly distributed in the cytoplasm of GC. Among those cases of GC, 83 (55.33%) GC tissues showed high WISP1 expression, whereas in the remaining 67 (44.67%) cases, WISP1 expression was low. The Chi-square test was used to determine whether there was a statistically significant difference in WISP1 expression between different groups of GC patients based on the following parameters: Age, gender, tumor differentiation, T stage, N stage, TNM stage, and chemotherapy outcome. The results are presented in Table 1. The differential expression of WISP1 protein (high versus low) was dramatically related to T stage and chemotherapy outcome of GC patients ($P < 0.05$).

Upregulated WISP1 expression predicts a poor prognosis of GC patients

We next investigated whether overexpression of WISP1 is associated with the prognosis of GC patients. First, through mining the TCGA database and Kaplan-Meier Plotter, we found that compared to patients with low WISP1 mRNA expression, GC patients with high WISP1 mRNA expression had a significantly lower overall survival ($P = 0.023$, Figure 3). Furthermore, GC patients with high expression of WISP1 protein had a dramatically lower overall survival compared to those with low expression of WISP1 protein ($P = 0.0019$, Figure 3B). In addition, Cox univariate and multivariate survival analyses were performed on WISP1 expression levels and patients' clinicopathological parameters. Cox univariate analysis showed that TNM stage (III *vs* I-II), T stage (T3-T4 *vs* T2-T1), N stage (N1-N3 *vs* N0), chemotherapy outcome (SD/PD *vs* CR/PR), and WISP1 (High *vs* Low) were critical parameters affecting the survival time of GC patients (Table 2). Furthermore, Cox multivariate survival analysis indicated that high expression of WISP1 (High *vs* Low), TNM stage (III *vs* I-II), and N stage (N1-N3 *vs* N0) were predictors of unfavorable prognosis in patients with GC ($P < 0.05$, Table 2). Taken together, these findings suggested that WISP1 could be a novel biomarker for overall survival.

Inhibiting WISP1 expression reverses drug resistance by inducing DNA damage in gastric cancer cells

In this study, the best interference primers for MKN45 and AGS cells were selected and used in the oxaliplatin resistance assay. The half maximal IC50 was 183.6 ng/mL in MKN45, and the IC50 was 51.28 after silencing of WISP1. In AGS cells, the IC50 was 423.3 ng/mL, and after silencing of WISP1, the IC50 was 140.4 ng/mL. These findings suggested that inhibition of WISP1 expression significantly enhanced oxaliplatin sensitivity. Oxaliplatin is a cytotoxic drug that induces DNA damage in cells, γH2AX is a marker for DNA double-strand breaks, and XRCC1 is a multidrug resistance marker that plays a crucial role in the repair of DNA single-strand breaks. In MKN45 and AGS cells, inhibition of WISP1 significantly increased γH2AX expression, and inhibited XRCC1 expression. This suggested that inhibition of WISP1 reversed resistance by enhancing DNA damage repair.

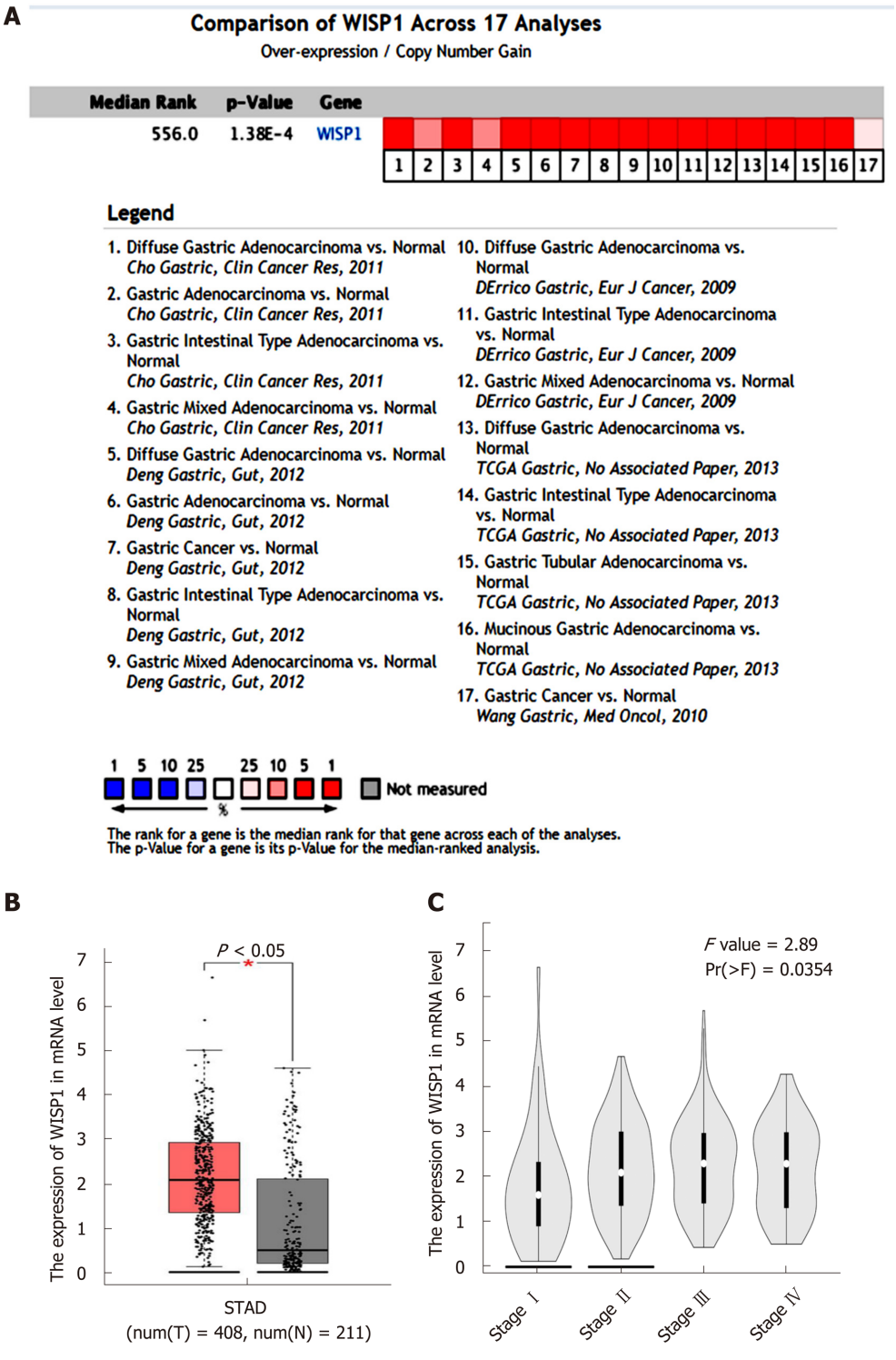

Figure 1 High expression of Wnt1-inducible signaling pathway protein 1 mRNA in gastric cancer predicted based on Oncomine and Coexpedia databases. A: Meta-analysis of 17 datasets from five studies on Wnt1-inducible signaling pathway protein 1 (WISP1) mRNA levels in gastric cancer (GC) *vs* normal gastric tissue based on the Oncomine database; B: WISP1 mRNA levels of GC *vs* normal gastric tissue in Coexpedia databases from The Cancer Genome Atlas (TCGA) ($P < 0.05$); C: The expression of WISP1 mRNA was associated with clinical stage in Coexpedia database from TCGA ($P < 0.05$). WISP1: Wnt1-inducible signaling pathway protein 1; TCGA: The Cancer Genome Atlas; STAD: Stomach adenocarcinoma.

DISCUSSION

The molecular mechanisms underlying the carcinogenesis and development of GC have not yet been elucidated. Therefore, GC remain a heavy health issue for decades, and novel anticancer targets and drugs with prognostic value are imperatively needed to explore. WISP1, encoded by the WISP1 gene, is also known as CCN4[7]. The WNT1 inducible signaling pathway (WISP) protein subfamily regulates diverse

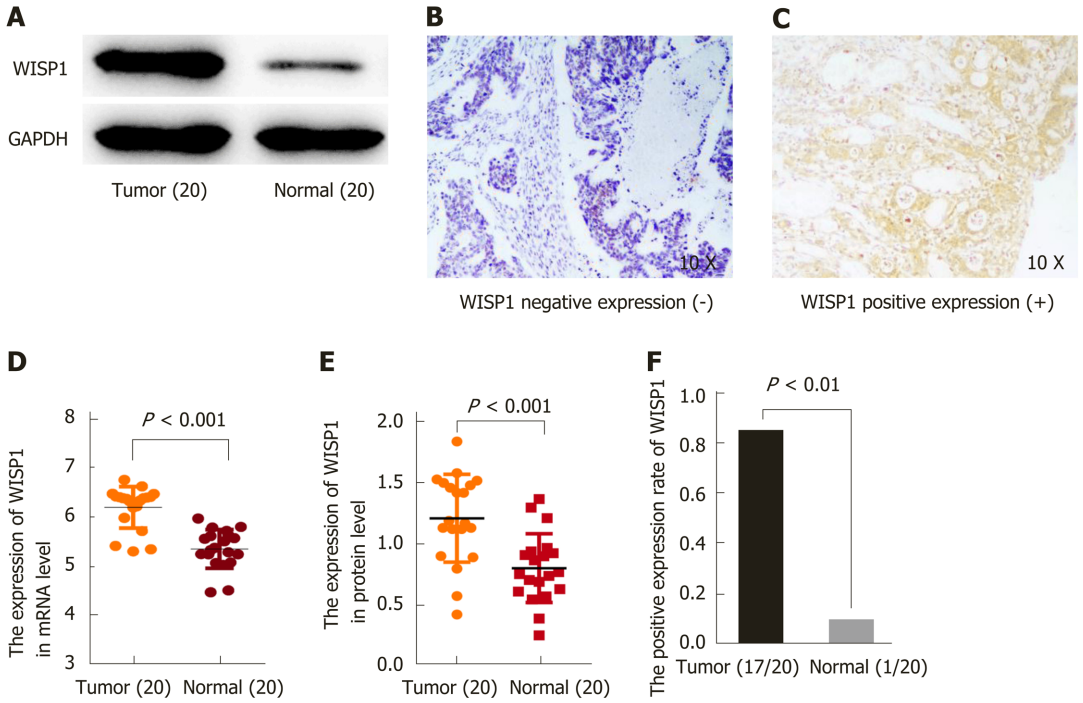

Figure 2 Expression of Wnt1-inducible signaling pathway protein 1 at the mRNA and protein levels is upregulated in gastric cancer patients. A: Western blot analysis showed the expression of Wnt1-inducible signaling pathway protein 1 (WISP1) at the protein level in 20 samples; B: Negative expression of WISP1 in gastric cancer by immunohistochemical staining; C: Positive expression of WISP1 in gastric cancer by immunohistochemical staining; D: Statistical results of WISP1 expression at the mRNA level in 20 samples; E: Statistical results of WISP1 expression at the protein level in 20 samples; F: Statistic results of the immunohistochemical staining for WISP1 at the protein level in 20 samples. WISP1: Wnt1-inducible signaling pathway protein 1.

cellular functions, including cell adhesion, migration, proliferation, differentiation, and survival. WISP1 expression has been shown to promote proliferation, and high WISP1 expression correlates with advanced tumors of the breast, colon, brain, and lung. WISP1 acts as an oncogene by promoting proliferation, migration, and invasion in GC cells[9]. Although the role of WISP1 has been demonstrated in cancer progression, its prognostic value in clinical samples of GC remains unclear.

To address this issue, we first evaluated the expression of WISP1 in GC compared to normal tissues through bioinformatics analysis. The mRNA levels of WISP1 in GC and normal tissues were analyzed using the Oncomine database (www.oncomine.org). The meta-analysis of all datasets from five studies in the Oncomine database demonstrated that WISP1 mRNA levels in GC versus normal gastric tissues were significantly upregulated. Coexpedia is an analysis platform for exploring biomedical hypotheses *via* co-expression associated with medical subject headings. Coexpedia demonstrated that WISP1 was not only up-regulated in GC, but also positively associated with clinical stage ($P < 0.05$). Next, to verify these findings in Chinese GC patients, we analyzed the expression of WISP1 at the mRNA level using RT-qPCR. At the protein level, WISP1 expression was detected by Western blot analysis and IHC. Our results confirmed that WISP1 was significantly up-regulated in GC compared to normal tissues at both the mRNA and protein levels. Taken together, these findings strongly showed that WISP1 expression is remarkably upregulated in GC at both the mRNA and protein levels.

Next, we explored the relationship of WISP1 protein expression with the clinicopathological parameters of GC. The Kaplan-Meier plotter is a meta-analysis tool to assess the effect of 54675 genes on survival using 10461 cancer samples[17]. In Kaplan-Meier Plotter analysis for GC, we found that GC patients with high WISP1 mRNA expression had a significantly lower overall survival ($P < 0.05$, Figure 3A), suggesting that WISP1 might be a novel prognostic biomarker. In addition, IHC was performed to determine WISP1 protein expression in GC tissues. We analyzed the association of WISP1 protein expression and clinicopathological factors of GC patients by the chi-square test. The expression of WISP1 was significantly associated with T stage and chemotherapy outcome. However, no significant differences were found between age, gender, tumor differentiation, N stage, or TNM stage. The clinical prognostic significance of WISP1 expression in patients with GC was investigated using Kaplan-Meier survival analysis, which showed that patients with high WISP1 expression had a significantly poor overall survival when compared to those with low

Table 1 Correlation between Wnt1-inducible signaling pathway protein 1 expression and clinicopathological variables in gastric cancer patients

Variable		Number	Low expression (*n* = 67)	High expression (*n* = 83)	*P* value
Age (yr)					
	< 60	63	25	38	0.299
	≥ 60	87	42	45	
Gender					
	Male	69	30	39	0.789
	Female	81	37	44	
Tumor differentiation					
	Well/moderate	80	33	47	0.372
	Poor	70	34	36	
T stage					
	T1-T2	81	45	36	0.003
	T3-T4	69	22	47	
N stage					
	N0	39	16	23	0.598
	N1-N3	111	51	60	
TNM stage					
	I-II	109	49	60	0.909
	III	41	18	23	
Chemotherapy outcome					
	CR/PR	67	38	29	0.019
	SD/PD	83	30	53	

CR: Complete response; PR: Partial response; SD: Stable disease; PD: Progressive disease; TNM: Tumor node metastasis.

WISP1 expression. Cox multivariate analysis showed that high expression of WISP1, advanced TNM stage, and N stage were independent unfavorable risk factors in GC. Overall, these findings suggested that WISP1 might be a prognostic biomarker for GC, and might be involved in chemotherapy outcome of GC.

Because WISP1 was associated with the effects of chemotherapy treatment, we speculated that WISP1 might be a primary drug-resistant gene for chemotherapy. Oxaliplatin is commonly used in chemotherapy for GC, which is used for DNA damage, and is cross-linked to DNA, antagonizing its replication and transcription[18,19]. The sensitivity of MKN45 and AGS cells to oxaliplatin was significantly enhanced after inhibiting WISP1 (Figure 4). γH2AX is a sensitive molecular marker of DNA damage and repair[20,21]. XRCC1 is a mediator of single-strand breaks DNA repair[22-24], playing a pivotal role in drug resistance by promoting DNA damage repair in cancer cells. After inhibiting WISP1, γH2AX significantly increased and XRCC1 significantly decreased. These findings suggested that inhibiting WISP1 and enhancing the toxicity of oxaliplatin on GC cells enhanced the sensitivity of oxaliplatin by reducing DNA repair and enhancing cell DNA damage.

This study had some limitations. First, the sample size used in this research was limited and future studies from multiple centers and an increased sample number would be needed. Second, the effect of WISP1 siRNA on the sensitivity of the standard regimen, *i.e.*, 5-FU and cisplatin, warrants further investigation. Third, the detailed molecular mechanisms of WISP1 in GC need further exploration.

Table 2 Univariate and multivariate analyses of prognostic factors for gastric cancer

Variable	Univariate analysis		Multivariate analysis	
	HR (95%CI)	*P* value	HR (95%CI)	*P* value
Age (> 60 *vs* < 60)	1.003 (0.618-1.628)	0.991		
Gender (Female *vs* Male)	0.874 (0.639-1.195)	0.400		
Differentiation (Poorly *vs* Well/moderately)	1.236 (0.760-2.009)	0. 393		
TNM stage (III *vs* I-II)	3.714 (2.261-6.102)	< 0.001	2.676 (1.236-5.793)	0.013
T stage (T3-T4 *vs* T2-T1)	1.931 (1.178-3.165)	0.009		
N stage (N1-N3 *vs* N0)	2.666 (1.599-4.448)	< 0.001	2.089 (1.169-3.732)	0.013
Chemotherapy outcome (SD/PD *vs* CR/PR)	3.254 (1.885-5.616)	< 0.001		
WISP1 (High *vs* Low)	2.341 (1.385-3.956)	0.0019	2.180 (1.187-4.003)	0.012

CR: Complete response; PR: Partial response; SD: Stable disease; PD: Progressive disease; TNM: Tumor-node-metastasis; CI: Confidence interval; HR: Hazard ratio.

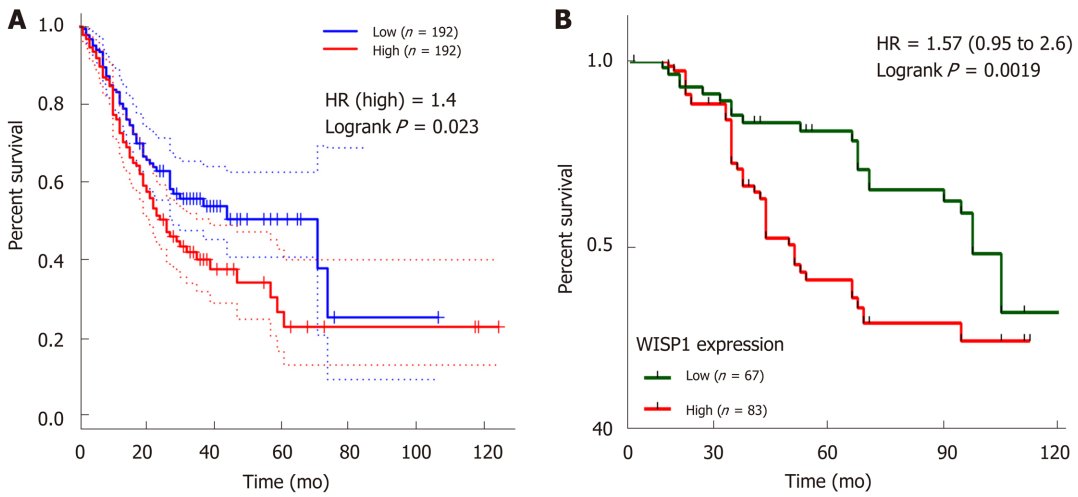

Figure 3 Wnt1-inducible signaling pathway protein 1 expression is associated with overall survival in gastric cancer. A: Kaplan-Meier survival curves plotted with Coexpedia by stratifying patients into high and low Wnt1-inducible signaling pathway protein 1 (WISP1) expression groups based on the median expression value at the mRNA level; B: Kaplan-Meier survival curves generated by WISP1 expression at the protein level. CI: Confidence interval; HR: Hazard ratio; WISP1: Wnt1-inducible signaling pathway protein 1.

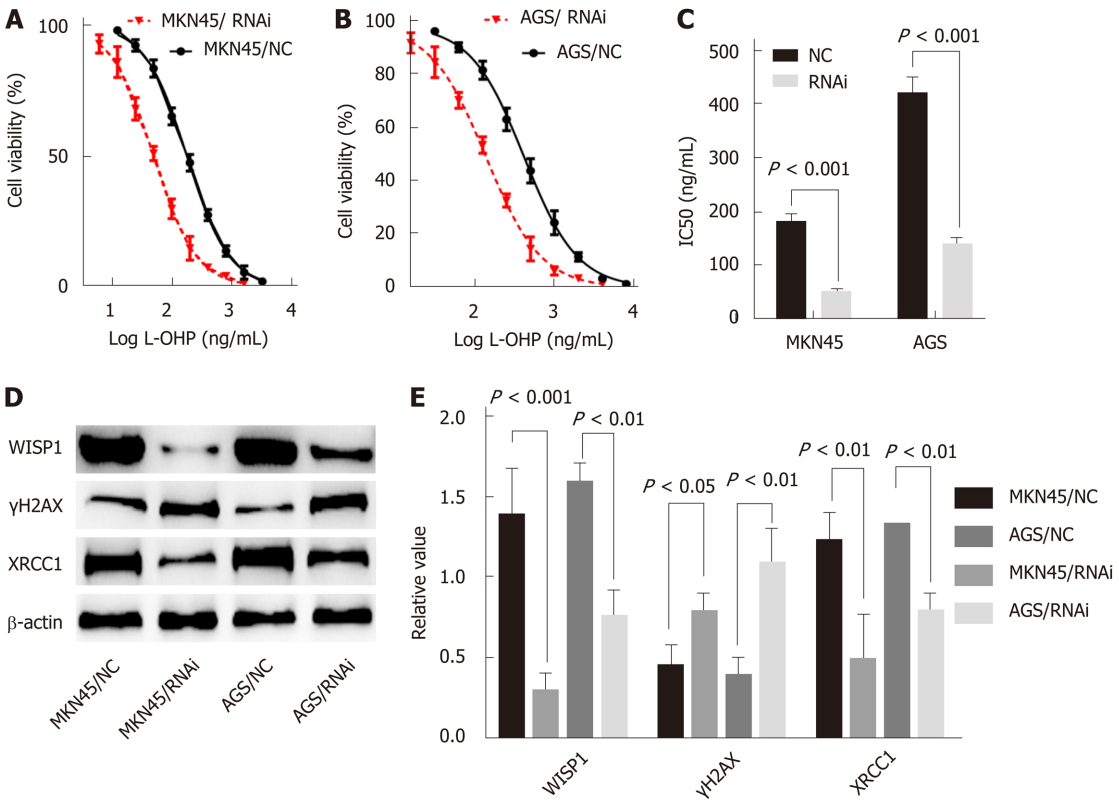

Figure 4 Mechanistic role of Wnt1-inducible signaling pathway protein 1 in gastric cancer cells. A and B: Silencing of Wnt1-inducible signaling pathway protein 1 (WISP1) has effects on cell viability of MKN45 and AGS cells; C: Silencing of WISP1 results in a significant decrease in the IC50 value; D and E: Silencing of WISP1 results in γH2AX overexpression and XRCC1 downregulation in MKN45 and AGS cells. NC: The matching scramble control siRNAs were transfected into cell lines as normal controls; RNAi: The siRNAs were transfected into cell lines as RNA interference; WISP1: Wnt1-inducible signaling pathway protein 1.

ARTICLE HIGHLIGHTS

Research background

Gastric cancer (GC) is the most prevalent gastrointestinal tract malignancy. The prognosis of GC patients remains relatively poor. Through bioinformatics data mining and integrated analysis, we found that Wnt1-inducible signaling pathway protein 1 (WISP1) mRNA was upregulated in GC tissues relative to normal gastric tissues. However, it needs to be further verified clinically.

Research motivation

There are insufficient reports about the correlation between WISP1 and GC.

Research objectives

The aim of the present study was to explore the expression pattern and clinical significance of WISP1 in GC.

Research methods

Public data portals, including Oncomine, the TCGA database, COEXPEDIA, and Kaplan-Meier plotter were analyzed for the expression and clinical significance of WISP1 mRNA levels in GC. One hundred and fifty patients who underwent surgery for GC between February 2010 and October 2012 at the Affiliated Hospital of Jiangnan University were selected for validation study. WISP1 expression was measured at both the mRNA and protein levels by RT-qPCR, Western blot analysis, and immunohistochemistry (IHC). The correlation of WISP1 expression status with patient prognosis was then determined by univariate and multivariate Cox regression analyses. WISP1 was knocked down by RNA interference. IC50 was detected by CTB assay.

Research results

WISP1 expression at both the mRNA and protein levels was remarkably upregulated in GC tissues compared to normal tissues. Moreover, IHC revealed that WISP1 expression was associated with T stage and chemotherapy outcome, but not with lymph node metastasis, distant metastasis, age, sex, histological grade, or histological type. GC patients with high WISP1 expression showed a poor overall survival. Multivariate survival analysis indicated that WISP1 was an important prognostic factor for GC patients. The IC50 of oxaliplatin in MKN45 and AGS cell lines were significantly reduced after WISP1 knock-down. In addition, WISP1 knock-down enhanced γH2AX expression and reduced XRCC1 expression.

Research conclusions

WISP1 is overexpressed in GC tissues and is associated with a poor prognosis, indicating its potential as a novel prognosis biomarker for GC. Mechanistically, knock-down of WISP1 expression enhances oxaliplatin sensitivity by reducing DNA repair and enhancing DNA damage. WISP1 may be a potential therapeutic target for GC treatment.

Research perspectives

The present study suggested that WISP1 is a novel prognostic biomarker for GC, and the significance of WISP1 as a promising therapeutic target for GC is highlighted.

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ORIGINAL ARTICLE

Retrospective Study

Hepatitis C virus clearance and less liver damage in patients with high cholesterol, low-density lipoprotein cholesterol and *APOE ε4* allele

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Author contributions: Panduro A conceived the study and clinically evaluated all patients. Gonzalez-Aldaco K carried out experimental work, statistical analysis, and drafted the manuscript. Roman S revised all experimental data and copyedited the English version. Torres-Valadez R carried out clinical and laboratory work. Ojeda-Granados C performed the clinical nutrition evaluation, and Torres-Reyes LA revised all biostatistical analysis. All authors contributed intelligently, critically revised, and approved the final version of the manuscript.

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Abstract

BACKGROUND

Cholesterol is related to improvements in the rate of sustained virological response and a robust immune response against the hepatitis C virus (HCV). *APOE* gene polymorphisms regulate cholesterol levels modifying the course of the HCV infection. The relationship between cholesterol, *APOE* alleles, and the outcome of HCV infection has not been evaluated in the admixed population of Mexico.

AIM

To investigate the role of *APOE* -ε2, -ε3, and -ε4 alleles and the metabolic profile in the outcome of HCV infection.

METHODS

A total of 299 treatment-naïve HCV patients were included in this retrospective study. Patients were stratified in chronic hepatitis C (CHC) (n = 206) and spontaneous clearance (SC) (n = 93). A clinical record was registered. Biochemical tests were assessed by dry chemistry assay. *APOE* genotypes were determined using a Real-Time polymerase chain reaction assay.

RESULTS

Total cholesterol, low-density lipoprotein cholesterol (LDL-c), triglycerides, and hypercholesterolemia were higher in SC than CHC patients as well as the frequency of the *APOE* ε4 allele (12.4% *vs* 7.3%). SC patients were overweight (54.8%). The ε4 allele was associated with SC (OR = 0.55, 95%CI: 0.31-0.98, P = 0.042) and mild fibrosis (F1-F2) in CHC patients (OR 0.091, 95%CI 0.01-0.75, P =

0.020). LDL-c ≥ 101.5 mg/dL (OR = 0.20, 95%CI: 0.10-0.41, $P < 0.001$) and BMI ≥ 26.6 kg/m^2 (OR= 0.37, 95%CI: 0.18-0.76, $P < 0.001$) were associated with SC status; while ALT ≥ 50.5 IU/L was negatively associated (OR = 5.67, 95%CI: 2.69-11.97, $P < 0.001$).

CONCLUSION

In SC patients, the *APOE ε4* allele and LDL-c conferred a protective effect in the course of the HCV infection in the context of excess body weight.

Key words: Liver damage; Body mass index; Spontaneous hepatitis C virus clearance; Low-density lipoprotein; Cholesterol

Core tip: Cholesterol is a metabolic regulator of the hepatitis C virus (HCV) life cycle. Genetic polymorphisms in the *APOE* gene can regulate cholesterol and modify the outcome of the HCV infection. Our findings suggest that *APOE ε4* allele and low-density lipoprotein cholesterol (LDL-c) confer a protective effect in the course of the HCV infection in the context of high body mass index (BMI). Levels of LDL-c, BMI, and ALT may estimate the risk of chronicity in HCV-infected patients. An individualized therapy accounting the host´s genetic, environmental, and metabolic factors could aid in the clinical management of HCV infection, especially in populations with a high prevalence of overweight and obesity.

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INTRODUCTION

Hepatitis C virus (HCV) is a significant health problem causing chronic liver diseases worldwide. According to the World Health Organization (WHO), 71 million people are chronically infected, and 399,000 deaths each year are related to HCV infection[1]. Estimates are that up to 90% of the infected individuals are unaware of their status of infection[2]. In approximately 25-30 years, chronic HCV infection may progressively lead to a broad spectrum of clinical outcomes such as fibrosis, cirrhosis, and in some cases, hepatocellular carcinoma[3]. However, some patients (20%-40%) may resolve an acute infection by self-spontaneous clearance of the virus, evidenced by positive anti-HCV antibodies and negative viral RNA in the serum[4]. This rate is variable due to a combination of the immunologic, metabolic, and genetic factors of the host[5].

In particular, plasmatic levels of total cholesterol (TC) and low-density lipoprotein cholesterol (LDL-c) have been reported as predictors of the response to interferon therapy during HCV infection[6]. Likewise, the Apolipoprotein E (*APOE*) gene encoding the glycoprotein component of the low-density lipoprotein has also been implicated in the outcome of HCV infection and associated comorbidities[7]. HCV binds to the ApoE ligand entering the hepatocyte via the low-density lipoprotein receptor (LDLR)[8]. Two functional polymorphisms rs429358 and rs7412 in the *APOE* gene lead to three common alleles, *ε2*, *ε3* and *ε4*, encoding the corresponding major isoforms, ApoE -E2, -E3 and -E4[9]. ApoE3 is the wild-type isoform with a natural affinity for the LDLR, while ApoE2 and ApoE4 present opposed binding abilities. ApoE2 isoform has a significantly decreased attachment to the LDLR. Conversely, the ApoE4 isoform confers increased binding to LDLR compared to ApoE2 and ApoE3[10]. These relative binding properties are consistent with findings that suggest a protective effect of *APOE ε4* in the progression of liver damage as revealed by histopathological analysis[11], whereas *APOE ε3* has been associated with the persistence of the infection[12].

There is growing evidence of the occurrence of dyslipidemia in HCV-infected patients[13]. Therefore, changes in body weight may have a meaningful impact on the

management of these patients. Currently, Mexico and the United States are experiencing a significant adult obesity health problem[14]. In Mexico, 72.5% of the adult population present overweight or obesity[15]. This increase in body mass index (BMI) is associated with the development of several metabolic abnormalities including dyslipidemias such as, hypercholesterolemia (HChol), which is one of the eight most important risk factors of mortality in Mexico[16]. Both obesity and dyslipidemia are associated with environmental risk factors such as diet. Recently, we described that the dietary pattern of the general Mexican population and HCV-infected patients promote the development of lipid abnormalities[17]. On the other hand, the *APOE ε4* allele that is associated with HChol has a heterogeneous prevalence at the national level ranging from 0-20.3%[18]. However, the relationship between *APOE* alleles and lipid metabolism, as well as its potential implication in HCV infection among the Mexican population is currently unknown.

West Mexico is a region characterized by a genetically admixed population with Amerindian, European, and less extensively African ancestries[19]. Given the variability of *APOE* alleles observed by ethnicity[20,21], it is plausible that differences in the genetic and environmental factors of the Mexican population may influence the relationship between *APOE*, lipid abnormalities and outcome of HCV infection. Therefore, this study aimed to investigate the role of *APOE ε*2, - *ε3*, and - *ε4* alleles and the metabolic profile in the outcome of HCV-infected patients in West Mexico.

MATERIALS AND METHODS

Patients and study design

In this retrospective study, adult patients who were anti-HCV positive, un-related, and treatment-naive were enrolled from January 2014 to December 2016 at the Department of Molecular Biology in Medicine, Civil Hospital of Guadalajara "Fray Antonio Alcalde". The exclusion criteria were chronic hepatitis B virus infection or human immunodeficiency virus infection, autoimmune disease, Child-Pugh class B and C, Wilson´s disease, hemochromatosis, drinkers, and use of hypolipidemic drugs.

