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FRONTIER

Antimicrobial peptides and the gut microbiome in inflammatory bowel disease

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Abstract

Antimicrobial peptides (AMP) are highly diverse and dynamic molecules that are expressed by specific intestinal epithelial cells, Paneth cells, as well as immune cells in the gastrointestinal (GI) tract. They play critical roles in maintaining tolerance to gut microbiota and protecting against enteric infections. Given that disruptions in tolerance to commensal microbiota and loss of barrier function play major roles in the pathogenesis of inflammatory bowel disease (IBD) and converge on the function of AMP, the significance of AMP as potential biomarkers and novel therapeutic targets in IBD have been increasingly recognized in recent years. In this frontier article, we discuss the function and mechanisms of AMP in the GI tract, examine the interaction of AMP with the gut microbiome, explore the role of AMP in the pathogenesis of IBD, and review translational applications of AMP in patients with IBD.

Key Words: Antimicrobial peptides; Inflammatory bowel disease; Ulcerative colitis; Crohn's disease; Gut microbiome; Biomarkers

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infection while maintaining intestinal homeostasis to support commensalism with the gut microbiome. AMPs have broad spectrum antimicrobial activity with diverse mechanisms of action and regulate gut microbiome composition. Defects in endogenous AMP expression and function have been linked with animal models of inflammatory bowel disease (IBD). Exogenous delivery of AMPs such as defensins, cathelicidin, and elafin attenuates intestinal inflammation in murine models of IBD. AMPs such as calprotectin and lactoferrin are useful biomarkers for patients with IBD. Challenges with AMP stability, bioavailability, and selectivity are major barriers to their application as potential therapies.

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INTRODUCTION

The gastrointestinal (GI) tract is a highly complex and dynamic ecosystem consisting of a protective epithelial barrier in constant exposure to commensal microorganisms that are collectively known as the gut microbiome[1]. An intricate balance between tolerance to commensal microorganisms and protection against enteric pathogens is required to maintain intestinal homeostasis. A breakdown in this balance has been recognized to play a role in the pathogenesis of inflammatory disorders of the GI tract such as inflammatory bowel disease (IBD)[2]. Antimicrobial peptides (AMPs) are diverse and bioactive compounds that play critical roles in host defense and maintaining tolerance to commensal microorganisms[3,4]. Here we provide a comprehensive review of the significant AMP functions in the GI tract and the gut microbiome, potential roles of AMPs in the pathogenesis and treatment of IBD based on preclinical animal models, and translational applications of AMPs in patients with IBD.

ANTIMICROBIAL PEPTIDES IN THE GASTROINTESTINAL TRACT

Human defensins

Table 1 summarizes the major classes of AMPs in the GI tract. Defensins, which consist of small cationic peptides, protect against bacterial infections by directly disrupting bacterial membranes. The two major classes of defensins include α-defensins and β-defensins which differ structurally in their cysteine pairings[5]. Human α-defensins are also known as human neutrophil peptides (hNP). Human defensin 5 and 6 (HD5 and HD6) are the only α-defensins produced in the GI tract by Paneth cells, highly specialized secretory epithelial cells with antimicrobial function[6]. Known functions of HD5 include conferring resistance to oral challenge with enteric pathogens[7] and regulating the intestinal microbiota by reducing levels of segmented filamentous bacteria[8]. HD6 has been shown to restrict infection by limiting intestinal epithelial cell invasion[9]. β-defensins are expressed by enterocytes of the small and large intestine. The most relevant intestinal β-defensins include human β-defensins 1–4 (hBD-1, hBD-2, hBD-3, and hBD-4). hBD-2 and hBD-3 expression increases in response to infectious stimuli, whereas hBD-1 is constitutively expressed by the GI tract[10]. β-defensins hBD-2-4 have antimicrobial activity against *Escherichia coli* (*E. coli*), *Pseudomonas aeruginosa, Staphylococcus aureus, and Streptococcus pyogenes*, whereas hBD-1 only has activity against gram positive commensals[11-13].

Cathelicidin

Cathelicidin is another class of cationic peptides that mediates its bactericidal effects through direct disruption and lysis of bacterial membranes. Cathelicidin, also known as LL-37 or hCAP18, is an 18 kDa antimicrobial peptide involved in innate immune defenses and is encoded by the CAMP gene in humans[14]. Cathelicidin has a broad-

Table 1 Antimicrobial peptides in the gastrointestinal tract

Antimicrobial peptide class	Gene	Specific antimicrobial peptides	Tissue expression	Biologic function
α-defensins (human neutrophil peptides)[5-9]	*DEFA*	Human defensin 5 and 6 (HD5 and HD6)	Paneth cells	Confers resistance to oral challenge with enteric pathogens, regulates the intestinal microbiota by reducing levels of segmented filamentous bacteria, restricts infection by limiting intestinal epithelial cell invasion
β-defensins[5,10-13]	*DEFB*	Human β-defensins 1–4 (hBD-1, hBD-2, hBD-3, and hBD-4)	Intestinal epithelial cells	Antimicrobial activity (hBD-2-4) against bacterial pathogens including *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Streptococcus pyogenes*, antimicrobial activity (hBD-1) against gram-positive commensals
Cathelicidin[14-22]	*CAMP*	Cathelicidin (LL-37/hCAP18)	Colonic epithelial cells, neutrophils, monocytes, macrophages, mast cells	Cationic peptide that directly disrupts bacterial cell membranes, deficiency increases susceptibility to infection with enterohemorrhagic *E. coli (EHEC)*
Regenerating (Reg) protein [23-29]	*REG*	RegIII; Hepatocarcinoma-intestine pancreas (HIP)/pancreatitis-associated protein (PAP)	Paneth cells, intestinal epithelial cells	Regulates intestinal homeostasis by maintaining a physical separation between epithelial cells and the microbiota, selective for gram-positive bacteria through interaction with cell wall peptidoglycan
Lactoferrin[30]	*LTF*	Lactoferrin	Epithelial cells	Secreted iron binding protein, sequesters free iron required for bacterial growth
Lipocalin[31,129]	*LCN2*	Lipocalin-2 (neutrophil gelatinase-associated lipocalin, GAL)	Neutrophils, granulocytes, macrophages, epithelial cells	Binds to bacterial siderophore enterobactin and inhibits bacterial growth by sequestering iron
Calprotectin[32]	*S100A8, S100A9*	Calprotectin	Intestinal epithelial cells, neutrophils	Chelates and sequesters metal co-factors (manganese, zinc, iron) during infection and inhibits bacterial growth
Hepcidin[33]	*HAMP, LEAP1*	Hepcidin antimicrobial peptide	Intestinal epithelial cells	Regulates iron absorption and homeostasis, inhibits bacterial growth by limiting iron availability
Galectin[34,35]	*LGALS*	Galectin-3, Galectin-4, Galectin-8	Intestinal epithelial cells	Galectins has bactericidal activity against bacteria expressing blood group antigen, Gal-8 targets damaged vesicles for autophagy during bacteria invasion
Lysozyme[36]	*LYZ*	Lysozyme	Paneth cells	Enzymatic degradation of bacterial membranes, preference towards Gram-positive pathogens
Elafin[37]	*PI3*	Elafin (peptidase inhibitor 3)	Intestinal epithelial cells	Binds to bacterial lipopolysaccharide (LPS) and modulates innate immunity
Secretory Leukocyte Protease Inhibitor (SLPI)[38,39]	*SLPI*	SLPI	Intestinal epithelial cells, paneth cells, neutrophils, macrophages	Protease inhibitor binds to bacterial mRNA and DNA, dose-dependent bactericidal properties of SLPI against both Gram-positive and Gram-negative bacteria, has fungicidal properties

spectrum activity against bacteria, enveloped viruses, and fungi[15]. It is expressed by differentiated colonic epithelial cells as well as resident immune cells in the GI tract including neutrophils, monocytes, and macrophages, and mast cells[16,17]. Cathelicidin expression has been reported to be increased in inflamed and noninflamed mucosa in ulcerative colitis patients[18]. Butyrate[18] and vitamin D[19,20] are known inducers of cathelicidin expression on colonic epithelial cells and immune cells. Cathelicidin deficiency increases susceptibility to infection with enterohemorrhagic *E. coli* (EHEC)[21]. Vitamin D induction of cathelicidin in human colonic epithelial cells has been shown to inhibit *in vitro E. coli* growth[21]. Likewise, cathelicidin protects against colonization with epithelial adherent bacterial pathogens [22].

Regenerating protein

Another class of antimicrobial peptides expressed in the GI tract include the soluble lectins belonging to the regenerating (Reg) Protein family. RegIIIγ and its human counterpart RegIIIα, also known as Hepatocarcinoma-Intestine Pancreas/Pancreatitis-Associated Protein (HIP/PAP), are expressed by enterocytes and Paneth cells in response to microbial and inflammatory stimuli[23,24]. RegIIIα selectively binds to cell wall peptidoglycan in gram-positive bacteria to induce pore formation[25]. RegIIIβ interacts with surface Lipid A structures to target gram-negative bacteria[26]. In mice, RegIIIγ maintains physical separation between the gut microbiota and the intestinal epithelial surface and regulates bacterial colonization and intestinal immune responses by the microbiota[27]. In mice, RegIII is strongly induced in gut epithelial cells following bacterial reconstitution and colitis[28]. In human studies, Reg Iα, Reg Iβ, and Reg IV are overexpressed in colon mucosa with ulcerative colitis, whereas Reg IV is overexpressed in Crohn's disease[29].

Metal sequestering antimicrobial peptides

Some antimicrobial peptides function by sequestering metal micronutrients which are required as co-factors for microbial growth. Lactoferrin is a secreted iron binding protein that is expressed by intestinal epithelial cells. Lactoferrin mediates its antimicrobial activity by sequestering free iron required for bacteria growth[30]. Lipocalin-2 (neutrophil gelatinase-associated lipocalin, GAL) is expressed by intestinal epithelial cells after stimulation by proinflammatory cytokines IL-17 and IL-22. Lipocalin-2 sequesters the siderophore enterobactin which then prevents bacteria cells from binding iron[31]. Calprotectin, a heterodimer consisting of S100A8 and S100A9, is produced by intestinal epithelial cells and neutrophils. Calprotectin inhibits bacterial growth by sequestering zinc and manganese during infection[32]. The cationic peptide hepcidin plays a key role in regulating iron homeostasis through its binding to the iron exporter ferroportin. During infection and inflammation, hepcidin is upregulated and subsequently limits iron availability to bacterial pathogens. Hepcidin has antimicrobial activity against *E. coli*, *Pseudomonas aeruginosa*, and group A *Streptococcus*[33].

Antimicrobial peptides with different mechanisms of action

Other AMPs of various mechanisms of action have also been characterized. Galectins are β-galactoside-binding lectins that can bind to galactose-containing glycans on glycoproteins and glycolipids. They are highly expressed by intestinal epithelial cells and innate immune cells. Galectin-3, -4, and-8 recognize human blood group B antigen-like determinants on the surface of *E. coli* O86 and have bactericidal activity. Galectin-3 can bind to lipopolysaccharide (LPS) on gram-negative bacteria. Galectin-8 targets damaged vesicles for autophagy during bacteria invasion[34,35]. Another mechanism involves enzymatic degradation of bacterial membranes. Lysozyme which is secreted by Paneth cells preferentially binds to gram-positive bacteria and degrades bacterial membranes by hydrolyzing peptidoglycan linkages[36]. AMPs also function as protease inhibitors such as elafin and secretory leukocyte protease inhibitor (SLPI). Elafin is produced by epithelial cells of mucosal surfaces including the GI tract. Elafin mediates its antimicrobial activity by binding to LPS from gram-negative bacteria and modulating macrophages[37]. SLPI is a major serine proteinase inhibitor that is expressed and apically secreted by human intestinal epithelium as well as Paneth cells, neutrophils, and macrophages. SLPI has antimicrobial activity against the enteric pathogen *Salmonella typhimurium* as well as gram-positive and gram-negative bacteria and fungi[38,39].

ANTIMICROBIAL PEPTIDES AND THE GUT MICROBIOME

The appropriate maintenance of the gut microbiome is critical for health. In addition to offering competitive protection against pathogen growth, the microbiome regulates gut development[40] modulates digestion[41] and provides nutrients[42]. Thus, the microbiome must be carefully cultivated, without being permitted to proliferate excessively. However, the rapid renewal of epithelial layers, particularly in the gut where renewal rates are amongst the most rapid[43,44], poses a unique challenge for maintaining microbial composition and distribution. AMPs are a critical mechanism for regulating the microbiome, and act as part of a complex interplay between the gut microbiome, the innate immune system, and epithelium renewal. Reduced AMP production is associated with disorders such as IBD[45], which will be discussed in more depth in section III. In wounds or acute infections, multiple classes of AMPs are

rapidly upregulated, frequently through PAMP-dependent induction. Above threshold doses, they achieve rapid bacterial killing by synergistically targeting diverse yet critical microbial functions[46]. In contrast, direct interactions between AMPs and the gut microbiome occur at sub-lethal doses[47], though AMPs also act indirectly on the gut microbiome through the local modulation of immune response [48].