A physician elaborated all medical records in which demographics, clinical data, risk factors for the acquisition of viral hepatitis, and laboratory test results were registered. Patients were serologically tested for anti-HCV antibodies (Third-generation ELISA, AxSYM®, Abbott Laboratories, IL, United States) and quantitative assessment of serum RNA was performed by a standardized quantitative reverse PCR assay (Roche COBAS® AmpliPrep and COBAS® TaqMan 48 HCV test, Pleasanton, CA, United States). After testing, the study population was divided into two groups: Spontaneous clearance (SC) patients (n = 93) who had at least two undetectable serum HCV RNA results in the last 12 months with a six-month interval between each test. Chronic hepatitis C infection (CHC) patients (n = 206) had two detectable serum HCV RNA results during the preceding 12 months with a six-month interval between each test.

Time of evolution was estimated as the elapsed time between the date of diagnosis and first exposure to risk. Patients had not been previously diagnosed at the time of the study.

Anthropometric assessment

Body mass index (kg/m^2) was estimated using electrical bio-impedance (InBody3.0, Analyzer Body Composition, Biospace, South Korea). Normal weight was > 18.5-24.99 kg/m^2, overweight > 25-29.99 kg/m^2 and obesity > 30 kg/m^2 as defined by the WHO[22].

Liver stiffness measurement by transitional elastography

Liver stiffness measurement was assessed by a certified physician using transitional elastography (TE) (FibroScan®, Echosens, Paris, France). Liver stiffness was calculated as the median value of ten valid TE measurements expressed in kilopascals (kPa) indicating liver fibrosis according to the following classification: F1, mild fibrosis (7.1-8.7 kPa), F2, moderate fibrosis (8.8-9.4 kPa), F3, severe fibrosis (9.5-12.4 kPa) and F4, cirrhosis (> 12.5 kPa)[23].

Biochemical measurements

Ten mL of blood samples were drawn after a 12-h fast. Biochemical measurements of TC, triglycerides (TG), high-density lipoprotein cholesterol (HDL-c), aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were performed using a Vitros 250 Analyzer (Ortho-Clinical Diagnostic, Johnson & Johnson, Rochester, NY, USA). Commercial control serum and human pooled serum were used to ensure the accuracy of the biochemical measurements. LDL-c concentration was calculated using

the Friedewald formula[24], and very-low-density lipoprotein cholesterol (VLDL-c) concentration was calculated as TC-(LDL-c + HDL-c). Fasting insulin levels were measured by an enzyme-linked immunosorbent assay (Monobind Inc, Texas, United States). The homeostatic model assessment of insulin resistance (HOMA-IR) was calculated as the following formula: (fasting insulin (μU/mL) x fasting glucose (mg/dL)/405[25].

Lipid abnormalities

Lipid abnormalities were defined according to the National Cholesterol Education Program ATP III criteria and the Mexican Official Norm-037 (NOM-037)[26,27]. Hypertriglyceridemia (HTG) ≥ 150 mg/dL, HChol ≥ 200 mg/dL, hypo-alphalipoproteinemia (HALP) ≤ 40 mg/dL for men and ≤ 50 mg/dL for women, high LDL ≥ 130 mg/dL. Insulin resistance was defined as HOMA-IR > 2.5.

APOE genotyping

Genomic DNA was extracted from peripheral whole blood leukocytes using the salting-out method and stored at -80 °C until use. The *APOE* genotype was determined using a 5′ allelic discrimination method[28]. The reactions were carried out using two TaqMan® SNP Genotyping Assays (rs429358 C_3084793_20 and rs7412 C_904973_10, Applied Biosystems, Foster, CA, USA). Cycle conditions were an initial enzyme activation for 10 min at 95 °C followed by 40 cycles of denaturalization for 15 s at 95 °C and alignment/extension for 1 min at 60 °C in a StepOnePlus thermocycler (Applied Biosystems, Foster, CA, USA). Genotypes were verified using positive and negative controls. Twenty percent of the samples were genotyped in duplicate, and 100% of concordance was observed.

Statistical analysis

Quantitative variables are expressed as mean ± SD and were compared by student´s *t*-test. Categorical variables are expressed as number and percentage and were analyzed by Chi-square or Fisher´s exact test. The normal distribution of the quantitative variables was tested with Kolmogorov-Smirnov or Shapiro-Wilks test if the number of cases was more or less than 30, respectively. The *APOE* allelic frequencies were obtained by direct counting method. The Hardy-Weinberg Equilibrium (HWE) expectation was assessed by the software Arlequin version 3.1[29]. The contribution of the *APOE* alleles to lipid profile in SC and CHC patients was analyzed as *APOE* genotype groups: *E2*: *ε2ε2* + *ε2ε3* + *ε2ε4*, *E3*: *ε3ε3* and *E4*: *ε3ε4* + *ε4ε4*.

The variables associated with HCV status were identified using univariate and multivariate logistic regression analysis. The results were expressed as odds ratio (OR) with a 95% confidence interval (CI). We also tested the goodness of fit of the regression model using the Hosmer-Lemeshow method[30]. The area under the receiver-operating characteristic (ROC) curve analysis was computed to select the corresponding thresholds for variables associated with SC. The sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) were computed for the selected cutoffs using viral load as a reference variable. Statistical analyses were performed using Epi Info™ 7.1.2.0 (CDC, Atlanta, USA) and IBM SPSS Statistics version 21.0 for Windows (IBM Corp, Inc., Chicago, IL, United States). A *P* value <0.05 was considered statistically significant. An expert biostatistician revised the statistical analysis.

Ethics

The study protocol complied with the ethical guidelines established in the Declaration of Helsinki and was approved by the Institutional Review Board, Health Sciences Center, University of Guadalajara, Certificate #CI-00612. All participants signed informed consent before participating in the study.

RESULTS

Characteristics of the study population

The demographic and clinical features of the study population were compared, as shown in Table 1. No significant differences in age, gender, and BMI were found between CHC and SC groups. Risk factors were essentially similar in both groups except for body piercing in SC. Notably, CHC patients were more normal weight than SC (36.4% *vs* 19.3%, *P* = 0.003), whereas a higher rate of overweight was observed in SC compared to CHC patients (54.8% *vs* 42.2%, *P* = 0.042). HOMA-IR tended to be comparatively higher in CHC than in SC patients (55.4% *vs* 43.0%, *P* = 0.072). According to the TE, 62.5% and 29.5% of the SC and CHC patients, respectively had fibrosis stage F1 (*P* = 0.001). On the other hand, 12.5% and 38.5% of the SC and CHC

patients, respectively presented fibrosis stage F4 (P = 0.002). The levels of LDL-c, TC, and TG, as well as the rate of lipid abnormalities (HChol, abnormal LDL-c, and HTG), were higher in SC patients compared to CHC patients (P < 0.001). Conversely, both AST and ALT were significantly increased in CHC patients than SC patients.

Distribution of APOE alleles, association of APOE ε4 allele with SC and fibrosis stage

Overall, *APOE ε4* allele was present in 8.8% of the study population, as shown in Table 2. The frequency of the *APOE* alleles was concordant with the HWE (P > 0.05). A higher prevalence of the *ε4* allele was found in SC (12.4%) compared to CHC (7.3%) patients, and it was associated with an increased likelihood of SC (OR = 0.55, 95%CI: 0.31-0.98, P = 0.042). Also, the *APOE ε4* allele was associated with mild fibrosis (F1-F2) in CHC patients (OR = 0.091, 95%CI: 0.01-0.75, P = 0.020). In contrast, the *APOE ε3* allele was associated with 2.99-fold risk (95%CI: 1.13-7.87, P = 0.021) for severe liver damage (F3-F4). CHC patient carriers of *APOE ε4* allele had lower serum levels of AST and ALT than the *APOE ε3* allele carriers (59.7 IU/L *vs* 79.1 IU/L, P = 0.041 and 53.2 IU/L *vs* 88.36 IU/L, P = 0.046, respectively) (data not shown).

Effect of APOE genotype groups on the lipid profile of CHC and SC patients

In SC patients, being a carrier of the *E4* genotype increased the plasma levels of TC and LDL-c (P < 0.05). Meanwhile, in the CHC patients, the *E4* genotype increased the levels of HDL-c and the prevalence of HChol (Table 3).

Effect of lipid profile on spontaneous HCV clearance status

Univariate and multivariate analysis of TC, LDL-c, BMI, and other relevant biochemical variables were performed to clarify whether they were related to SC status (Table 4). Multivariable analysis identified LDL-c, BMI, TG, and ALT as significantly associated with SC status (P < 0.05). A ROC curve analysis was performed to determine the optimal threshold values of LDL-c, BMI, TG, and ALT and their association with SC status. For practical applications, sensitivity, specificity, NPV, and PPV were also calculated (Table 5). Finally, the cutoffs were used to convert these variables into dichotomous variables, and a new multivariate analysis was carried out. This final model identified that LDL-c ≥ 101.5 mg/dL and BMI ≥ 26.6 kg/m^2 were better predictors of SC, whereas ALT ≥ 50.5 IU/L was negatively associated with SC status (Figure 1).

DISCUSSION

The interplay between lipids/lipoproteins and HCV can modulate HCV infection. For example, cholesterol improves the rate of sustained virological response and immune response against HCV[6]. Also, cell entry is achieved by the virus in the form of lipo-viro-particles associated with ApoE. On the other hand, the three *APOE* alleles (*ε2*, *ε3*, and *ε4)* portray distinct biological properties that mediate lipid levels by interacting with environmental factors such as diet. These alleles also have a heterogeneous distribution worldwide[20]. Currently, a high prevalence of lipid alterations in the context of the obesity epidemic and an uneven distribution of the *APOE* alleles is notorious among the Mexican population. These factors prompted us to seek if the differences in the *APOE* alleles and lipid profile were associated with the outcome of HCV infection. To the best of our knowledge, this study is the first in reporting the effect of *APOE* alleles in the course of HCV infection in a Native American-derived population. Our results showed that the *APOE* allele distribution in the admixed population of West Mexico agrees with our previous work[31], and the overall high frequency of the *APOE ε4* allele was consistent with the Native Amerindian ancestry of the study group[32]. However, on further analysis, the prevalence of *APOE ε4* allele was higher in SC patients compared to CHC patients and correlated with the lipid profile and fibrosis stage.

Lipo-viro-particles bind to hepatic receptors such as LDLR and Scavenger Receptor class B type 1[33]. In the case of the *APOE ε4* allele, it indirectly regulates lipoprotein levels by reducing the expression of LDLR in the hepatocyte surface[34]. Increased LDL-c has been demonstrated in healthy carriers of this allele[35]. In this study, the SC group had a higher *ε4* allele prevalence than the CHC patients and was associated with increased levels of TC and LDL-c. These high levels of LDL-c may compete with the lipo-viro-particles for the binding to the LDLR, thus decreasing the entry of the virus. Also, downregulation of the LDLR may hinder viral entry, thus preventing the early stages of infection and diminishing the progression of liver damage.

In agreement with these biological mechanisms mentioned above, in this study, the SC and CHC patients who were ε4 allele carriers also had less liver damage.

Table 1 Demographic and clinical characteristics of hepatitis C virus patients, *n* (%)

Variable	Chronic, *n* = 206	Clearance, *n* = 93	*P* value
Demographic and clinical data			
Age, yr (mean ± SD) (range)	51.0 ± 12.0 (20-78)	47.1 ± 13.0 (21-74)	0.100
Female sex	123 (60)	48 (52)	0.236
Time of evolution, yr	18.0 ± 14.5	20.2 ± 15.6	0.156
BMI, kg/m^2 (mean ± SD)	27.0 ± 6.0	28.0 ± 4.1	0.098
Normal weight	76 (36.4)	19 (19.3)	0.003
Overweight	87 (42.2)	51 (54.8)	0.042
Obesity	43 (20.8)	23 (24.7)	0.456
Glucose, mg/dL	105.6 ± 43.6	101.6 ± 33.0	0.46
HOMA-IR > 2.5	114 (55.4)	40 (43.0)	0.072
Type 2 diabetes	27 (13.1)	7 (7.5)	0.183
Biochemistry			
AST, IU/L	74.2 ± 53.4	30.9 ± 14.4	< 0.001
ALT, IU/L	76.4 ± 66.7	31.5 ± 19.8	< 0.001
Lipid profile			
Total cholesterol, mg/dL	148.1 ± 43.3	184.1 ± 43.1	< 0.001
LDL-c, mg/dL	83.7 ± 37.2	112.4 ± 35.4	< 0.001
Triglycerides, mg/dL	127.7 ± 61.3	168.2 ± 80.3	< 0.001
VLDL-c, mg/dL	25.8 ± 14.3	33.3 ± 15.8	0.001
HDL-c, mg/dL	39.7 ± 13.5	41.9 ± 17.7	0.766
Lipid abnormality			
Hypercholesterolemia	20 (9.8)	30 (32.2)	< 0.001
High LDL-c	20 (9.7)	24 (25.8)	< 0.001
Hypertriglyceridemia	60 (29.1)	45 (48.3)	0.001
Hypoalphalipoproteinemia	85 (41.3)	44 (47.3)	0.328
Viral genotype			
HCV genotype 1	138 (66.9)	Not determined	-
Non-genotype 1	68 (33.1)		
Fibrosis stage[1]			
F1	26 (29.5)	30 (62.5)	< 0.001
F2	19 (21.8)	11 (22.9)	0.883
F3	9 (10.3)	1 (2.1)	0.083
F4	33 (38.5)	6 (12.5)	0.002
Risk factors for HCV infection			
Surgeries	144 (69.9)	62 (66.7)	0.164
Blood transfusion	119 (58)	36 (38.7)	0.169
Tattooing	49 (23.7)	19 (20.4)	0.375
Dental procedure	49 (23.7)	18 (19.3)	0.28
Sexual promiscuity	41 (19.9)	14 (15.0)	0.702
Acupuncture	28 (13.5)	8 (8.6)	0.464
Injection drug use	27 (13.1)	10 (10.7)	0.932
Body piercing	4 (1.9)	8 (8.6)	0.002

[1]Liver damage was assessed in 87 chronic hepatitis C and 48 spontaneous clearance patients. BMI: Body mass index; HOMA-IR: Homeostatic model assessment insulin resistance; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase.

Furthermore, CHC patients who were carriers of the *APOE ε4* allele had the lowest levels of AST and ALT in comparison with the *APOE ε3* allele carriers. The protective effect of *APOE ε4* found in this study agrees with data reported from other populations with African and European ancestries[11,36,37]. Conversely, *APOE ε3* allele was associated with advanced fibrosis in CHC. This observation agrees with previous data reporting that specifically, ApoE ε3 mediates the HCV immune escape mechanism by blocking the innate immunity-activated ficolin-2 protein, thus

Table 2 *APOE* allele distribution among the study population and stages of fibrosis in chronic hepatitis C patients, *n* (%)

	HCV-patients			Fibrosis stage in CHC patients[4]		
	Chronic (*n* = 206)	Clearance (*n* = 93)	*P* value	F1-F2 (*n* = 49)	F3-F4 (*n* = 38)	*P* value
Genotypes						
ε2ε2	2 (1.0)	0	-	0	0	-
ε2ε3	15 (7.4)	8 (8.6)	0.691	5 (10.5)	2 (5.3)	0.400
ε2ε4	2 (1.0)	0	-	0	0	-
ε3ε3	160 (77.5)	64 (68.8)	0.102	29 (59.2)	34 (89.5)	0.001
ε3ε4	26 (12.7)	19 (20.4)	0.080	14 (28.6)	1 (2.6)	0.001
ε4ε4	1 (0.5)	2 (2.2)	0.181	1 (2)	1 (2.6)	0.969
Alleles						
ε2	21 (5.1)	8 (4.3)	0.674	4 (5.1)	2 (2.6)	0.603
ε3	361 (87.6)	155 (83.3)	0.158	78 (78.6)	70 (93.4)	0.022[2]
ε4	30 (7.3)	23 (12.4)	0.042[1]	16 (16.3)	4 (4)	0.023[3]
HWE	0.438	0.892	-	0.910	0.286	-

[1]ε4 allele was associated with SC OR = 0.55, 95%CI: 0.31-0.98, *P* = 0.042.
[2]ε3 allele was associated with severe fibrosis (F3-F4) OR = 2.99, 95%CI: 1.13-7.87, *P* = 0.021.
[3]ε4 allele was associated with mild fibrosis (F1-F2) OR = 0.091, 95%CI: 0.01-0.75, *P* = 0.020.
[4]Liver damage was assessed in 87 chronic hepatitis C patients. CHC: Chronic hepatitis C; HWE: Hardy-Weinberg Equilibrium.

promoting the progression of the infection[38]. On the other hand, cholesterol and cholesterol derivatives have an immunomodulatory effect against HCV[39,40]. In this study, *APOE ε4* increased the levels of total cholesterol and LDL-c in SC patients and the prevalence of HChol in CHC, thus confirming its participation in the modulation of cholesterol in the course of HCV infection as previously reported[37].

An interesting observation was that LDL-c and BMI were the main variables predicting SC status. This finding is concordant with the higher prevalence of overweight in SC than in CHC patients who were mainly normal weight. Overweight and obesity are conditions that lead to lipid alterations of cholesterol and triglycerides that in turn, evoke insulin resistance[41]. In this study, CHC patients tended to have a better lipid profile but depicted a higher level of HOMA-IR than patients with SC. This data is consistent with the higher prevalence of type 2 diabetes in the CHC patients, in contrast with those who were SC. Moreover, insulin resistance is the hallmark of liver fibrosis. Notably, in this study, the CHC patients who were non-*ε4* allele carriers had a higher stage of fibrosis than their peers with SC. Since the levels of LDL-c, BMI, and ALT were the best predictors of SC, these determinants may be used in the early detection of chronicity in HCV-infected patients.

Diet is another crucial interacting factor related to BMI and lipid alterations. Mexico has experienced a nutrition transition in the past three decades, shifting from a traditional food pattern to a westernized diet, a known factor involved in the obesity epidemic[42]. Current diets are hepatopatogenic containing high amounts of simple sugars and saturated fats that result in HChol and hypertriglyceridemia[17,43,44]. Due to this fact and the estimated time of evolution of the patients, we hypothesized that high BMI and cholesterol levels, which are key factors for SC, might have been present at the time of the acute phase of HCV infection, and that some SC patients remained overweight years after clearing the virus. Also, in the context of HCV infection, high levels of LDL-c correlate with interferon sensitivity which is detected by the production of IFN-gamma-induced protein, a chemokine produced by T cells, natural killer cells, and monocytes[45]. Furthermore, high levels of LDL-c are related to interferon sensitivity in genotype 1[46], allowing an adequate innate immune response against HCV that facilitates spontaneous viral clearance. Nevertheless, further investigation is needed to clarify the mechanisms involved in this association as well as designing prospective studies in patients with acute infection.

The relationship between lipid alterations and the dynamics of HCV infection are also influenced by other genetic polymorphisms. In this sense, *IFNL4* has been associated with SC by modulating LDL-c levels[47]. *CD36* rs1761667 polymorphism was associated with fat perception and advanced fibrosis in Mexican patients with CHC[48]. On the other hand, it may be interesting to investigate if changes in lifestyle such as nutritional interventions could cause cholesterol metabolism disturbances that modify HCV life cycle[44]. In perspective, genetic and environmental factors affecting cholesterol levels may vary significantly worldwide; therefore, we advocate that these

Table 3 Effect of *APOE* alleles on lipid profile and lipid abnormalities of hepatitis C virus patients

	Chronic			Clearance		
	E2 (*n* = 19)	E3 (*n* = 160)	E4 (*n* = 27)	E2 (*n* = 8)	E3 (*n* = 64)	E4 (*n* = 21)
Lipid profile						
TC, mg/dL	140.6 ± 34.1	150.9 ± 45.7	158.3 ± 45.5	142.7 ± 40.3	184.3 ± 41.4[3]	188.9 ± 42[4]
TG, mg/dL	134.4 ± 78.4	130.2 ± 59.6	114.2 ± 53.4	151.2 ± 81.6	169.1 ± 81.1	165.8 ± 79.4
HDL-c, mg/dL	36.9 ± 7.7	38.8 ± 12.9	45.9 ± 17.6[1]	34.7 ± 6.5	42.6 ± 21.1	42.0 ± 9.7
VLDL-c, mg/dL	24.3 ± 11.4	26.7 ± 15.3	22.1 ± 10.0	30.1 ± 16.7	33.2 ± 15.7	33.1 ± 15.9
LDL-c, mg/dL	82.6 ± 29.2	85.6 ± 38.2	98.7 ± 43.5	77.7 ± 29.0	110.1 ± 33.1[5]	121.6 ± 34.7[6]
Lipid abnormalities, *n* (%)						
Hypercholesterolemia	1 (5.3)	13 (8.1)	6 (22.2)[2]	0	19 (29.6)	8 (38.1)
Hypertriglyceridemia	7 (36.8)	43 (26.9)	6 (22.2)	3 (37.5)	26 (40.6)	9 (42.8)
Hypoalphalipoproteinemia	10 (52.6)	75 (46.9)	9 (33.3)	7 (87.5)	30 (46.9)	10 (47.6)
High LDL-c	1 (5.3)	14 (8.7)	5 (18.5)	0	12 (18.7)	7 (33.3)

[1]E4 *vs* E3, $P = 0.033$;
[2]E4 *vs* E3, $P = 0.024$;
[3]E3 *vs* E2, $P = 0.018$;
[4]E4 *vs* E2, $P = 0.014$;
[5]E3 *vs* E2, $P = 0.012$;
[6]E4 *vs* E2, $P = 0.005$. E2: *ε2ε2* + *ε2ε3* + *ε2ε4*; E3: *ε3ε3*; E4: *ε3ε4* + *ε4ε4*. TC: Total cholesterol; TG: Triglycerides; Hchol: Hypercholesterolemia; HTG: Hypertriglyceridemia; HALP: Hypoalphalipoproteinemia.

factors be considered by population for the management of HCV infection. Furthermore, understanding the molecular mechanisms by which LDL-c is implicated in the course of HCV infection could provide valuable information for controlling HCV infection and limiting its expansion.

In conclusion, *APOE ε4* allele and LDL-c confer a protective effect in the course of the HCV infection in the context of high BMI. An individualized therapy accounting the host´s genetic, environmental, and metabolic factors is required to achieve better control of HCV infection, especially in populations with a high prevalence of overweight and obesity.

Table 4 Logistic regression analysis of variables associated with spontaneous hepatitis C virus clearance

Variable	Univariate			Multivariate		
	OR	95%CI	*P* value	OR	95%CI	*P* value
AST, IU/L	1.058	1.040-1.076	< 0.001			
ALT, IU/L	1.04	1.026-1.054	< 0.001	1.037	1.019-1.056	< 0.001
LDL-c, mg/dL	0.981	0.973-0.988	< 0.001	0.977	0.963-0.992	0.002
Total cholesterol, mg/dL	0.983	0.977-0.989	< 0.001			
Triglycerides, mg/dL	0.993	0.989-0.996	< 0.001	0.992	0.986-0.999	0.027
VLDL-c, mg/dL	0.97	0.953-0.987	0.001			
Age, (yr)	1.024	1.003-1.045	0.023			
BMI, kg/m^2	0.962	0.913-1.013	0.138	0.874	0.790-0.966	0.008
Female, sex	1.358	0.833-2.213	0.22			
HDL-c, mg/dL	0.994	0.977-1.011	0.481			

Hosmer and Lemeshow test: Chi-square = 4.53, P = 0.806. Only significant variables (P < 0.2) in the univariate analysis were introduced in the multivariate analysis when P < 0.05 was significant. AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; LDL-c: Low-density lipoprotein cholesterol; VLDL-c: Very low-density lipoprotein cholesterol; BMI: Body mass index; HDL-c: High density-lipoprotein cholesterol; OR: Odds ratio; CI: Confidence interval.

Table 5 Receiver operating characteristic analysis of variables associated with spontaneous hepatitis C virus clearance

Variable	Cutoff	AUC	*P* value	Sensitivity, %	Specificity, %	PPV	NPV
ALT, IU/L	50.5	.79	< 0.001	62%	83%	88%	52%
LDL-c, mg/dL	101.5	.72	< 0.001	60.7%	78%	79%	58%
Triglycerides, mg/dL	117.5	.64	< 0.001	69%	55%	78%	42%
BMI, kg/m^2	26.6	.59	0.018	63%	54%	76%	38.7%

PPV: Positive predictive value; NPV: Negative predictive value; AUC: Area under the curve; ALT: Alanine transaminase; LDL-c: Low-density lipoprotein cholesterol; BMI: Body mass index.

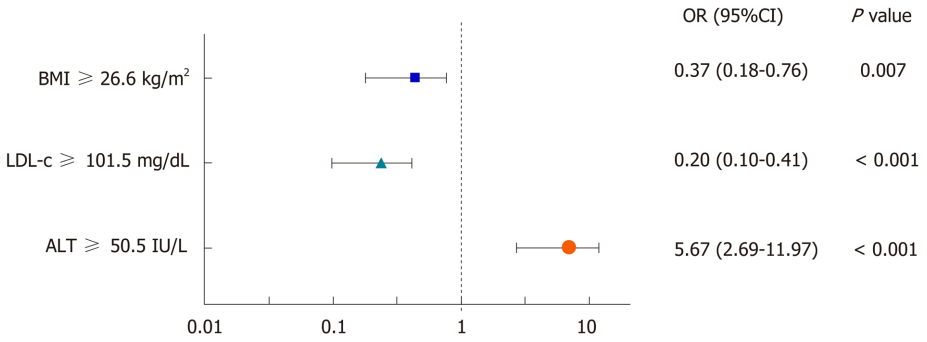

Figure 1 Odds Ratio of the multivariate analysis of dichotomous variables associated with spontaneous clearance (95% confidence interval). BMI: Body mass index; LDL-c: Low-density lipoprotein cholesterol; ALT: Alanine aminotransferase.

ARTICLE HIGHLIGHTS

Research background

The interplay between lipids and hepatitis C virus (HCV) can modulate the course of HCV infection. Cholesterol improves the rate of sustained virological response and immune response against HCV. On the other hand, the three *APOE* alleles mediate lipid levels by interacting with environmental factors such as diet. Currently, a high prevalence of lipid alterations, obesity, and an uneven distribution of the *APOE* alleles is notorious among the Mexican population. Herein, we investigate the effect of *APOE* polymorphisms and the lipid profile on the outcome of the HCV infection in patients from Mexico. To the best of our knowledge, this study is the first in reporting the effect of *APOE* alleles in the course of HCV infection in a Latin American

population.

Research motivation
HCV is a leading cause of chronic liver disease worldwide. Although it is expected to be eliminated by 2030, HCV infection still represents an unsolvable problem in many developing countries. At present, the factors impacting on the clinical outcome of HCV infection in Latin American countries are not fully known. Understanding the role of metabolic abnormalities and the participation of cholesterol and *APOE* polymorphisms in the outcome HCV infection could favor the implementation of earlier strategies of detection and treatment in these populations.

Research objectives
This study aimed to investigate the effect of *APOE* polymorphisms and the lipid profile on the outcome of the HCV infection in patients with an admixture genetic background living in West Mexico.

Research methods
A total of 299 positive anti-HCV positive patients were enrolled from January 2014 to December 2016. Clinical records were elaborated by a physician. Quantitative assessment of serum RNA was performed by a standardized quantitative reverse PCR assay. After testing, the study population was divided into two groups: Spontaneous clearance (SC) and chronic hepatitis C infection (CHC) patients. Biochemical determinations were tested through a Vitros 250 analyzer, and liver stiffness was assessed by a certified physician using transitional elastography. The *APOE* genotype was determined using a 5′ allelic discrimination method. Data analysis was performed using IBM SPSS Statistics version 21.0 for windows.

Research results
Patients who presented SC were mainly overweight, had higher levels of total cholesterol, LDL-c, and triglycerides than CHC patients. The *APOE* ε4 allele was significantly associated with spontaneous HCV clearance status and with less fibrosis than non- ε4 alleles carriers among chronic patients. Levels of LDL-c ≥ 101.5 mg/dL and BMI ≥ 26.6 kg/m^2 were associated with SC status; while ALT ≥ 50.5 IU/L was negatively associated.

Research conclusions
The present study suggests that *APOE ε4* allele and LDL-c confer a protective effect in the course of the HCV infection in the context of high BMI. Levels of LDL-c, BMI, and ALT may help in the estimation of the risk of chronicity in HCV-infected patients.

Research perspectives
In our view, an individualized therapy accounting the host´s genetic, environmental, and metabolic factors could aid in the clinical management of HCV infection, especially in populations with a high prevalence of overweight and obesity.

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ORIGINAL ARTICLE

Retrospective Study

Nomogram to predict prolonged postoperative ileus after gastrectomy in gastric cancer

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Author contributions: Chen L, Wei B, Liang WQ, Zhang KC, and Cui JX designed the study; Liang WQ, Zhang KC, and Cui JX wrote the manuscript; Xi HQ and Cai AZ contributed to the patient material; Li JY and Liu YH collected the clinical data; Liu J, Zhang W, and Wang PP contributed to data analysis and validation; Liang WQ, Zhang KC, and Cui JX contributed equally to this work.

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Informed consent statement: All study participants provided written consent prior to study

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Abstract

BACKGROUND

Prolonged postoperative ileus (PPOI) is one of the common complications in gastric cancer patients who underwent gastrectomy. Evidence on the predictors of PPOI after gastrectomy is limited and few prediction models of nomogram are used to estimate the risk of PPOI. We hypothesized that a predictive nomogram can be used for clinical risk estimation of PPOI in gastric cancer patients.

AIM

To investigate the risk factors for PPOI and establish a nomogram for clinical risk estimation.

METHODS

Between June 2016 and March 2017, the data of 162 patients with gastrectomy were obtained from a prospective and observational registry database. Clinical data of patients who fulfilled the criteria were obtained. Univariate and multivariable logistic regression models were performed to detect the relationship between variables and PPOI. A nomogram for PPOI was developed and verified by bootstrap resampling. The calibration curve was employed to detect the concentricity between the model probability curve and ideal curve. The clinical usefulness of our model was evaluated using the net benefit curve.

RESULTS

enrollment.

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This study analyzed 14 potential variables of PPOI in 162 gastric cancer patients who underwent gastrectomy. The incidence of PPOI was 19.75% in patients with gastrectomy. Age older than 60 years, open surgery, advanced stage (III–IV), and postoperative use of opioid analgesic were independent risk factors for PPOI. We developed a simple and easy-to-use prediction nomogram of PPOI after gastrectomy. This nomogram had an excellent diagnostic performance [area under the curve (AUC) = 0.836, sensitivity = 84.4%, and specificity = 75.4%]. This nomogram was further validated by bootstrapping for 500 repetitions. The AUC of the bootstrap model was 0.832 (95%CI: 0.741–0.924). This model showed a good fitting and calibration and positive net benefits in decision curve analysis.

CONCLUSION

We have developed a prediction nomogram of PPOI for gastric cancer. This novel nomogram might serve as an essential early warning sign of PPOI in gastric cancer patients.

Key words: Prolonged postoperative ileus; Gastric cancer; Complication; Nomogram; Bootstrap

Core tip: Prolonged postoperative ileus (PPOI) is one of the common complications in gastric cancer patients who underwent gastrectomy. Evidence on the predictors of PPOI after gastrectomy is limited. This study investigated the risk factors for PPOI and established an easy-to-use nomogram model for clinical risk estimation. This nomogram had an excellent diagnostic performance and showed superior effects when used in the clinical setting based on the results of the decision curve analysis. This novel nomogram might serve as an essential early warning sign of PPOI for medical practitioners.

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INTRODUCTION

Postoperative ileus (POI) is an iatrogenic gastrointestinal dysfunction following abdominal surgery[1]. The clinical manifestations of POI are characterized by abdominal distension and pain, nausea and vomiting, lack of bowel sounds, accumulation of gas and fluid, inability to pass stools, and accumulation of gas and fluid[2-6]. Usually, it resolves within 2-4 d, although it may persist for longer days or reoccur. When the symptoms extend beyond the expected duration, it is called prolonged postoperative ileus (PPOI). However, the period of POI to PPOI remains unclear. A systematic review and global survey proposed that PPOI is best defined as ileus that occurs 96 h after surgery based on the results of the previous literature, which has been acknowledged by many investigators[7]. PPOI is a frequent complication of abdominal surgery that results in severe disease burden and pain[8,9]. A multicenter survey of 17876 patients undergoing colectomy showed that the frequency of PPOI was 15.3%, which prolonged hospitalization and increased health care resource utilization[10]. However, the majority of the previous studies on PPOI were based on patients referred to colonic or rectal resection, and little data existed on gastrectomy[11,12].