Evolutionary analysis of AMPs offers insights into AMP function

Across a wide array of species, the regional control of which AMP classes are expressed acts in concert with local environmental conditions to fine-tune both the microbiome's spatial heterogeneities as well as bacterial phenotype[49]. The requirements for broad-spectrum pathogen resistance, coupled with carefully tuned microbiome maintenance, lead to fascinating AMP evolutionary behavior. While genes associated with immune defense are associated with rapid evolution, AMP amino acid sequences evolve more slowly than the genome average. Indeed, they can be highly conserved across multiple species[50]. The relatively slow evolution rate of AMP amino acid sequences therefore suggests that pathogen control is likely a result of a complex AMP mixture, and that any individual AMP exerts minimal co-evolutionary pressure[51,52].

Given the importance of microbiome composition for health, and in light of the highly conserved AMP amino acid sequences, one might expect strict control over AMP copy number and regulation. Unexpectedly, this is not what has been observed. While AMP coding sequences are highly conserved within a species, there is substantial variability in both copy number and regulatory sequences, as reviewed in [53]. This is particularly intriguing given that there is a high evolutionary cost associated with AMPs; when model organisms are propagated in germ-free environments, AMPs are rapidly lost[54]. Together, these data strongly suggest that the regulatory variability that is observed within humans may be a function of geography, specifically long-term local diets, local pathogens, and/or candidate microbiome components.

Dynamics of AMP-microbiome interactions

AMP serve as key regulators of host-gut microbiota interactions in a bi-directional and highly dynamic process[55]. AMP can shape the composition of the gut microbiome. For example, sublethal doses of AMPs could prime *E. coli* to develop tolerance and increase persistence by production of curli or colonic acid[56]. Prior studies have demonstrated that species-specific AMP profiles in animals maintains species-specific bacterial communities. Loss-of-function experiments have also shown that antimicrobial peptide composition is a predictor of bacterial colonization[57]. Furthermore, AMP resistance patterns maintains the resilience of prominent gut commensals during perturbations such as inflammation[58]. Conversely, the gut microbiome produces a complex array of metabolites[59] that directly regulate AMP production and function [60,61]. For example, the microbiota metabolite short chain fatty acid promoted the production of the AMP RegIIIγ and β-defensins by intestinal epithelial cells[62].

Manipulation of gut microbiome composition has been shown to control AMP production and function. Cazorla *et al*[63] demonstrated that oral administration of probiotics in mice increased Paneth cell and intestinal antimicrobial activity. In addition, treatment of mice with VSL #3, a common probiotic used in patients with IBD, was associated with restoration of *AMP* gene expression in the small intestine and increased abundance of bacterial commensals in the gut[64]. Some probiotic strains produce AMP and has been proposed as a strategy to improve immune responses in immunocompromised patients[65]. Finally, fecal microbial transplant also modulates AMP expression in the GI tract. Teng *et al*[66] demonstrated that fecal microbial transplant of piglets resulted in increased expression in porcine beta-defensins in the jejunum and subsequent increased gut *Firmicutes* and decreased *Bacteroides*.

Gut microbiome effects of different antimicrobial peptides

Different locations and cellular origins of AMP production are superimposed along the GI tract. Defensins, the most abundant AMPs in the gut, are notable for their multiple disulfide bridges which confer substantial structural resistance to bacterial-derived peptidases[67]. Defensins exert antimicrobial activity through forming pores in target bacterial membranes. Above sufficient thresholds, this results in cell death. Although the effect of sub-lethal concentrations is still undergoing characterization in humans, it is notable that a similar strategy is used by plants[50]. Here, pore-forming AMPs are used to facilitate the release of endosymbiotic microbe-derived nutrients.

Local immune cell populations such as macrophages, T cells, and B cells[68] secrete both classes of defensins. The highly spatially restricted secretion of α-defensins, in comparison to the ubiquitous secretion of β-defensins, strongly suggests that their role is likely to prevent bacterial overgrowth[61]. Indeed, Paneth cells are positioned just beneath the actively proliferating epithelial stem cells which are critical for epithelium renewal. Single-crypt studies show that Paneth cell degranulation of α-defensins is induced by both gram-negative and gram-positive bacteria, regardless of whether they are alive or dead, as well as bacterial components such as lipopolysaccharide, lipoteichoic acid, lipid A, and muramyl dipeptide[69]. Furthermore, the antimicrobial products of Paneth cells are protective against *in vitro* microbial challenges many orders of magnitude (> 10^6) higher than those encountered *in vivo*. Notably, degranulation is not induced by eukaryotic pathogens, including live fungi and protozoa[69]. While α-defensin deficiencies in mouse models do not affect total bacterial load, they do result in reduced *Bacteroides* abundance and increased *Firmicutes* abundance[70].

β-defensins act in the gut as a two-layered, ubiquitous defense system. β-defensin-1 is constitutively expressed at low levels, even in the gut of germ-free models[71]. β-defensin-2 and β-defensin-3 can be further induced by the local microbiome, and additionally act as potent chemo-attractants for neutrophils and memory T cells[72]. In contrast to α-defensins, cell culture models suggest that gut β-defensin induction may rely on live bacteria; pre-incubation of Caco-2 epithelial cells with *Enterococcus faecium* reduced *Salmonella typhimurium* uptake, while pre-incubation with heat-killed *E. faecium* did not[72]. Unlike α-defensins, at least one (β-defensin-3) has anti-fungal activity[72].

Cathelicidins (in humans: LL-37) have broad anti-microbial and immunomodulatory function, and act to maintain epithelial barrier integrity[73,74]. Cathelicidins also have a two-tiered anti-microbial activity. While their primary mechanism of activity at high concentrations is to disrupt bacterial membranes, their immunomodulatory functions occur at substantially lower concentrations. Epithelial barrier integrity maintenance is accomplished primarily through increasing tight junction protein expression, as well as post-translational effects including the redistribution of tight junctions[75]. Together, this suggests that cathelicidins are primarily used when the epithelial barrier becomes compromised. Furthermore, LL-37 has also been shown to alter the composition of the gut microbiome in mice. Cathelicidin knockout mice had significantly more OTUs belonging to the phylum *Verrucomicrobia* and had lower amount of OTUs belonging to phylum *Proteobacteria* and the genus *Lactobacillus* than the other genotypes[76].

Reg III AMPs, primarily secreted by Paneth cells and epithelial cells[28,61], are soluble lectins that appear to primarily govern spatial relationships between the microbiome host tissues *via* the mucosa. In mice, Reg IIIβ/γ are co-regulated; Reg IIIα is the human ortholog[27,77]. Thinning of the mucosa driven by dietary restrictions in microbiota-accessible carbohydrates resulted in increased Reg IIIβ[78], as did increased mucosal inflammation[28]. Reg IIIγ-/- mice exhibited increased mucosal bacterial burden and impaired spatial relationships between bacteria and their host tissues[27].

FUNCTION AND MECHANISMS OF ANTIMICROBIAL PEPTIDES IN THE PATHOGENESIS OF IBD

Alpha defensins: HNP-1

Several prior studies have linked defects or alterations in GI tract AMPs with the pathogenesis of IBD. Table 2 summarizes studies exploring the function and mechanisms of AMPs in IBD. HNPs and their role in IBD continues to be investigated. Maeda *et al*[79] found that mild transgenic overexpression of HNP-1 reduces the susceptibility to murine dextran sulfate sodium (DSS) induced colitis. Not only did the colon of HNP-1 transgenic mice show less tissue damage, but mice also had significantly lower disease activity index (DAI) scores when compared to wild type mice. Additionally, the authors found intraperitoneal injection of low dose HNP-1 mitigates DSS-induced colitis and results in reduced expression of pro-inflammatory cytokines in the colon of mice. This improvement of colitis from low-dose HNP-1 could be from its antimicrobial activity[79].

Furthermore, Hashimoto *et al*[80] found that intraperitoneal injection of high concentrations of HNP-1 exacerbate DSS-induced colitis in pathogen free (BALB/c) mice and severe combined immunodeficient (SCID) mice. Clinically, HNP-1 treated BALB/c mice had significantly decreased weight and colon length as well as significantly increased DAI score, histologic score and myeloperoxidase (MPO)

Table 2 Antimicrobial peptides in preclinical models of inflammatory bowel disease

Ref.	Antimicrobial peptides (expression location)	Antimicrobial peptide delivery	Preclinical models (animal, human cell culture)	Key findings
Maeda *et al* [79]	Alpha defensins: Human neutrophil peptide-1 (HNP-1) murine colon	Genetic overexpression, intraperitoneal	Murine dextran sulfate sodium (DSS) colitis	Mild transgenic overexpression of HNP-1 reduces the susceptibility to DSS-induced colitis; Intraperitoneal injection of low-dose HNP-1 ameliorates DSS-induced colitis; The amelioration of colitis by low-dose HNP-1 may be explained by its indirect antimicrobial activity
Hashimoto *et al*[80]	Alpha defensins: Human neutrophil peptide-1 (HNP-1): Murine colon, human colon cells	Intraperitoneal	Murine dextran sulfate sodium (DSS) colitis, SCID mice, human colon cell cultures	Body weight and colon length significantly decreased, and the disease activity index score, histologic score, and myeloperoxidase activity significantly increased in HNP-1-treated mice compared with PBS-treated mice. High concentrations of HNP-1 aggravate DSS-induced colitis, including upregulated expression of such macrophage-derived cytokines as IL-1β
Han *et al*[82]	Porcine β-defensin (pBD)2: Murine colon	Intrarectal	Murine dextran sulfate sodium (DSS) colitis, human colon cell cultures	Administration of pBD2 effectively attenuated colonic inflammation in mice with DSS induced colitis. pBD2 reduced the increased serum and colon levels of TNF-a, IL-6 and IL-8 all caused by DSS. The effects of pBD2 appeared to be through upregulation of the expression of genes associated with tight junctions and mucins
Koeninger *et al*[81]	Beta defensins: human beta defensin 2 (HBD-2): Murine colon	Subcutaneous	Murine dextran sulfate sodium (DSS) colitis, 2,4,6-trinitrobenzenesulfonic acid (TNBS) colitis, T cell transfer colitis model	Treatment improved disease activity index and hindered colitis-induced body weight loss on par with anti-TNF-α and steroids. Mechanistically, hBD2 engaged with CCR2 on its DC target cell to decrease NF-κB, and increase CREB phosphorylation, hence curbing inflammation
Koon *et al*[73]	Cathelicidin (LL-37): Murine colon	Genetic knockouts	Murine dextran sulfate sodium (DSS) colitis	Increased expression of cathelicidin in the colon of DSS-exposed mice; Compared with WT mice, cathelicidin KO mice developed a more severe form of DSS-induced colitis; Cathelicidin protects against induction of colitis in mice; Increased expression of cathelicidin in monocytes and experimental models of colitis involves activation of TLR9-ERK signaling by bacterial DNA
Fabisiak *et al* [83]	Cathelicidin (LL-37) KR-12 (active fragment of LL-37): Murine colon	Intraperitoneal	Murine dextran sulfate sodium (DSS) colitis, 2,4,6-trinitrobenzenesulfonic acid (TNBS) colitis, T cell transfer colitis model	LL-37 and KR-12 (1 mg/kg, ip, twice daily) showed a decrease in macroscopic and ulcer scores in the acute TNBS-induced model of colitis. KR-12 (5 mg/kg, ip, twice daily) reduced microscopic and ulcer scores in the semi-chronic and chronic TNBS-induced models of colitis compared with inflamed mice
Yoo *et al*[84]	Cathelicidin (LL-37): Murine colon	Intracolonic, intravenous	2,4,6-trinitrobenzenesulfonic acid (TNBS) Colitis,	Intracolonic cathelicidin (mCRAMP peptide) administration or intravenous delivery of lentivirus-overexpressing cathelicidin gene significantly reduced colonic col1a2 mRNA expression in TNBS-exposed mice compared with vehicle administration. Cathelicidin can reverse intestinal fibrosis by directly inhibiting collagen synthesis in colonic fibroblasts
Tai *et al*[85]	Cathelicidin (LL-37): Murine colon	Genetic knockouts, intrarectal	Murine dextran sulfate sodium (DSS) colitis	Cathelicidin knockout mice had more severe symptoms and mucosal disruption than the wild-type mice in response to DSS colitis. Intrarectal administration of plasmids encoding cathelicidin reversed colitis in cathelicidin knockout mice
Gubatan *et al* [21]	Cathelicidin (LL-37): Murine colon, human colon cells	Intrarectal	Murine dextran sulfate sodium (DSS) colitis, human colon cell cultures	Vitamin D-induced cathelicidin in human colonic epithelial cells suppressed *Escherichia coli* growth. Intrarectal cathelicidin reduced the severity of DSS colitis but did not mitigate the impact of colitis on microbial composition
Motta *et al*[91]	Elafin: Murine colon	Transgenic expression, adenoviral delivery	Murine dextran sulfate sodium (DSS) colitis, 2,4,6-trinitrobenzenesulfonic acid (TNBS) colitis	In mice given TNBS or DSS, transgenic expression of elafin protected against the development of colitis. Similarly, adenoviral delivery of Elafin significantly inhibited inflammatory parameters. Elafin modulated a variety of inflammatory mediators *in vitro* and *in vivo* and strengthened intestinal epithelial barrier
Ogawa *et al* [28]	RegIII (HIP/PAP): Murine colon	Endogenous expression	Murine dextran sulfate sodium (DSS) colitis	Epithelial expression of Reg III or HIP/PAP was induced under mucosal inflammation initiated by exposure to commensal bacteria or DSS as well as inflamed IBD colon

Jiang *et al*[93]	Donkey milk lysozyme (DML): Murine colon	Oral	Murine dextran sulfate sodium (DSS) colitis	DML ameliorated weight loss, colon damage and mucosal inflammation in DSS colitis mice. DML improved mechanical barrier function and increased gut microbiota composition diversity, promoting growth of probiotics and inhibiting pernicious bacteria
Reardon *et al*[92]	Secretory leukocyte peptidase inhibitor (SLPI): Murine colon	Genetic SLPI deficiency, oral	Murine dextran sulfate sodium (DSS) colitis, T cell transfer colitis model	Tslp−/− mice lead to endogenous SLPI deficiency which exacerbated DSS colitis. Treatment with recombinant SLPI (rSLPI) reduced DSS-induced mortality in Tslp−/− mice
Togawa *et al*[95]	Lactoferrin: Rat colon	Oral	Rat dextran sulfate sodium (DSS) colitis	DSS-induced colitis was attenuated by oral administration of lactoferrin in a dose-dependent manner. Reduced inflammation in response to lactoferrin was correlated with the significant induction of the anti-inflammatory cytokines and with significant reductions in the pro-inflammatory cytokines
Shanmugam *et al*[96]	Hepcidin: Murine colon	Endogenous expression	Murine dextran sulfate sodium (DSS) colitis, T cell transfer Colitis model	TNFα inhibits hepcidin expression in two distinct types of innate colitis, with down-regulation of Smad1 protein playing an important role in this process

activity when compared to control mice. Furthermore, inflammatory cytokines IL-1β and TNF-α were significantly higher in colon of HNP-1 treated mice. In both murine models, an increased recruitment of F4/80-positive macrophages in the inflamed colonic mucosa after HNP-1 injection has been observed. This enhanced disease activity is thought to be due in part to HNP-1 induced cytokine production in macrophages.

Beta defensins: Porcine B-defensin and hBD-2

Beta defensins are epithelial cell derived AMPs that have immunomodulating properties. Koeninger *et al*[81] found that subcutaneous recombinant hBD-2 reduced intestinal inflammation in three distinct animal models of IBD: chemically induced mucosal injury (DSS), loss of mucosal tolerance (TNBS), and T cell transfer into immunodeficient recipient mice. Mice treated with hBD-2 had less weight loss, better stool score and improved DAI scores in comparison to the T cell colitis control group. Additionally, mice given hBD-2 had less mucosal damage and inflammation as they maintained crypt anatomy and had reduced colon weight.

In addition to the protective effects of hBD-2, Han *et al*[82] found that intrarectal administration of porcine beta-defensin 2 (pBD2) ameliorated colonic inflammation in mice during the induction of DSS-induced colitis. Mice in the pBD2 plus DSS group had less symptoms, including less weight loss, firmer and less bloody stools compared to the DSS-treated group. Mice treated with pBD2 plus DSS also had less evidence of macroscopic and histological colitis in addition to reduced production of TNF-a, IL-6 and IL-8 when compared to the DSS-treated group. Through colon cell culture, the effects of pBD2 seemed to occur *via* an upregulation of genes associated with tight junctions and mucins. This may explain how pBD2 can improve DSS-induced changes in the mucosa and paracellular permeability through possible activation of the NF-kB signaling.

Cathelicidin (LL-37)

Koon *et al*[73] demonstrated that genetic knockout of LL-37 in mice had more severe forms of DSS-induced colitis and that inflamed colon in wild type mice in DSS colitis

models had increased cathelicidin expression. The authors suggested that this upregulation of cathelicidin involves activation of TLR9-ERK signaling from bacterial DNA, which may play a role in the development of colitis. In addition to its protection against the induction of colitis, Fabisiak *et al*[83] showed that intraperitoneal injection of LL-37, and its shortest active metabolite, KR-12, decreases ulcer and macroscopic scores in DSS-induced and TNBS-induced models of colitis. The study showed that intraperitoneal injection of KR-12 altered the microbiomes of TNBS-induced colitis mice by reducing total and *E. coli* group bacteria.

In addition to the protective and antimicrobial properties of LL-37, Yoo *et al*[84] found that intracolonic cathelicidin or intravenous delivery of lentivirus-overexpressing cathelicidin gene significantly reduced colonic collagen deposition TNBS-induced colitis mice when compared to TNBS-induced mice not receiving LL-37. These results suggest that cathelicidin reverses fibrosis in the intestines *via* inhibition of collagen synthesis in colonic fibroblasts.

Another unique property of LL-37 was investigated by Tai *et al*[85], who describe that intrarectal administration of plasmids containing cathelicidin to DSS-induced colitis mice reestablished colonic mucus thickness *via* increased expression of mucin genes and reduced severe symptoms compared to cathelicidin knockout mice with DSS-induced colitis. This increase in mucin genes protected against mucosal damage and was linked to the activation of MAP kinase.

Gubatan *et al*[21] found that cathelicidin is a key mediator of the protective role of vitamin D in ulcerative colitis (UC). The authors found higher levels of 25(OH)D correlate with increased levels of both serum and colonic LL-37 in UC patients, and these higher levels are associated with decreased histologic inflammation and probability of clinical relapse. Intrarectal LL-37 reduced the severity of DSS-induced colitis in mice, but did not alter the intestinal microbial imbalance, whereas 25(OH)D-induced cathelicidin in human colonic epithelial cells suppressed *E.coli* growth. The study demonstrated that 25(OH)D is an independent predictor of cathelicidin in UC patients in remission and may protect against microbial associated gut inflammation.

Arachidonic acid and its metabolism also play a role in the regulation of antimicrobial peptides in inflammatory bowel disease. Arachidonic metabolites such as leukotrienes and are elevated in both animal models of colitis and patients with IBD [86]. Leukotrienes have been shown to trigger release of human cathelicidin from neutrophils[87], whereas prostaglandins suppress cathelicidin in human macrophages [88]. In addition, cyclooxygenase-2 (COX-2), an enzyme that metabolizes arachidonic acid, is also induced in colonic epithelial cells in IBD[89]. Cox-2 selective inhibitors have been shown to inhibit production of human beta defensins but not cathelicidin [90].

Elafin

Motta *et al*[91] showed that in TNBS or DSS-induced mouse models of colitis, transgenic expression of elafin or disruption of enzymes that elafin inhibits protected against development of colitis. Transgenic mice expressing elafin had reduced inflammation as measured by a reduction in macroscopic tissue damage and myeloperoxidase (MPO) activity when compared to TNBS or DSS-induced mice that were not expressing elafin. Authors showed that adenoviral delivered elafin inhibited inflammatory parameters. The authors demonstrated that elafin is involved in inflammatory mediators and its protective effect could in part be from a bolstering of epithelial and mucosal barriers.

SLPI

Reardon *et al*[92] reported that thymic stromal lymphopoietin-deficient (TSLP-/-) mice led to endogenous SLPI deficiency, which prevented recovery from DSS-induced colitis and resulted in death. The authors demonstrated that the mechanism by which the absence of SLPI prevents healing of the colon is from increased neutrophil elastase (NE) activity in TSLP-/- mice. When TSLP-/- mice were treated with oral recombinant SLPI (rSLPI) there was reduced DSS-induced mortality.

Reg III (HIP/PAP)

Ogawa *et al*[28] aimed to identify genes that were modulated by bacterial flora to better understand mucosal inflammation in IBD patients. The authors found that expression of Reg III (HIP/PAP) was increased in DSS-induced colitis. Furthermore, the upregulation of Reg III may be due to an increase in the acute phase reactant IL-6 that occurs during gut inflammation.

Donkey milk lysozyme

Donkey milk contains high lysozyme levels and was studied by Jiang *et al*[93] due to its antimicrobial properties. Authors found that mice given donkey milk lysozyme (DML) orally in a DSS-induced colitis model had improved symptoms of colitis measured by a reduction in weight loss, loose stools, rectal bleeding and mucosal inflammation**.** The authors showed that 50% DML treatment brought cytokines, TNF-a and IL-13, a pleiotropic cytokine that has proinflammatory effects on intestinal epithelial cells resulting in apoptosis and epithelial barrier dysfunction in intestinal inflammation[94] back to basal levels similar to control mice. They hypothesized that DML improves the intestinal barrier by increasing expression of tight junction proteins in the colon. They also presume that DML increases gut microbiota diversity and reduces detrimental bacteria thereby restoring the gut microflora.

Lactoferrin

Lactoferrin, a known immunomodulator, was studied by Togawa *et al*[95] and was found to reduce DSS-induced colitis in a dose-dependent manner after oral administration to rats. The DAI, shortening of colon length, histological/macroscopic damage score, tissue levels of MPO activity, WBC, and reduction in hemoglobin were decreased when DSS-induced colitis rats were treated with lactoferrin. The authors postulate that the protective properties of lactoferrin were tied to its modulation of the immune system by reducing pro-inflammatory cytokines TNF-a, IL-1B and IL-6 as well as the augmented levels of anti-inflammatory cytokines IL-4 and IL-10 in colonic tissue of DSS-induced colitis rats given lactoferrin.

Hepcidin

Hepcidin is regulator of iron metabolism and is upregulated during the inflammation in IBD, often resulting in anemia. Shanmugam *et al*[96] investigated the mechanisms that control hepcidin during periods of inflammation. They showed that the pro-inflammatory cytokine TNF-a inhibits hepcidin in both a DSS-induced colitis and T cell transfer colitis model in mice with downregulation of Smad1 protein mediating this effect.

TRANSLATIONAL APPLICATIONS OF ANTIMICROBIAL PEPTIDES AS BIOMARKERS IN PATIENTS WITH IBD

The diagnosis and long-term monitoring of IBD commonly involve invasive and costly endoscopy combined with histologic screening. Consequently, a biomarker that reflects the ongoing severity of disease is attractive as a non-invasive, cost-effective, and convenient alternative for diagnosing new IBD cases and identifying flares of disease. Given their involvement in disease pathophysiology, AMPs represent such potential markers, and several have been studied to determine their utility in differentiating CD and UC from other conditions, such as celiac disease and IBS, as well as active from quiescent disease states. In addition to reflecting ongoing severity of inflammation, several AMPs have shown promise as predictors of relapse, complication risk, and treatment response in the setting of IBD. Table 3 summarizes the application of AMPs as biomarkers in IBD.

Calprotectin

Among all known AMPs, calprotectin is the one most frequently used in the clinical diagnosis and monitoring of IBD. It has been known for decades that fecal calprotectin (FC) concentrations are markedly increased in the setting of both CD and UC[97-100]. Elevated FC is a highly sensitive marker and is thus a particularly useful tool in the initial diagnosis and discrimination of IBD from non-inflammatory causes of abdominal discomfort and bowel dysfunction like IBS[97-103]. Based on this diagnostic utility, current practice guidelines from the World Gastroenterology Organization support measuring FC in the initial work-up of suspected IBD in both adult and pediatric patients[101,102]. Recent research has supported using FC measurements for the early diagnosis of IBD in at-risk populations, such as patients with ankylosing spondylitis[104].