Gastric cancer (GC) is a major health issue worldwide, which remains the third leading cause of cancer death[13]. Immunologic impairment, surgical trauma, inflammatory responses, and tract stasis can increase the frequency of PPOI and bacterial overgrowth and translocation, potentially leading to bacteremia and systemic sepsis[11]. Therefore, to identify the risk indicators for PPOI and determine optimal management strategies, a risk prediction model is urgently required. Of all the available models, a nomogram can provide a highly accurate, individualized evidence-based risk estimation[14,15]. Nomograms predicting survival of patients with

unresectable or metastatic GC were well established[16]. To date, various risk indicators have been suggested to be associated with an increased risk of PPOI[17-20]. However, to our knowledge, few prediction models of a nomogram were used to estimate the risk of PPOI after abdominal surgery, especially in patients who underwent radical gastrectomy.

The present study aimed to investigate the pre-, intra-, and postoperative risk factors for PPOI as well as develop and validate a nomogram using clinicopathological variables of patients who underwent radical gastrectomy for GC.

MATERIALS AND METHODS

Study patients

Between June 2016 and March 2017, 203 patients who underwent gastrectomy were identified from a prospectively collected registry database of PPOI in the Chinese People's Liberation Army (PLA) General Hospital. The process for patient selection is presented in Figure 1. Patients diagnosed with resectable gastric cancer who were able to provide written informed consent were eligible for this study. All of the included patients were scheduled to receive gastrectomy with curative intent according to the 2010 Japanese GC treatment guidelines (v. 3)[21]. All resections were performed by a specialized gastric surgical team at the Department of General Surgery, Chinese People's Liberation Army General Hospital. During the study period, 41 patients who underwent the following types of surgery were excluded to avoid the confounding bias: Resection at urgent operation (n = 12), palliative surgery (n = 11), planned laparoscopic surgery converted to open surgery (n = 9), open-close operation (n = 5), and multi-visceral resection (n = 4). Finally, a total of 162 patients were included in the final analysis.

All the included patients were informed of the clinical trial process and signed an informed consent form before surgery. This study was conducted in accordance with the Declaration of Helsinki. The protocol of this study was reviewed and approved by the Institutional Review Board of the Chinese PLA General Hospital, and all information was obtained with appropriate Institutional Review Board waivers (registration number: S2016-092-01).

Definition of PPOI

A systematic review and global survey proposed a definition of PPOI[7], which was supported by numerous studies[19,22,23]. PPOI was diagnosed if patients met two or more of the following five criteria on day 4 or more postoperatively: Nausea or vomiting for 12 h or more without relief, intolerance to a solid or semi-solid oral diet, persistent abdominal distension, absence of passage of both stool and flatus for 24 h or more, and ileus noted on plain abdominal films or CT scans. We adopted this definition, and the diagnosis of PPOI must independently concur based on two experienced surgeons.

Data collection

Clinical data of patients who fulfilled the criteria were obtained from the prospective registry database before the assessment of PPOI. Such steps ensured the authenticity and reliability of the data. Patient's baseline data were collected upon admission as following: Sex, age, body mass index (BMI), and history of previous abdominal surgery. The operation time, surgical bleeding volume, intraoperative blood transfusion, surgical procedure, lymph node dissection, and type of surgical approach (open or laparoscopic) were also obtained. All patients were operated under standard general anesthesia, and the tumor–node–metastasis stage was staged according to the 7th edition of the International Union Against Cancer tumor–node–metastasis classification of malignant tumors. Over the study period, the results of patients' postoperative physical examination, hematopoietic levels, and biochemical levels were examined within 24 h after surgery. White blood cell (WBC) count and body temperature on the first postoperative day were measured. Patients' albumin levels improved after receiving postoperative oral feeding and enteric nutrition, which were evaluated in this study. Postoperative potassium plays an essential role in smooth muscle autoregulation and is associated with the development of PPOI[24]. Postoperative potassium level was monitored in our study. Opioid analgesic could induce bowel dysfunction, which usually occurred immediately after the first dose and persisted within the duration of therapy. Opioid analgesic was reported as an essential indicator of PPOI[25,26]. Whether opioid analgesics were used postoperatively was also evaluated as a consequence of pain tolerance of patients on the first day after surgery.

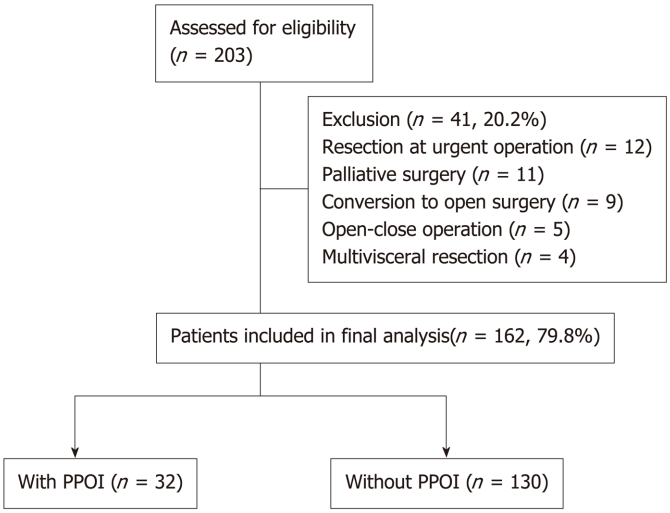

Figure 1 Flowchart of the process of patient enrollment. PPOI: Prolonged postoperative ileus.

Model establishment and validation

Univariate and multivariable logistic regression models were used to detect the relationship between variables and PPOI. In the univariate analysis, crude analyses were performed to identify potential risk factors. All variables having a bivariate association with PPOI with $P < 0.1$ were included in the multivariable model. A collinearity screening was performed on all independent variables to eliminate the variable with a variance inflation factor > 10. A stepwise nomogram model of PPOI was developed using a multivariate logistic regression. The nomogram model was performed following a backward step-down selection process using a threshold of $P < 0.05$. We can explain the nomogram by the following steps: First, determine the value of the variable on the corresponding axis; second, draw a vertical line to the total points axis to determine the points; third, add the points of each variable; and finally, draw a line from the total point axis to determine the PPOI probabilities at the lower line of the nomogram. The discriminatory ability of the model was evaluated using receiver operating characteristic (ROC) curve analysis. The accuracy of our model was further verified by bootstrap validation using computer resampling for 500 repetitions of simple random sampling with replacement. The calibration curve was employed to detect the concentricity between the model probability curve and ideal curve. The clinical usefulness of our model was evaluated using the net benefit curve, which was derived by Vickers *et al*[27].

Statistical analysis

Continuous variables are expressed as the mean ± SD or median (min-max value), while categorical data are expressed as number and percentage. The associations between PPOI and variables were assessed using χ^2 tests, Fisher exact tests, and logistic regression models. Statistical analyses were two tailed with 95% confidence intervals (CI). A P value < 0.05 was considered significant. All statistical analyses were performed using SPSS version 22.0 (IBM, New York), R software (http://www. R-project.org), and Empower Stats software (www.empowerstats.com, X&Y Solutions, Inc., Boston, Boston, Massachusetts).

RESULTS

Patient characteristics

We retrospectively analyzed data from a prospective registry database developed and updated by the Department of General Surgery, Chinese People's Liberation Army General Hospital. The patient, operation, tumor, and postoperative characteristics of 162 GC patients who underwent gastrectomy from June 2016 to March 2017 are summarized in Table 1. Overall, the mean age at diagnosis was 59.5 ± 10.9 years, and 124 (76.54%) patients were men. Thirty-one (19.14%) patients previously underwent abdominal surgery, while 61.11% underwent laparoscopic gastrectomy. Opioid analgesic was used for postoperative pain relief in 62 (38.27%) patients. Of 162 patients, PPOI occurred in 36 (19.75%, 95%CI: 14.1%-26.8%) patients.

Table 1 Patient, operation, tumor, and postoperative characteristics

Characteristic	Category	*n* = 162	Percentage (%)
Sex	Female	38	23.46
	Male	124	76.54
Age(yr)	Range 30-89	–	–
	Mean 59.5, median 59.0	–	–
BMI (kg/m^2)	Range 22.30-26.80	–	–
	Mean 24.66, median 24.95	–	–
Previous abdominal surgery	No	131	80.86
	Yes	31	19.14
Operation method	Open surgery	63	38.89
	Laparoscopic surgery	99	61.11
Operation time (min)	Range 120-433	–	–
	Mean 236.4, median 230.0	–	–
Intraoperative blood loss (mL)	Range 10-1800	–	–
	Mean 229.4, median 200.0	–	–
Blood transfusion	No	131	80.86
	Yes	31	19.14
Surgical procedure	Proximal gastrectomy	21	12.96
	Distal gastrectomy	56	34.57
	Total gastrectomy	85	52.47
Lymph node dissection	D1+	40	24.69
	D2	122	75.31
Tumor stage	I	39	24.07
	II	50	30.86
	III	72	44.44
	IV	1	0.62
Postoperative body temperature (°C)	Range 36.4-39.1	–	–
	Mean 37.6, median 37.5	–	–
Postoperative WBC count (×10^9/L)	Range 5.43-22.02	–	–
	Mean 12.76, median 12.70	–	–
Postoperative albumin (g/L)	Range 25.5-40.3	–	–
	Mean 31.93, median 31.80	–	–
Postoperative K^+ (mmol/L)	Range 2.67-5.15	–	–
	Mean 3.75, median 3.74	–	–
Postoperative opioid analgesic	No	100	61.73
	Yes	62	38.27
PPOI	No	130	80.25
	Yes	32	19.75

Data are presented as number of patients unless indicated otherwise. BMI: Body mass index; WBC: White blood cell; PPOI: Prolonged postoperative ileus.

Risk factors for PPOI

Table 2 shows the results of the univariate and multivariable logistic regression analyses performed to detect the relationship between variables and PPOI. The risk of PPOI among patients aged ≤ 60 years was lower than that of patients aged > 60 years (OR = 0.43, 95%CI: 0.19-0.95, P = 0.033) and the risk increased 5% for per year increase in age. Compared with the laparoscopic group, more patients in the open surgery group developed PPOI, with a significantly increased risk (OR = 2.44, 95%CI: 1.11-5.26, P = 0.025). Patients with early-stage (I and II) gastric carcinoma were less likely to suffer from PPOI than those with advanced-stage GC (III and IV), with a decreased risk of 59% (OR = 0.41, 95%CI: 0.19-0.92, P = 0.027). Besides, avoiding the use of opioid analgesics during the postoperative period reduced the frequency of PPOI by 71% (OR = 0.29, 95%CI: 0.13-0.64, P = 0.002). For postoperative albumin and potassium levels, there was no relationship with PPOI when considered as categorical variables; however, significant differences were found when they were regarded as

continuous variable, and these results need to be further excavated in the following studies. In addition, there was no significant difference in the incidence of PPOI between the two groups in terms of sex, BMI, previous abdominal surgery, operation time, intraoperative blood loss, blood transfusion, surgical procedure, lymph node dissection, postoperative body temperature, and postoperative WBC count. All variables having a bivariate association with PPOI with $P < 0.1$ were included in the multivariable logistic regression, which yielded the adjusted ORs shown in Table 2. In the multivariable model, the significant predictors of PPOI were: Age older than 60 years (OR = 2.70, 95%CI: 1.10-6.66, $P = 0.030$), open surgery (OR = 3.45, 95%CI: 1.33-9.09, $P = 0.010$), advanced III-IV stage (OR = 3.23, 95%CI: 1.32-7.90, $P = 0.010$), and postoperative use of opioid analgesic (OR = 5.84, 95%CI: 2.25-15.16, $P < 0.001$). All possible two-way interactions among variables in the multivariable model were examined, but no statistically significant ($P > 0.05$) interaction was found.

Nomogram for PPOI

Fourteen clinicopathological variables were analyzed to determine their association with PPOI. Of the initial 14 variables, 5 were filtered out: Age, postoperative opioid analgesic, postoperative K^+, operation methods, and tumor stage. In this study, the stepwise selected model was computed as follows: 3.24671 + 0.07000 × (age) + 1.55342 × (postoperative opioid analgesic = yes) - 2.60385 × (postoperative K^+) - 1.59227 × (operation methods = laparoscopic surgery) + 1.58622 × (tumor stage = III-IV). The probability of PPOI can be estimated using the stepwise nomogram, as described in Figure 2. The performance of this nomogram was measured using ROC curve analysis, and the area under the ROC curve (AUC) of this model was 0.836, indicating a good diagnostic performance (Figure 3) with a sensitivity of 84.4% and a specificity of 75.4% at the optimal cutoff value.

Model validation

The stepwise nomogram was further validated using internal bootstrap validation. The ROC curve was measured by bootstrapping for 500 repetitions, and the AUC of the bootstrap stepwise model was 0.832 (95%CI: 0.741-0.924), with a statistical power similar to that of the initial stepwise model (Figure 4A). The internal bootstrap validation calibration curve demonstrated that at a probability of 0-0.5, the nomogram-derived curve may underestimate the risk of PPOI (Figure 4B). When the probability was higher than 0.5, the nomogram may overestimate the probability. In general, our model showed a good fitting and calibration with the ideal curve. In addition, decision curve analysis demonstrated good positive net benefits in the predictive model under a threshold probability of 0.8, indicating the favorable potential clinical effect of the predictive model (Figure 5).

DISCUSSION

This study analyzed 14 potential variables of PPOI in 162 GC patients who underwent gastrectomy. The following independent risk factors were identified: Age older than 60 years, open surgery, advanced stage (III-IV), and postoperative use of opioid analgesic. A simple and easy-to-use prediction nomogram for PPOI after gastrectomy using multivariate analyses was developed for the first time. Five variables were filtered out for the nomogram using stepwise regression. This nomogram had an excellent diagnostic performance (AUC = 0.836, sensitivity = 84.4%, and specificity = 75.4%) and was validated internally using the bootstrap sampling method. Besides, this prediction model showed superior performance when used in the clinical setting based on the results of the decision curve analysis.

Knowledge on the incidence of PPOI could make a vital contribution to the development of new strategies to prevent or decrease such incidence. A total of 36 patients were diagnosed with PPOI in the present study, accounting for 19.75% of the total patients who underwent radical gastrectomy. The frequency of PPOI in our study was lower than that in the study of Huang *et al*[12] (32.4%), which was conducted in patients with GC, and the study of Mao *et al*[28] (27%), which was conducted in patients who underwent elective colorectal surgery, and was similar to that reported in the study of Wolthuis *et al*[29] (15.9%), which was conducted in patients after colorectal resection. A meta-analysis of 54 studies revealed a PPOI incidence of 10.3% after colorectal surgery[19]. Notably, the frequency of PPOI varied in the previous studies, depending on the type of abdominal surgery and definitions of PPOI. There is no widely accepted precise cutoff time over which ileus should persist before being regarded as prolonged, which varied from 3 d to 7 d in different studies[8,22,30]. A standardized and universally accepted definition of the exact point in time when normal POI changes to PPOI should be identified in future research. In the present

Table 2 Association of prolonged postoperative ileus with background, operative, and postoperative variables in bivariate analysis and in multivariable models

Variable	Category	Number (%) with PPOI	Univariate OR(95%CI)	*P* value	Multivariable OR (95%CI)	*P* value
Sex	Female	9/38 (23.7)	1.36 (0.57, 3.27)	0.487	–	–
	Male	23/124 (18.5)	Ref.	–	–	–
Age (yr)	Continuous variable	–	1.05 (1.01, 1.09)	0.009	–	–
	≤ 60	12/88 (13.6)	0.43 (0.19, 0.95)	0.033	Ref.	0.030
	> 60	20/74 (27.0)	Ref.	–	2.70 (1.10, 6.66)	–
BMI (kg/m^2)	Continuous variable	–	0.91 (0.81, 1.02)	0.110	–	–
	≤ 24.66	17/78 (21.8)	1.02 (0.42, 2.49)	0.529	–	–
	> 24.66	15/84 (17.9)	Ref.	–	–	–
Previous abdominal surgery	No	27/131 (15.1)	1.35 (0.47, 3.85)	0.573	–	–
	Yes	5/31 (16.1)	Ref.	–	–	–
Operation method	Open surgery	18/63 (20.6)	2.44 (1.11, 5.26)	0.025	3.45 (1.33, 9.09)	–
	Laparoscopic surgery	14/99 (14.1)	Ref.	–	Ref.	0.010
Operation time (min)	Continuous variable	–	0.99 (0.99, 1.00)	0.532	–	–
	≤ 236.4	16/89 (18.0)	0.78 (0.36, 1.69)	0.531	–	–
	> 236.4	16/73 (21.9)	Ref.	–	–	–
Intraoperative blood loss (mL)	Continuous variable	–	1.00 (0.99, 1.00)	0.693	–	–
	≤ 229.4	16/87 (18.4)	1.02 (0.42, 2.49)	0.639	–	–
	> 229.4	8/75 (21.3)	Ref.	–	–	–
Blood transfusion	No	23/131 (17.6)	0.52 (0.21, 1.28)	0.149	–	–
	Yes	9/31 (29.0)	Ref.	–	–	–
Surgical procedure	Total gastrectomy	21/85 (24.7)	Ref.	–	–	–
	Proximal gastrectomy	3/21(14.3)	0.51 (0.14, 1.89)	0.314	–	–
	Distal gastrectomy	8/56 (14.3)	0.51 (0.21, 1.25)	0.138	–	–
lymph node dissection	D1+	5/40 (12.5)	Ref.	–	–	–
	D2	27122 (22.1)	1.99 (0.71, 5.59)	0.191	–	–
Tumor stage	I-II	12/89 (13.5)	0.41 (0.19, 0.92)	0.027	Ref.	0.010
	III-IV	20/73 (27.4)	Ref.	–	3.23 (1.32, 7.90)	–
Postoperative body temperature (°C)	Continuous variable	–	0.99 (0.47, 2.05)	0.969	–	–
	≤ 37.6	19/97 (19.6)	0.97 (0.44, 2.14)	0.948	–	–
	> 37.6	13/65 (20.0)	Ref.	–	–	–
Postoperative WBC count (×10^9/L)	Continuous variable	–	1.04 (0.92, 1.17)	0.572	–	–
	≤ 12.76	18/82 (22.0)	1.33 (0.61, 2.89)	0.477	–	–
	> 12.76	14/80 (17.5)	Ref.	–	–	–
Postoperative albumin (g/L)	Continuous variable	–	0.83 (0.72, 0.95)	0.007	–	–
	≤ 31.93	21/86 (24.4)	1.91 (0.85, 4.28)	0.113	–	–
	> 31.93	11/76 (14.5)	Ref.	–	–	–
Postoperative K^+ (mmol/L)	Continuous variable	–	0.26 (0.08, 0.81)	0.020	–	–
	≤ 3.75	20/85 (23.5)	1.67 (0.75, 3.69)	0.205	–	–
	> 3.75	12/77 (16.0)	Ref.	–	–	–
Postoperative opioid analgesic	No	12/100 (12.0)	0.29 (0.13, 0.64)	0.002	Ref.	< 0.001
	Yes	20/62 (32.3)	Ref.	–	5.84 (2.25, 15.16)	–
Postoperative opioid analgesic	No	12/100 (12.0)	0.002		0.29 (0.13, 0.64)	–

BMI: Body mass index; WBC: White blood cell; PPOI: Prolonged postoperative ileus; OR: Odds ratio; CI: Confidence Interval.

study, advanced age (> 60 years) was identified as an independent predictor of PPOI. This finding is in line with those of several previous studies[12,31], which indicated that physicians should pay more attention to those patients. Older patients usually have

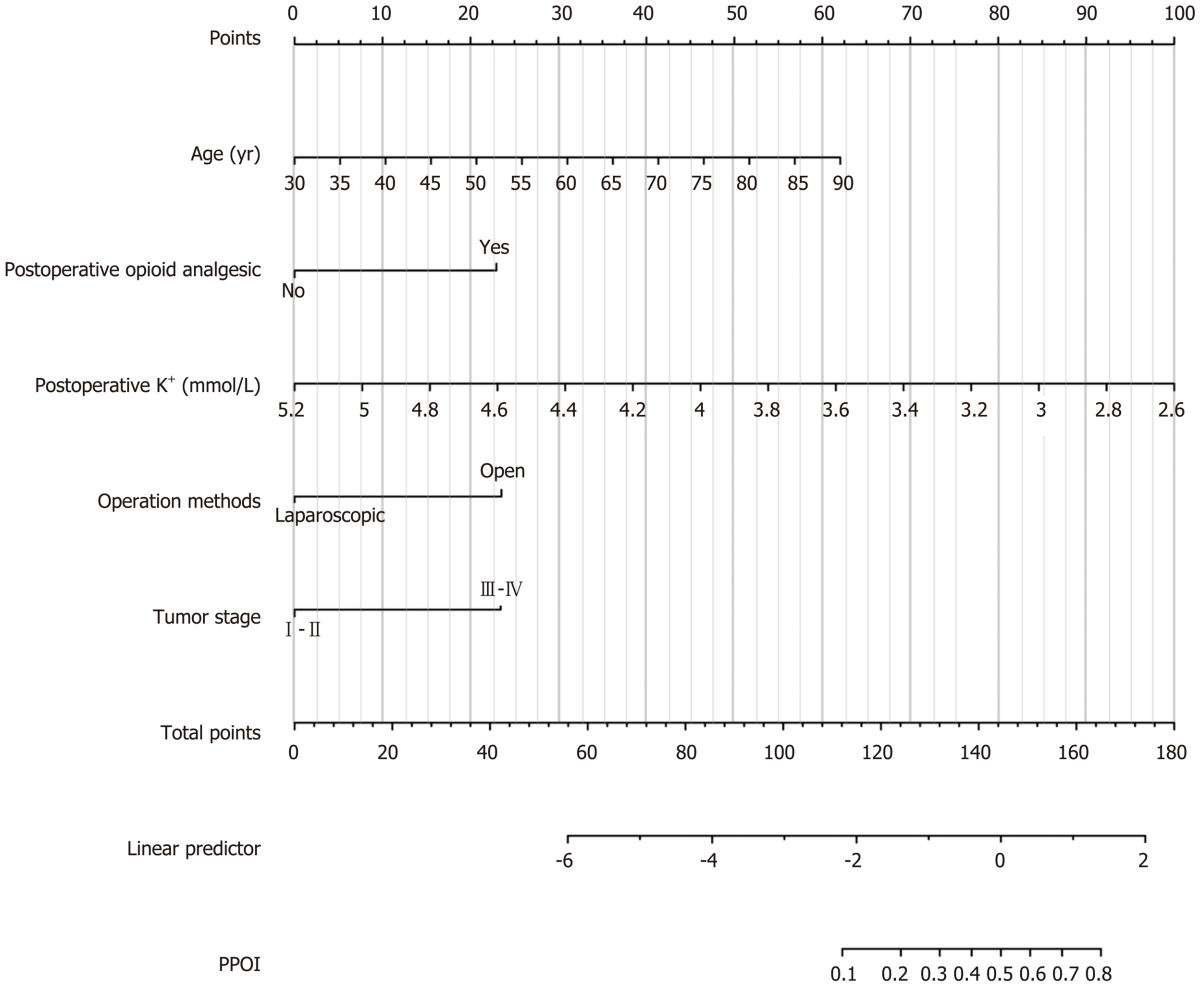

Figure 2 Nomogram prediction of prolonged postoperative ileus. The steps are: Determine the value of the variable on the corresponding axis, draw a vertical line to the total points axis to determine the points, add the points of each variable, and draw a line from the total point axis to determine the PPOI probabilities at the lower line of the nomogram. PPOI: Prolonged postoperative ileus.

reduced peristalsis and need more time for postoperative recovery[32]. Low albumin has been identified as an independent risk factor for the development of PPOI[6] and older patients generally have a poor nutritional and functional status. Our study emphasizes the need for perioperative dietary intervention in older patients who underwent gastrectomy for advanced GC.

We identified the laparoscopic approach as a way to limit PPOI, and this finding is consistent with the results reported in other studies[29-31]. The long-term oncologic outcomes of laparoscopic gastrectomy for patients with GC were comparable to those of open gastrectomy in a large-scale, multicenter, retrospective clinical study conducted in 2976 patients[33]. With the development of minimally invasive techniques, experienced surgeons can safely perform laparoscopic gastrectomy with D2 lymphadenectomy for advanced GC[34]. The gastrointestinal function of patients who underwent open abdominal surgery took 2 d to recover compared with that of patients who underwent laparoscopic surgery[6]. Laparoscopy is recommended as a feasible and reproducible procedure in the diagnosis and treatment of patients with GC, which results in decreased PPOI, faster recovery, and definite clinical effect.

Opioid-related dysmotility is thought to play a central role in postoperative gut dysfunction, and the effect of opioid analgesic on gastrointestinal function has been well elucidated in previous studies[25,26]. Opioid analgesic was also identified as an independent risk factor for PPOI in the present study. Opioid analgesic usually activates peripheral μ-opioid receptors located in the myenteric plexus, further inhibits acetylcholine, and impairs the gut motility[35]. Peripherally acting μ-opioid receptor antagonists methylnaltrexone and alvimopan, which are potentially used for the prevention of PPOI, are a new class of drugs designed to reverse opioid-induced side effects on the gastrointestinal system without compromising pain relief[25,36,37].

The nomogram was used to calculate the overall probability of PPOI for an

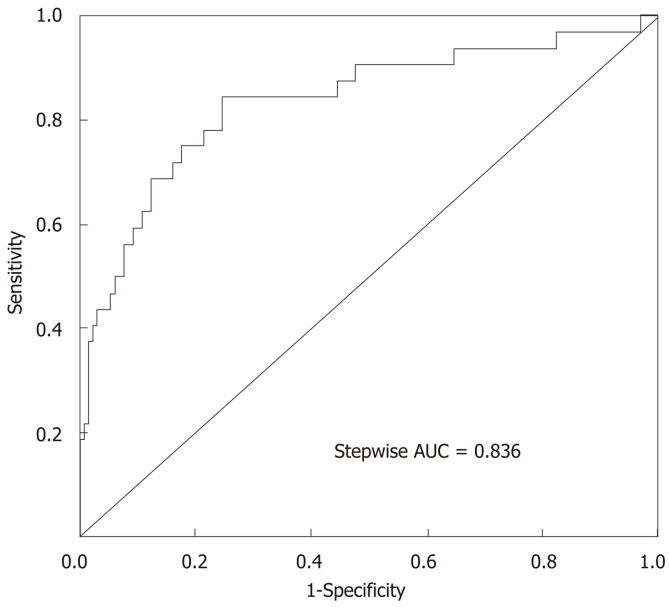

Figure 3 Receiver operating characteristic curve. AUC: Area under the receiver operating characteristic curve.

individual patient in the present study. This prediction model is important for risk estimation, improving the communication between patients and physicians, and clinical decision-making. In the present study, five independent variables were filtered out using stepwise regression, and the nomogram was established to predict the risk of PPOI in GC patients. The nomogram showed an excellent diagnostic performance (AUC = 0.836) and yielded a sensitivity of 84.4% and specificity of 75.4% at the optimal cutoff value. To our knowledge, this is the first study to evaluate a nomogram for predicting PPOI in GC patients. The nomogram might serve as a statistical tool to calculate the overall probability of PPOI in patients who underwent gastrectomy. This novel nomogram might serve as an essential early warning sign of PPOI in gastric cancer patients. If patients are associated with higher risk estimates, doctors and nurses may take appropriate measures including postoperative management and adjustments in pharmacological treatment.

The present study has some strengths. First, the majority of the previous studies focused on investigating the incidence of PPOI in patients with colonic or rectal cancer, and only a few studies were conducted among GC patients. This study provided novel evidence of PPOI in GC. Second, a nomogram prediction model of PPOI was first established for GC patients, which had great potential value for the clinical recommendation. Besides, the nomogram was confirmed to be constant by internal bootstrap validation and was found to have good positive net benefits by decision curve analysis. By contrast, the present study has several limitations. First, the retrospective nature of the study and the relatively small sample size may have weakened the results of the analyses. Second, the nomogram lacked a robust external validation. Therefore, these results need further validation in the subsequent studies.

In conclusion, PPOI is one of the common complications in GC patients who underwent gastrectomy. Age, postoperative opioid analgesic, operation methods, and tumor stage are independent risk factors for PPOI. Less traumatic operative technique and avoidance of postoperative pain medications are encouraged for GC patients. This study has established an easy-to-use nomogram model for predicting PPOI in GC patients. The novel nomogram might serve as an essential early warning sign to help doctors and nurses take appropriate measures.

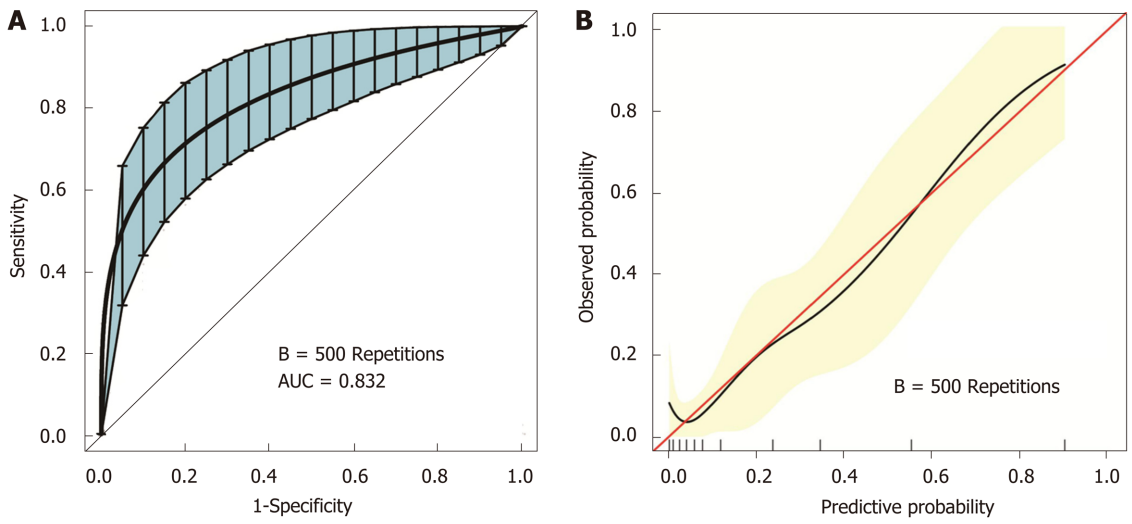

Figure 4 Internal validation of the nomogram using the bootstrap sampling. A: The ROC curve was measured by bootstrapping for 500 repetitions, and the AUC of the bootstrap stepwise model was showed; B: Calibration curve for predicted probability of the PPOI nomogram. The X axis is the predicted probability of the nomogram, and the Y axis is the observed probability. The red line shows the ideal calibration line, while the yellow area shows the 95% confidence interval of the prediction model. AUC: Area under the receiver operating characteristic curve; ROC: Receiver operating characteristic; PPOI: Prolonged postoperative ileus.

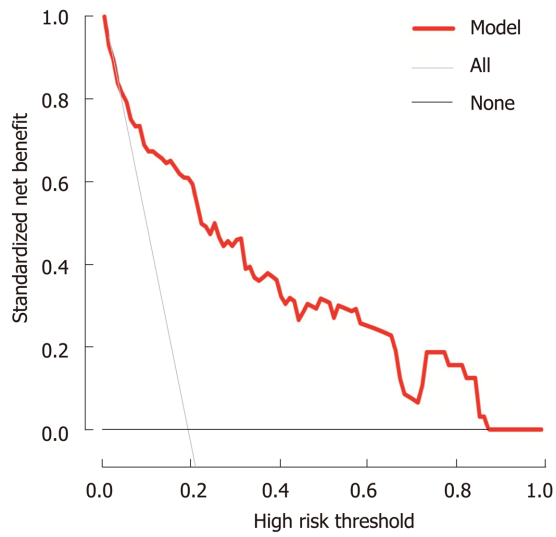

Figure 5 Decision curve analysis for the prediction model. Red solid line: Prediction model. Tin slash line: Assume all patients have PPOI. Solid horizontal line: Assume no patients have PPOI. The graph indicates the expected net benefit per patient relative to the nomogram prediction of PPOI. PPOI: Prolonged postoperative ileus.