FC is also particularly useful in the evaluation of IBD severity and the early identification of disease flares[104-106]. Data suggest that FC concentrations positively correlate with histologic inflammation in IBD, and assays can be used to accurately classify inactive, mild, moderate, and severe disease[102,103]. Cut-off values of fecal

Table 3 Biomarker applications of antimicrobial peptides in patients with inflammatory bowel disease

Ref.	Antimicrobial peptides	Type of IBD	Biomarker application	Key findings
Holgersen *et al* [110]	Alpha defensins 5 and 6 (DEFA5/DEFA6)	UC	IBD diagnosis	Marked upregulation of DEFA5 and DEFA6 in terminal ileal biopsies of inflamed ulcerative colitis relative to normal controls
Wehkamp *et al* [111]	Alpha defensin (HD -5/6)	UC/CD	IBD diagnosis	HD-5/6 both decreased in ileal Crohn's, and this correlated with a decrease in transcription factor Tcf-4, a known regulator of Paneth cell differentiation. Normal levels were observed in UC and colonic Crohn's
Yamaguchi *et al* [112]	Alpha defensin (HNP1-3), beta-defensin (HBD-2)	UC/CD	Disease activity	HNP-1-3 all elevated in IBD patients, while HBD-2 levels normal; serum HNP1-3 levels correlated with disease severity for Crohn's
Kanmura *et al* [113]	Alpha defensin (HNP)	UC/CD	Disease activity	Fecal-HNP levels were markedly elevated in both UC and Crohn's, but slightly more so in Crohn's; F-HNP was significantly higher during flares of UC than remission. For UC, HNP levels correlated with Mayo endoscopic score
Cunliffe *et al* [114]	Alpha defensin (HNP 1-3)	UC/CD	Disease activity	Surface epithelial cells strongly immunoreactive for neutrophil defensins and lysozyme were seen in active ulcerative colitis and Crohn's disease (but not normal or inactive IBD) mucosal samples. Many of these cells coexpressed both antimicrobial proteins.
Tran *et al*[116]	Cathelicidin	UC/CD	Disease activity	Cathelicidin levels were significantly increased in IBD patients and were inversely correlated with CD activity. In moderate to severe IBD, higher cathelicidin levels before treatment correlated with better prognosis.
Krawiec *et al* [115]	Cathelicidin	UC/CD	IBD diagnosis	Cathelicidin was significantly increased in patients with ulcerative colitis (1073.39 ± 214.52 ng/mL) and Crohn's disease (1057.63 ± 176.03 ng/mL) patients compared to controls (890.56 ± 129.37 ng/mL) (P = 0.0003)
Gubatan *et al*[21]	Cathelicidin	UC	Disease activity, clinical relapse	In ulcerative colitis patients, serum 25(OH)D positively correlated with serum and colonic cathelicidin. Higher serum cathelicidin is associated with decreased risk of histologic inflammation and clinical relapse but not independent of 25(OH)D or baseline inflammation
Borkowska *et al* [118]	Lactoferrin	UC/CD	IBD diagnosis, disease activity	Fecal concentration of lactoferrin in children with IBD was significantly higher than in the controls. The sensitivity and specificity were 80.7% and 92.7%, respectively, and its positive and negative prognostic values were 96.8% and 63.3%, respectively
Sugi *et al*[119]	Lactoferrin, lysozyme	UC/CD	Disease activity	Lactoferrin and lysozyme were significantly increased in the active phases of CD and UC relative to inactive. They both correlated with fecal Hb concentration in UC, and with alpha 1-AT concentration in CD
Sidhu *et al*[120]	Lactoferrin	UC/CD	IBD diagnosis, disease activity	Lactoferrin levels were significantly higher in IBD patients compared with IBS/healthy controls (P < 0.001). The sensitivity, specificity, positive and negative predictive values of lactoferrin in distinguishing active IBD from IBS/healthy controls were 67% and 96%, 87% and 86.8% respectively
Wang *et al*[121]	Lactoferrin	UC/CD	IBD diagnosis	FL test has a high sensitivity (82%) and specificity (95%) for the discrimination of patients with IBD against non-IBD patients
Kane *et al*[122]	Lactoferrin	UC/CD	Disease activity	Fecal lactoferrin was 90% specific for identifying inflammation in patients with active IBD. Elevated fecal lactoferrin was 100% specific in ruling out IBS
Turner *et al*[123]	Lactoferrin	UC	IBD diagnosis	Lactoferrin levels significantly were elevated in pediatric UC patients, but were not responsive to change or predictive of response to corticosteroids
Wang *et al*[132]	Elafin	CD	Disease activity, intestinal strictures	High serum elafin levels were associated with a significantly elevated risk of intestinal stricture in CD patients. Serum elafin levels had weak positive correlations with clinical disease activity but not endoscopic disease activity
Zhang *et al*[133]	Elafin	UC/CD	Disease activity	The expression of elafin mRNA in peripheral blood in active IBD patients is decreased, which may be correlated with the activity of IBD, and negatively correlated with corresponding disease activity score
Motta *et al*[130]	Elafin	UC	Disease activity	Study identified a previously unrevealed production of elastase 2A (ELA2A) by colonic epithelial cells, which was enhanced in IBD patients.

				Study demonstrated that ELA2A hyperactivity is sufficient to lead to a leaky epithelial barrier and modified the cytokine gene expression profile with an increase of pro-inflammatory cytokine transcript
Schmid *et al*[134]	Elafin and SLPI	UC/CD	Disease activity	Levels of mRNA and immunostaining of the antiproteases elafin and SLPI were enhanced strongly in inflamed versus noninflamed UC
Frol'ová *et al*[124]	Galectin-3	UC/CD	Disease activity	Serum concentrations were significantly increased in specimen of patients with active and remission-stage ulcerative colitis and Crohn's disease (relative to healthy controls)
Yu *et al*[125]	Galectin-1, -3	UC/CD	IBD diagnosis	Serum level of galectin-1 and -3, but not galectins-2, -4, -7 and -8, were significantly higher in IBD patients than in healthy people. None of the galectins however were able to distinguish active disease from remission in UC or CD
Tibble *et al*[97]	Calprotectin	CD	IBD diagnosis	The cross-sectional study showed a sensitivity of 96% for calprotectin in discriminating between normal subjects and those with Crohn's disease. With a cutoff point of 30 mg/L fecal calprotectin has 100% sensitivity and 97% specificity in discriminating between active CD and irritable bowel syndrome
Moniuszko *et al*[100]	Calprotectin	UC/CD	Disease activity, progression	Rapid bedside FC test reliably detected disease flares in patients with both UC and CD. FC levels increased even with early signs of inflammations; values were lower in isolated small bowel disease for CD patients
Pous-Serrano *et al*[101]	Calprotectin	CD	Disease activity	FC was the only inflammatory marker significantly associated with the degree of histologic inflammation in surgical specimens
Scheopfer *et al*[102]	Calprotectin	CD	Disease activity	FC correlates more closely with endoscopic disease activity that CRP, blood leukocytes, and CDAI. It was the only marker that reliably discriminated inactive from mild, moderate, and highly active disease, underscoring its value in disease monitoring
Ferreiro-Iglesias *et al*[103]	Calprotectin	UC/CD	Relapse	In IBD patients under Infliximab maintenance therapy, high FC levels allow predicting relapse within the following 2 mo. Long-term remission is associated with low calprotectin levels
Klingberg *et al*[104]	Calprotectin	CD	IBD diagnosis, treatment monitoring	FC was a useful predictor of the development of CD in patients with ankylosing spondylitis; NSAIDs increase FC levels; FC levels drop following TNF-blocker treatments
Godny *et al*[109]	Calprotectin	CD	Treatment monitoring	FC decreases following successful diet-based treatment of active CD
Karaskova *et al*[126]	Hepcidin	UC/CD	IBD diagnosis	Serum hepcidin concentration was significantly decreased in IBD children compared with controls; levels did not differ significantly between patients with CD and UC
Martinelli *et al*[128]	Hepcidin	UC/CD	IBD diagnosis, iron deficiency Monitoring	Serum hepcidin was significantly higher in IBD patients with active disease versus healthy and celiac patients. Hepcidin levels corresponded with iron malabsorption and other inflammatory biomarkers like ESR
Aksan *et al*[129]	Hepcidin	UC/CD	Response to iron supplementation	Higher hepcidin and other inflammatory markers correlated with decreased iron absorption follow supplementation
Zollner *et al*[127]	Lipocalin	CD	Clinical and endoscopic activity	Fecal lipocalin-2 levels of 78.4 and 0.56 μg/g in Crohn's disease patients for clinical and endoscopic activity, respectively, corresponded well with fecal calprotectin levels in UC patients (R = 0.87, $P < 0.001$)

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

calprotectin to differentiate active disease *vs* remission in patients with IBD have been previously evaluated[107]: A cutoff value of 50 mg/g had a pooled sensitivity of 0.92 and specificity of 0.60 (0.52–0.67), a cutoff value of 100 mg/g had a pooled sensitivity of 0.84 and specificity of 0.66, a cutoff value of 250 mg/g had a pooled sensitivity of 0.80 (0.76–0.84) and specificity of 0.82 (0.77–0.86). Decreased levels of FC after therapy are associated with clinical, endoscopic and histological improvement with a normal-

ization of FC (< 50 mg/g) signifying deeper remission[108].

Notably, FC has been found to correlate more strongly with IBD activity than other markers of inflammation, including C-reactive protein and blood leukocytes[104,105]. FC elevations are more pronounced in patients with pan-colonic CD than in those with isolated small bowel disease, indicating that concentrations may reflect disease location[105]. Rapid bedside and at-home FC assays are currently available as tools for monitoring IBD activity, with elevated concentrations detectable early in disease flares [104,109]. FC can be used to predict the risk of relapse for patients with quiescent CD and UC[105]. FC monitoring also plays a role in the treatment of IBD, as levels decrease following effective medical and diet-based management of disease[107,110].

Despite its clear clinical utility, FC remains an imperfect biomarker for the diagnosis and monitoring of IBD. Like many other inflammatory biomarkers, FC is not 100% specific for IBD. Other factors, including the use of NSAIDs, can also result in elevated FC, thereby introducing potential inaccuracy when using the biomarker to evaluate IBD[104,105].

Defensins

Previous studies have revealed increased defensin concentration at the intestinal surface epithelium in the setting of IBD, and dysregulation of defensin gene expression has been proposed as one pathogenic mechanism of disease[110,111]. Thus, defensins have been explored as potential biomarkers of IBD[112,113]. Among the 10 known human defensins, the alpha defensins HNP-1, HNP-2, and HNP-3 have been found to be significantly elevated in the sera of both UC and CD patients[114,115]. In CD, serum HNP-1-3 Levels have been shown to correlate with disease severity, as measured by Crohn's disease Activity Index (CDAI)[114]. In UC, these levels are significantly greater in active disease than in inactive disease, and serum HNP-1-3 Levels decrease following successful treatment with corticosteroids[113]. Notably, serum HNP-1-3 Levels do not decrease following corticosteroid administration in non-responders, signifying the potential use of defensins in the monitoring of treatment efficacy[114]. Fecal HNP-1-3 Levels are also significantly elevated in both CD and UC as well, with greater elevations measured during UC flares than in remission[113]. In the same study, fecal HNP-1-3 Levels correlated more closely with endoscopic severity than calprotectin. Results involving the ability to differentiate between UC and CD using defensin levels remain mixed[110-113].

Cathelicidin

Significantly elevated levels of serum LL-37 have been detected in both adult and pediatric IBD cohorts[115,116]. Multiple studies have indicated that cathelicidin can be used to reliably differentiate both CD and UC from healthy controls, reflecting the AMP's potential diagnostic utility[115,116]. While cathelicidin levels are increased in both active and remission-stage IBD patients relative to controls, these levels seem to inversely correlate with disease activity, histologic inflammation, and risk of clinical relapse[21,116,117]. In moderate to severe IBD, higher serum cathelicidin prior to treatment is associated with better prognosis and may therefore serve as a predictor of treatment response[21]. Cathelicidin may also be a useful indicator of complication risk, as reduced serum levels correlate with significantly increased risk of intestinal stricture in CD[117]. Serum levels positively correlate with 25(OH)D levels, and the apparent protective effect of elevated cathelicidin is likely at least partially dependent on this increase in vitamin D[21].