ARTICLE HIGHLIGHTS

Research background

Prolonged postoperative ileus (PPOI) is one of the common complications in gastric cancer patients who underwent gastrectomy. PPOI is an essential contributor to cause the increase of hospitalization expense and extension of hospitalization time.

Research motivation

For the research of PPOI, most of previous studies were focused on colorectal cancer. Evidence in gastric cancer is scanty and needs further study.

Research objectives

This study aimed to evaluate the risk factors for PPOI after gastrectomy in gastric cancer and put forward a prediction model for clinical practitioners.

Research methods

In this retrospective study, we performed univariate and multivariable logistic regression

analyses to detect the relationship between variables and PPOI. We established a nomogram model for PPOI following a backward step-down selection process.

Research results

The incidence of PPOI was 19.75% in patients with gastrectomy. Age, postoperative opioid analgesic, surgical methods, and tumor stage were independent risk factors of PPOI. A nomogram was established and had a good performance. The nomogram was further validated using internal bootstrap validation, and the decision curve analysis demonstrated good positive net benefits of this model.

Research conclusions

The novel nomogram might serve as an essential early warning sign of PPOI in gastric cancer patients and thus will help doctors and nurses take appropriate measures.

Research perspectives

Further studies are needed to validate this predictive nomogram model, and some basic medical studies are meaningful to investigate the mechanism of PPOI.

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ORIGINAL ARTICLE

Retrospective Study

Nucleoside diphosphate-linked moiety X-type motif 15 R139C genotypes impact 6-thioguanine nucleotide cut-off levels to predict thiopurine-induced leukopenia in Crohn's disease patients

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Author contributions: Zhu X and Zheng H detected the metabolite concentrations and genotypes; Chao K, Li M, and Gao X enrolled the patients and collected the clinical data; Wang XD and Zhang JX finished the data analysis; Zhu X and Wang XD wrote the manuscript; Hu PJ and Huang M supervised the study; Xie W reviewed this paper.

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Abstract

BACKGROUND

Thiopurine-induced leukopenia (TIL) is a life-threatening toxicity and occurs with a high frequency in the Asian population. Although nucleoside diphosphate-linked moiety X-type motif 15 (*NUDT15*) variants significantly improve the predictive sensitivity of TIL, more than 50% of cases of this toxicity cannot be predicted by this mutation. The potential use of the 6-thioguanine nucleotide (6TGN) level to predict TIL has been explored, but no decisive conclusion has been reached. Can we increase the predictive sensitivity based on 6TGN by subgrouping patients according to their *NUDT15* R139C genotypes?

AIM

To determine the 6TGN cut-off levels after dividing patients into subgroups according to their *NUDT15* R139C genotypes.

METHODS

Patients' clinical and epidemiological characteristics were collected from medical records from July 2014 to February 2017. *NUDT15* R139C, thiopurine S-

methyltransferase, and 6TGN concentrations were measured.

RESULTS

A total of 411 Crohn's disease patients were included. TIL was observed in 72 individuals with a median 6TGN level of 323.4 pmol/8 × 10^8 red blood cells (RBC), which was not different from that of patients without TIL (P = 0.071). Then, we compared the 6TGN levels based on *NUDT15* R139C. For CC (n = 342) and CT (n = 65) genotypes, the median 6TGN level in patients with TIL was significantly higher than that in patients without (474.8 *vs* 306.0 pmol/8 × 10^8 RBC, $P = 9.4 \times 10^{-5}$; 291.7 *vs* 217.6 pmol/8 × 10^8 RBC, P = 0.039, respectively). The four TT carriers developed TIL, with a median 6TGN concentration of 135.8 pmol/8 × 10^8 RBC. The 6TGN cut-off levels were 411.5 and 319.2 pmol/8 × 10^8 RBC for the CC and CT groups, respectively.

CONCLUSION

The predictive sensitivity of TIL based on 6TGN is dramatically increased after subgrouping according to *NUDT15* R139C genotypes. Applying 6TGN cut-off levels to adjust thiopurine therapies based on *NUDT15* is strongly recommended.

Key words: Crohn's disease; Thioguanine nucleotide levels; Nucleoside diphosphate-linked moiety X-type motif 15; Thiopurine-induced leukopenia

Core tip: Thiopurine-induced leukopenia (TIL), a life-threatening toxicity in inflammatory bowel disease, occurs with a high frequency in Asia. Although nucleoside diphosphate-linked moiety X-type motif 15 (*NUDT15*) variants significantly increase the prediction sensitivity of TIL, more than 50% of cases cannot be predicted by this mutation. The potential use of steady-state thioguanine nucleotide (6TGN) levels to predict TIL has been explored for decades, but no decisive conclusion has been reached. Can we increase the predictive sensitivity based on 6TGN by subgrouping patients according to their *NUDT15* genotypes? Yes! According to our research, applying 6TGN levels to adjust thiopurine therapies based on *NUDT15* is strongly recommended.

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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.

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INTRODUCTION

Thiopurines, including mercaptopurine (MP) and azathioprine (AZA), are immunosuppressive drugs that have been regularly used to maintain remission in patients with Crohn's disease (CD)[1,2]. Although thiopurine therapy is clinically effective, up to 10%-30% of patients discontinue treatment due to adverse reactions[3,4], among which thiopurine-induced leukopenia (TIL) is the most common and life-threatening toxicity, especially in Asian populations[5,6].

Neither AZA nor MP has an intrinsic activity. They must undergo extensive metabolic transformation into the active metabolites 6-thioguanine nucleotides (6TGN) and 6-methylmercaptopurine ribonucleotides (6MMPR), to exert their pharmacological effect or cytotoxicity[7,8]. The potential use of a steady-state 6TGN level above 450 pmol/8 × 10^8 red blood cells (RBC) to predict toxicity is still controversial[9-14]. Several studies have reported that 6TGN levels are not significantly correlated with TIL, and no applicable therapeutic range for 6TGN has been determined[15-18]. For example, a prospective study found that only three out of ten Japanese patients with TIL exhibited high 6TGN levels (> 450 pmol/8 × 10^8 RBC)[17]. In addition, a few groups have reported that extremely elevated 6MMPR concentrations are associated with TIL[12,19]. Therefore, further investigation of the exact relationship between the 6TGN and 6MMPR levels and TIL is warranted, especially in the Asian

population, since Asians are more sensitive than Caucasians to thiopurine toxicity[6,20,21].

Nucleoside diphosphate-linked moiety X-type motif 15 (*NUDT15*) has been considered as the key gene which can highly predict thiopurine toxicity in Asian that is comparable to thiopurine S-methyltransferase (*TPMT*) in Europe[22-24]. *TPMT* variants with deficient enzyme activity were observed to have a preferential accumulation of 6TGN in TIL, so the guidelines have suggested adjusting the starting doses of thiopurine for variants based on the level of 6TGN[24]. However, *NUDT15* variants have been discovered to develop TIL with normal or even lower 6TGN levels, suggesting that *NUDT15* has an unfavorable effect on thiopurine metabolism[25,26]. *NUDT15* is an enzyme responsible for catalyzing deoxy-6-thioguanine triphosphate (dTGTP) and 6-thioguanine triphosphate (TGTP) into their inactive monophosphates, preventing TGTP and dTGTP from incorporating into DNA[27]. Moriyama *et al*[28,29] discovered that *NUDT15* R139C variants CT and TT were associated with a much higher TGTP/TGMP ratio and DNA-incorporated thioguanine (DNA-TG)/6TGN ratio in children with acute lymphoblastic leukemia (ALL). According to this mechanism, *NUDT15* R139C variants are assumed to cause cytotoxicity with TGTP and dTGTP accumulation independent of the total 6TGN levels due to decreases in 6-thioguanine monophosphate (TGMP) and deoxy-6-thioguanine monophosphate (dTGMP). Thus, the guidelines applied to *TPMT* variants would not be suitable for patients with *NUDT15* variants. Can we determine 6TGN cut-off levels thereafter dividing patients into subgroups according to *NUDT15* R139C genotypes?

In this study, we investigated the relationship between the levels of 6TGN and 6MMPR and thiopurine-induced TIL based on patients' *NUDT15* R139C genotypes and obtained specific 6TGN cut-off levels to guide thiopurine therapy.

MATERIALS AND METHODS

Patient recruitment

Patients diagnosed with CD according to the criteria of Lennard-Jones and prescribed thiopurines at a steady dose for more than 1 mo were recruited at the Sixth Affiliated Hospital, Sun Yat-sen University from July 1, 2014 to February 1, 2017. The exclusion criteria included blood transfusion or administration of cyclosporine or methotrexate; insufficient function of the heart, liver, or kidney; active infection; and pregnancy.

Weight-based thiopurine dosing was provided in a step-wise approach (the initial dose of AZA was 1.0 mg/kg daily or 0.5 mg/kg daily for MP and gradually increased to the target dose of 2.0 mg/kg or 1.0 mg/kg, respectively). If patients developed adverse effects, such as leukopenia [white blood cell count (WBC) < 3.5 × 10^9/L], gastric intolerance, hepatotoxicity, flu-like symptoms, pancreatitis, rash, or others, the dose was reduced. If the laboratory abnormalities did not subside, the treatment was discontinued.

A total of 2 mL of venous blood samples (EDTA anticoagulation) were obtained for determination of the erythrocyte 6TGN/6MMPR concentrations and *NUDT15* R139C and *TPMT**3C genotypes at the Institute of Clinical Pharmacology, Sun Yat-sen University[30,31].

NUDT15 R139C and TPMT*3C genotyping

We used allele-specific polymerase chain reaction (PCR)-restriction fragment length polymorphism (RFLP) to test the genotypes of *NUDT15* R139C (rs116855232) and *TPMT**3C (rs1142345). The sequences of the primers for *NUDT15* R139C are: forward, 5-AGCTTACCCAAATAAACACCCT-3 and reverse, 5-TGGGGGATACATTAAGAGACTGC-3, and those for *TPMT**3C are: forward, 5-AAGTGTTGGGATTACAGGTG-3 and reverse, 5-TCCTCAAAAACATGTCAGTGTG-3. PCR amplification started at 94 °C for 5 min, and continued with 30 cycles of 94 °C for 60 s, 59 °C for 30 s, and 72 °C for 30 s. Final extension was performed at 72 °C for 10 min. The PCR product was digested with restriction enzyme HpyCH4III (New England Biolabs, Hertfordshire, United Kingdom) for *NUDT15* R139C testing and the enzyme AccI (New England Biolabs, Hertfordshire, United Kingdom) for *TPMT**3C testing.

6TGN and 6MMPR determination

6TGN and 6MMPR concentrations in patients' erythrocyte lysates were determined using the Dervieux *et al*'s method[32] by high performance liquid chromatography. First, 6TGN were hydrolyzed (100 °C for 45 min) into their bases (6-thioguanie) and 6MMPR were converted into their derivatives with perchloric acid. Then, they were separated using a C18 column with the mobile phase consisting of methanol-20

mmol/L potassium dihydrogenphosphate-triethylamine (pH adjusted to 3.2 using phosphoric acid) (5:95:0.1) at a flow rate of 1.0 mL/min. Finally, the wavelength for detection was set at 345 nm for 6TGN and at 303 nm for 6MMPR.

The present study was approved by the Ethics Committee of the Sixth Affiliated Hospital of the Sun Yat-sen University.

Statistical analysis

Statistical analyses and calculations were performed with SPSS 21.0 (SPSS, Inc., Chicago, IL, United States) and Prism 6 (GraphPad Software, La Jolla, CA, United States). Quantitative variables are expressed as medians and ranges. Categorical variables are summarized as frequencies. Data such as sex, *NUDT15* R139C and *TPMT**3C* diplotypes, disease location, and co-medication were compared using the *X2* method or Fisher's test. Data such as age and thiopurine dose were compared using the nonparametric Kruskal-Wallis *H*-test. The nonparametric Kruskal-Wallis *H*-test was also used to evaluate the relationship between the 6TGN level and TIL. A receiver operating characteristic (ROC) curve was obtained to calculate the sensitivity and specificity of various 6TGN concentrations to predict the development of TIL. Multivariate logistic regression was used to identify the variables associated with TIL. *P*-values less than 0.05 were considered statistically significant.

RESULTS

Clinical characteristics of CD patients

A total of 411 patients with CD were included in this study. Among them, 100 (24.3%) patients had adverse effects during the AZA maintenance treatment, including TIL (n = 72, 17.5%), gastric intolerance (n = 21, 5.1%), flu-like symptoms (n = 6, 1.5%), hepatitis (n = 5, 1.2%), pancreatitis (n = 3, 0.7%), rash (n = 2, 0.5%), and others (n = 3, 0.7%). The characteristics of the patients are summarized in Table 1. The TIL rates were higher in patients carrying the *NUDT15* R139C allele T (CT + TT) compared with those carrying the CC genotype ($P = 9.04 \times 10^{-20}$, OR = 8.04, 95%CI: 4.84-13.34). The median dosage was significantly lower for patients with TIL than for those without TIL (1.5 mg/kg per day *vs* 1.7 mg/kg per day, P = 0.03). Overall, there were no significant differences in gender, age, disease location, or co-medication between individuals with or without TIL ($P > 0.05$) (Table 1). No significant association of *TPMT**3C* with TIL was observed. Neither *NUDT15* R139C nor *TPMT**3C* had significant associations with the other adverse effects ($P > 0.05$).

6TGN and 6MMPR levels and their ratios to the thiopurine dose according to NUDT15 R139C and TPMT*3C diplotype

For *NUDT15* R139C, the median 6TGN was 312.4 pmol/8 × 10^8 RBC in the CC group, 240.5 pmol/8 × 10^8 RBC in the CT group, and 135.8 pmol/8 × 10^8 RBC in the TT group. The level of 6TGN was much higher in the NUDT CC group than in the CT and TT groups ($P = 9.0 \times 10^{-6}$) (Figure 1), while the median level of 6MMPR was also significantly higher in CC patients (P = 0.030). To assess the levels of 6TGN and 6MMPR with equal doses of thiopurine, the ratios of the levels of 6TGN and 6MMPR to the thiopurine dose were compared across groups. There was no difference in the ratios between these three groups (P= 0.29, P= 0.58) (Figure 1). For *TPMT**3C*, the patients carrying *TPMT**3C* variants had excessively higher 6TGN ($P = 2.0 \times 10^{-6}$) and lower 6MMPR levels ($P = 3.7 \times 10^{-4}$), which could not be offset by the thiopurine dose ($P = 4.8 \times 10^{-5}$, $P = 7.7 \times 10^{-5}$) (Supplemental Figure 1).

Associations between thiopurine metabolite concentrations and TIL based on NUDT15 R139C genotypes

In this study, neither the concentration of 6TGN nor 6MMPR was significantly different between patients with or without TIL (P = 0.071, P = 0.95). According to the *NUDT15* R139C genotypes, the samples were divided into three subgroups. In the CC group (n = 342), the median 6TGN concentration was significantly higher in patients who developed TIL than in patients who did not [$P = 9.4 \times 10^{-5}$, 474.8 (174.2-1133.6) pmol/8 × 10^8 RBC *vs* 306.0 (62.2-1823.0) pmol/8 × 10^8 RBC] (Figure 2). In the CT genotype (n = 65), the level of 6TGN was significantly higher in patients who developed TIL [P = 0.039, 291.7 (80.6-701.5) pmol/8 × 10^8 RBC *vs* 217.6 (62.9-631.0) pmol/8 × 10^8 RBC] (Figure 2). All patients with the TT genotype (n = 4) developed TIL, with a median 6TGN concentration of 135.8 (90.0-291.3) pmol/8 × 10^8 RBC. No correlations between the 6-MMPR concentrations and TIL were found in the subgroups of CC (P = 0.55) and CT (P = 0.30), respectively.

Table 1 Characteristics of the 411 Crohn's disease patients included in this study

Characteristic	All patients	With leukopenia	Without leukopenia	*P* value
No. of subjects (%)	411	72 (17.5)	339 (82.5)	-
Gender				
Male, *n* (%)	305 (74.0)	52 (17.0)	253 (83.0)	0.180
Female, *n* (%)	106 (26.0)	20 (18.9)	86 (81.1)	
NUDT15 R139C				
CC, *n* (%)	342 (80.4)	35 (10.2)	307 (89.8)	2.40×10^{-15}
CT, *n* (%)	65 (17.9)	33 (50.2)	32 (49.8)	
TT, *n* (%)	4 (1.7)	4 (100)	0	
*TPMT**3C				0.26
AA, *n* (%)	398 (96.8)	68 (17.1)	330 (82.9)	
AG, *n* (%)	13 (3.2)	4 (30.8)	9 (69.2)	
Age in years, median (range)	28 (12-70)	26(12-70)	27(12-60)	0.734
Medication				
Azathiopurine	337 (82.0)	57 (17.3)	273 (82.7)	0.113
Mercaptopurine	74 (18.0)	14 (18.9)	60 (81.1)	
Thiopurines dose, mg/kg per day, median (range)	1.7 (0.2-3.1)	1.5 (0.5-2.6)	1.7 (0.2-3.1)	0.030
CD				
Ileal L1	34	6	28	0.495
Colorectal L2	21	6	15	
Ileocolonic L3	292	48	244	
Upper gastrointestinal L4	61	12	49	
Co-medication	211	45	166	0.74
Corticosteroids	79	15	64	
Thalidomide	51	13	38	
Anti-TNF agent	64	13	51	
5-ASA	4	0	4	
Allopurinol	13	4	9	

Patients carrying the nucleoside diphosphate-linked moiety X-type motif 155 R135C CT and TT genotypes were more likely to develop thiopurine-induced leukopenia ($^{b}P < 0.01$). *NUDT15*: Nucleoside diphosphate-linked moiety X-type motif 15; CD: Crohn's disease.

6TGN cut-off values to predict TIL

Based on ROC analysis, we defined the cut-off level of 6TGN to be 411.5 pmol/8 × 10^8 RBC in the CC group and 319.2 pmol/8 × 10^8 RBC in CT group. After considering the level of 6TGN in different subgroups, the area under the curve (AUC) was increased from 0.57 to 0.65 and 0.70. Moreover, in the CT group, the specificity was as high as 96.9%, with a sensitivity of 42.4%, and in the CC group the specificity was 73.3%, with a high sensitivity of 60.0%, compared with the values in the total samples (Table 2).

Multivariable prediction model for TIL

After performing the multivariate regression analysis, we found that the *NUDT15* R139C genotypes and 6TGN concentration were relevant determinants for the development of TIL. Table 3 presents the prevalence of TIL and the odds of developing it for each category compared with the reference group of patients with the CC genotype and a 6TGN level below 411.5 pmol/8 × 10^8 RBC. Patients carrying the *NUDT15* TT allele, irrespective of the 6TGN concentration, were at the highest risk, as all of them developed TIL in this study (Table 3). Patients with the CT genotype and a 6TGN concentration above the cut-off value of 319.2 pmol/8 × 10^8 RBC were at the second highest risk of developing TIL [OR 225.0 (95%CI: 27.6-1836.2)], followed by the CT genotype with a 6TGN concentration below 319.2 pmol/8 × 10^8 RBC [OR 9.9 (95%CI: 4.5-21.6)] and the CC genotype with a 6TGN concentration above 411.5 pmol/8 × 10^8 RBC [OR 4.1 (95%CI: 2.0-8.5)] (Table 3). The area under the ROC curve for the obtained predicted probabilities based on *NUDT15* and the 6TGN level was 0.79 (95%CI: 0.76-0.92) (Figure 3).

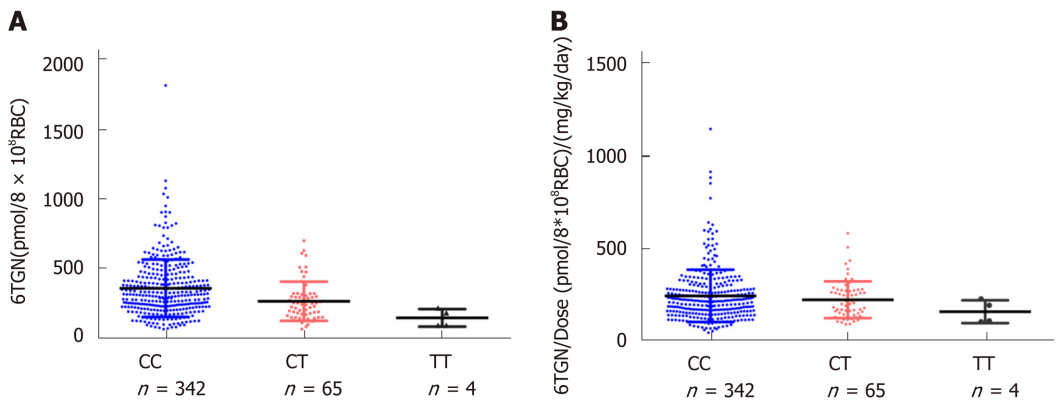

Figure 1 6-thioguanine nucleotide level and its ratio to thiopurine dose according to nucleoside diphosphate-linked moiety X-type motif 15 R139C diplotype. A: The level of 6-thioguanine nucleotide (6TGN) was much higher in the nucleoside diphosphate-linked moiety X-type motif 15 CC group than in the CT and TT groups [$^{b}P < 0.01$, 312.4 pmol/8 × 10^8 red blood cells (RBC) *vs* 312.4 pmol/8 × 10^8 RBC *vs* 135.8 pmol/8 × 10^8 RBC]. B: There was no difference in the ratio of the level of 6TGN to the thiopurine dose among these three groups. 6TGN: 6-thioguanine nucleotide; RBC: Red blood cells.

DISCUSSION

In this retrospective study, for the first time, we found that the level of 6TGN level was significantly associated with thiopurine-induced TIL in different *NUDT15* R139C genotypes. After combing *NUDT15* R139C genotypes and different 6TGN cut-off levels, 80% of TIL cases could be explained by an ROC_{AUC} of 0.79.

The incidence of thiopurine-induced TIL is more common in Asians than in Caucasians, even at the lower standard dose[6,33]. The discovery of *NUDT15* R139C as a major genetic determinant predicting TIL in the Asian population was a milestone[22,23,34]. In our study, there was a strong relationship between *NUDT15* R139C genotypes and TIL ($P = 9.04 \times 10^{-20}$, OR = 8.04, 95%CI: 4.84-13.34) (Table 1). However, in our study, no significant association of *TPMT**3C with TIL was observed (Table 1), with only four (30.7%, 4/13) variants experiencing TIL, which is similar to our previous report[33]. Thus, in the Asian population, *NUDT15* R139C might be a better biomarker to predict thiopurine toxicity.

NUDT15 is a gene that encodes the purine-specific Nudix hydrolase, which dephosphorylates the active metabolites TGTP and dTGTP into their inactive monophosphates, thus preventing TGTP from binding to Rac1and dTGTP from incorporating into DNA[27,28]. When the function of *NUDT15* is decreased, the excessive accumulation of TGTP and dTGTP may induce TIL, with decreased levels of TGMP and dTGMP[28]. Thus, the total 6TGN remains apparently unchanged. In our study, there were lower median levels of 6TGN in the CT and TT groups compared with the CC group ($P = 9.0 \times 10^{-6}$) (Figure 1). A study on Korean children with ALL also found that the level of 6TGN was negatively correlated with thenumber of *NUDT15* risk alleles ($P = 5.3 \times 10^{-6}$), and theassociation remained significant after adjusting for the MP dosage($P = 1.7 \times 10^{-6}$)[26]. However, in our study, this difference was counteracted by the thiopurine dosage ($P = 0.29$) (Figure 1), suggesting lower 6TGN and 6MMPR levels in patients carrying the T allele due to lower thiopurine administration. Contrary to the function of *NUDT15*, the patients carrying *TPMT* variants had excessively higher 6TGN and lower 6MMP levels, which could not be offset by the thiopurine dose (Supplemental Figure 1). These data suggested different mechanisms of *TPMT* and *NUDT15*, which made us believe that considering the 6TGN level alone may overlook *NUDT15*-deficient Asian patients who are prone to TIL[14,26,27]. In our study, we found no difference in the 6TGN concentration between patients with and without TIL in the total samples ($P = 0.071$). Thus, we subgrouped the entire sample based on patients' *NUDT15* R139C genotypes.

Few studies have analyzed the relationship of the level of 6TGN with TIL according to patients' *NUDT15* diplotypes[31,26]. Based on our previous research, we found that the concentration of 6TGN was significantly correlated with TIL in patients with the CC genotype, while no correlation was found in patients with the CT genotype due to a small sample size (24 patients). However, in this study, which had a larger sample size ($n = 65$), the level of 6TGN was significantly associated with TIL in the CC and CT groups. In the CC group ($n = 342$), the median 6TGN concentration was significantly higher in patients who developed TIL than in those who did not [$P = 9.4 \times 10^{-5}$, 474.8 (174.2-1133.6) pmol/8 × 10^8 RBC *vs* 306.0 (62.2-1823.0) pmol/8 × 10^8 RBC] (Figure 2). Similarly, in the CT genotype ($n = 65$), the level of 6TGN was higher in patients who

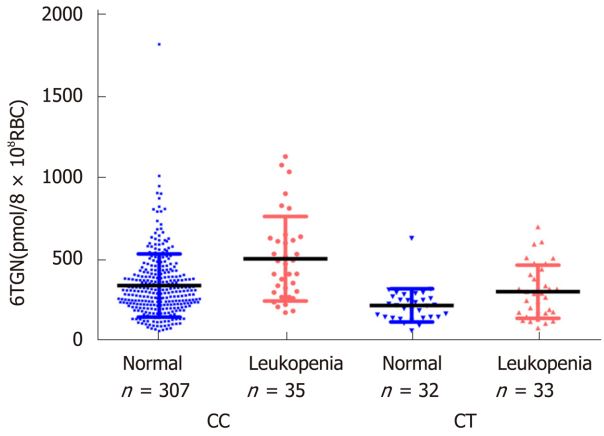

Figure 2 Relationship between thiopurine-induced leukopenia and 6-thioguanine nucleotide concentrations in different nucleoside diphosphate-linked moiety X-type motif 15 R139C genotypes. In the CC group (n = 342), the median 6-thioguanine nucleotide (6TGN) concentration in patients who developed leukopenia was significantly higher than that in patients who did not [^{b}P < 0.01, 474.8 (174.2-1133.6) pmol/8 × 10^8 red blood cells (RBC) *vs* 306.0 (62.2-1823.0) pmol/8 × 10^8 RBC]. In the CT group (n = 65), 6TGN level was also significantly higher in patients who developed leukopenia [^{a}P < 0.05, 291.7 (80.6-701.5) *vs* 217.6 (62.9-631.0) pmol/8 × 10^8 RBC]. 6TGN: 6-thioguanine nucleotide; RBC: Red blood cells.

developed TIL than in patients who did not [P = 0.039, 291.7 (80.6-701.5) pmol/8 × 10^8 RBC *vs* 217.6 (62.9-631.0) pmol/8 × 10^8 RBC] (Figure 2). According to ROC analysis, we defined the cut-off level of 6TGN to be 319.2 pmol/8 × 10^8 RBC in the CT group, which was significantly lower than that in the CC group (411.5 pmol/8 × 10^8 RBC). Moriyama *et al*[28,29] reported that all patients with deficient *NUDT15* diplotypes had a higher DNA-TG/dosage ratio, even for the lower level of 6TGN. Thus, in our cohort, CT individuals might have a higher percentage of active dTGTP incorporated into their DNA (DNA-TG), even with a lower TGN level compared with that of CC genotype individuals, which needs to be further investigated.

Although *NUDT15* genotype screening can inform the initial drug dosage of thiopurine, therapeutic drug monitoring of its metabolites still serves as a valuable tool for further fine-tuning of the treatment, especially to reduce the incidence of TIL. Dubinsky *et al*[9] reported that excessive 6TGN levels (> 450 pmol/8 × 10^8 RBC) were associated with a higher risk of TIL in Caucasians. However, in our study, the sensitivity of 450 pmol/8 × 10^8 RBC to predict TIL was 34.7% (25/72), as 79% of patients in the *NUDT15* CT group and all patients in the TT group developed TIL with a 6TGN level far below 450 pmol/8 × 10^8 RBC, which indicated that *NUDT15* deficient Asian patients were more sensitive to thiopurines. Feng *et al*[20] reported that the cut-off level for 6TGN in Chinese IBD was from 180-355 pmol/8 × 10^8 RBC, but patients' with *NUDT15* genotypes were not incorporated and the sample size of TIL was small (n = 12), indicating that the result was not conclusive. Based on our study including 411 patients, the sensitivity of the 6TGN cut-off level to TIL was increased to 42.4% in the CT group and 60.0% in the CC group compared with the threshold values in the studies of Dubinsky and Feng. Thus, different 6TGN cut-off levels should be considered in different *NUDT15* genotypes in CD patients.

Finally, multivariable regression analysis showed that both the *NUDT15* genotypes and 6TGN level were associated with TIL. Based on the combinations of these two parameters, patients can be categorized to assess their degree of risk for the development of TIL. Patients with the TT genotype, irrespective of the 6TGN level, developed TIL. Patients with the CT genotype and a 6TGN level above 319.2 pmol/8 × 10^8 RBC exhibited the highest risk of TIL [OR = 225.0 (95%CI: 27.6-1836.2)], followed by patients carrying either the CT genotype (< 319.2 pmol/8 × 10^8 RBC) or the CC genotype (> 411.5 pmol/8 × 10^8 RBC). In a Japanese report, the AUC of the model based on *NUDT15* to predict TIL was 0.71[23]. Cao *et al*[33] reported a similar AUC value of 0.69 in a Chinese multicenter study. However, in our study, the AUC of the combination model to predict TIL was increased to 0.79, and 80% of TIL cases could be predicted. Based on this model, we can distinguish the thiopurine refractoriness if the patient has the steady-state 6TGN levels above the cut-off values without efficacy, which suggests that the thiopurine dose should not be increased and the alternative therapeutic agent should be chose to avoid the incidence of TIL.

There were some limitations in our research. The concentration of DNA-TG was not detected in our study. DNA-TG, the final active metabolite of thiopurines, has

Table 2 Relationship between concentrations of 6-thioguanine nucleotide and thiopurine-induced leukopenia in different nucleoside diphosphate-linked moiety X-type motif 15 R139C genotype groups among 411 patients according to receiver operating characteristic curves

NUDT15 genotype (No. of patients)	6TGNs (pmol/8 × 10^8 RBC)	*P* value	AUC	95%CI	Sensitivity (%)	Specificity (%)
CT + CC	≥ 474.7	0.071	0.57	(0.49-0.65)	34.7	82.6
CC (342)	< 474.7	9.4 × 10^{-5}		(0.61-0.79)	60.0	73.3
	≥ 411.5		0.70			
	< 411.5					
CT (65)	≥ 319.2	0.039	0.65	(0.51-0.79)	42.4	96.9
	< 319.2					

In subgroups, the area under the curve was increased from 0.57 to 0.65 and 0.70. Moreover, in the CT group, the specificity was as high as 96.9%, with a sensitivity of 42.4%, and in the CC group, the specificity was 73.3%, with a high sensitivity of 60.0%, compared with the values in the total samples. AUC: Area under the curve; *NUDT15*: Nucleoside diphosphate-linked moiety X-type motif 15; RBC: Red blood cells.

been reported to be a new biomarker to predict relapse and is correlated with the concentration of 6TGN in childhood acute lymphoblastic TIL based on a prospective study[35]. Curffari *et al*[36] also discovered that the levels of DNA-TG metabolites were correlated with the erythrocyte 6TGN level, but not the total leukocyte level. However, a recent study performed by Coulthard *et al*[37] showed contradictory results, indicating that the exact function of DNA-TG still requires further validation in IBD. On the other hand, we performed this retrospective research to determine the cut-off levels of 6TGN for predicting TIL, which need to be validated in further prospective studies.

In conclusion, this study found that the level of 6TGN was significantly correlated with thiopurine-induced TIL in Chinese CD patients with different *NUDT15* genotypes, and specific cut-off values were determined. It is strongly recommended that these specific 6TGN cut-off levels be applied to adjust thiopurine therapy to ensure the therapeutic effects and decrease the incidence of TIL.