Lactoferrin

Lactoferrin is among the most thoroughly explored AMPs in the diagnosis and clinical evaluation of IBD. Fecal concentrations of lactoferrin are consistently elevated among both children and adults with IBD relative to healthy controls[118-123]. While estimates of fecal lactoferrin sensitivity in identifying CD and UC vary, several studies have confirmed the AMP's utility as a highly specific marker of IBD-related inflammation[120-122]. This specificity makes lactoferrin a particularly valuable biomarker for differentiating IBD from IBS, with studies indicating that lactoferrin levels can discriminate between the two conditions with a specificity at or near 100%[119-121]. Lactoferrin levels positively correlate with disease activity, with significantly higher fecal concentrations found in those with moderate to severe IBD relative to those with mild or inactive disease[122]. Unlike some of the other AMPs, lactoferrin has not been shown to predict responsiveness to corticosteroid treatment, and only insignificant concentration changes have been detected following both effective and ineffective treatment regimens[123].

Galectin

Many members of the galectin family of proteins have been studied as potential biomarkers of IBD. Though several galectins are known to be expressed by intestinal epithelial cells, only galectin-1 and -3 have been shown to be significantly elevated in the serum of IBD patients[124,125]. Unlike those of galectin-1 and -3, serum levels of galectins-2, -4, -7, and -8 have not been shown to differentiate IBD patients from healthy controls[125]. Of note, galectin-1 and -3 Levels cannot reliably distinguish active from remission-stage CD or UC, nor can they distinguish CD and UC from each other[125,126]. Evidence also suggests that galectin-1 is a slightly more sensitive marker of IBD than galectin-3[125]. Nevertheless, galectins-1 and -3 may have use as biomarkers either alone or when combined with other molecules, and their upregulation in the intestinal cells of IBD patients may indicate their potential as therapeutic targets[124,125].

Hepcidin

Data regarding the utility of hepcidin as a diagnostic biomarker remain mixed[126-129]. However, given hepcidin's crucial role in regulating iron absorption, the AMP may be useful in the monitoring of iron deficiency and related anemia, which are two common comorbidities seen in IBD patients[126,127]. These comorbidities are most frequently seen in pediatric IBD patients[126,127]. Consequently, multiple studies have aimed to elucidate the relationship between hepcidin expression and these comorbidities in pediatric IBD cohorts. In pediatric patients with IBD, elevated hepcidin levels negatively correlate with iron absorption and serum iron levels[125,126]. Elevated hepcidin corresponds with decreased response to iron supplementation in these patients, suggesting that the biomarker may serve a role in predicting response to oral iron supplementation in the setting of IBD[129].

Elafin

Elafin is known to be markedly upregulated in the intestinal mucosa of UC patients [130,131]. Intestinal expression seems to correlate closely with disease progression, as elevated concentrations are detectable in the right colon of patients with pan-colonic disease, but not those with exclusively left-sided disease[130]. This finding is further supported by enhanced colonic mRNA immunostaining in inflamed relative to non-inflamed UC samples[131]. While serum elafin levels are increased in UC patients relative to healthy controls, some evidence suggests an inverse correlation between serum elafin and disease severity within UC cohorts[131,132]. Among UC patients, significantly elevated serum elafin tends to correlate with decreased disease activity scores, with the highest elafin levels measured during disease remission[133,134]. Data involving elafin as a biomarker in CD remain mixed, with most results indicating only weak correlations between elafin and CD activity[132-134]. However, serum elafin measurements may play a role in the evaluation of complication risk in CD, as elevations are significantly associated with increased risk of intestinal stricture[132].

CONCLUSION

AMPs produced by innate immune cells of the GI tract and cells that support barrier function such intestinal epithelial cells and Paneth cells play critical roles in protecting against enteric pathogens while maintaining tolerance to support a complex ecosystem of commensal gut microbiota. These highly dynamic molecules have broad spectrum antimicrobial activity against bacteria, fungi, and enveloped viruses and mediate their protective effects through diverse mechanisms of action from disrupting cell membranes, binding microbial components such as LPS, and sequestering metal co-factors to limit microbial growth. AMPs also play major roles in regulating gut microbiome composition and spatial relationships between the microbiota and intestinal barrier.

Defects in endogenous AMP expression and function have been linked with intestinal inflammation in mice. Conversely, exogenous delivery of AMPs such as defensins, cathelicidin, and elafin have been shown to attenuate intestinal inflammation in murine models of IBD. AMPs such as calprotectin and lactoferrin have found clinical applications as biomarkers of intestinal inflammation in patients with IBD. Other AMPs including alpha- and beta-defensins, cathelicidin, and elafin may be useful biomarkers for disease activity and predicting clinical outcomes in patients with IBD. Although the protective effects of AMPs have been demonstrated in murine models of IBD, there are currently no AMP-based therapies approved or in clinical

trials for IBD. Future studies should focus on translation of AMPs as potential therapies in patients with IBD. Several challenges with AMPs including limited stability due to enzymatic degradation by endogenous proteases[135,136] and cross-reactivity of AMPs with host cells leading to cytotoxicity[137] pose major barriers to their application as therapies. Biochemical modifications to enhance AMP stability, selectivity, and delivery are being explored[46,137].

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FRONTIER

Idiopathic chronic pancreatitis: Beyond antioxidants

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Abstract

Chronic pancreatitis (CP) is a complex disease associated with gene-gene or gene-environment interactions. The incidence of idiopathic CP has shown an increasing trend, withits phenotypeshaving changed considerably in the last two decades. The diseaseitself can be regulated before it reaches the stage of established CP; however, the etiopathogenesis underlying idiopathic CP remains to be established, making the condition difficult to cure. Unfortunately, there also remains a lack of consensus regarding the beneficial effects of antioxidant therapiesfor CP. It is known that antioxidant therapy does not reduce inflammatory and fibrotic cytokines, making it unlikely that they could modulate the disease process. Although antioxidants are safe, very few studies to date have reported the long-term beneficial effects in patients with CP. Thus, studies are being performed to identify drugs that can improve symptoms and alter the natural history of CP. Statins, with their numerous pleiotropic effects, may play a role in the treatment of CP, butin 2006, their use was found to be associated with the undesirable side effect of promoting pancreatitis. Latter studies showed favourable effects of statins in CP, highlighting the particular benefits of lipophilic statins, such as lovastatin and simvastatin, over the hydrophilic statins, such as rosuvastatin. Ultimately, studies to repurpose N-acetylcysteine as a CP therapy areyielding very promising results.

Key Words: Chronic pancreatitis; Simvastatin; Antioxidants; Quality of life; N-acetylcysteine; Acute pancreatitis

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Core Tip: The clinical management of a majority of chronic diseases has seen a paradigm shift over the last two decades. To date, however, a well-defined standard of care has not been established for patients with chronic pancreatitis (CP). Lack of sufficient scientific evidence regarding the use of antioxidant supplementation, in particular, provides opportunities to repurpose drugs and study their efficacy and safety in clinical trials. Statins and N-acetylcysteine represent two of the most promising molecules for the treatment of CP, today.

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INTRODUCTION

Biography

Rajiv M Mehta, MD, is Professor and Head of the Department of Gastroenterology and Clinical Research at Surat Institute of Digestive Sciences (SIDS) Hospital and Research Centre in Surat, India. He received his undergraduate and postgraduate degrees from Baroda University in Vadodara, India, in 1996 and 2000, respectively. He received his advanced degree in gastroenterology from Amrita Institute of Medical Sciences (AIMS) in Kochi, India. Only 5 years later, in 2005, he was awarded the prestigious presidential gold medal from the National Board of Examination, New Delhi, in Gastroenterology. In his continued efforts to advance the overall field of gastroenterology, Dr. Mehta authored the very popular "Clinical Gastroenterology" book for undergraduate and post-graduate students, withits fourth edition published in December 2019 at the Asia Pacific Digestive Disease Week held in Kolkata, India. He has also published more than 50 articles in various prestigious journals, to date. Dr. Mehta has also been collaborating with Dr. Stephan Pandol and his team from Cedars-Sinai Medical Center in Los Angeles, CA, United States over the past 3 years, focusing on chronic pancreatitis (CP). Collectively, this group isinvolved in the development of novel biomarkers for the diagnosis of "early CP". Additionally, Dr. Mehta has investigated the effects of simvastatin on the histology of L-arginine-induced pancreatitis in mouse models, in association with the Jay Research Foundation (JRF) in Vapi, Gujarat, India. Defining the overall role of simvastatin and N-acetylcysteine (NAC) in CPtreatment remains Dr. Mehta's seminal work, while defining the role of genetic polymorphisms in patients with idiopathic CP is his focused area of interest. Further, he is diligently working towards the development of "New Chemical Entity" in pancreatic cancer.

Background

CP is a fibro-inflammatory disorder of the exocrine pancreas occurring in individuals with genetic, environmental and other risk factors who develop persistent pathological responses to parenchymal injury or stress[1]. CP is characterized by acinar cell damage, ductal dysfunction, persistent inflammation, atrophy, fibrosis, and neuroimmune responses. The clinical course of CP involves significant abdominal pain, exocrine function deficiency (manifested as maldigestion), and endocrine deficiency (manifested as diabetes). The causes of CP include alcohol intake, smoking, metabolic derangements, genetic disorders, autoimmune factors, obstructive mechanisms, and idiopathic aetiologies[2]. Unfortunately, it is difficult to determine the exact prevalence of CP, since the early diagnosis of CP continues to be challenging.

Alcoholic chronic pancreatitis (ACP) is most commonly observed in Western countries, whereas idiopathic chronic pancreatitis (ICP) is observed more frequently in developing countries, like India; reportedly, ICP accounts for 57.3%–69.6% of the cases of CP in India[3]. The cause of ICP remains unknown[4]. In genetically susceptible individuals, environmental factors initiate the fibroinflammatory process (gene-gene or gene-environment interplay), which leads to the development of CP[5]. The subtle pathophysiological changes pose challenges to the early diagnosis of CP, and the parameters for early diagnosis are ill-defined. Late-stage CP is characterized by variable fibrosis and calcification in the pancreatic gland, leading to parenchymal and

ductal changes[6].

Abdominal pain is a well-recognized and debilitating symptom, prompting patients with CP to seek medical assistance. Among all the complications of CP, abdominal pain was found to be strongest predictor of poor quality of life (QoL)[7]. Endoscopic and surgical treatments are often performed to relieve pain, but these methods are invasive and are beneficial only in a specific subgroup of patients with CP. The therapeutic strategies currently used for the management of CP include a combination of analgesics, pancreatic enzymes, adequate nutrition, and antioxidants[8]. However, the effect of antioxidants on providing sustained pain relief or reversing disease activity has not been established, thus far. As such, further studies are warranted to address this unmet need for an alternative therapeutic approach for CP.

PATHOPHYSIOLOGIC BASIS OF ANTIOXIDANT THERAPY

The use of antioxidant therapy is based on observations that CP tissues show marked oxidative changes.

Oxidative stress

Oxidative stress is caused by an imbalance between production and accumulation of reactive oxygen species (ROS) in cells and tissues and the ability of a biological system to detoxify these reactive products[9]. Xenobiotics, such as alcohol and smoking molecules, are detoxified in the body through phase I and phase II pathways. The phase I reaction involves cleaving the parent molecule by enzymes into a less toxic molecule. The phase II reaction adds an endogenous molecule to the compound at the end of phase I to make it more polar and excretable. Increased exposure to xenobiotics may overwhelm the capacity of phase I and phase II detoxification pathways and result in oxidative stress. Production of ROS is a particularly destructive aspect of oxidative stress. ROS are mainly produced by mitochondria, during both physiological and pathological conditions[10]. When the production of ROS increases, they exert harmful effects on important cellular structures, like proteins, lipids and nucleic acids, leading to destruction of the cell membrane, depletion of cellular antioxidants, and alteration in various signalling pathways. Previous studies have indicated that oxidative stress is involved, to a varying extent, in the onset and/or progression of several diseases[9].

Oxidative stress in CP

Pancreatic acinar cells are the main site for generation of oxidant stress and, therefore, are exposed to its detrimental effects. Intra-acinar oxidative stress leads to impairment of the transsulfuration pathway, which is required for zymogen exocytosis[11,12]. This leads to recurrent intra-acinar zymogen activation. Methionine and ascorbic acid appear to be important components in maintaining the transsulfuration pathway[13]. Several studies conducted in the 1990s showed that deficiency of essential antioxidants, such as vitamin A, ascorbic acid, methionine, vitamin E and selenium, is particularly prevalent among patients with ACP. Persistent exposure to xenobiotics *via* smoking and consumption of alcohol increase the levels of oxidative stress. Thus, the role of oxidative stress and micronutrient deficiency in patients with CP exposed to high levels of xenobiotics has been established. "Tropical pancreatitis" has been typically associated with protein and micronutrient deficiencies; however, the role of malnutrition in the etiopathogenesis of CP has been discarded[14].

Over the past one and a half decades, results of several studies conducted in India have shown that the phenotype of patients with ICP has changed significantly. A complex gene-environment interplay is now known to be involved in the development of ICP[5]. Our experience has revealed that the levels of antioxidant micronutrients are normal in patients with ICP who carry normal genetic polymorphisms (in press as of the writing of this paper).

ANTIOXIDANT SUPPLEMENTATION IN CP: THE STORY, SO FAR

In 1990, Uden *et al*[15] performed a double-blind, placebo controlled, crossover trial in 20 patients to determine the efficacy of the combination of 600 mcg of organic selenium, 9000 IU of b carotene, 0.54 g of vitamin C, 270 IU of vitamin E and 2 g of methionine. Results of this trial indicated that antioxidants were superior to placebo in

relieving pain. Several non-randomized studies in small patient populations have also shown the benefits of antioxidant treatments in patients with CP. In a study by Bharadwaj *et al*[16], 147 patients were randomized to an antioxidant therapy or placebo group, and the results indicated a beneficial effect of antioxidant therapy in reducing "painful days" and in improving the markers of oxidative stress. Although the study by Bharadwaj *et al*[16] was conducted in a large and heterogenous patient population (including patients with ACP as well as those with ICP), validated pain scores were not used and formal analysis of QoL was not performed. Results of the ANTICIPATE study showed that administration of antioxidants to patients with painful ACP does not reduce pain or improve QoL, despite a sustained increase in blood levels of antioxidants[17]. The results of a randomized controlled trial by Singh *et al*[18] showed that antioxidant supplementation increased the blood antioxidant levels but produced no additional benefit on endocrine and exocrine functions, markers of fibrosis, inflammation, nutritional status, pain or QoL.

Gooshe *et al*[19] performed a meta-analysis to determine the efficacy and adverse effects of antioxidant therapy in patients with acute pancreatitis (AP), CP and post-endoscopic retrograde cholangiopancreatography pancreatitis (commonly known as PEP). This meta-analysis provided evidence to support the efficacy of antioxidant therapy only in AP, whereas its effects in CP and PEP were less clear. The meta-analysis by Rustagi *et al*[20] demonstrated a benefit of antioxidants; however, the investigators did not control for a heterogenous study population and the use of different types of antioxidants among such. The most recent Cochrane Systematic Review of 18 studies concluded that antioxidants could result in a slight reduction in pain in patients with CP but there were no conclusive data reported for analgesic requirement and QoL[21]. Rupjoyti *et al*[22] showed that treatment with a methionine-containing antioxidant was associated with a significant increase in the number of pain-free patients and a trend towards decreased requirement for hospital visits or admissions. Thus, methionine may help to restore the transsulfuration pathway and decrease intrapancreatic inflammation. Rupjoyti *et al*[22] performed a meta-analysis based upon data from January 1980 to August 2014, encompassing eight studies (six randomized controlled trials and two non-randomized trials). Although the overall results supported the efficacy of methionine supplementation, when the two non-randomized studies (by Shah *et al*[23] and Castasno *et al*[24]) were excluded, the antioxidant combination was no longer statistically significant for decreasing the pain score.

A recent meta-analysis by Mohta *et al*[25] showed negative results for antioxidants' ability to reduce pain and improve QoL in patients with CP. These findings are important because all studies included in the meta-analysis had been performed using a similar type of antioxidant and were based on a combination of commercially-available antioxidants and those used in clinical practice; therefore, the findings of that meta-analysis are more relevant to clinical practice. However, the meta-analysis itself had some limitations. First, there was variation in the method of reporting of pain among the studies included and, as such, the analysis had to be performed with two different parameters, namely the visual analogue scale (VAS) score and pain-free participants. This resulted in a decrease in the number of patients that could be simultaneously included in the analysis. Second, limited information was available regarding the QoL.

Although the micronutrient antioxidant therapy was proposed for relieving the pain associated with CP more than three decades ago, this treatment has been used sporadically; moreover, the optimal formulation and duration of the antioxidant regimen has not been completely elucidated. Further, a majority of the clinical trials were not well-designed and did not include a homogenous study population. In view of the fluctuating nature of this disease, a well-defined method to determine the pain scores and measure the QoL was not developed and validated. Differences in the assessment and reporting of pain (*i.e.*, VAS score, numeric rating score, brief pain inventory scores, vocabulary score sheet, and "painful days") in various clinical studies make study comparisons and meta-analysis difficult. These featurescan be attributable to the inconsistent results obtained in the various meta-analysis[20-22,25,26].

The lack of alternative therapies for patients with CP warrants an urgent need for a well-designed study to evaluate the effect of antioxidant therapy in a clearly defined patient population.

STATINS: THE ROAD AHEAD

Antioxidant therapy has inconsistent efficacy in patients with CP. Therefore, a reliable, effective, safe and predictable agent represents an unmet (but required) need for the treatment of CP. A statin appears to be the most appropriate candidate.

Non-lipid-lowering effects of statins

Several primary and secondary prevention trials have shown the clinical benefits of statins in coronary artery disease[27]. An increasing number of studies indicate that statins have many non-lipid-lowering effects, known as pleotropic effects[28,29]. Some of the effects include anti-inflammatory actions and improvement of endothelial function by prevention of lipid peroxidation. Statins exert antioxidant effects by increasing the bioavailability of nitric oxide[30], decreasing the production of ROS[31] and inhibiting the distinct oxidation pathways[32]. Antiproliferative and immunomodulatory properties of statins suggest novel applications of statins in various diseases apart from dyslipidaemia[33].

Statins and pancreatitis

The relationship between statins and AP is controversial, considering simvastatin-induced AP was found in a previous study[34]. Statins are among the most widely prescribed medications worldwide for cardiovascular diseases. Thus, it is important to understand the relationship between statins and pancreatitis. A population-based study from Denmark found no association between the risk of AP and use of statins [35]. In contrast, a recent meta-analysis of 21 high-quality randomized controlled trials showed an overall decrease in the risk of pancreatitis among patients treated with statins compared with those treated with placebo[36]. However, since pancreatitis was not the primary outcome of the 21 trials included in that meta-analysis, these results may not be a true indicator of the protective effects. The protective effects of simvastatin and ezetimibe were shown in the Study of Heart and Renal Protection (SHARP) study[37]. The result of a recent study by Wu *et al*[38] also indicated that simvastatin and atorvastatin were associated with an overall decrease in the risk of AP. Moreover, subgroup analysis in the same study showed a decrease in the risk of pancreatitis in patients with chronic alcohol abuse, suggesting the possible role of simvastatin in preventing recurrent AP and subsequent progression to CP.

Statins and experimental studies

The effects of simvastatin pre-treatment on 10 Wistar rats was published by a group from Brazil in 2008[39]. They reported no beneficial effects on pancreatic inflammation but a trend towards improved survival rate in the simvastatin group. Of interest, the lovastatin treatment was found to successfully inhibit *invitro* activation of pancreatic stellate cells[40]. This is an important observation, as activated stellate cells mediate the fibroinflammatory response of CP. In a similar study of L-arginine-induced pancreatitis in rats, performed by Metalka *et al*[41], levels of malondialdehyde (commonly known as MDA) were significantly reduced in the pancreas tissues of the simvastatin treatment group.

Another considerable pathologic process that occurs in pancreatitis is impaired autophagy[42-44]. Autophagy is the process of removal of damaged cellular compounds, including dysfunctional mitochondria (mitophagy) during stress conditions[45]. Because dysfunctional mitochondria are a significant source of ROS generation, as indicated above, their dysfunction can be an important source of cellular stress that can promote pancreatitis. During autophagy, organelles such as mitochondria and cytoplasmic materials are sequestered by the autophagosomes (a double–membrane structure) and transported to the lysosome for digestion[46]. Autophagosome-lysosome fusion is impaired in pancreatitis, resulting in incomplete autophagy andmitophagy[47]. Piplani *et al*[48] showed that simvastatin restores autophagy and mitophagy, resulting in improvement in pancreatitis pathology in the cerulein-induced model of experimental AP.

Statins and pancreatic cancer

Autophagosome accumulation during pancreatitis can promote pancreatic cancer[49]. CP is the strongest identified risk factor for pancreatic cancer[50]. Intriguingly, the results of a study involving 250 patients with pancreatic cancer showed an improved survival among those patients with diabetes who were subject to statin treatment[51]. Based on these results, Jeon *et al*[52] retrospectively analysed a cohort of elderly patients with pancreatic cancer using the Surveillance, Epidemiology and End Results

(commonly known as SEER) database of patients in the United States. They studied the use of statins in patients after diagnosis of pancreatic ductal adenocarcinoma. Simvastatin was the most prescribed statin. The patients who used statins were found to live longer after their cancer diagnosis (median overall survival of 4.7 mo; interquartile range of 1.9-11.7 mo) as opposed to those who were not prescribed statins (median overall survival of 2.4 mo; interquartile range of 1.5-7.3 mo). There was a favourable impact of statin use on survival in those who had undergone pancreatectomy *vs* those who had undergone no surgery (HR = 0.80, 95%CI: 0.66, 0.97). Of interest, simvastatin treatment was associated with significantly lower hazard of death compared to no statin treatment (HR = 0.91, 95%CI: 0.84, 0.99). The results of that study were confirmed by another, showing the beneficial effect of statins in pancreatic cancer patients in a large health care system in Southern California, United States[53].

The mechanism of action of statins in pancreatic cancer remains poorly understood. Statins are known to decrease the expression of inflammatory cytokines and to modulate the expression of several genes involved in angiogenesis and inflammation, which may protect against carcinogenesis. Statins also inhibit protein prenylation. This prevents the proper functioning of guanosine triphosphatase proteins, such as Ras and Rho, thereby inhibiting downstream pathways that are involved in cell growth, proliferation, survival, motility and invasion, which leads to cell cycle arrest in G1. Furthermore, statins impair cancer cell proliferation by inhibiting the synthesis of cholesterol, which is essential for new membrane formation in rapidly proliferating cells[54].

OUR EXPERIENCE IN CP AND SIMVASTATIN

On the basis of the experimental and limited retrospective/population-based data, we performed two prospective studies to assess the role of simvastatin in patients with ICP. In the first prospective study, patients were assigned to receive either the standard antioxidant preparation (a tablet containing 3000 IU β carotene, 550 mg methionine, 200 μg selenium, 40 mg vitamin C, and 10 mg vitamin E administered thrice daily) or the combination of the standard antioxidant preparation and simvastatin (40 mg/d). Improvement in pain was assessed using the VAS. At the end of 12 mo, the decrease in the VAS score was significantly greaterin the simvastatin group (P = 0.032)[55]. In the other pilot study (under publication), health-related QoL was assessed using the European Organization for Research and Treatment of Cancer (referred to as EORTC) QLQ PAN 28 and QLQ C30 scoring. The results of the study showed that at 6 mo patients who received a combination of simvastatin and antioxidants showed a significant improvement in the pancreatic pain score compared to those who received antioxidants alone (P= 0.01) (Figure 1). Patients who received simvastatin and antioxidants also consumed fewer analgesics (P = 0.03) (Figure 2) and required less hospitalization (P = 0.04) than those who received antioxidants alone. Our findings indicate that, compared to antioxidants alone, simvastatin in combination with antioxidants significantly improves the overall QoL (Figure 3) (P = 0.01), which is consistent with the findings reported in several preclinical studies. Thus, simvastatin may emerge to modulate the disease process in CP.

Considering the complexity of CP, we have also identified NAC as another molecule that can be repurposed for CP. NAC exerts antioxidant and antifibrotic effects *via* inhibition of tissue growth factor signalling in fibrogenic cells[56]. In the environment of inflamed pancreatic tissue, activation of pancreatic stellate cells produces abundant extracellular matrix proteins, leading to fibrosis as well as inflammatory cytokines. Beneficial roles of the combination of simvastatin and NAC on the pathophysiology of inflammation and necrosis of the acinar cell involve inhibition of pancreatic stellate cells. Thus, we designed a prospective, randomized, open-labelled clinical trial entitled "The safety and efficacy of simvastatin plus standard of care or simvastatin plus NAC plus standard of care *vs* only standard of care in patients with idiopathic acute recurrent pancreatitis and chronic pancreatitis" (*i.e.*, the SNAPstudy). This study aims to assess not only the QoL but also various biomarkers for inflammation in CP.