Table 3 Multivariable prediction model for thiouprine-induced leukopenia, including the nucleoside diphosphate-linked moiety X-type motif 1 R139C genotypes and 6-thioguanine nucleotide cut off levels

Category	Leukopenia		% of total	OR (95%CI)	*P* value
	Yes	No			
NUDT15 R139C TT	4	0	4/4 (100%)	↑↑↑	-
NUDT15 R139C CT + 6TGN > 319.2	14	1	14/15 (93.3%)	225.0 (27.6-1836.2)	1.5×10^{-14}
NUDT15 R139C CT + 6TGN < 319.2	19	31	19/50 (38.0%)	9.9 (4.5-21.6)	8.1×10^{-11}
NUDT15 R139C CC + 6TGN > 411.5	21	82	21/103 (20.4%)	4.1 (2.0-8.5)	4.8×10^{-5}
NUDT15 R139C CC + 6TGN < 411.5	14	225	14/239 (5.9%)	Reference	-
Total	72	339	72/411 (17.5%)		

Patients carrying the TT allele, irrespective of the concentration of 6-thioguanine nucleotide (6TGN), were at the highest risk, as all of them developed leukopenia in this study. Patients with the CT genotype and a 6TGN concentration above the cut-off value of 319.2 pmol/8 × 10^8 red blood cells (RBC) were at the second highest risk of developing leukopenia [OR 225.0 (95%CI: 27.6-1836.2)], followed by those carrying the CT genotype with a 6TGN concentration below 319.2 pmol/8 × 10^8 RBC [OR = 9.9 (95%CI: 4.5-21.6)] and those carrying the CC genotype with a 6TGN concentration above 411.5 pmol/8 × 10^8 RBC [OR 4.1 (95%CI: 2.0-8.5)]. *NUDT15*: Nucleoside diphosphate-linked moiety X-type motif 15; 6TGN: 6-thioguanine nucleotide.

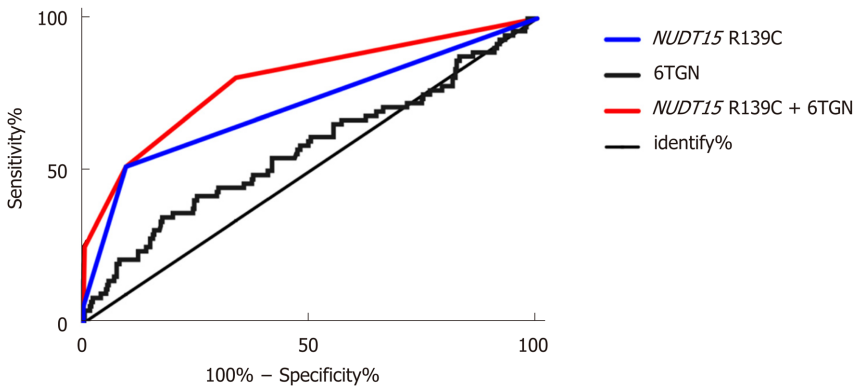

Figure 3 Receiver operating characteristic curves for the concentration of 6-thioguanine nucleotide with the threshold parameters and nucleoside diphosphate-linked moiety X-type motif 15 R139C genotypes. The area under the receiver operating characteristic curve for the obtained predicted probabilities based on nucleotide concentrations in different nucleoside diphosphate-linked moiety X-type motif 15 R139C genotypes and the level of 6-thioguanine nucleotide was 0.79 (95%CI: 0.76-0.92). 6TGN: 6-thioguanine nucleotide; *NUDT15*: Nucleoside diphosphate-linked moiety X-type motif 15.

ARTICLE HIGHLIGHTS

Research background

Thiopurine-induced leukopenia (TIL) is life-threatening and occurs with a high frequency in Asia. Although nucleoside diphosphate-linked moiety X-type motif 15 (*NUDT15*) variants improve the predictive sensitivity of TIL, more than 50% of TIL cannot be predicted by this mutation. The potential use of the 6-thioguanine nucleotide (6TGN) level to predict TIL is controversial.

Research motivation

Can we increase the predictive sensitivity based on 6TGN by subgrouping patients according to their *NUDT15* R139C genotypes?

Research objectives

To obtain a better model with combination of 6TGN levels and *NUDT15* R139C genotypes to predict thiopurine-induced TIL and improve the safety for the thiopurine treatment.

Research methods

A total of 411 patients diagnosed with Crohn's disease at the Sixth Affiliated Hospital of the Sun Yat-sen University were included in this study. Peripheral blood from patients was collected to detect the *NUDT15* R139C/*TPMT**3C genotypes and 6TGN concentrations at School of Pharmaceutical Sciences, Sun Yat-sen University. The X^2 method or Fisher's exact test was used to check the association of TIL with *NUDT15* R139C/*TPMT**3C diplotypes. A receiver operating characteristic (ROC) curve was used to obtain 6TGN cut-off levels to predict the development of TIL.

Research results

TIL was observed in 72 individuals with a median 6TGN level of 323.4 pmol/8 × 10^8 red blood cells (RBC), which was not different from that of patients without TIL (P = 0.071). After comparing the 6TGN levels based on *NUDT15* R139C, for CC (n = 342) and CT (n = 65), the median 6TGN level in patients with TIL was significantly higher than that in patients without (P = 9.4 × 10^{-5}, 474.8 pmol/8 × 10^8 RBC *vs* 306.0 pmol/8 × 10^8 RBC and P = 0.039, 291.7 pmol/8 × 10^8 RBC *vs* 217.6 pmol/8 × 10^8 RBC, respectively). The four TT carriers developed TIL, with a median 6TGN concentration of 135.8 pmol/8 × 10^8 RBC. The 6TGN cut-off levels were 411.5 and 319.2 pmol/8 × 10^8 RBC for the CC and CT groups, respectively. The area under the ROC curve for the obtained predicted probabilities based on *NUDT15* R139C and the 6TGN level was 0.79 (95%CI: 0.76-0.92).

Research conclusions

The predictive sensitivity of TIL based on 6TGN is dramatically increased after subgrouping patients according to *NUDT15* R139C genotypes.

Research perspectives

Applying these specific 6TGN cut-off levels to adjust thiopurine therapies based on *NUDT15* R139C is strongly recommended during the thiopurine treatment.

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ORIGINAL ARTICLE

Observational Study

Quality of life, work productivity impairment and healthcare resources in inflammatory bowel diseases in Brazil

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Abstract

BACKGROUND

Inflammatory bowel diseases (IBD) have been associated with a low quality of life (QoL) and a negative impact on work productivity compared to the general population. Information about disease control, patient-reported outcomes (PROs), treatment patterns and use of healthcare resources is relevant to optimizing IBD management.

AIM

To describe QoL and work productivity and activity impairment (WPAI), treatment patterns and use of healthcare resources among IBD patients in Brazil.

METHODS

A multicenter cross-sectional study included adult outpatients who were previously diagnosed with moderate to severe Crohn's disease (CD) or ulcerative colitis (UC). At enrolment, active CD and UC were defined as having a Harvey Bradshaw Index ≥ 8 or a CD Activity Index ≥ 220 or calprotectin > 200 μg/g or previous colonoscopy results suggestive of inadequate control (per investigator criteria) and a 9-point partial Mayo score ≥ 5, respectively. The PRO assessment included the QoL questionnaires SF-36 and EQ-5D-5L, the Inflammatory Bowel Disease Questionnaire (IBDQ), and the WPAI questionnaire. Information about healthcare resources and treatment during the previous 3 years was collected from medical records. Chi-square, Fisher's exact and Student's *t*-/Mann-Whitney *U* tests were used to compare PROs, treatment patterns and the use of healthcare resources by disease activity ($\alpha = 0.05$).

RESULTS

Of the 407 patients in this study (CD/UC: 64.9%/35.1%, mean age 42.9/45.9 years, 54.2%/56.6% female, 38.3%/37.1% employed), 44.7%/25.2% presented moderate-to-severe CD/UC activity, respectively, at baseline. Expressed in median values for CD/UC, respectively, the SF-36 physical component was 46.6/44.7 and the mental component was 45.2/44.2, the EQ-visual analog scale score was 80.0/70.0, and the IBDQ overall score was 164.0/165.0. Moderate to severe activity, female gender, being unemployed, a lower educational level and lower income were associated with lower QoL ($P < 0.05$). Median work productivity impairment was 20% and 5% for CD and UC patients, respectively, and activity impairment was 30%, the latter being higher among patients with moderate to severe disease activity compared to patients with mild or no disease activity (75.0% *vs* 10.0%, $P < 0.001$). For CD/UC patients, respectively, 25.4%/2.8% had at least one surgery, 38.3%/19.6% were hospitalized, and 70.7%/77.6% changed IBD treatment at least once during the last 3 years. The most common treatments at baseline were biologics (75.3%) and immunosuppressants (70.9%) for CD patients and 5-ASA compounds (77.5%) for UC patients.

CONCLUSION

Moderate to severe IBD activity, especially among CD patients, is associated with a substantial impact on QoL, work productivity impairment and an increased

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number of IBD surgeries and hospitalizations in Brazil.

Key words: Inflammatory bowel disease; Crohn's disease; Ulcerative colitis; Quality of life; Healthcare resources

Core tip: In a large multicenter sample of patients with ulcerative colitis or Crohn's disease (CD), disease activity, female gender, unemployment, and lower education and income were associated with a poorer quality of life. Approximately one-third of patients had some work and activity impairment, the latter increasing with disease activity. CD patients used more health resources, with 25.4% having at least one surgery and 38.3% being hospitalized in the previous 3 years. Inflammatory bowel disease prevalence is increasing, and health services should be prepared to provide an adequate response, including optimal therapies, to manage the care of such patients.

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INTRODUCTION

Crohn's disease (CD) and ulcerative colitis (UC) are chronic inflammatory bowel diseases (IBD) with periods of remission and relapse[1,2]. The incidence and prevalence of IBD have been increasing around the world, particularly in developing countries[3-7]. Furthermore, the impact on patients' quality of life (QoL) can be relevant when considering the onset at usually younger ages and the severity of IBD signs and symptoms, such as abdominal pain, rectal bleeding, diarrhea, and fatigue[1,8,9].

QoL refers to a person's physical functioning, social and emotional well-being, ability to work and freedom from disease symptoms, and has been reported to be significantly lower in IBD patients compared to the general population[10]. In 2007, a European survey observed that 75.6% of IBD patients reported having symptoms that interfered with their ability to enjoy leisure activities, and almost 70% stated that their symptoms negatively affected work performance[11]. Patients with an active disease usually have poor QoL[2,12], but other studies[13,14] have observed that stress level, anxiety or depression, female gender, and fatigue can also contribute to poor QoL in IBD patients. Several studies[12,15,16] have also shown that IBD impacts work productivity.

Knowing the distribution of IBD features such as disease control, patient-reported outcomes (PRO) and treatment patterns is of paramount relevance when optimizing IBD management and improving QoL[17,18]. Data from Latin American countries are needed, although the few studies available seem to indicate that the IBD burden is relevant[19]. In Brazil, epidemiological information about IBD is also scarce[6,9,12,20,21]. The Real-world Data of Moderate to Severe Inflammatory Bowel Disease in Brazil (RISE BR) study was a noninterventional study designed to evaluate disease control and treatment patterns and to compare the burden of disease and QoL in patients with moderate to severe IBD activity *vs* those with mild or no activity. In this work, we describe QoL and work and activity impairment experienced by IBD patients enrolled in the RISE BR study. In addition, we characterize treatment patterns and the use of healthcare resources in this setting.

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MATERIALS AND METHODS

Study design and participants

The RISE BR study was a multicenter, noninterventional study with a cross-sectional evaluation (baseline) and a 3-year retrospective data collection. In the study reported here, we recorded PROs at baseline and assessed the use of IBDs treatment and healthcare resources in the previous 3 years (*i.e.*, baseline-3 years). The study protocol was reviewed and approved by the Ethics Committees of the participant centers. All study participants provided written informed consent prior to study enrollment (ClinicalTrials.gov Identifier: NCT02822235).

Patients were enrolled consecutively from October 2016 to February 2017, when attending scheduled clinical appointments at one of the 14 reference IBD hospitals distributed across Brazilian geographical regions. Eligible patients were ≥ 18 years old, with a diagnosis, by a gastroenterologist, of moderate-to-severe CD or UC for at least 6 mo. Patients with indeterminate/unclassified colitis, who were hospitalized at baseline or who had participated in an experimental study within the 3 years prior to baseline were excluded.

Study variables

Sociodemographic data (age, gender, educational level and professional status), smoking habits, family history of IBD and extraintestinal manifestations were collected from medical records. Other variables included time since IBD diagnosis, location and severity/behavior of UC and CD (Montreal classification) and steroid behavior (dependent or refractory).

CD activity at baseline was evaluated with the Harvey Bradshaw Index (HBI)[22] and/or the Crohn's Disease Activity Index (CDAI)[23], according to local clinical practice. Moderately to severely active CD at baseline was considered when patients had an HBI ≥ 8 or a CDAI ≥ 220 or fecal calprotectin > 200 μg/g or previous colonoscopy results suggestive of inadequate control (as per investigator criteria)[22,24,25]. UC activity at baseline was assessed with the 9-point partial Mayo (pMayo) score[26] (moderate-to-severe activity: pMayo ≥ 5), according to local clinical practice[2,26].

QoL was assessed with three self-administered scales validated for the Brazilian population: The 36-item Short-Form Health Survey (SF-36)[27], the 5-dimensional EuroQoL measure (EQ-5D-5L)[28], and the Inflammatory Bowel Disease Questionnaire (IBDQ)[29]. The SF-36 evaluates eight health dimensions: Physical functioning, physical role functioning, bodily pain, general health perceptions, vitality, social role functioning, emotional role functioning, and mental health. Two summary scores (Physical Component Score and Mental Component Score) are obtained through a standardization of each dimension score and computation of aggregate scores. The component summary ranges from 0 to 100, with higher scores reflecting more a favorable health status[30-32]. The EQ-5D-5L considers five attributes (mobility, self-care, usual activity, pain/discomfort, anxiety/depression) scored with five possible levels (1-no problems, 2-slight problems, 3-moderate problems, 4-severe problems, 5-extreme problems) and a visual analog scale (VAS) where "100" indicates the best health imagined and a "0" represents the worst health imagined[2,15]. The IBDQ score comprises four domains: Bowel function, Emotional status, Systematic symptoms, and Social function[29,33]. Each IBDQ domain scores from 1 (poorest QoL) to 7 (best QoL), and the overall IBDQ score ranges from 32 to 224.

In addition, the impact of IBD on work productivity and daily activities was assessed with the Brazilian version of the Work Productivity and Activity Impairment (WPAI) questionnaire[34]. The WPAI topics are as follows: (1) If the patient was currently employed; (2) How many hours were missed due to the disease; (3) How many hours were missed for other reasons, (4) How many hours were worked; (5) To what degree did the disease affect productivity while working; and (6) To what degree did the disease affect regular activities. Based on the WPAI questionnaire, total work productivity impairment due to IBD (TWPI) was defined as the subjects' total percentage of impaired work time that resulted from both absenteeism (work time missed due to IBD) and presenteeism (impairment while working due to IBD)[16]. Total activity impairment (TAI) due to IBD was also estimated.

Utilization of healthcare resources during the previous 3 years was retrieved from medical records regarding imaging and laboratory testing, surgeries, hospitalizations, and consultations with gastroenterologists or other medical specialists. The type and duration of IBD treatment ongoing at baseline and received during the retrospective 3-year period was also collected.

Statistical analysis

The sample size was estimated for the primary objective of the RISE BR study to

estimate the prevalence of IBD activity. Hence, a sample size of 400 IBD patients was defined to allow estimates of disease activity at baseline with a 95% confidence interval (CI) and a margin of error less than 5%.

All analyses were presented by IBD type (UC or CD). Descriptive statistics were used to analyze sociodemographic, anthropometric and clinical variables. To compare QoL and WPAI according to disease activity at baseline (moderate to severe *vs*. mild or no activity), demographic characteristics and the presence of extraintestinal manifestations, the Chi-square or Fisher's exact tests were used for qualitative variables (*i.e.*, domain items) and the t-test for independent samples, the ANOVA test, the Kruskal-Wallis test or the Mann-Whitney *U* test were used for quantitative variables (*i.e.*, SF-36 domain scores or component summary scores, EQ-5D VAS, IBDQ domain scores or total score, and WPAI scores). The correlation of IBDQ scores and EQ-5D dimensions/VAS with SF-36 domain scores was determined using Spearman's correlation coefficient. There was no imputation of missing data in the study. Statistical tests were two-tailed, and significance was set at 5%. Statistical analysis was performed using SAS® (version 9.4, SAS Institute Inc., Cary).

RESULTS

Study participants and IBD characteristics

Of the 421 screened patients, 407 (96.7%) fulfilled the eligibility criteria and were included, 264 (64.9%) presented with CD and 143 (35.1%) had UC. The mean age (± standard-deviation, sd) was 42.9 ± 13.0 and 45.9 ± 13.8 years for CD and UC patients, respectively (Table 1). Most patients were female (54.2% CD and 56.6% UC patients), and over one-third were employed. The median time since the first diagnosis of CD and UC was 11.4 [range: 0.5-45.0] and 10.4 [range: 0.5-31.0] years, respectively. At baseline, CD patients had a median HBI of 2.0 [range: 0.0-37.0], a median CDAI of 137.0 [range: 25.0-495.0], and 44.7% (95%CI: 38.7-50.7%) presented moderate to severe activity. When considering UC patients, the median pMayo score was 1.0 [range: 0.0-9.0], and 25.2% (95%CI: 18.1-32.3%) presented moderate to severe activity.

QoL evaluation by IBD type and disease activity

Regarding the SF-36 results (Table 2), the median scores of the physical and mental components were 46.6 [range: 20.6-68.6] and 45.2 [range: 5.8-67.2] for CD patients and 44.7 [range: 23.4-63.8] and 44.2 [range: 7.9-65.0] for UC patients, respectively. CD patients with moderate to severe activity had significantly lower scores in all SF-36 domains except for the vitality domain and had a lower physical component score (median: 44.0 *vs* 48.6, $P < 0.001$) and mental component score (median: 42.3 *vs*. 48.4, $P = 0.022$) compared to CD patients with mild or no activity. For UC patients, those with moderate to severe activity also presented lower SF-36 scores in all domains, and both the physical component score (median: 40.5 *vs* 46.2, $P = 0.007$) and mental component score (median: 35.0 *vs* 46.6, $P < 0.001$) were lower than those of UC patients with mild or no activity.

The median [range] EQ-VAS scores for CD and UC patients were 80.0 [5.0-100.0] and 70.0 [0.0-100.0], respectively (Table 2). The majority of CD and UC patients had pain/discomfort (66.9% and 78.7%, respectively), anxiety/depression (63.9% and 63.8%), and problems with conducting their usual activities (52.5% and 53.2%). CD patients with moderate to severe activity had lower EQ-VAS scores (median: 70.0 *vs* 80.0, $P = 0.003$), and more patients reported problems of pain/discomfort ($P = 0.036$) and anxiety/depression ($P = 0.039$) compared to CD patients with mild or no activity. UC patients with moderate to severe activity had lower EQ-VAS scores (median: 50.0 *vs* 80.0, $P < 0.001$), and more patients reported problems in usual activities ($P = 0.009$) and pain/discomfort ($P = 0.012$) compared to UC patients with mild or no activity.

In both IBD types, EQ-VAS scores, as well as the EQ-5D dimensions, were statistically correlated with all SF-36 domains and component summary measures, except for the self-care and mental health dimensions in UC patients (Figure 1). Related dimensions presented larger coefficients, such as physical functioning in SF-36 and mobility in EQ-5D (CD: $r_s = -0.60$ and UC: $r_s = -0.69$) or body pain in SF-36 and pain/discomfort in EQ-5D (CD: $r_s = -0.64$ and UC: $r_s = -0.66$).

The median overall scores of the IBDQ for CD and UC patients were 164.0 and 165.0, respectively (Table 2). The lowest score was obtained for the systemic symptoms' domain (median: 4.8 for both CD and UC patients), and the highest score was observed for the bowel symptoms domain (median: 5.7/5.4 for CD/UC patients, respectively). In both IBD types, patients with moderate to severe activity at baseline presented statistically lower median scores in all IBDQ domains and overall score compared to patients with mild or no activity (CD: 153.5 *vs* 178.0, $P < 0.001$ and UC:

Table 1 Sociodemographic characteristics and clinical features of included patients

	CD patients (*n* = 264)	UC patients (*n* = 143)
Age (yr), mean ± SD	42.9 ± 13.0	45.9 ± 13.8
Female	143 (54.2)	81 (56.6)
Educational level		
Primary school	55 (26.6)	38 (38.8)
Secondary school	83 (40.1)	37 (37.7)
Higher education	69 (33.3)	23 (23.5)
Missing	57	45
Professional situation		
Employed	101 (44.3)	53 (42.7)
Unemployed	61 (26.8)	33 (26.6)
Student	10 (4.4)	5 (4.0)
Retired	30 (13.2)	15 (12.1)
Other	26 (11.4)	18 (14.5)
Missing	36	19
Current smokers[1]	24 (9.9)	3 (2.3)
Missing	22	11
Time since IBD diagnosis (yr), median [range]	10.0 [0.5-45.0]	10.0 [0.5-31.0]
Time since moderate-to-severe diagnosis (yr), median [range]	6.0 [0.5-30.0]	5.0 [0.5-25.0]
Steroid response[2]		
Steroid-dependent	31 (14.8)	23 (19.3)
Steroid-refractory	16 (7.7)	11 (9.2)
Not applicable (no previous use)	87 (41.6)	36 (30.3)
Unknown	75 (35.9)	49 (41.2)
Missing	55	24
Any extraintestinal manifestations	54 (37.8)	30 (38.0)
Family IBD history	33 (12.5)	15 (10.5)
Moderately to severely active disease at baseline[3]	118 (44.7)	36 (25.2)
UC location [*Montreal classification*]		
E1- distal UC	--	43 (30.1)
E2- left-sided	--	26 (18.2)
E3- pancolitis	--	74 (51.7)
UC severity [Montreal classification]		
S0- asymptomatic	--	57 (39.9)
S1- mild UC	--	32 (22.4)
S2- moderate UC	--	40 (28.0)
S3- severe UC	--	14 (9.8)
CD location [Montreal classification]		
L1- ileal	67 (25.4)	--
L2- colonic	42 (15.9)	--
L3- ileocolonic	150 (56.8)	--
L4- upper GI tract disease	17 (6.4)	--
CD behavior [Montreal classification]		
B1- Nonstricturing/nonpenetrating	58 (22.0)	--
B2- Stricturing	110 (41.7)	--
B3- Penetrating	91 (34.5)	--
Perianal disease	105 (39.8)	--
Ileal surface involved ≥ 1 m [*n* = 201]	38 (18.9)	--

Data are shown as *n* (%), except where otherwise mentioned.
[1]Patients were smoking at baseline and has smoked 100 cigarettes in his/her lifetime.
[2]Steroid-dependent disease: patients who either (1) Were unable to reduce steroids below the equivalent of prednisolone 10 mg/d within 3 mo of starting steroids, without recurrent disease, or (2) Had a relapse within 3 mo of stopping glucocorticoids. Steroid-refractory disease: active disease despite prednisolone up to 0.75 mg/kg/d over 4 wk.
[3]Moderate-to-severe activity at baseline was defined as pMayo ≥ 5 (UC) and having HBI ≥ 8 or CDAI ≥ 220 (CD). IBD: Inflammatory bowel disease; UC:

Ulcerative colitis; CD: Crohn's disease.

106.0 *vs* 178.5, $P < 0.001$). The IBDQ dimensions and overall score presented a significantly strong correlation with all SF-36 domains and component summary measures (Figure 1). The correlation coefficient of mental health in SF-36 and emotional health in IBDQ was higher than 0.8 (CD: $r_s = 0.81$ and UC: $r_s = 0.84$).

QoL vs. demographic and clinical characteristics

The SF-36 mental component score was statistically higher among CD/UC male and employed subjects, CD patients aged ≥ 60 years and UC patients with higher income (Table 3). The SF-36 physical component score was statistically higher among CD/UC male, employed subjects and those with higher income, and UC patients with higher education. For both IBD types, the EQ-VAS score was statistically higher among men. The EQ-VAS score was significantly different according to the professional situation of CD patients, with employed patients presenting higher scores. The IBDQ overall score was statistically higher in CD patients who were ≥ 60 years, male, and students. Considering UC patients, higher IBDQ scores were observed in males, employed individuals and those with a higher income.

Work productivity impairment due to IBD

Considering employed patients (CD: $n = 111$, 42.0%; UC: $n = 58$, 40.8%), the median TWPI was 20.0% (CD) and 5.0% (UC) (Table 2). The median TAI was 30.0% for both CD and UC patients. CD patients with moderate to severe disease activity presented higher absenteeism (median: 4.7% *vs* 0.0%, $P = 0.009$) and TAI (median: 50.0% *vs* 20.0%, $P < 0.001$) than those with mild or no disease activity. Moderately to severely active UC patients had higher TAI (75.0% *vs* 10.0%, $P < 0.001$).

Women presented higher activity impairment due to CD than men ($P = 0.014$). Activity impairment due to CD was significantly different by age group (p=0.007) and professional situation ($P < 0.001$) (Table 4). Higher TAI due to UC was observed in women when compared to men ($P = 0.043$). TAI due to UC presented statistically significant differences by professional situation ($P = 0.001$) and by income level ($P = 0.032$) (Table 4).

Healthcare utilization

A total of 108 surgeries were performed in 67 (25.4%) CD patients over the 3-year retrospective period. The median number of surgeries per CD patient was 1.0, and most (20.4%) were anal procedures (fistulectomy) (Table 5). Seven surgeries were performed in 4 (2.8%) UC patients (median 2.0), namely, 2 (28.6%) total colectomies and 2 (28.6%) enterostomy closures, among other interventions ($n = 3$, 42.9%). No statistically significant differences were observed when comparing patients by disease activity.

Regarding IBD hospitalizations, 101 (38.3%) CD patients had a total of 168 hospitalizations (median frequency: 1.0; median duration: 6 d), and 28 (19.6%) UC patients had 43 hospitalizations (median: 1.0; mean duration: 4 days). CD patients with moderate to severe disease activity at baseline had more hospitalizations (median: 2.0 *vs* 1.0 hospitalizations/patient, $P = 0.031$) than those with mild or no disease activity; no statistically significant differences were observed for UC patients by disease activity.

CD and UC patients attended 3192 (median 11.0) and 1541 (median 10.0) medical appointments, respectively. More than 90% of consultations were with IBD specialists for both IBD types. The assessment of disease activity through common scores, such as CDAI and HBI for CD patients and Partial Mayo Score for UC patients, was not performed in most medical appointments. When comparing by disease activity, no statistically significant differences were observed regarding the number or type of consultations for both IBD types. Changes in treatment occurred in 16.4% and 22.9% of CD and UC consultations, respectively, and CD patients with moderate to severe activity had a higher proportion of medical appointments with treatment changes (18.3% *vs* 14.7%, $P = 0.005$) than those with mild or low disease activity.

A total of 5674 imaging/laboratory tests were performed by 260 CD patients (median 19.0) and 2509 by 141 UC patients (median 15.0). Hemograms were the most frequent test, followed by quantification of C-reactive protein. Colonoscopies accounted for 6.1% (CD) and 9.1% (UC) of the total tests. No statistically significant differences were observed in IBD activity at baseline.

Most surgeries (CD/UC: 44.4%/71.5%) and hospitalizations (CD/UC: 47.6%/69.8%) occurred among IBD patients < 5 years since the first diagnosis of moderate to severe disease compared to patients diagnosed 5 to 10 years or 10 or more years prior (Figure 2). In addition, UC patients diagnosed for less than 5 years

Table 2 Quality of life according to type of inflammatory bowel disease and disease activity

	CD				UC			
	Total	Moderate to severe activity	No or mild activity	*P* value	Total	Moderate to severe activity	No or mild activity	*P* value
n	263	118	145		143	36	107	
Missing value[1]	1	0	1		0	0	0	
SF-36 scores								
Physical component	46.6 [20.6-68.6]	44.0 [20.6-62.3]	48.6 [27.1-68.6]	< 0.001	44.7 [23.4-63.8]	40.5 [23.4-58.8]	46.2 [23.9-63.8]	0.007
Physical functioning	75.0 [10.0-100.0]	70.0 [10.0-100.0]	85.0 [15.0-100.0]	< 0.001	70.0 [0.0-100.0]	55.0 [0.0-100.0]	77.5 [0.0-100.0]	0.043
Physical role	68.8 [0.0-100.0]	50.0 [0.0-100.0]	75.0 [0.0-100.0]	< 0.001	59.4 [0.0-100.0]	40.6 [0.0-100.0]	75.0 [0.0-100.0]	< 0.001
Body pain	52.0 [0.0-100.0]	51.0 [0.0-100.0]	62.0 [10.0-100.0]	< 0.001	51.0 [0.0-100.0]	31.0 [0.0-100.0]	60.5 [0.0-100.0]	0.001
General health	52.0 [0.0-100.0]	46.0 [0.0-100.0]	57.0 [10.0-100.0]	< 0.001	51.0 [5.0-100.0]	31.0 [5.0-100.0]	55.0 [5.0-100.0]	< 0.001
Mental component	45.2 [5.8-67.2]	42.3 [14.0-63.6]	48.4 [5.8-67.2]	0.022	44.2 [7.9-65.0]	35.0 [7.9-64.1]	46.6 [7.9-65.0]	< 0.001
Vitality	56.3 [0.0-100.0]	50.0 [6.3-100.0]	56.3 [0.0-100.0]	0.074	50.0 [0.0-100.0]	37.5 [0.0-93.8]	56.3 [0.0-100.0]	0.002
Social role functioning	62.5 [0.0-100.0]	50.0 [0.0-100.0]	75.0 [0.0-100.0]	0.002	62.5 [0.0-100.0]	50.0 [0.0-100.0]	62.5 [0.0-100.0]	0.002
Emotional role	75.0 [0.0-100.0]	66.7 [0.0-100.0]	79.2 [0.0-100.0]	0.012	75.0 [0.0-100.0]	45.8 [0.0-100.0]	75.0 [0.0-100.0]	0.002
Mental health	65.0 [0.0-100.0]	55.0 [0.0-100.0]	72.5 [0.0-100.0]	0.008	60.0 [0.0-100.0]	45.0 [5.0-100.0]	70.0 [0.0-100.0]	< 0.001
EQ-VAS (cm)	80.0 [5.0-100.0]	70.0 [5.0-100.0]	80.0 [15.0-100.0]	0.003	70.0 [0.0-100.0]	50.0 [0.0-100.0]	80.0 [0.0-100.0]	< 0.001
EQ-5D [no problems], *n* (%)								
Mobility	193 (73.4)	80 (67.8)	113 (77.9)	0.080[2]	85 (59.9)	17 (47.2)	68 (64.2)	0.073[2]
Self-care	225 (85.6)	95 (80.5)	130 (89.7)	0.052[2]	120 (84.5)	29 (80.6)	91 (85.8)	0.448[2]
Usual activities	125 (47.5)	47 (39.8)	78 (53.8)	0.158[2]	66 (46.8)	10 (27.8)	56 (53.3)	0.009[2]
Pain/discomfort	87 (33.1)	31 (26.3)	56 (38.6)	0.036[2]	30 (21.3)	3 (8.3)	27 (25.7)	0.012[2]
Anxiety/depression	95 (36.1)	31 (26.3)	64 (44.1)	0.039[2]	51 (36.2)	9 (25.0)	42 (40.0)	0.114[2]
IBDQ score	164.0 [50.0-224.0]	153.5 [50.0-222.0]	178.0 [57.0-224.0]	< 0.001	165.0 [47.0-224.0]	106.0 [48.0-217.0]	178.5 [47.0-224.0]	< 0.001
Bowel symptoms	5.7 [1.4-7.0]	5.3 [1.4-7.0]	6.0 [1.8-7.0]	< 0.001	5.4 [1.3-7.0]	3.7 [1.3-7.0]	6.1 [1.5-7.0]	< 0.001
Emotional health	4.8 [1.2-7.0]	4.4 [1.2-6.9]	5.3 [1.5-7.0]	0.001	4.9 [1.0-7.0]	2.9 [1.2-6.6]	5.3 [1.0-7.0]	< 0.001
Systemic symptoms	4.8 [1.0-7.0]	4.4 [1.2-7.0]	5.0 [1.0-7.0]	< 0.001	4.8 [1.0-7.0]	3.2 [1.0-7.0]	5.2 [1.0-7.0]	< 0.001
Social function	5.4 [1.2-7.0]	5.0 [1.2-7.0]	5.8 [1.4-7.0]	< 0.001	5.4 [1.2-7.0]	3.8 [1.2-7.0]	6.0 [1.2-7.0]	< 0.001
WPAI scores								
% TWPI	20.0 [0.0-100.0]	30.0 [0.0-100.0]	19.7 [0.0-100.0]	0.053	5.0 [0.0-100.0]	34.8 [0.0-100.0]	0.0 [0.0-100.0]	0.082
% work time missed	0.0 [0.0-100.0]	4.7 [0.0-100.0]	0.0 [0.0-64.0]	0.009	0.0 [0.0-100.0]	5.9 [0.0-100.0]	0.0 [0.0-100.0]	0.287
% impairment while working	10.0 [0.0-100.0]	10.0 [0.0-80.0]	10.0 [0.0-100.0]	0.336	0.0 [0.0-80.0]	20.0 [0.0-80.0]	0.0 [0.0-80.0]	0.08
% TAI	30.0 [0.0-100.0]	50.0 [0.0-100.0]	20.0 [0.0-100.0]	< 0.001	30.0 [0.0-100.0]	75.0 [0.0-100.0]	10.0 [0.0-100.0]	< 0.001

[1]The missing data is due to a patient who has not completed SF-36 at Day 1. All p-values from Mann-Whitney *U* test, except
[2]Chi-square test. Data are shown as median [range], except otherwise mentioned. CD: Crohn's disease; UC: Ulcerative colitis. SF-36: Short-form 36; EQ-5D: Euro quality of life – 5 dimensions; EQ-VAS: EQ-5D visual analog scale. IBDQ: Inflammatory bowel disease questionnaire. WPAI: Work Productivity and Activity Impairment Questionnaire. TWPI: Total work productivity impairment; TAI: Total activity impairment.

had the most medical appointments (45.0%), with 25.1% occurring in patients diagnosed between 6 months and 3 years prior. Medical appointments with treatment changes occurred most frequently (CD/UC: 38.5%/46.7%) among patients diagnosed for less than 5 years, as did imaging/laboratory tests (CD/UC: 44.5%/36.5%),

	EQ-5D						IBDQ				
	Mobility	Self-care	Usual activities	Pain / discomfort	Anxiety / depression	EQ-VAS	Bowel systems	Emotional health	Systematic systems	Social function	IBDQ score
Crohn's disease											
Physical functioning	-0.6	-0.4	-0.6	-0.54	-0.42	0.57	0.51	0.56	0.66	0.64	0.63
Physical role	-0.47	-0.29	-0.68	-0.54	-0.44	0.57	0.63	0.63	0.72	0.74	0.73
Body pain	-0.49	-0.32	-0.54	-0.64	-0.44	0.54	0.59	0.57	0.63	0.56	0.64
General health	-0.35	-0.28	-0.56	-0.58	-0.51	0.63	0.59	0.65	0.67	0.6	0.68
Vitality	-0.32	-0.24	-0.5	-0.54	-0.61	0.57	0.56	0.73	0.74	0.6	0.73
Social functioning	-0.4	-0.3	-0.6	-0.56	-0.57	0.51	0.62	0.73	0.72	0.65	0.74
Emotional role	-0.41	-0.24	-0.54	-0.5	-0.6	0.53	0.55	0.69	0.66	0.59	0.7
Mental health	-0.35	-0.27	-0.49	-0.58	-0.72	0.53	0.54	0.81	0.69	0.56	0.74
SF-36 summary											
Mental component score	-0.31	-0.23	-0.48	-0.55	-0.71	0.52	0.56	0.81	0.71	0.57	0.75
Physical component score	-0.55	-0.38	-0.66	-0.6	-0.34	0.61	0.61	0.53	0.68	0.69	0.66
Ulcerative colitis											
Physical functioning	-0.69	-0.38	-0.64	-0.63	-0.4	0.48	0.62	0.57	0.65	0.68	0.65
Role physical	-0.53	-0.28	-0.75	-0.62	-0.4	0.65	0.72	0.66	0.75	0.78	0.75
Body pain	-0.58	-0.36	-0.57	-0.66	-0.54	0.47	0.62	0.58	0.6	0.62	0.64
General health	-0.47	-0.26	-0.56	-0.52	-0.45	0.62	0.62	0.69	0.58	0.6	0.68
Vitality	-0.52	-0.25	-0.51	-0.59	-0.61	0.6	0.65	0.72	0.73	0.62	0.73
Social functioning	-0.45	-0.24	-0.58	-0.56	-0.51	0.54	0.64	0.71	0.67	0.69	0.72
Role-emotional	-0.43	-0.24	-0.54	-0.58	-0.58	0.51	0.64	0.71	0.64	0.66	0.71
Mental Health	-0.41	-0.16	-0.45	-0.53	-0.72	0.56	0.66	0.84	0.66	0.64	0.77
SF-36 summary											
Mental component score	-0.37	-0.16	-0.46	-0.53	-0.69	0.54	0.66	0.82	0.67	0.65	0.77
Physical component score	-0.67	-0.4	-0.73	-0.65	-0.34	0.57	0.67	0.56	0.67	0.72	0.68

Spearman's correlation coeficient:

0.10-0.29	Very low
0.30-0.49	Low
0.50-0.69	Moderate
⩾0.70	High

Figure 1 Spearman's correlation coefficients between domains and summary measures of the different quality of life measures. SF-36: Short-form 36; EQ-5D: Euro quality of life – 5 dimensions; EQ-VAS: EQ-5D visual analog scale; IBDQ: Inflammatory bowel disease questionnaire.

compared to patients diagnosed 5 to 10 years or 10 or more years.

IBD treatment at baseline and changes over the previous 3 years

At baseline, the majority of CD (95.1%) and UC (90.2%) patients were on IBD treatment (Table 6). The median number of concurrent medicines at baseline, by patient, was 2.0 for both CD and UC patients. The most common treatments at baseline were biologics (75.3%) and immunosuppressants (70.9%) for CD patients and 5-ASA compounds (77.5%) for UC patients. Considering each IBD medicine (Figure 3), azathioprine was used by 65.7% CD patients, followed by infliximab (42.2%) and adalimumab (31.1%). Among UC patients, 5-ASA (72.9%), azathioprine (45.7%) and infliximab (24.0%) were the three most frequently used treatments.

Most CD patients with moderate to severe disease activity were receiving an immunosuppressant (72.1%) and/or biologic (71.2%). Among CD patients with mild or no disease activity, most were receiving a biologic (78.6%) and/or immunosuppressant (70.0%) as well. With regard to UC patients, the majority were receiving at least one 5-ASA compound (84.4% *vs* 75.3%, in moderate to severe *vs*. mild or no disease activity, respectively) (Table 6).

The majority of CD (70.7%) and UC (77.6%) patients changed treatment at least once during the previous 3 years, including changes in antibiotics and corticosteroids. CD patients had fewer treatment changes than UC patients (median: 1.0 *vs* 2.0; P = 0.036), and changes were not statistically associated with disease activity in both IBD types. Most of the treatment changes were discontinuations (CD/UC: 43.5%/38.1%). When considering only biologics, immunosuppressants and 5-ASA compounds, most dose changes and discontinuations occurred in immunosuppressants (50.7% and 39.8%) for CD patients and 5-ASA compounds (69.5% and 64.1%) for UC patients. With regard to biologic therapy, dose changes were due to poor effectiveness

Table 3 Quality of Life scores by sociodemographic and clinical characteristics and by type of inflammatory bowel disease

	CD								UC							
	SF-36 mental component		SF-36 physical component		EQ-VAS		IBDQ score		SF-36 mental component		SF-36 physical component		EQ-VAS		IBDQ score	
	mean ± SD	*P* value	mean ± SD	*P* value	mean ± SD	*P* value	mean ± SD	*P* value	mean ± SD	*P* value	mean ± SD	*P* value	mean ± SD	*P* value	mean ± SD	*P* value
Age (years)		0.018		0.895		0.514		0.002		0.155[3]		0.263		0.534		0.847[3]
18-39	44.8 ± 13.0		43.1 ± 10.5		72.4 ± 20.3		158.9 ± 41.0		46.5 ± 10.0		46.0 ± 10.0		67.7 ± 22.2		151.6 ± 43.8	
40-59	41.9 ± 12.9		43.5 ± 14.3		73.4 ± 20.5		151.0 ± 43.4		43.1 ± 10.1		45.0 ± 9.8		65.5 ± 25.6		153.0 ± 54.8	
≥60	48.8 ± 9.4		42.0 ± 16.8		76.1 ± 19.8		178.7 ± 30.1		46.1 ± 9.7		48.2 ± 7.9		69.9 ± 30.5		157.2 ± 46.8	
Gender		< 0.001[1]		0.027[1]		0.004[1]		< 0.001[1]		0.004[1]		0.032[1]		0.042[1]		< 0.00[1]
Male	48.0 ± 11.3		45.9 ± 13.6		77.5 ± 18.0		169.5 ± 38.0		47.5 ± 9.7		47.3 ± 8.9		72.4 ± 22.0		169.7 ± 43.3	
Female	41.2 ± 12.9		41.0 ± 13.1		69.8 ± 21.4		149.9 ± 42.1		42.7 ± 9.9		44.8 ± 10.0		62.8 ± 26.8		140.7 ± 50.8	
Professional situation		0.002		0.030 [2]		0.003		< 0.001		< 0.001[2]		< 0.001		0.130		0.001
Employed	47.5 ± 11.5		47.6 ± 11.4		78.1 ± 19.4		168.1 ± 40.4		49.4 ± 8.9		49.2 ± 8.0		73.2± 23.2		175.7 ± 39.3	
Unemployed	40.0 ± 14.0		39.2 ± 13.5		67.6 ± 21.2		145.3 ± 40.6		41.2 ± 9.8		42.9 ± 9.3		64.9± 25.9		135.8 ± 51.5	
Student	43.0 ± 16.0		44.2 ± 11.2		75.5 ± 18.6		172.6 ± 42.8		42.0 ± 12.6		54.2 ± 9.8		61.6± 15.2		139.6 ± 35.3	
Retired	46.4 ± 8.2		39.3 ± 18.3		75.2 ± 15.8		165.2 ± 33.5		41.7 ± 10.7		45.4 ± 7.8		55.2± 31.4		135.7 ± 53.3	
Other	39.0 ± 14.0		40.3 ± 12.7		64.8 ± 23.4		135.5 ± 50.7		41.2 ± 8.9		39.8 ± 10.2		62.2± 24.3		138.4 ± 52.2	
Educational level		0.802		0.176 [2]		0.990		0.398 [2]		0.122 [2]		0.028[2]		0.510		0.185
Primary school	43.7 ± 11.4		43.7 ± 11.4		73.2 ± 20.8		156.3 ± 40.8		44.0 ± 9.3		44.0 ± 9.3		66.6 ± 27.9		143.0 ± 56.8	
Secondary school	43.0 ± 13.3		43.0 ± 13.3		73.1 ± 20.7		152.9 ± 44.9		45.3 ± 9.9		45.3 ± 9.9		66.4 ± 25.8		160.0 ± 47.6	
Higher education	44.1 ± 13.2		44.1 ± 13.2		72.8 ± 20.1		162.2 ± 39.1		48.4 ± 8.9		48.4 ± 8.9		76.0 ± 6.5		171.4 ± 42.3	
Income		0.112		0.007[2]		0.547		0.087[2]		< 0.001		0.095[2]		0.070[2]		0.002[2]
> 3x MW	46.2 ± 11.9		47.7 ± 9.2		74.6 ± 21.4		169.2 ± 39.6		52.3 ± 9.4		49.3 ± 8.0		75.3 ± 16.2		179.0 ± 39.7	
> 1x – 3x MW	43.6 ± 11.8		46.1 ± 14.0		73.9 ± 20.2		157.6 ± 40.2		44.1 ± 9.0		46.2 ± 9.1		69.8 ± 24.6		158.7 ± 49.3	
< 1x MW	40.4 ± 13.6		36.2 ± 13.3		70.9 ± 20.8		149.4 ± 43.9		39.2 ± 10.0		45.9 ± 9.8		58.8 ± 26.2		124.0 ± 50.7	

Any EIM	0.470[3]	0.924[3]	0.687[1]	0.750[1]	0.343[3]	0.242[3]	0.601[1]	0.701[1]
Yes	44.8 ± 13.0	42.0 ± 14.9	70.7 ± 19.6	158.0 ± 42.2	42.6 ± 8.6	44.4 ± 7.9	69.6 ± 19.0	149.6 ± 48.5
No	43.2 ± 11.9	42.3 ± 12.9	71.3 ± 22.0	161.0 ± 40.7	44.7 ± 10.4	46.2 ± 10.4	64.1 ± 27.8	152.2 ± 49.8

All *P*-values from Kruskal-Wallis test, except
[1]Mann-Whitney *U* test,
[2]ANOVA test, and
[3]*t*-test. EIM: Extraintestinal Manifestations; MW: Minimum wage.

according to physician criteria in 22 (41.5%) CD patients and poor effectiveness and serum level of the biologic drug in 3 (each) UC patients. Discontinuations of biologics were mainly due to adverse reactions (CD/UC: 29.2%/27.5%).

DISCUSSION

The RISE BR study is the first study with a real-world characterization of the burden of moderate to severe IBD in Brazil, both in the patient and payer perspectives. Overall, 407 (143 UC and 264 CD) patients diagnosed with moderate to severe disease were included, and at enrolment, 25% UC and 45% CD patients had moderately to severely active disease.

QoL was assessed with both generic and disease-specific questionnaires. Irrespective of IBD type, the SF-36 summary scores were low (*i.e.*, scores less than 50, in a range from 1-100). Other Brazilian single-center studies have observed no low SF-36 scores[29] or that SF-36 was low regarding physical limitations and emotional aspects domains[35,36]. Our results may reflect that patients with a previous diagnosis of moderate to severe IBD, even though treated, still perceive their general health as poor and that IBD physically and emotionally impacts their life. These findings are supported by the EQ-5D results, with the most compromised dimensions being pain/discomfort and anxiety/depression and better results in the mobility and self-care dimensions. Not surprisingly, a correlation was observed between these two general questionnaires, especially when considering the EQ-VAS and the SF-36 summary scores and comparing between related dimensions (*e.g.*, SF-36 physical functioning *vs*. EQ-5D mobility).

The IBDQ results are in line with those of the general QoL questionnaires, and a significant correlation was observed between SF-36 and IBDQ, as reported by other Brazilian studies[29,36]. Considering that the overall IBDQ score ranged between 32 and 224, the observed scores were slightly above the midpoint value, with poorer results for the emotional health and systemic symptoms domains, irrespective of IBD type. Pontes *et al*. observed a higher range of IBDQ overall scores (min-max: 114-222), probably due to the inclusion of mostly patients with no active IBD[29].

Notably, patients with moderate to severe active disease had lower QoL scores in SF-36 domains and summary measures, EQ-VAS and IBDQ dimensions and overall score versus those with mild or no disease activity. In fact, patients with moderate to severe disease activity had a median IBDQ greater than 170 and scored more than 16 points of difference when compared to patients with mild or no disease activity[37]. Other clinically significant differences were also observed in the SF-36 summary measures (more than 2 points of difference) and EQ-VAS scores (more than 8 points)[37,38]. This pattern has been described in several studies across different world regions[38,39]. For instance, one recent French survey reported that the risk of low QoL was significantly increased with greater disease activity[40]. A Polish study showed that CD patients in remission had lower QoL and work productivity impairment compared to patients with active disease[41]. The same trend was observed in one local single-site study conducted in Mato Grosso, Brazil[36]. Parra *et al*. also showed that during maintenance treatment with infliximab, adequate serum levels are associated with higher rates of clinical remission, mucosal healing and QoL[12]. In line with other studies[13,39,40,42,43], female gender, being unemployed, lower educational level and lower income were associated with poor QoL in almost all domains and summary measures of the different QoL scales. Pain and the intensity of other symptoms during relapses are disruptive of daily life and are particularly relevant for younger and more socially active patients. However, disease activity is not the only determinant of QoL, as other sociodemographic characteristics play a role in the way patients perceive their disease. To provide the best care to IBD patients, subgroups of patients at higher risk

Table 4 Work productivity and activity impairment scores by sociodemographic and clinical characteristics and by type of inflammatory bowel diseases

	CD								UC							
	% TWPI		% work time missed		% impairment while working		% TAI		% TWPI		% work time missed		% impairment while working		% TAI	
	mean ± SD	*P* value	mean ± SD	*P* value	mean ± SD	*P* value	mean ± SD	*P* value	mean ± SD	*P* value	mean ± SD	*P* value	mean ± SD	*P* value	mean ± SD	*P* value
Age (yr)		0.522		0.208		0.541		0.007		0.803		0.453		0.929		0.433
18-39	31.8 ± 32.4		13.8 ± 23.6		23.3 ± 26.4		36.3 ± 34.1		28.5 ± 34.6		13.7 ± 27.1		19.6 ± 28.0		44.3 ± 36.1	
40-59	35.2 ± 35.1		12.9 ± 25.8		27.4 ± 30.8		41.2 ± 34.2		23.7 ± 33.6		11.7 ± 29.3		15.9 ± 25.0		37.6 ± 37.5	
≥ 60	19.6 ± 25.8		4.2 ± 11.8		15.6 ± 21.3		21.2 ± 25.4		19.6 ± 29.4		3.6 ± 6.2		18.0 ± 26.8		37.4 ± 40.9	
Gender		0.186 [1]		0.914[1]		0.148 [1]		0.014[1]		0.465[1]		0.649[1]		0.092[1]		0.043[1]
Male	27.8 ± 30.5		13.0 ± 24.4		20.0 ± 23.4		30.4 ± 32.6		22.5 ± 32.0		13.4 ± 27.9		12.4 ± 21.9		31.8 ± 36.2	
Female	37.6 ± 35.5		12.2 ± 23.2		29.6 ± 32.0		40.3 ± 33.6		29.5 ± 35.1		9.3 ± 25.2		24.6 ± 29.8		45.9 ± 37.5	
Professional situation		0.198		0.753		0.078		< 0.001		-		-		-		0.001
Employed	30.1 ± 31.4		11.0 ± 20.5		23.0 ± 27.8		24.2 ± 30.6		23.4 ± 31.5		9.4 ± 23.8		17.7 ± 26.2		23.7 ± 30.7	
Unemployed	54.7 ± 18.0		5.4 ± 6.7		56.0 ± 16.7		49.8 ± 33.3		-		-		-		52.4 ± 39.1	
Student	-		-		-		23.0 ± 27.9		-		-		-		56.0 ± 24.1	
Retired	62.5 ± 17.7		25.0 ± 35.3		33.3 ± 28.9		32.3 ± 32.2		0.0		0.0		0.0		48.7 ± 43.4	
Other	45.0 ± 63.6		33.3 ± 47.1		35.0 ± 49.5		56.2 ± 29.1		-		-		-		51.1 ± 36.3	
Educational level		0.608		0.591		0.455		0.114		0.331		0.571		0.186		0.252
Primary school	37.4 ± 30.8		12.3 ± 15.6		28.6 ± 29.0		39.6 ± 34.9		15.7 ± 29.1		8.0 ± 17.7		8.0 ± 19.3		39.2 ± 40.8	
Secondary school	37.1 ± 33.4		9.0 ± 17.7		29.1 ± 30.5		43.6 ± 36.6		26.3 ± 32.5		10.1 ± 28.6		21.2 ± 26.0		42.2 ± 37.5	
Higher education	30.0 ± 33.6		16.0 ± 26.6		20.5 ± 25.6		29.1 ± 28.4		10.6 ± 19.4		1.1 ± 2.7		10.0 ± 18.3		25.7 ± 31.7	
Income		0.293		0.708		0.042		0.079		0.341		0.648		0.453		0.032
> 3x MW	33.0 ± 35.5		13.4 ± 25.6		25.8 ± 30.2		25.9 ± 30.8		23.0 ± 33.0		9.3 ± 27.5		22.0 ± 28.6		25.8 ± 36.7	
> 1x – 3x MW	28.0 ± 28.5		13.9 ± 23.8		16.8 ± 20.1		38.6 ± 34.2		27.6 ± 27.4		7.1 ± 16.4		16.7 ± 22.7		38.9 ± 36.8	
< 1x MW	60.0 ± 10.8		7.3 ± 8.9		50.0 ± 24.5		42.3 ± 35.1		0.0 ± 0.0		0.0 ± 0.0		0.0 ± 0.0		56.8 ± 35.8	
Any EIM		0.005[1]		0.004[1]		0.199 [1]		0.656[1]		0.345[1]		0.824[1]		0.214 [1]		0.896[1]
Yes	53.9 ± 33.4		23.3 ± 28.2		32.9 ± 31.8		37.2 ± 32.1		28.5 ± 31.2		1.8 ± 4.4		27.3 ± 31.0		37.0 ± 36.4	

No	23.5 ± 31.9	6.2 ± 15.4	21.0 ± 28.4	35.1 ± 33.8	19.0 ± 31.6	9.4 ± 26.6	13.7 ± 23.9	36.7 ± 38.4

All *P*-values from Kruskal-Wallis test, except
[1]Mann-Whitney *U* test. EIM: Extraintestinal manifestations; MW: Minimum wage; TWPI: Total work productivity impairment; TAI: Total activity impairment.

of poor QoL should be identified and offered additional coping strategies and social support.

IBD has a relevant impact on work productivity and daily activities. In our study, patients had approximately 30% impaired worktime, with approximately 12% absenteeism and 18%-24% presenteeism, and approximately 36%-40% TAI. Of note, unemployment frequency in IBD patients (23%) was higher than that of the general population of Brazil (12.6%), in 2017[44]. In addition, TAI was higher among IBD patients with moderate to severe disease activity but also among women, middle-aged patients (40-59 years old, CD only) and patients with lower income (UC only). Other studies have reported the same association, with a TAI of approximately 30%[15,40]. Froes *et al*. have described that IBD in Brazil leads to frequent and prolonged disability periods and contributes to early retirement, especially among CD patients[45]. A European survey conducted in 2010-2011 with 4670 IBD patients showed that, during previous year, only 25% had not been absent from work due to IBD and that 25% had been absent for more than 25 d[46].

To the best of our knowledge, this is the first study to report the use of healthcare resources for IBD patients in Brazil, even though studies from other countries have observed the high economic burden of these conditions[2,47-51]. Approximately one-quarter of CD patients had at least one surgery during the last 3 years. The proportion of UC patients with previous surgeries was considerably smaller (3%), but approximately 30% underwent a high-cost colectomy. Almost half of CD patients and approximately 35% of UC patients had at least one previous hospitalization, with a median duration of 6 and 4 days, respectively. Gibson *et al*. observed a higher frequency (43.5%) of hospitalization in UC patients in Australia, which may reflect differences in the access of hospital care[2]. On the other hand, the large majority of the medical appointments in our study were with IBD specialists, while in other countries, IBD follow-up is also performed by general practitioners[2,52-54]. Interestingly, CD patients with moderate to severe disease activity at baseline and those with less time since the first diagnosis of moderate to severe disease had more previous hospitalizations and medical appointments with treatment changes, which may reflect the difficulty of maintaining or achieving remission.

In our study, almost all patients had at least one imaging or laboratory test, with almost half having more than 20 tests in the previous 3 years. However, colonoscopy only accounted for approximately 6%-9% of the procedures, and other IBD-specific tests had an even smaller frequency. This finding, aligned with the rare use of clinical scores during medical appointments, make us speculate that other information (such as specific symptoms or patient-reported disease activity) may be more relevant for IBD specialists in Brazil when deciding about treatment[55].

Almost all IBD patients were on some form of treatment at baseline, mainly with immunosuppressants and biologics among CD patients and with 5-ASA compounds in UC patients. In fact, the treatment pattern at baseline is in line with other studies from Latin America[19]. International guidelines recommend the use of 5-ASA compounds (especially in proctitis and left-sided UC) and/or corticosteroids (preferred in CD patients) for the induction phase and, in more severe or refractory cases, azathioprine and biological agents, while salicylates, thiopurines and biologics are usually recommended for the maintenance period[56-58].

Some study limitations should be addressed. The retrospective data collection may bias the estimates of treatment changes and healthcare use due to missing data on patient's medical records. For instance, patients could have more than one IBD specialist, with medical appointments that are not registered, thus underestimating healthcare use. On the other hand, the cross-sectional evaluation does not enable us to conclude if patients with higher disease activity at baseline should have received a more effective treatment, especially when considering the relapsing and remission nature of IBD. We cannot exclude that the inclusion of patients with a previous diagnosis of moderate to severe disease, although deliberate, may have contributed to a higher rate of active disease at baseline, thus affecting the generalization of results to all IBD patients. Moreover, selection bias was minimized by the consecutive enrolment at scheduled clinical appointments, but patients with an active disease have a higher chance of having a medical appointment during the recruitment period. Finally, the smaller number of UC patients may have compromised the power of

Table 5 Utilization of healthcare resources during the retrospective 3-year period

	CD				UC			
	Total	Moderate to severe activity	No or mild activity	*P* value	Total	Moderate to severe activity	No or mild activity	*P* value
n	264	118	146		143	36	107	
IBD surgeries								
At least one IBD surgery	67 (25.4)	32 (27.1)	35 (24.0)	0.559 [1]	4 (2.8)	1 (2.8)	3 (2.8)	--
Surgeries, *n*	108	45	63		7	2	5	
Surgeries/pt, median [range]	1.0 [1-5]	1.0 [1-4]	2 [1-5]	0.074	2.0 [1-2]	2	2.0 [1-2]	--
More than one IBD surgery	28 (41.8)	10 (31.2)	18 (51.5)		3 (75.0)	1 (100.0)	2 (66.7)	
Type [frequency ≥ 5%]								
Partial colectomy	13 (12.0)	5 (11.1)	8 (12.7)		0 (0.0)	0 (0.0)	0 (0.0)	
Total colectomy	1 (0.9)	0 (0.0)	1 (1.6)		2 (28.6)	1 (50.0)	1 (20.0)	
Drainage of anorectal abscess	6 (5.6)	3 (6.7)	3 (4.8)		0 (0.0)	0 (0.0)	0 (0.0)	
Fistulectomy	22 (20.4)	9 (20.0)	13 (20.6)		0 (0.0)	0 (0.0)	0 (0.0)	
Enterostomy	13 (12.0)	6 (13.3)	7 (11.1)		0 (0.0)	0 (0.0)	0 (0.0)	
Enterostomy closure	5 (4.6)	5 (11.1)	0 (0.0)		2 (28.6)	0 (0.0)	2 (40.0)	
IBD hospitalizations								
At least one IBD hospitalization	101 (38.3)	51 (43.2)	50 (34.2)	0.136 [1]	28 (19.6)	11 (30.6)	17 (15.9)	0.055 [1]
Hospitalizations, *n*	168	93	75		43	18	25	
Hospitalizations/pt, median [range]	1.0 [1-5]	2.0 [1-4]	1.0 [1-5]	0.031	1.0 [1-5]	1.0 [1-5]	1.0 [1-3]	0.978
More than one hospitalization	47 (46.5)	29 (56.8)	18 (36.0)		10 (35.7)	4 (36.4)	6 (35.3)	
Duration (d), median [range]	6 [1-98]	5 [0-76]	4.5 [0-97]		4 [1-737]	2.5 [0-20]	5 [0-737]	
IBD medical appointments								
At least one IBD consultation	263 (99.6)	117 (99.2)	146 (100.0)		143 (100.0)	36 (100.0)	107 (100.0)	
Consultations, *n*	3192	1466	1726		1541	423	1118	
Consultations/pt, median [range]	11.0 [1-45]	12.0 [1-45]	11.0 [1-35]	0.801	10.0 [1-39]	9.5 [1-39]	10.0 [2-30]	0.896
More than 20 consultations	27 (10.3)	15 (12.8)	12 (8.2)		9 (6.3)	4 (11.1)	5 (4.7)	
Type								
IBD specialist	2989 (93.6)	1356 (92.4)	1633 (94.6)		1426 (92.5)	394 (93.1)	1032 (92.3)	
Emergency	43 (1.3)	25 (1.7)	18 (1.0)		34 (2.2)	20 (4.7)	14 (1.3)	
Other specialist	160 (5.0)	85 (5.8)	75 (4.3)		81 (5.3)	9 (2.1)	72 (6.4)	

Consultations with registered score								
HBI	11 (0.3)	1 (0.1)	10 (0.6)		--	--	--	
CDAI	103 (3.2)	54 (3.7)	49 (2.8)		--	--	--	
Other CD scores	30 (0.9)	21 (1.4)	9 (0.5)		--	--	--	
pMayo score	--	--	--		95 (6.2)	12 (2.8)	83 (7.4)	
Consultations with change of IBD treatment	522 (16.4)	269 (18.3)	253 (14.7)	0.005 [1]	353 (22.9)	86 (20.3)	267 (23.9)	0.139 [1]
IBD imaging and laboratory testing								
At least one IBD test	260 (98.5)	116 (98.3)	144 (98.6)	--	141 (98.6)	36 (100.0)	105 (98.1)	--
IBD tests, *n*	5674	2722	2952		2509	643	1866	
Tests/pt, median [range]	19.0 [1-143]	20.0 [1-143]	16.0 [1-94]	0.372	15.0 [1-82]	14.5 [2-82]	16 [1-75]	0.767
More than 20 tests	118 (45.4)	56 (48.3)	62 (43.1)		57 (40.4)	15 (41.7)	42 (40.0)	
Type [frequency ≥ 5%]								
Complete blood cell count	2317 (40.8)	1077 (39.6)	1240 (42.0)		973 (38.8)	250 (38.9)	723 (38.7)	
C-reactive protein	1773 (31.2)	837 (30.7)	936 (31.7)		748 (29.8)	214 (33.3)	534 (28.6)	
Serum albumin	647 (11.4)	346 (12.7)	301 (10.2)		356 (14.2)	59 (9.2)	297 (15.9)	
Colonoscopy	346 (6.1)	183 (6.7)	163 (5.5)		228 (9.1)	57 (8.9)	171 (9.2)	

All *P*-values from Mann-Whitney *U* test, except
[1]Chi-square test. Data are shown as *n* (%), except otherwise mentioned. CD: Crohn's disease; UC: Ulcerative colitis; IBD: Inflammatory bowel disease; pt: Patient.

statistical analysis by subgroup.

This was the first multicenter real-world study in Brazil that assessed several PROs, providing insight about the patient and payer perspectives that can contribute to optimizing IBD treatment. Based on clinical and patient-reported assessments, we conclude that moderate-to-severe disease activity, especially among CD patients, is associated with a substantial impact on QoL, work productivity impairment and consumption of healthcare resources (namely, IBD hospitalizations and surgeries) in Brazil. The large sample size selected from reference centers of the most populated regions of Brazil allows a good comprehension of IBD burden and management in the Brazilian context. However, the low use of clinical scores and laboratory examinations of recognized biomarkers (such as fecal calprotectin) should be addressed through specific medical education programs. The frequency of IBD is increasing, and health services should be prepared to provide an adequate response, including by addressing unmet medical needs regarding the access and use of more effective therapies, to help patients with IBD.