CONCLUSION

There is a scarcity of scientific evidence to substantiate the use of antioxidants in the treatment of CP. Simvastatin and NAC appear to be promising candidates for the treatment of CP.

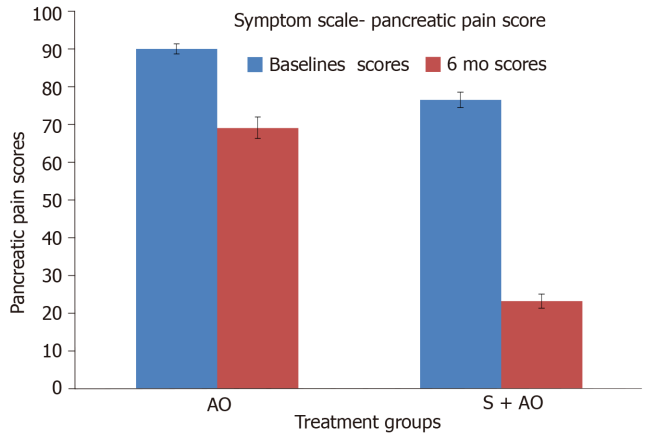

Figure 1 Reduction in pain score observed in both treatment groups. AO: Antioxidants; S: Simvastatin.

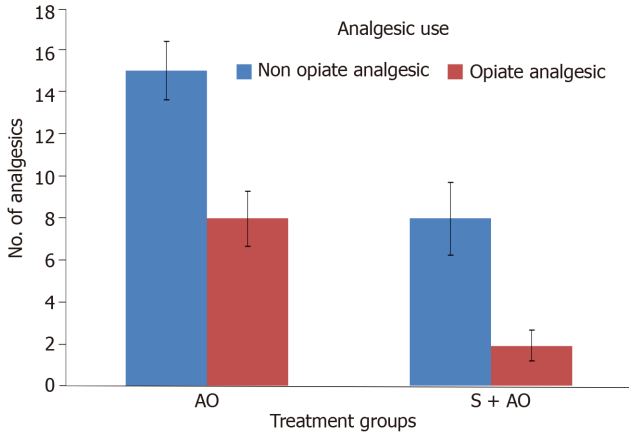

Figure 2 Reduction in analgesic requirement observed for both treatment groups. AO: Antioxidants; S: Simvastatin.

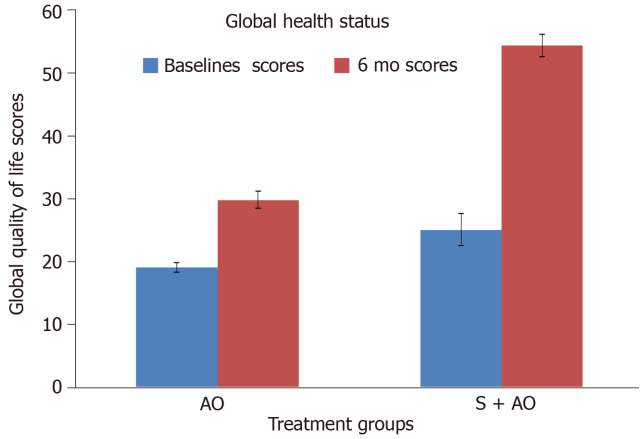

Figure 3 Global health status observed in both treatment groups. AO: Antioxidants; S: Simvastatin.

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OPINION REVIEW

COVID-19 as a trigger of irritable bowel syndrome: A review of potential mechanisms

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Abstract

In December 2019 a novel coronavirus disease 2019 (COVID-19), caused by the severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2), started spreading from Wuhan city of Chinese Hubei province and rapidly became a global pandemic. Clinical symptoms of the disease range from paucisymptomatic disease to a much more severe disease. Typical symptoms of the initial phase include fever and cough, with possible progression to acute respiratory distress syndrome. Gastrointestinal manifestations such as diarrhoea, vomiting and abdominal pain are reported in a considerable number of affected individuals and may be due to the SARS-CoV-2 tropism for the peptidase angiotensin receptor 2. The intestinal homeostasis and microenvironment appear to play a major role in the pathogenesis of COVID-19 and in the enhancement of the systemic inflammatory responses. Long-term consequences of COVID-19 include respiratory disturbances and other disabling manifestations, such as fatigue and psychological impairment. To date, there is a paucity of data on the gastrointestinal sequelae of SARS-CoV-2 infection. Since COVID-19 can directly or indirectly affect the gut physiology in different ways, it is plausible that functional bowel diseases may occur after the recovery because of potential pathophysiological alterations (dysbiosis, disruption of the intestinal barrier, mucosal microinflammation, post-infectious states, immune dysregulation and psychological stress). In

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this review we speculate that COVID-19 can trigger irritable bowel syndrome and we discuss the potential mechanisms.

Key Words: SARS-CoV-2; COVID-19; Irritable bowel syndrome; Microbiota; Dysbiosis; Gut-brain axis

Core Tip: Coronavirus disease 2019 (COVID-19) is not only a respiratory tract illness, as it may involve other systems, including the gastrointestinal tract. Persistent symptoms after the resolution of the infection are described, but there is almost no mention on the possible consequences on bowel function. However, some aspects concerning COVID-19, its management, and psychological aspects, may contribute to trigger disorders of the gut-brain interaction, among which the irritable bowel syndrome is the most frequent.

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INTRODUCTION

In December 2019 a cluster of acute atypical respiratory infections were reported in the Wuhan city of Hubei province by the Chinese authorities to the World Health Organization (WHO). The responsible pathogen was identified as a new member of the family Coronaviridae, and it was called severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) due to its similarity to the SARS coronavirus, previously involved in the 2002-2003 pandemic. The SARS-CoV-2-related disease was named coronavirus disease 2019 (COVID-19) and rapidly spread worldwide. Indeed, COVID-19 became a public health emergency on January 30, 2020 and, subsequently, a pandemic state was declared on March 11, 2020 by the WHO[1].

SARS-CoV-2 is a positive-sense single-stranded RNA virus, whose genome encodes for four major structural proteins: Spike (S) protein, envelope protein, membrane protein and nucleocapsid protein. The S protein mediates the entering of SARS-CoV-2 in the host cells by binding to the peptidase angiotensin receptor 2 (ACE2)[2].

COVID-19 is a contagious and highly lethal illness, especially for individuals with chronic comorbidities (such as diabetes mellitus, hypertension, cardiorespiratory disorders, chronic hepatic and renal diseases), elderly, oncological and immunosuppressed patients[2]. The infection is predominately transmitted by person to person through respiratory droplets, although many other modes of potential transmission have been postulated, which include through faecal-oral transmission. The average incubation period for COVID-19 is 5.2 d, but it can last up to 15.5 d.

The infection can have an asymptomatic course or it can present with fever, malaise and dry cough in the initial phase, during the invasion and infection of the upper respiratory tract. Patients may also experience gastrointestinal symptoms such as abdominal pain, vomiting and diarrhoea, and signs of systemic involvement (mainly neurological, cardiological, renal, and hepatological manifestations). Subsequently, the disease can involve the lower respiratory tract in approximately 20% of the cases and, in most severe situations, it can culminate in acute respiratory distress syndrome. This condition is characterized by a surge in circulatory inflammatory cytokines [mainly interleukin (IL)-1, IL-6, IL-8, tumor necrosis factor (TNF)-α], termed 'cytokine storm', which is responsible for the subsequent inflammation and lung injury[1,3].

Treatments for COVID-19 change according to the disease severity: They include symptomatic and supportive therapy (such as oxygen supplementation, fluid resuscitation and vasopressors in case of septic shock), broad-spectrum antibiotics for prevention/management of secondary bacterial infections or sepsis, steroids if respiratory failure occurs, and prophylactic low molecular weight heparin in patients

with moderate to severe disease because of the high risk of thromboembolism. The efficacy of antivirals (predominantly remdesivir and lopinavir/ritonavir combination), immunomodulatory drugs (including tocilizumab, chloroquine and hydroxychloroquine) and other treatments in reducing mortality and exacerbation of COVID-19 pneumonia is controversial and needs further evidence. However, these drugs are frequently used in clinical practice in the absence of any alternative[1].

As in other infectious diseases, recovered patients often continue to suffer from various long-term sequelae involving the respiratory system, as dyspnoea and cough, as well as less defined disabling manifestations; the latter include neuropsychiatric sequelae such as fatigue, anxiety, depression, post-traumatic stress disorder and insomnia[3-7]. It is still unknown whether these symptoms derive from the infection itself, from its general management (mainly medical therapies) or from the disease itself through mechanisms that have yet to be determined.

As COVID-19 affects also the gastrointestinal tract, some sequelae may derive from a disequilibrium of the intestinal homeostasis, but current evidence is almost absent[5,8].

In this review we hypothesised that the direct involvement of the gut and the one derived from COVID-19-related circumstantial conditions can predispose to the development of irritable bowel syndrome (IBS). To support this idea, we analysed the mechanism through which SARS-CoV-2 perturbs the intestinal physiology in infected individuals, went through the physiopathology of IBS and finally considered the possible factors that can subsequently trigger IBS after the COVID-19 recovery. For this aim, PubMed and Google Scholar were searched using various combinations of the terms "SARS-CoV-2", "COVID-19", "gastrointestinal", "gut", "symptoms", "irritable bowel syndrome", "microbiota", and "microbiome". Subsequently, we selected the most pertinent articles in support of reasonable common factors between COVID-19 and IBS enhancement and summarised current evidence.

GASTROINTESTINAL INVOLVEMENT OF COVID-19

Gastrointestinal manifestations of COVID-19 can be present with variable incidence (40%-50%), and include mainly diarrhoea, nausea, anorexia, vomiting, abdominal pain and belching. These symptoms may arise even in the absence of respiratory involvement or may appear after the onset of respiratory symptoms[2,9,10]. SARS-CoV-2 is also associated with other gastrointestinal symptoms. One of these include liver injury, which can manifest as increased serum aminotransferases, bilirubin and γ-glutamyl transferase[2,10]. Furthermore, elevated blood levels of amylase and lipase have been described, but a strict causality of pancreatic damage with SARS-CoV-2 infection has not been ascertained. Importantly, the drugs used to treat COVID-19 may also have impact on the gastrointestinal tract[11].

Gastrointestinal involvement in COVID-19 may be due to the capacity of SARS-CoV-2 to directly infect the intestinal tract: This hypothesis is supported by detection of the virus in enterocytes and in stool samples of affected patients, and also in faecal samples of individuals with negative nasopharyngeal tests[12-14]. As previously mentioned, SARS-CoV-2 attaches to the ACE2 to enter into human cells and to infect the host. This receptor exists in two forms: The full-length mACE2, which is located on cell membranes with a transmembrane anchor and an extracellular domain, and the sACE2, a soluble form released into blood circulation. The N-terminal domain of the mACE2 is the target of the S protein of SARS-CoV-2[15]. The S protein consists of two different subunits: The S1, which binds to the cell receptors of the host, and the S2, which mediates the fusion of the viral and cell membranes[16]. Two transmembrane protease serines, TMPRSS2 and TMPRSS4, are essential to cleave the S protein at S1/S2 and S2 sites, to enhance the S fusogenic activity, the entry and replication of the virus in mature small intestinal enterocytes[17,18].

The ACE2 is expressed in several tissues within the human body with specific localization on different cells, including enterocytes, renal tubules, gallbladder, cardiomyocytes, male reproductive cells, placental trophoblasts, ductal cells, eye, vasculature and others[15,19]. Concerning the digestive system, the expression of ACE2 gene is highest in the small intestine, but it is also present among other sites, such as colon, stomach, oesophagus, liver, biliary tract and pancreas[19-22]. Specifically, this receptor is expressed in the muscularis mucosa and mucosa of the intestine, including the epithelial cells, cholangiocytes, hepatocytes, pancreatic ductal, acinar and islet cells, and in the gastrointestinal vasculature[11,23,24]. ACE2 seems to play a key role in the intestinal homeostasis and functions. Indeed, it can regulate the

blood flow perfusion by increasing the vascular resistance (primarily the mesenteric vasculature). Moreover, it is possible that ACE2 is capable of enhancing the mucosal nitric oxide production, which regulates the properties of the epithelial barrier, and of modulating the ion transport and the paracellular permeability. It can also induce duodenal secretory responses of mucosal bicarbonate against the luminal acid from the stomach and stimulate sodium and water absorption. It seems plausible that ACE2 is involved in the relaxation of the gastrointestinal wall musculature. Nonetheless, current evidence suggests that ACE2 is involved in inflammation and immunomodulation, and in the pathophysiology of IBS for contributing to enhance low-grade inflammation in the enteric nerve plexa[24,25]. The ACE2 can also regulate the intestinal amino acid homeostasis and absorption, the production of antimicrobial peptides, the intestinal motility and the gut microbiota independently of the renin-angiotensin system[24,26]. It is also reported that the deficiency of this receptor in a murine model of colitis leads to an increased susceptibility to intestinal inflammation. This effect seems to be mediated by an impaired epithelial immunity and induced dysbiosis, defined as the impairment of the diversity and function of intestinal microbes. This is suggested by the increased propensity to develop severe colitis after the faecal microbiota transplantation of an impaired intestinal microbiota from mice with genetic inactivation of ACE2 into germ-free wild-type animals[26]. Moreover, preclinical evidence indicates that ACE2 can impair the electrophysiological and synaptic functions of the neurons of the enteric nervous system, thus influencing the gastrointestinal motility, sensitivity and the pathways of inflammation[27].