Table 6 Treatment for inflammatory bowel diseases at baseline and changes during the previous 3 years

	CD				UC			
	Total	Moderate to severe activity	No or mild activity	*P* value	Total	Moderate to severe activity	No or mild activity	*P* value
n	264	118	146		143	36	107	
IBD treatment at baseline								
Treated patients	251 (95.1)	111 (94.1)	140 (95.9)		129 (90.2)	32 (88.9)	97 (90.7)	
IBD medicines/pt, median [range][2]	2.0 [1-7]]	2.0 [1.6]	2.0 [1-7]		2.0 [1-6]	3 [1-6]	2 [1-5]	
Main IBD therapy[3]								
5-ASA compounds	39 (15.5)	20 (18.0)	19 (13.6)		100 (77.5)	27 (84.4)	73 (75.3)	
Biologic therapy	189 (75.3)	79 (71.2)	110 (78.6)		41 (31.8)	11 (34.4)	30 (30.9)	
Immunosup-pressants	178 (70.9)	80 (72.1)	98 (70.0)		63 (48.8)	17 (53.1)	46 (47.4)	
Any corticosteroid	30 (12.0)	17 (15.3)	14 (10.0)		26 (20.2)	13 (40.6)	13 (13.4)	
Any antibiotic	14 (5.6)	11 (9.9)	3 (2.1)		7 (5.4)	5 (15.6)	2 (2.1)	
Changes to IBD treatment								
Treatment changes /pt, median [range][2]	1 [0-23]	1 [0-23]	1 [0-9]	0.526	2 [0-15]	1 [0-15]	2 [0-10]	0.226
At least one change on IBD treatment[2]	186 (70.7)	81 (69.2)	105 (71.9)	0.634[1]	111 (77.6)	24 (66.7)	87 (81.3)	0.068[1]
Type of change[2]								
Ongoing or beginning	437 (35.6)	190 (31.6)	247 (39.5)		265 (36.1)	75 (38.7)	190 (35.2)	
Dose change	256 (20.9)	128 (21.3)	128 (20.4)		189 (25.7)	39 (20.1)	150 (27.8)	
Discontinued	534 (43.5)	283 (47.1)	251 (40.1)		280 (38.1)	80 (41.2)	200 (37.0)	
Dose change by main IBD therapy	146	71	75		128	31	97	
5-ASA compounds	19 (13.0)	5 (7.0)	14 (18.7)		89 (69.5)	20 (64.5)	69 (71.1)	
Biologic therapy	53 (36.3)	27 (38.0)	26 (34.7)		12 (9.4)	5 (16.1)	7 (7.2)	
Immunosup-pressants	74 (50.7)	39 (54.9)	35 (46.7)		27 (21.1)	6 (19.4)	21 (21.6)	
Discontinua-tion by main IBD therapy	226	123	103		128	36	92	
5-ASA compounds	64 (28.3)	33 (26.8)	31 (30.1)		82 (64.1)	20 (55.6)	62 (67.4)	
Biologic therapy	72 (31.9)	44 (35.8)	28 (27.2)		11 (8.6)	8 (22.2)	3 (3.3)	
Immunosup-pressants	90 (39.8)	46 (37.4)	44 (42.7)		35 (27.3)	8 (22.2)	27 (29.3)	
Reason for dose change of biologics [frequency ≥ 5%][4]								

Adverse reaction	0 (0.0)	0 (0.0)	0 (0.0)	2 (16.7)	2 (40.0)	0 (0.0)
Patient decision/adherence	1 (1.9)	1 (3.7)	0 (0.0)	2 (16.7)	0 (0.0)	2 (28.6)
Poor effectiveness	22 (41.5)	12 (44.4)	10 (38.5)	3 (25.0)	1 (20.0)	2 (28.6)
Remission	5 (9.4)	2 (7.4)	3 (11.5)	0 (0.0)	0 (0.0)	0 (0.0)
Serum level of biologic drug	2 (3.8)	1 (3.7)	1 (3.8)	3 (25.0)	1 (20.0)	2 (28.6)
Reason for discontinuation of biologics [frequency ≥ 5%]						
Adverse reaction	21 (29.2)	13 (29.5)	8 (28.6)	3 (27.5)	2 (25.0)	1 (33.3)
Contraindication	6 (8.3)	5 (11.4)	1 (3.6)	1 (9.1)	0 (0.0)	1 (33.3)
Patient decision/adherence	5 (6.9)	4 (9.1)	1 (3.6)	2 (18.2)	1 (12.5)	1 (33.3)
Poor effectiveness	6 (8.3)	2 (4.5)	4 (14.3)	0 (0.0)	0 (0.0)	0 (0.0)

All *P*-values from Mann-Whitney *U* test, except
[1]Chi-square test. Data are shown as *n* (%), except otherwise mentioned.
[2]Including antibiotics and corticosteroids.
[3]Patients with at least one of the main IBD drug classes, excluding antibiotics, corticosteroids and other medications.
[4]More than one possible reason for dose change. CD: Crohn's disease; UC: Ulcerative colitis; IBD: Inflammatory bowel disease; pt: Patient.

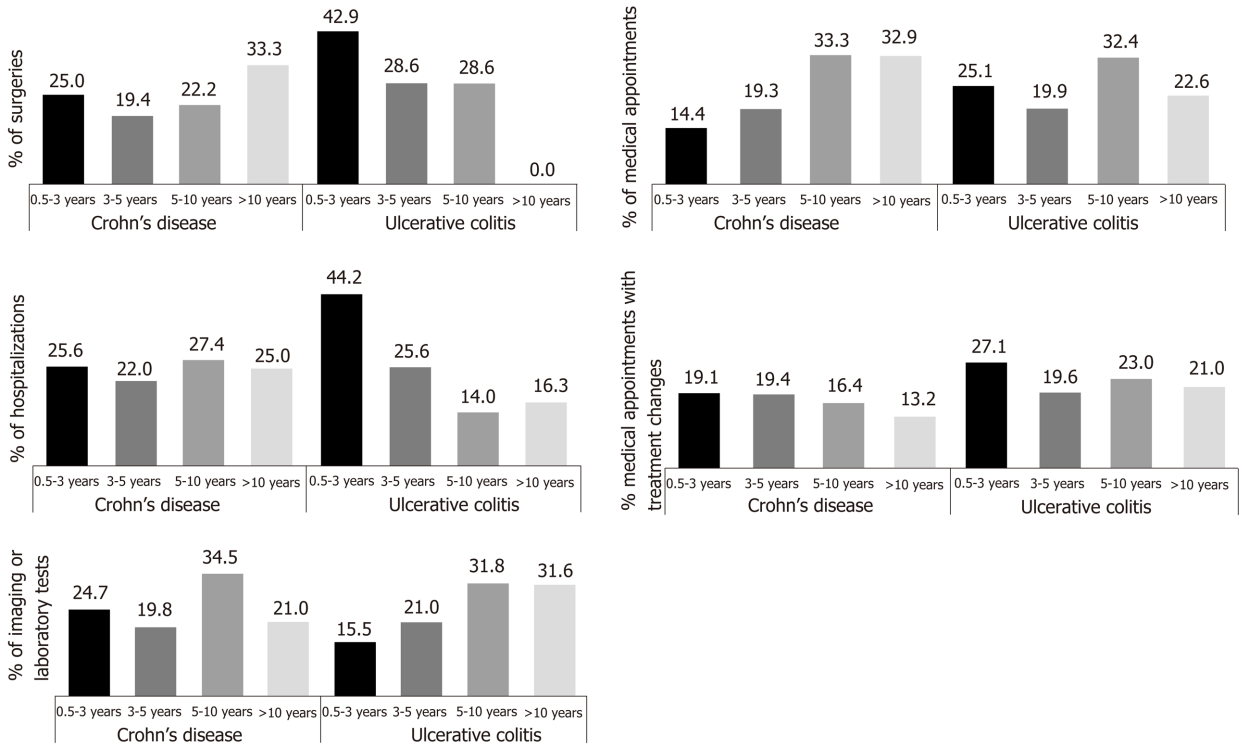

Figure 2 Healthcare resource utilization by time since the first diagnosis of moderate to severe inflammatory bowel diseases.

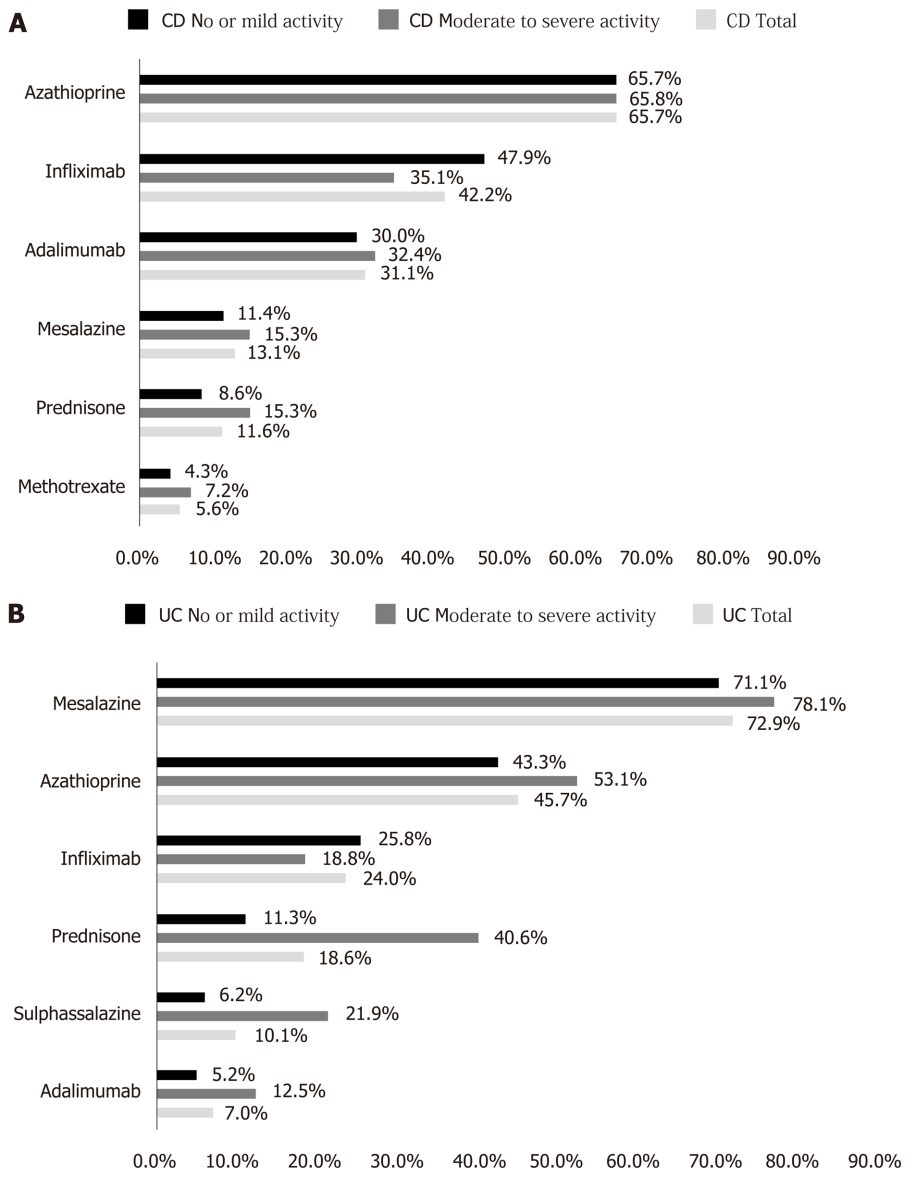

Figure 3 Most frequently used drugs for inflammatory bowel diseases (overall frequency ≥ 5%). A: Drugs used by patients with Crohn's disease (total and by disease activity); B: Drugs used by patients with ulcerative colitis (total and by disease activity). Note: % refers to patients using at least one medicine. CD: Crohn's disease; UC: Ulcerative colitis.

ARTICLE HIGHLIGHTS

Research background

Inflammatory bowel diseases (IBD) have been associated with a low quality of life (QoL) and a negative impact on work productivity compared to the general population, across several world regions.

Research motivation

Information about the impact of IBD on QoL and work productivity in Latin American countries is scarce and, in Brazil, emerges mostly from single-center studies. It is important to describe IBD control, patient-reported outcomes (PROs), treatment patterns and use of healthcare resources, so that clinicians and health services can optimize IBD management.

Research objectives

To describe QoL and work productivity and activity impairment (WPAI), treatment patterns and use of healthcare resources among IBD patients in Brazil. The association of disease activity with these outcomes was also evaluated.

Research methods

We conducted a multicenter cross-sectional study in several Brazilian IBD centers, with adult IBD outpatients, with clinical evaluation of disease activity at enrolment, chart review of the

previous 3-years for collection of treatment and healthcare use, and an extensive collection of PROs, namely: General measures of QoL such as short-form 36 and EQ-5D-5L questionnaires, the Inflammatory and Bowel Disease Questionnaire (IBDQ, a disease-specific QoL measure) and the WPAI questionnaire.

Research results

In a large sample (n = 407) of patients with ulcerative colitis (n = 143) or Crohn's disease (n = 264), almost half of CD patients (44.7%) and about a quarter of UC patients (25.2%) presented active disease at baseline. Irrespective of IBD type, QoL scores (SF-36, EQ-5D and IBDQ) were low. Disease activity, female gender, unemployment, and lower education and income were associated with a poorer QoL. IBD patients with active disease had a median IBDQ score 16 points higher (i.e., power QoL) than patients with mild or no disease activity, as well as in SF-36 summary measures (more than 2 points of difference) and EQ-VAS scores (more than 8 points). In our study, patients had approximately 30% impaired worktime, with approximately 12% absenteeism and 18%-24% presenteeism, and approximately 36%-40% total activity impairment. Patients with active IBD showed higher total activity impairment. With regard to use of healthcare resources, approximately one-quarter of CD patients had at least one surgery during the last 3 years. The proportion of UC patients with previous surgeries was considerably smaller (3%), but approximately 30% underwent a high-cost colectomy. Almost half of CD patients and approximately 35% of UC patients had at least one previous hospitalization. Almost all IBD patients were on some form of treatment at baseline, mainly with immunosuppressants and biologics among CD patients and with 5-ASA compounds in UC patients.

Research conclusions

Active IBD, especially among CD patients, is associated with a substantial impact on QoL, work productivity impairment and an increased number of IBD surgeries and hospitalizations in Brazil. This is particularly relevant due to the large proportion of IBD patients with active disease at enrolment and to the increase of IBD prevalence, suggesting the need to improve treatment but also the social support and follow-up of IBD patients in Brazil.

Research perspectives

Future research should address the evolution of PROs and its association with treatment changes and control of IBD activity, in a cohort of newly diagnosed patients in Brazil.

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ORIGINAL ARTICLE

Observational Study

Prevalence of hepatocarcinoma-related hepatitis B virus mutants in patients in grey zone of treatment

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Abstract

BACKGROUND

Antiviral treatment of patients with chronic hepatitis B (CHB) in the grey zone of treatment comands risk management in order to optimize the health outcome. In this sense, the identification of HBV mutants related with an increased risk of hepatocellular carcinoma (HCC) could be useful to identify subpopulations with potential indication of antiviral treatment.

AIM

To analyze the prevalence/persistence of hepatitis B virus (HBV) preS and basal core promoter (BCP)/precore/core variants associated to HCC development in CHB patients in the grey zone.

METHODS

Edition of the Gilead Fellowship Program, No. GLD13/00046 and Modificaciones de los niveles de expresión génica mediada por mutantes naturales de la región PreS del virus de la hepatitis B, y asociación con genes implicados en el desarrollo de hepatocarcinoma Efecto del tratamiento antiviral.

Institutional review board statement: The study was approved by the Ethical Committee of the Hospital Carlos III in Madrid, conforms to the ethical guidelines of the 1975 Declaration of Helsinki.

Informed consent statement: All the participants received and signed written consent for its participation.

Conflict-of-interest statement: All the authors have nothing to disclose.

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Work was designed as a longitudinal retrospective study, including 106 plasma samples from 31 patients with CHB in the grey zone of treatment: Hepatitis B e antigen negative, HBV-DNA levels between 12-20000 IU/mL, normal or discordant transaminase levels during follow up and mild/moderate necro-inflammatory activity in liver biopsy or Fibroscan (up to 9.5 kPa). Serum HBV-DNA was tested using the Abbott Real Time HBV Assay and the BCP/precore/core and the hepatitis B surface antigen (HBsAg) coding regions were analyzed in positive samples by PCR/bulk-sequencing to identify the HCC-related HBV mutants.

RESULTS

High-risk HCC related mutants were detected in 24 (77%) patients: 19 (61%) in the BCP/precore/core, and 7 (23%) in the HBsAg coding region (2 preS1 and 5 preS2 deletions). The prevalence of preS deletions was genotype-dependent: 3/5 (60%) patients with preS2 deletions and 1/2 with preS1 deletions were infected with the HBV-E genotype. Since HBV-E was the most prevalent in sub-Saharan patients, a correlation between preS deletions and ethnicity was also found: 6/8 (75%) sub-Saharan *vs* 1/19 (5%) Caucasian patients had preS deletions (P = 0.00016). Remarkably, this correlation was maintained in those patients infected with HBV-A, a minor genotype in sub-Saharan patients: 2/2 patients infected with HBV-A from West Africa *vs* 0/6 of Caucasian origin had preS deletions. The HCC related variants were the major strains and persisted over time (up to 48 mo). Patients with preS deletions had a significant higher prevalence of F2 fibrosis stage than the negatives (57% *vs* 10%, P = 0.0078).

CONCLUSION

HBV genetic analysis of selected populations, like sub-Saharans infected with HBV-E/A genotypes, will allow identification of subpopulations with risk of HCC development due to accumulation of high-risk HBV variants, thus commanding their increased clinical surveillance.

Key words: Hepatitis B virus; Hepatocellular carcinoma; PreS deletions; Hepatitis B virus treatment; Grey zone

Core tip: The antiviral treatment in patients with chronic hepatitis B in the "grey zone" of treatment is controversial and not clearly indicated. The genetic analysis of hepatitis B virus (HBV) basal core promoter/precore/core and preS regions has shown a high prevalence and persistence of preS deletions in the sub-Saharan population infected with HBV-E/A genotypes. By contrast, Caucasian patients, who have shown a good clinical evolution in previous studies, were negative for these variants. The recognition of these subpopulations warrant to increase the clinical surveillance in order to minimize the risk of liver cancer development due to accumulation of hepatocellular carcinoma-related HBV genetic variants.

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INTRODUCTION

In most cases, patients with chronic hepatitis B (CHB) treated with nucleos(t)ide analogues (NAs) such as tenofovir or entecavir achieve a maintained virological suppression[1-3]. Although the inhibition of viral replication has an evident beneficial effect on the progression of the liver disease, the antiviral treatment does not achieve eradication of the hepatitis B virus (HBV) infection due to the persistence of

intracellular covalently closed circular DNA in the nucleus of infected hepatocytes[4]. Thus, therapy is generally recommended for prolonged periods of time and the discontinuation of NAs is controversial because the early interruption of treatment is usually associated with a reactivation of viral replication that may induce severe flares if not controlled by the host immunity, requiring the re-start of the antiviral treatment[5]. According to EASL guidelines treatment may be discontinued in hepatitis B e antigen (HBeAg) negative patients only when hepatitis B surface antigen (HBsAg) loss is achieved or after 3 years of virological suppression[1]. Conversely, AASLD guidelines still recommends lifelong antiviral treatment in patients with HBeAg negative CHB[2].

The indications for CHB treatment are mainly based on the combination of three criteria: serum HBV-DNA levels, alanine aminotransferase (ALT) values and severity of liver disease. Patients should be considered for treatment when they have HBV-DNA levels above 2000 IU/mL, persistent or intermittent serum ALT abnormal levels and severity of the liver disease assessed by liver biopsy (or non-invasive markers validated in HBV-infected patients) showing moderate to severe active necroinflammation and/or at least moderate fibrosis using a standardised scoring system[1,2]. Conversely, there are HBeAg-negative patients with maintained minimal or mild fibrosis, for whom the treatment is not clearly indicated: A) patients with maintained normal ALT levels and low or undetectable serum HBV-DNA: B) patients with marginally elevated ALT and/or HBV-DNA titters ranging 2000-20000 IU/mL; and C) persistently normal ALT and HBV-DNA titters higher than 2000 IU/mL[6,7].

The indication of treatment in these patients, in the so named "grey zone" of treatment, requires to balance the risks and benefits for health outcomes. Although low levels of HBV replication and the absence of significant liver damage are good prognostic factors in the natural history of CHB, it cannot be ruled out that some of these patients may be infected with viral strains with mutations associated with high-risk of hepatocellular carcinoma (HCC) development, which are an additional risk of progression for liver disease[8,9]. There are several studies that associate certain mutations in the HBV genome with an increased risk of HCC development, but the results are controversial because the HBV genotypes or even different HBeAg status have different mutation patterns. One of the mechanisms by which HBV can promote direct carcinogenesis is the ability of wild-type and mutated/truncated viral proteins like hepatitis B x-protein, hepatitis B core-protein (HBc) and preS region (preS) to affect cell functions, activate oncogenic pathways and sensitize liver cells to mutagens[10]. The most common HBV specific mutations are at the preS2 start codon, preS deletions, and point mutations in the basal core promoter (BCP) and in the precore/core coding region[11-13].

For these reasons, the aim of this work is to analyse the prevalence and persistence over time of HBV mutants that predispose to the development of HCC in patients with CHB but without clear indication of treatment, and to analyze their role as a tool in the selection of suitable subpopulations for antiviral treatment.

MATERIALS AND METHODS

Study population

This work was designed as an observational, longitudinal, retrospective study including 106 plasma samples from 31 CHB patients in the grey zone of treatment: HBeAg negative, HBV-DNA levels between 12-20000 IU/mL, normal or discordant ALT levels during follow up and mild/moderate necro-inflammatory activity in liver biopsy or Fibroscan (up to 9.5 Kpa). Patients with chronic hepatitis C, D or HIV coinfection, or with toxic, alcoholic or autoimmune hepatitis were excluded. The average number of analysed samples per patient was [mean ± standard deviation (SD)] 3.2 ± 1.2 (ranging: 2-6 samples) and the follow-up time period per patient was (mean ± SD) 2.1 ± 2.2 years (ranging: 0.5-8.4 years). No patient was under antiviral treatment for HBV at the time in which samples were collected. The clinical features of the patients, obtained from the full clinical charts, with a follow-up time (mean ± SD) of 6.1 ± 3.9 years (ranging: 0.7-12.8 years) are shown in Table 1. The study was approved by the Ethical Committee of the Hospital Universitario La Paz/Carlos III in Madrid, according to the ethical guidelines of the 1975 Declaration of Helsinki. All the participants received and signed written consent for their participation.

Serum HBV-DNA analysis

Serum HBV-DNA was quantified using the Abbott Real Time HBV Assay (Abbott Laboratories, Abbott Park, IL, United States). In positive samples, the viral genome was purified from 200 L of serum using the QIAmp DNA Kit (QIAGEN GmbH,

Table 1 Demographic and clinical features of patients

Demographic data	Value
Male/female	16/15
Caucasian/sub-Saharan/chinese[1]	22/8/1
Age (years: mean ± SD)	45.1 ± 10.2
Virological data	
HBV-DNA positivity (persistent/fluctuant)[2]	28/3
HBV-DNA titers (IU/mL: Mean ± SD)[3]	$1.4 \times 10^3 \pm 3.5 \times 10^4$
Clinical data[4]	
Persistently normal/marginally altered AST or ALT	18/13
Persistently normal/marginally altered AST	25/6
Persistently normal/marginally altered ALT	20/11
AST (IU/L: mean ± SD)	25.6 ± 9.5
ALT (IU/L: mean ± SD)	28.5 ± 14.3
Fibrosis stage (Fibroscan)	
F0-F1	22
F2	6
Not available	3

[1]Origin country of sub-Saharan patients: 3 Equatorial Guinea, 2 Senegal, 1 Ivory Coast, 1 Ghana, and 1 Cameroon;
[2]Persistent/fluctuant: Positive results in more/less than the 90% of samples;
[3]In 16 samples from 8 patients, point flares of HBV-DNA titers (> 20000 IU/mL) were observed;
[4]Persistently normal/marginally altered: Normal values in more/less than the 90% of the samples. IU: International units; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; SD: Standard deviation.

Hilden, Germany), and BCP/precore/core and HBsAg coding regions were amplified using the PCR Master Mix (Promega Corporation, Fitchburg, WI, United States) using genotype-specific primers.

DNA sequencing

PCR products were purified using the Illustra Exo ProStar (GE Healthcare Life Sciences, Fairfield, Connecticut, United States), sequenced using Bright Dye Terminator Cycle Sequencing (NimaGen, Nijmegen, Netherlands) and analyzed with the DNA sequencer ABI PRISM 3730xl DNA Analyzer (Applied Biosystems, Foster City, CA, United States).

Sequence analysis

Analysis of the DNA sequences was done using Lasergene SeqMan Pro V7.1.0 (ADNSTAR, United States) software. The phylogenetic relations were established using the MEGA version 4.0 software (Center for Evolutionary Medicine and Informatic, Tempe, AZ, United States).

Assignation of the HCC risk development associated to each HBV mutation was performed according to the previously published data[11,13] (Table 2).

HBV genotyping

HBV genotype was analyzed in all cases using the line probe assay INNO-LiPA HBV Genotyping assay (Innogenetics N.V., Ghent, Belgium). Patients with negative results in this technique were typed by the sequence analysis of HBsAg coding region using the Geno2pheno[HBV] open access of the Genafor website (www.genafor.org).

Statistical analysis

Data were analyzed using the SPSS v13 software (SPSS Inc. North Chicago, IL, United States) on an intention to treat (ITT) basis. All parameters expressed as absolute number or percentages were analyzed using the Spearman´s ran-correlation, the Wilcoxon matched-pairs and the *U* Mann-Whitney systems. The mean comparisons were performed using the Student-*t* test.

Table 2 Assignment of risk of hepatocellular carcinoma development associated to each hepatitis B virus genetic variants

HCC risk	HBV region	Genetic variant
High		
	PreS2	PreS2 deletions
	BCP	C1653T, T1753V (A/C/G), A1762T + G1764A
	Precore	G1896A, G1899A
Suspect		
	PreS1	PreS1 deletions
	PreS2	T53C
	BCP	C1773T
	Precore	A1846T
	Core	C1914G, C2289A
Minor		
	PreS1	T3098C, T3139A
	PreS2	PreS2 start codon
	S	T766A, T791A
	BCP	T1674C, G1727, C1741, C1761, C1766T, T1768A
	Precore	C1858T, G1862T
	Core	C1909, A1934T, C2002T, T2003A, C2100A, A2159G, A2189C, A2246C

High evidences: HBV mutants with positive data for HCC correlation evidenced in at least 3 different papers, or in meta-analysis studies; Suspected correlation: Positive data for HCC correlation evidenced in at least 2 works or without meta-analysis evidences; Minor evidences: Positive data for HCC correlation in single works. HCC: Hepatocellular carcinoma.

RESULTS

HBV genotyping analysis

In only 3 (10%) patients no amplification of any of the HBV analyzed regions was achieved. In the remaining 28 patients the efficacy of the HBV-DNA amplification was different depending on the region of the HBV genome analyzed. Thus, we have data from the complete HBsAg and BCP/precore/core regions in at least one sample of the follow-up in 11 (35%) patients, and partial information in 17 (55%) patients (Figure 1A). HBV-DNA titters were significantly higher in the samples positive for the in-house PCR techniques than in the negatives (mean ± SD: $7.6 \times 10^3 \pm 1.3 \times 10^4$ IU/mL *vs* $3.1 \times 10^3 \pm 4.6 \times 10^3$ IU/mL, respectively. $P = 0.016$).

HBV genotype was determined in 30 (97%) patients [12 (39%) by LIPA and 27 (87%) by HBsAg sequence analysis]: 9/31 (29%) were infected with HBV-A genotype, 1 (3%) with HBV-C, 12 (39%) with HBV-D, 6 (19%) with HBV-E, 2 (6%) with HBV-F, 2 (6%) with HBV-H and 1 (3%) with a recombinant HBV-A/E. Serial changes of the genotyping results during the follow-up were observed in 4 patients: One sub-Saharan patient (who evolve from E to F genotypes), one Chinese (A to C), and 2 Caucasians (from D to A and from A to H, respectively). Differences in the genotype distribution among ethnic groups, especially in the frequency of D and E genotypes, were observed (Figure 1B). In only one case, corresponding to the A/E recombinant, discrepancies between LIPA and HBsAg analysis were remarkably noted.

Identification of mutants related with HBeAg negative status

In 21/23 (91%) patients in whom the BCP and/or precore/core regions were amplified, the presence of the most common genetic variants related with the HBeAg negative/anti-HBe status was confirmed. Only partial sequence data (BCP or precore/core sequences) were available for the remaining 2 patients lacking confirmation of these variants. The frequency of mutants related to the HBeAg negative status was higher in the precore/core region than in the BCP in all genotypes, especially in genotypes D and E (Figure 2A). Thus, in 10 patients with mutations in the precore/core region no changes were observed in the BCP region. In addition, coexistence of several mutations in the BCP/precore/core region was observed in the 21 patients (Figure 2B), with special importance for the combination of the A1846T + C1858T + G1896A mutations, which was present in the majority of the patients irrespective of the genotype (Figure 2C).

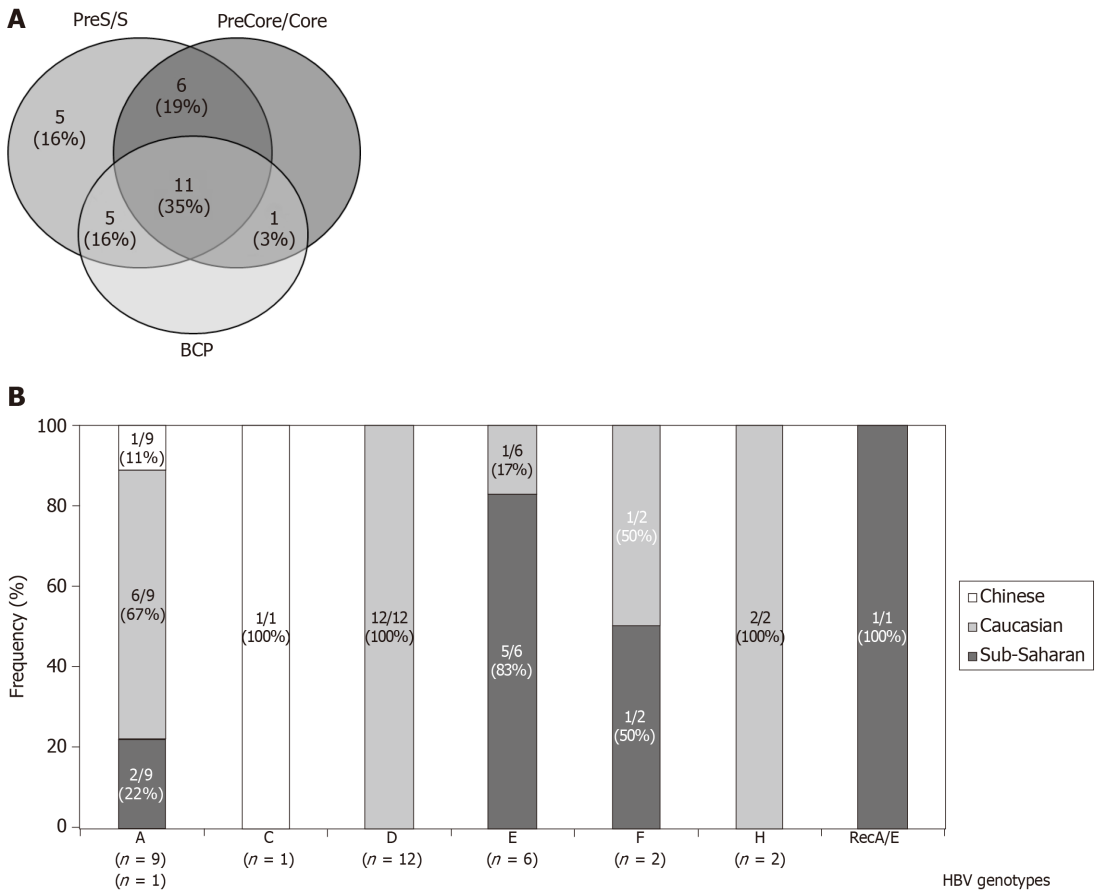

Figure 1 Features of samples analyzed. A: Number of patients with HBV-DNA amplification positive results in the different genomic regions analysed; B: Ethnic group distribution among the different HBV genotypes. The 4 patients in whom were confirmed sequential changes of the genotyping results during the follow-up were included in both genotype categories. BCP: Basal core promoter; HBV: Hepatitis B virus.

Identification of HCC related mutants

BCP/precore/core region: Taken globally, 19 (61%, on an ITT basis) patients have any high-risk variant (C1653T, T1753V, A1762T/G1764A, G1896A and G1899A) and this percentage increases up to 21 (68%) patients by including the genetic variants with suspect correlation with HCC (C1773T, A1846T, C1914G, C2289A and A2339T+C2340G) in the BCP or precore/core regions. The distribution of the individual mutations in the study population is shown in Figure 3A. Although the presence of single high-risk mutations was the most frequent situation (9 patients), the existence of coinfection with several high-risk mutations was also a common event (Figure 4A).

PreS1/preS2/S region: PreS1 deletions were found in 2 (6%), and preS2 deletions in 5 (16%) and the T53C mutation in 4 (13%) patients, respectively. The distribution of the individual mutations in the study population is shown in Figure 3A and the sequences of the deletions of the preS region in Figure 3B. In only 1/10 patient the simultaneous presence of preS1 deletion and the T53C mutation was confirmed. Moreover, the previously described mutation in the preS2 start codon was identified in 3 patients (1 HBV-A, 1 HBV-E and 1 HBV-F). A high correlation between lack of start codon and the preS2 deletion was found, with coexistence of both changes in 2 of the 3 patients with the preS2 start codon mutation. The distribution of HCC related mutations in the HBsAg coding region was genotype-dependent. In this way, 3/5 (60%) patients with preS2 deletions and 1/2 patient with preS1 deletions were infected with the HBV-E genotype. Moreover, it is very remarkable the correlation of these preS deletions with ethnicity: 6/8 (75%) sub-Saharan had preS deletions *vs* 1/19 (5%) of the Caucasian patients (P = 0.00016). This correlation was observed not only in the HBV-E, which is the predominant genotype in sub-Saharan population, but also in the HBV-A genotype, that is minoritarian within this ethnic group. So, among HBV-A infected patients, only those from West Africa (n = 2) had preS deletions, but none of the patients (n = 6) with Caucasian origin (Figure 4B). In 5/10 (50%) patients with high or medium risk mutations in the HBsAg, data of genetic analysis of the

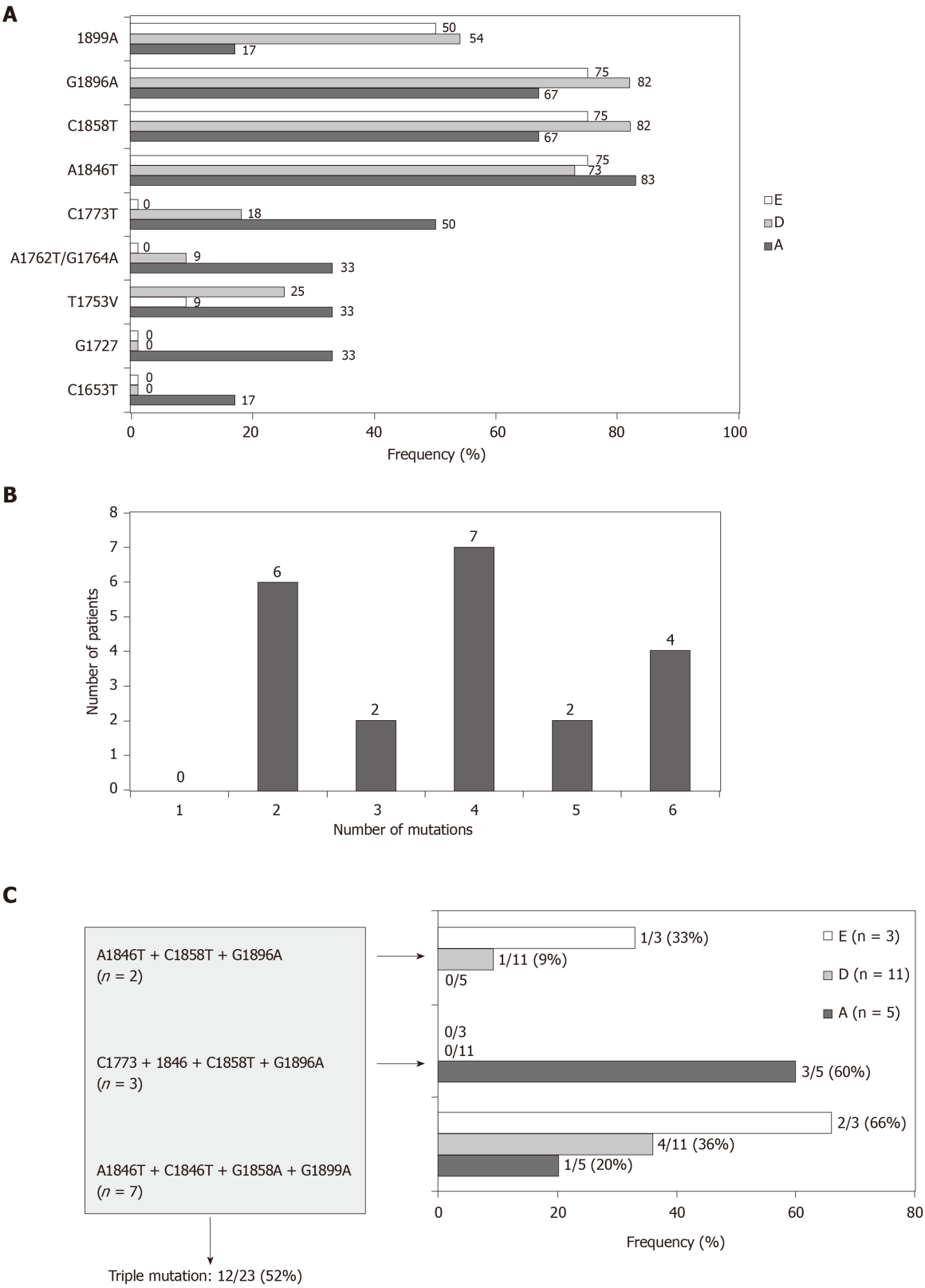

Figure 2 Analysis of basal core promoter /precore/core mutants related with the e-antigen negative status. A: Prevalence of individual mutations of the in the 3 most frequent HBV genotypes analyzed; B: Frequency of coexistence of more than one BCP/precore/core mutants; and C: Frequency of the most common combination of mutations in the different genotypes. The frequency calculation was performed including only the 23 patients with precore/core sequences data, not in on intention to treat basis. BCP: Basal core promoter; HBV: Hepatitis B virus; A: HBV-A; D: HBV-D; E: HBV-E.

precore/core region were also available. In 2/5 (40%) patients with high-risk deletion in the preS2 coding region, and in 3/4 (75%) patients with the T53C substitution, were simultaneously detected high-risk mutations in the BCP/precore region. Especially significant was the sub-Saharan patient infected with an HBV-E genotype who had a

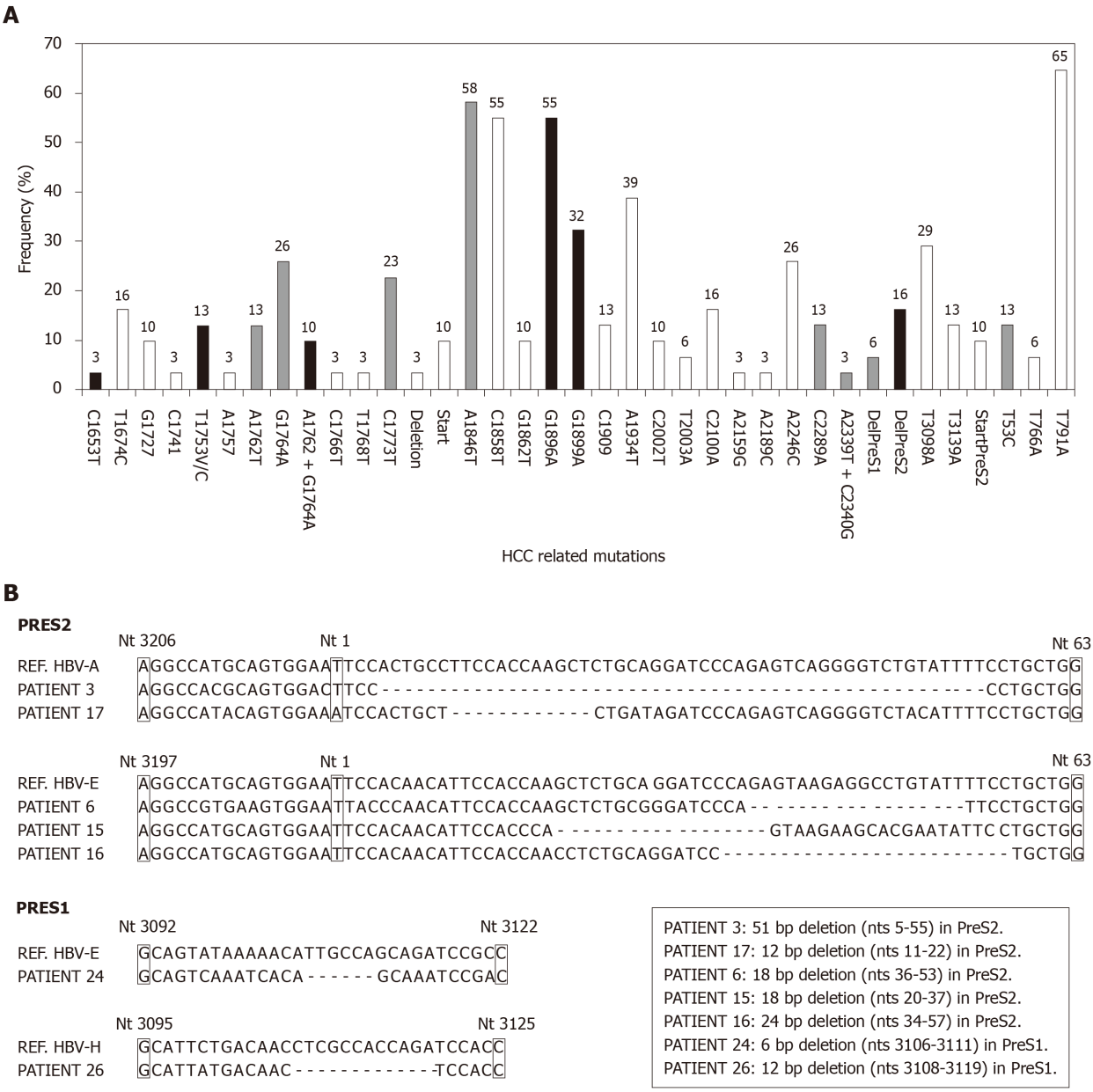

Figure 3 Analysis of basal core promoter/precore/core region mutants related with the hepatocellular carcinoma risk. A: Frequency distribution of BCP/precore/core and HBsAg mutations (Black, grey and white bars show the viral variants with the highest, median and lowest evidences of association with HCC, respectively); B: Sequences of the 7 patients with preS deletions. BCP: Basal core promoter; HCC: Hepatocellular carcinoma.

preS2 deletion and was simultaneously positive for 3 high-risk mutations in the BCP/precore/core region (T1753V, G1896A and G1899A) (Figure 4A).

Persistence of HCC related HBV strains**:** In 12 patients with HCC related mutations, it was possible to analyze the persistence of these changes over time by using bulk sequencing [follow-up time (mean ± SD): 24.9 ± 24.0 mo; ranging: 5-96 mo]. In 11 cases, the mutations persist during the complete follow-up period, or punctually disappear to latter re-appear in the last samples tested. In the remaining case, the G1899A mutation disappears in stable manner. No changes in the pattern of preS2 deletions were observed in none case (Figure 5).

Liver function tests: Although all the patients are in the grey zone of treatment, with low-moderate fibrosis levels (F0-F2, according the selection criteria of patients), it was observed a significant higher percentage of patients with F2 stage of fibrosis in patients with preS deletions than in the negative ones (Figure 6A). In addition, the behaviour of aspartate aminotransferase (AST) and ALT was quite different between patients with or without preS2 deletions. So, the percentage of patients of patients with fluctuant AST or ALT trend to be higher in patients with preS2, although these differences reached significant values only when analyzing AST behaviour (Figure 6B). Only one patient, with sub-Saharan origin, infected with HBV-E genotype, and

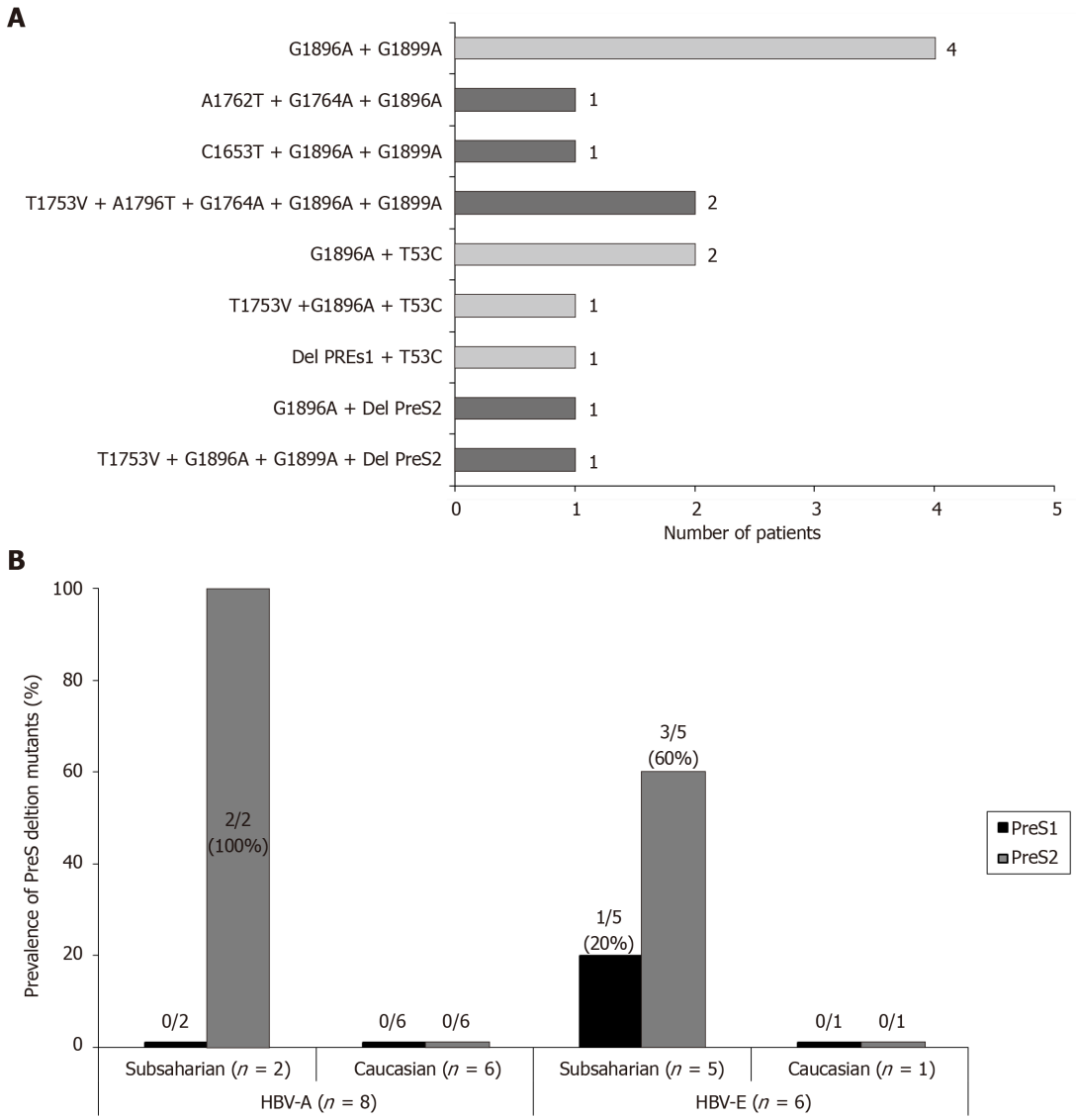

Figure 4 Analysis of preS deletion mutations related with the hepatocellular carcinoma risk. A: Frequency of coexistence of hepatocellular carcinoma related mutations in both BCP/precore and preS regions (Dark bars indicate the patients with theoretical highest risk of hepatocellular carcinoma development, based in the coexistence of mutations in the HBsAg and precore region, or the simultaneous detection of 3 or more mutation irrespective of the genomic region); B: Prevalence of preS deletions in different genotypes and ethnic groups. Only a Caucasian patient infected with HBV-H genotype had a preS1 deletion. BCP: Basal core promoter.

with a preS2 deletion, reported alpha fetoprotein abnormal values at the end of follow-up, although at the moment of its inclusion in this work no radiologic evidences of any neoplasic event was confirmed.

DISCUSSION

In this work we performed a longitudinal retrospective study to analyze the prevalence and persistence over time of HBV mutants related with risk of HCC development in HBeAg-negative/anti-HBe-positive patients within the grey zone of treatment, in order to identify subpopulations susceptible to be treated.

Our results confirm the presence of high-risk HCC mutations in BCP/precore or preS regions, in 19 (61%) patients, using a statistical analysis of ITT, with a higher prevalence in the BCP/precore that in the preS region. The most frequently detected high-risk mutations in the BCP/precore region were the G1896A and G1899A. Indeed, finding these mutations in this group of patients is common and frequently related with the Mediterranean variants in patients with HBeAg-negative serological profile[14]. Thus, the G1896A mutation introduces a stop codon at position 28 of the HBc protein that prevents the synthesis of HBeAg[15,16]. The simultaneous presence of A1762T and G1764A mutations that are related to a decrease in HBeAg levels[17-19] and

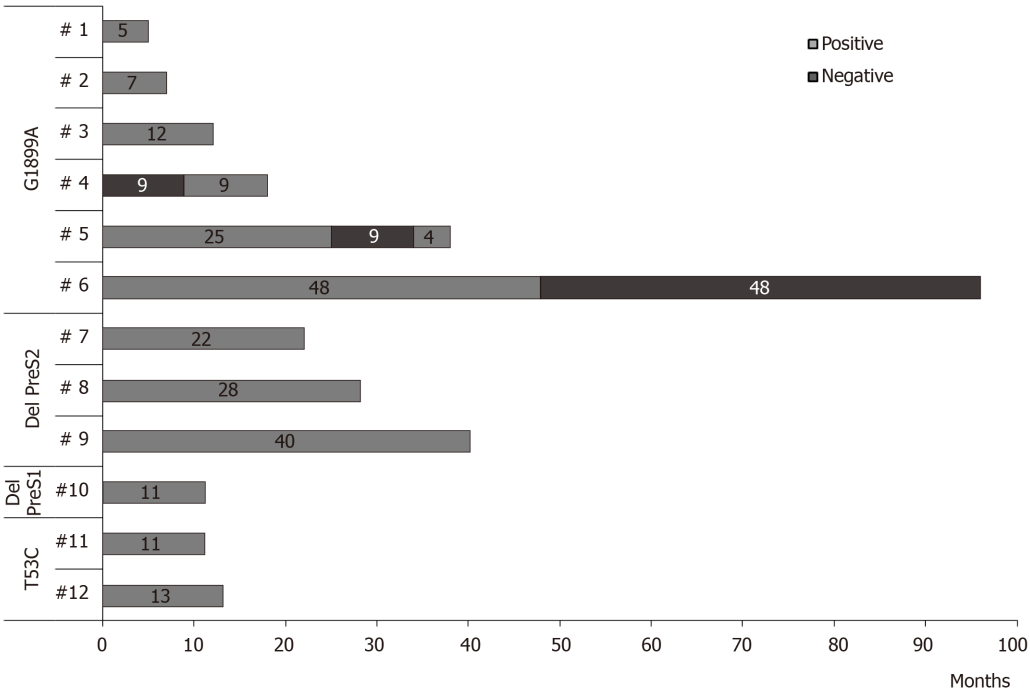

Figure 5 Persistence over time of the high-risk mutations as major population in the patients analyzed.

an increase of the HBV-DNA titters, was also confirmed in 10% of the patients[20]. The prevalence of these variants in our cohort of patients was genotype-dependent: BCP variants (C1653T, T1753V, A1762T/G1764A and C1773T) were more frequent in HBV-A genotype, as previously described, except for the C1653T mutation, which was more frequent in HBV-D. Precore/core variant G1899A was more frequent in HBV-E genotype in our cohort, tallying with the published data[21], but also found in a high proportion of patients infected with HBV-A genotype. The prevalence of the G1896A mutation in our patients is consistent with other studies carried out in Spanish population, in which this variant was found with similar frequency in genotypes A and D[22], contrasting with other works conducted in different geographical regions, in which no correlation with the HBV-A genotype was found[23,24]. These discrepancies probably reflect that the HBeAg-negative serological profile may present a different genetic basis in at least some HBV genotypes, with a greater representativeness than in other populations of the G1896A mutation in Mediterranean population, in which this serological pattern appears be more related to the simultaneous presence of A1762T/G1764A double mutation[22].

Although the BCP and precore/core variants has been usually associated to HCC development in meta-analysis studies, a recent work performed in HBeAg-negative Spanish Caucasian patients in the grey zone without antiviral treatment reflects that, in the long term, their clinical evolution is favorable, without progression of liver disease or HCC development in the vast majority of them[25]. Considering that the main objective of antiviral treatment is to prevent the fibrosis progression, these data support the idea that treatment does not provide substantial benefits in this group of patients. In this case, the low progression of HBV disease can be related with the low viral replication levels.

HBV-DNA titters have been classically considered as a prognostic marker of HCC development risk[26]. The maintenance of HBV-DNA levels undetectable or below 2,000 IU/mL can compensate the risk of harbouring HCC-related variants. However, it cannot be ruled that the accumulation of mutations in both BCP/precore/core region and other viral regions like HBsAg, maintained over time, in combination with persistence of viral replication, even at low or moderate levels are risk factors for HCC development[9]. For this reason, we have focused our work on the analysis of the prevalence and persistence over time of the BCP/precore/core and HBsAg regions mutants, present as main strains of HBV detectable by means of bulk sequencing techniques.

The prevalence of preS mutations, which have been correlated with HCC in previous meta-analysis, showed a strong correlation in our cohort with the

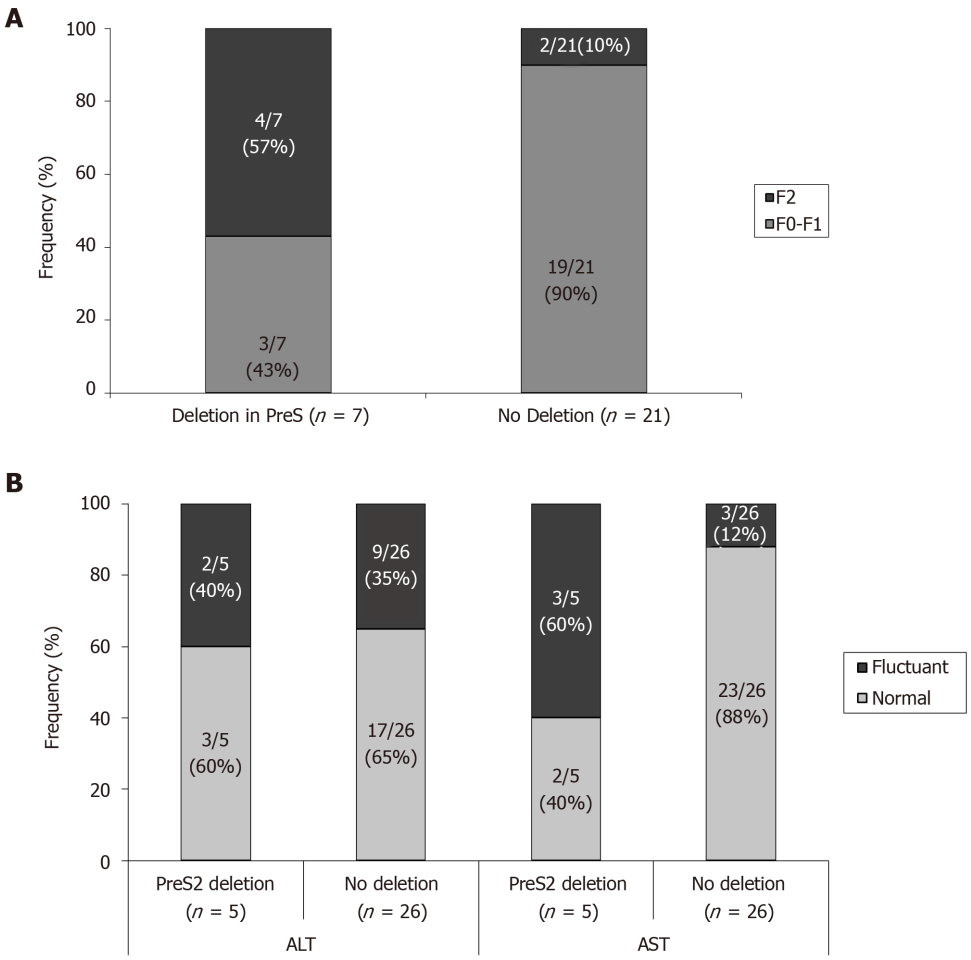

Figure 6 Differences between patients with or without preS deletion mutants. A: Differences between patients with or without preS deletion mutants at fibrosis level; B: Differences between patients with or without preS deletion mutants at evolution of transaminase levels during follow-up. AST: Aspartate aminotransferase; ALT: Alanine aminotransferase.

geographical origin and, in consequence, with the HBV genotypes: 75% of our sub-Saharan patients, the majority infected with HBV-E genotype, had deletions in the preS1 or preS2 regions. In addition, the only 2 sub-Saharan patients infected with HBV-A had also deletions in preS2, while this change was not found in any of the remaining patients infected with HBV-A, all of them with Caucasian origin. HBV-E genotype has not been extensively studied until now. Previous studies performed in Germany confirm our results showing that patients in the grey zone of treatment infected with the HBV-E genotype have a higher prevalence of preS1 and preS2 deletions in comparison with HBV-A and D genotypes[27]. However, the absolute prevalence rate of these mutations in HBeAg negative patients differs between different studies, ranging from 16% in the German series to 75% in our cohort. The phylogenetic analysis of HBV-E strains from different regions of Africa, indicates that the spread of HBV-E genotype in West Africa is a relatively recent event, as reflected by the conspicuously low genetic diversity of this genotype despite the higher prevalence in this geographical region[28,29]. This fact together with the high degree of isolation and scattering of human populations in these regions favours the appearance of viral strains relatively stable over time, limited to specific geographical locations. In this sense, it must be noted that the country origin of the sub-Saharan patients included in our study is located in the South/Central regions of West Africa, corresponding to the area in which the genetic variability of HBV-E is lower, among the countries with high prevalence of this viral genotype[30,31]. In this context, the striking high proportion of preS deletions found in sub-Saharan patients in our series could reflect the existence of isolated viral strains of HBV-E in this region with a higher prevalence than that described for neighbouring countries. It may constitute a subpopulation of patients with increased risk of developing HCC by simultaneously sharing high-risk mutations in different regions of HBV genome.

Especially significant was the persistence of the high-risk mutations over time, as

major strains of the viral *quasispecies*, as demonstrated by its identification with bulk sequencing techniques. As regards amino acid substitutions, these were generally very stable, although changes in the composition were observed. Thus, G1899A mutation remained continuously detectable during a follow-up period of up to 48 mo, with sporadic disappearances of the mutant strain in 3 patients and a definitive loss in only 1 patient. This persistence of point mutations in the BCP/precore/core region can be related to the low level of viral replication, which hinders the appearance and selection of mutations that restore the wild-type genotype in the population. Regarding deletion mutations in the preS region, these were maintained without sporadic eliminations during the follow-up in all the patients who carried them, with proven periods of up to 40 mo. The persistence, without fluctuations, of these deletions seems to influence the presence in the geographical origin of the patients in which these strains are majority. Under these conditions, exclusive infection with deleted strains would make the emergence of wild-type strains impossible due to the absence of a genetic template on which to act. The persistence over time of these mutations favours their accumulation, as major strains, in chronic infected patients. Thus, it was possible to confirm in our patients, not only the coexistence of the mutations A1762T/G1764A and G1896A/G1899A widely documented and associated with a high-risk of carcinogenesis, but also the accumulation of 3-5 risk mutations in other 5 patients, including a case that presented 3 risk mutations in the BCP/precore/core in combination with a deletion in the preS region. In any case, the persistence and accumulation over time of these mutations may have clinical relevance, since it perpetuates the molecular processes by which these viral variants favour the development of HCC.

The selection in this work of patients in the grey zone of treatment, with low-moderate fibrosis levels and normal or marginally elevated transaminase levels, makes it difficult to evaluate unless through a long-term follow-up, the possible harmful effect of the presence of these mutations on the progression of liver disease. However, and even recognizing the limitations of transient elastometry techniques for discriminate patients with low or moderate stages of fibrosis, our data showed a higher prevalence of F2 stage in patients with preS2 deletion than in those in which this mutation was not detected. On the other hand, the behaviour of AST and ALT was quite different between both types of patients, with is a higher percentage of patients with fluctuant AST or ALT levels during follow up in patients harbouring preS deletions.

These results focus an increased risk of HCC development in sub-Saharan patients. Africa represents one of the most HBV endemic regions in the world and HCC mainly due to CHB infection is a major cause of premature death, suggesting that viral strains located in Africa, which mostly belong to HBV-A, are more likely to cause HCC[32]. In this way, occult HBV infection was shown to be present in 75% of Black Africans with HCC, and genotype A was shown to be 4.5 times more likely than other genotypes to cause HCC in Black Africans, whilst tumours occurred at a significantly younger age[33]. Our findings also correlate a higher risk of HCC development with HBV-E, which almost exclusively occurs in African people. Moreover, it has been linked the distribution of HBV genotype E infection with African countries with high incidences of HCC[34].

In conclusion our data indicate that the presence of preS mutations should be assessed in patients with sub-Saharan origin, especially if they are infected with HBV-E and HBV-A genotype, in order to identify subpopulations of patients in which the antiviral treatment can be indicated to minimize the risk of HCC due to accumulation of high-risk HBV genetic variants. In this sense, the development of detection systems for deletion mutations in preS region is technically feasible with conventional techniques of Molecular Biology available for most centers. Its use, especially if it is restricted to preselected subpopulations, can help the treatment decision in patients in grey area of treatment.

The most important limitations of this study are: (1) The number of patients. It must be increased to elucidate the significance of prevalence data of some mutations; (2) The necessity to perform longitudinal studies with longer follow-up periods to clarify the progression of liver disease in this kind of patients. However, our data confirm the accumulation and persistence of HBV mutants related with HCC in different regions of HBV genome especially in the subpopulation of sub-Saharan origin.

ARTICLE HIGHLIGHTS

Research background

The indication of treatment in patients with chronic hepatitis B in the grey zone is not clear, and

it is necessary to balance the risks and benefits for health outcomes, including the evaluation of the risk of hepatocellular carcinoma development.

Research motivation
To optimize the management of patients in the grey zone of treatment in order to identify suitable subpopulations for antiviral treatment.

Research objectives
To analyze the prevalence, and persistence over time, of hepatitis B virus (HBV) mutants that predispose to the development of hepatocellular carcinoma in patients in the grey zone of treatment.

Research methods
We analyzed the presence of basal core promoter/precore/core and preS deletion mutants related with hepatocellular carcinoma development in 106 samples from 31 patient in the grey zone of treatment.

Research results
A significant number of the patients analyzed in this work shows hepatocellular carcinoma related mutations. All these hepatocellular carcinoma related mutants are major viral strains and persist over time. Some patients have hepatocellular carcinoma related mutants in basal core promoter/precore/core and preS regions simultaneously. The presence of preS deletions is associated with sub-Saharan subpopulations infected with HBV-A and HBV-E genotypes.

Research conclusions
The presence of preS mutations should be assessed in patients with sub-Saharan origin, especially if they are infected with HBV-E and HBV-A genotype, in order to identify subpopulations of patients in whom the antiviral treatment could be indicated to minimize the risk of hepatocellular carcinoma due to accumulation of high-risk HBV genetic variants.

Research perspectives
The analysis of preS deletions could be indicated in the management of sub-Saharan patients in grey zone of treatment. Analysis of higher sample size populations during long-time longitudinal follow-up should be further performed in these patient subpopulation.

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