Overall, it is plausible that the impairment of bowel physiology by SARS-CoV-2 may derive from a dysregulation of all these ACE2-mediated functions due to a competitive mechanism of the virus on this receptor or from a downregulation of its anti-inflammatory activity. Moreover, the gastrointestinal manifestations may arise from a direct cytopathic effect of the virus on the mucous epithelium, from a malabsorption secondary to the invasion of enterocytes, or from the triggered inflammatory response with plasma cells and lymphocytes infiltration in the intestinal lamina propria[2,28]. Accordingly, SARS-CoV-2 infection can be associated with microscopic bowel inflammation with infiltrating plasma cells and lymphocytes, and with interstitial edema in the lamina propria, as well as overt acute haemorrhagic colitis with endoscopically confirmed mucosal injury[28,29]. The hypothesis of intestinal inflammation is supported by the detection of significantly increased levels of faecal cytokines, as IL-8, in COVID-19 patients when compared to uninfected controls[30]. Additionally, a significant number of patients (approximately 30%–75%), more frequently those with gastrointestinal manifestations, has elevated values of faecal calprotectin, a protein released by neutrophils of the intestinal mucosa[31]. The occurrence of diarrhoea seems also higher among patients with higher SARS-CoV-2 RNA loads in stool samples[30]. Finally, the presence of virus-specific immunoglobulin A (IgA) in faecal samples suggests that the gastrointestinal tract may be immunologically active during SARS-CoV-2 infection[30].

It is likely that the gut homeostasis and the intestinal immunity play a major role in the pathogenesis of COVID-19 and in the enhancement of the systemic inflammation triggered by the SARS-CoV-2 infection, which is characterized by significantly higher serum IL-6, IL-8, IL-10 and TNF-α in severe cases[30]. Indeed, it is described that higher levels of faecal IL-23 correlate with more severe COVID-19 disease, as well as the finding of intestinal virus-specific IgA responses[30]. Interestingly, gut microbial alterations in COVID-19 patients can contribute to regulate systemic inflammation, as suggested by the correlation between specific changes in genera and inflammation indices[32].

IBS

IBS is the most common chronic disorder of the gut-brain interaction, and it is characterized by mild to severe recurrent abdominal pain and bloating associated to alterations in bowel habits in the absence of organic disease or biochemical abnormalities[33]. IBS is also often accompanied by other comorbidities, like psychiatric conditions, pain syndromes, overactive bladder, migraine, and visceral sensitivity[34]. The debilitating symptoms of IBS impose a significant burden on the quality of life of affected individuals, since it is associated with depression and suicidal ideation, reduces work productivity and increases the accesses to medical care[35,36]. The prevalence of IBS varies substantially between countries due to the different diagnostic criteria and survey methods used in worldwide studies, ranging from less

than 1% to more than 25%, with a predominance in women in comparison to men (12.0% *vs* 8.6% respectively; odds ratio 1.46)[37]. Moreover, it is more frequent in lower socioeconomic groups and individuals younger than 50 years[36]. IBS is diagnosed according to the Rome criteria, a clinical classification which includes four types of IBS according to the predominant bowel habits: IBS with predominant constipation, IBS with predominant diarrhoea (IBS-D), IBS with mixed bowel habits (IBS-M) and unclassified IBS[38]. IBS-M and IBS-D are reported to be the most prevalent subtypes[37]. For an accurate diagnosis of IBS, organic underlying conditions must be excluded, with an accurate patient history, physical examination, laboratory tests and, if necessary, endoscopic assessment. Common conditions which should be ruled out include celiac disease, microscopic colitis, inflammatory bowel disease, bile acid malabsorption, colorectal cancer, and dyssynergic defecation[38,39].

The physiopathology of IBS is currently not fully understood, but it is considered a complex multifactorial disorder with a still unknown molecular pathophysiology. Indeed, it has been hypothesised that an impairment of different functions (such as central and autonomic neurophysiology, visceral nociception, bowel motility, secretory activity and psycho-somatic balance) due to perturbing factors (*i.e.*, stress exposure, psychosocial conditions, food antigens, antibiotics and infections of various origin) leads to physiological abnormalities, which may be involved in the development and perpetuating of IBS. These include intestinal dysbiosis, increased intestinal permeability, immune cell hyper-reactivity with impaired expression and release of mucosal and immune mediators, microinflammation with altered mucosal functions, hyper-sensitivity of the enteric nervous system, dysregulation of the hypothalamus-pituitary-adrenal (HPA) axis and of the enteric nervous system. Increased levels of faecal bile acids and predisposing inheritable susceptibility are recognised as co-occurring factors as well[34,39,40]. Increasing evidence suggests that all these affected pathways are part of the microbiota-gut-brain axis, a bidirectional crosstalk between the brain, the bowel and the gut microbiota which occurs through nervous signalling, immune mediators, microbial products, tryptophan metabolites and other hormones[39,41].

Accordingly, dysbiosis may contribute to IBS by triggering the gut immune system and enhancing low-grade inflammation in susceptible individuals. This hypothesis is supported by a higher prevalence of small intestinal bacterial overgrowth and imbalances of the gut microbiota composition in patients with IBS compared with healthy controls in many recent studies, and by the benefit from the use of non-absorbable antibiotics on related symptoms. A reduction of the diversity and stability of the gut microbiota in patients with IBS has been described[42]. Increased Enterobacteriaceae, which includes several harmful genera (as *Escherichia*, *Shigella*, *Campylobacter*, and *Salmonella*), and Lactobacillaceae families, together with high levels of *Bacteroides* genus, reduced *Faecalibacterium* and *Bifidobacterium* genera and uncultured Clostridiales I are reported in patients with IBS in comparison with controls in a recent systematic review including 24 studies[43].

In 6%-17% of the patients suffering with IBS the onset of the symptoms occurs after a recent episode of gastrointestinal infection, which can increase up to 6-fold the risk of developing IBS. This phenomenon is characterised by the persistence of IBS-like disturbances (mainly diarrhoea and abdominal discomfort) after the resolution of the infection, and it is known as post-infectious IBS (PI-IBS). The prevalence of PI-IBS is approximately 4%-36% in patients with previous infectious gastroenteritis and is higher in females, young people, patients who experienced severe infections and individuals with psychological comorbidities. Moreover, some pathogens seem more predisposing than others; indeed, bacterial infections (particularly by *Campylobacter*, *Shigella*, *Escherichia coli* and *Salmonella*) are more likely to enhance PI-IBS than viruses and other microorganisms[34,44,45]. The pathogenesis of this condition is poorly understood, but it is hypothesized that the responsible pathogenic microorganism may trigger an immunologic and inflammatory response with low-grade inflammation and mucosal injury, which causes the prolongation of IBS symptoms in predisposed individuals. Furthermore, it is described that patients with PI-IBS may have increased macrophages and T lymphocytes in intestinal samples, together with high expression of IL-1 in rectal biopsies and elevated blood level pro-inflammatory cytokines (such as TNF-α, IL-6, IL-8, IL-10 and IL-1β)[44,46]. As in IBS, it is likely that an altered intestinal permeability, an impairment of the gut eubiosis and of the neuromuscular function are involved in PI-IBS as well[47].

COVID-19 AND ITS MANAGEMENT: WHAT ARE THE POSSIBLE TRIGGERS OF IBS?

The plausible mechanisms involved in the development of IBS in individuals who experienced COVID-19 are summarised in Figure 1.

Gastrointestinal disturbances are often associated with respiratory infections or to secondary complications, and the gut-lung axis, a hypothetical bidirectional pathway which works *via* biochemical and immunologic systemic signalling molecules, is possibly involved in the pathophysiology. Among the perturbing factors of the gut microbial environment, respiratory viral infections, including COVID-19, can play a relevant role[48]. As previously mentioned, an impairment of the gut microbiota composition, which is frequently associated to a dysregulation of the overall intestinal homeostasis and gut-brain axis, can participate to the development and maintaining of IBS[39,41,42]. An imbalance of the gut microbiota is described in SARS-CoV-2-infected individuals. Gu *et al*[32] found a significant reduction in mean community richness and bacterial diversity in COVID-19 patients in comparison with healthy controls according to the Shannon diversity index and Chao diversity index. A significantly higher relative abundance of *Streptococcus*, *Rothia*, *Veillonella*, and *Actinomyces*, which are opportunistic pathogens, and a lower relative abundance of beneficial symbionts were reported. Moreover, *Fusicatenibacter*, *Romboutsia*, *Intestinibacter*, *Actinomyces* and *Erysipelatoclostridium* were identified as biomarkers to discriminate the COVID-19 patients from healthy individuals[32]. Zuo *et al*[49] reported that even antibiotics-unexposed patients with COVID-19 have a significantly changed intestinal microbiota during the hospitalization, with enrichment of opportunistic pathogens (including *Clostridium hathewayi*, *Actinomyces viscosus*, and *Bacteroides nordii*) and depletion of beneficial commensals when compared to healthy individuals. Moreover, a correlation between the disease severity and the baseline abundance of certain genera and strains was found, suggesting that the gut microbiota may contribute to the systemic involvement in the immune system responses; specifically, a positive relation was observed with *Coprobacillus*, *Clostridium ramosum*, and *Clostridium hathewayi*, while a negative association was described with *Faecalibacterium prausnitzii*. The loss of beneficial bacteria persisted even after a negative throat swab and the disease resolution, suggesting a persistent deleterious effect on the gut microbiota[49]. The same working group also observed that an active intestinal infection is present in approximately half of COVID-19 patients even without gastrointestinal manifestation, and persisted even after respiratory clearance of SARS-CoV-2. Of interest, stool specimen with a signature of high SARS-CoV-2 infectivity were characterised by an enrichment of opportunistic pathogens (including *Collinsella aerofaciens*, *Collinsella tanakaei*, *Streptococcus infantis* and *Morganella morganii*). On the other hand, faecal samples with a signature of "low-to-none" SARS-CoV-2 infectivity displayed higher concentration of *Parabacteroides merdae*, *Bacteroides stercoris* and *Lachnospiraceae bacterium 1_1_57FAA*. The latter are short-chain fatty acid producing bacteria, which play a crucial role in boosting host immunity. A longitudinal follow-up revealed relevant alterations of the faecal microbiota composition in a subset of patients[50].

More generally, intestinal and pulmonary dysbiosis are described in various acute and chronic pulmonary diseases. For example, pulmonary viral infections, such as the ones caused by influenza virus and respiratory syncytial virus, can even directly impair the gut microbiome[49]. Moreover, patients suffering from asthma have functional and structural impairment of the intestinal mucosa, and patients with chronic obstructive pulmonary disease often have leaky gut[51]. Apart from the acute COVID-19 phase, respiratory sequelae and radiological abnormalities (such as dyspnoea, chronic cough, fibrotic lung disease, bronchiectasis, and pulmonary vascular disease) may persist in recovered patients, and the optimal management is still undefined[5,52,53]. Thus, it might be plausible that an impairment of the gut homeostasis may occur in patients during the acute COVID-19 illness and persist after the disease resolution, even in those who did not experience gastrointestinal disturbances. This can be hypothetically explained by the communication between the two systems through the gut-lung axis. The existence of this connection is not entirely understood, but it is strengthened by the occurrence of lung diseases worsening as a consequence to intestinal microbial imbalances, gut inflammation and increased intestinal permeability[54]. Accordingly, it is reported that elevated values of faecal calprotectin are associated with a pathological chest X ray in COVID-19 patients[55]. Of interest, the enriched presence in faecal samples of patients with COVID-19 with high SARS-CoV-2 infectivity of *Streptococcus infantis*, an upper respiratory tract and oral cavity colonizer bacterial pathogen, may indicate a translocation or transmission

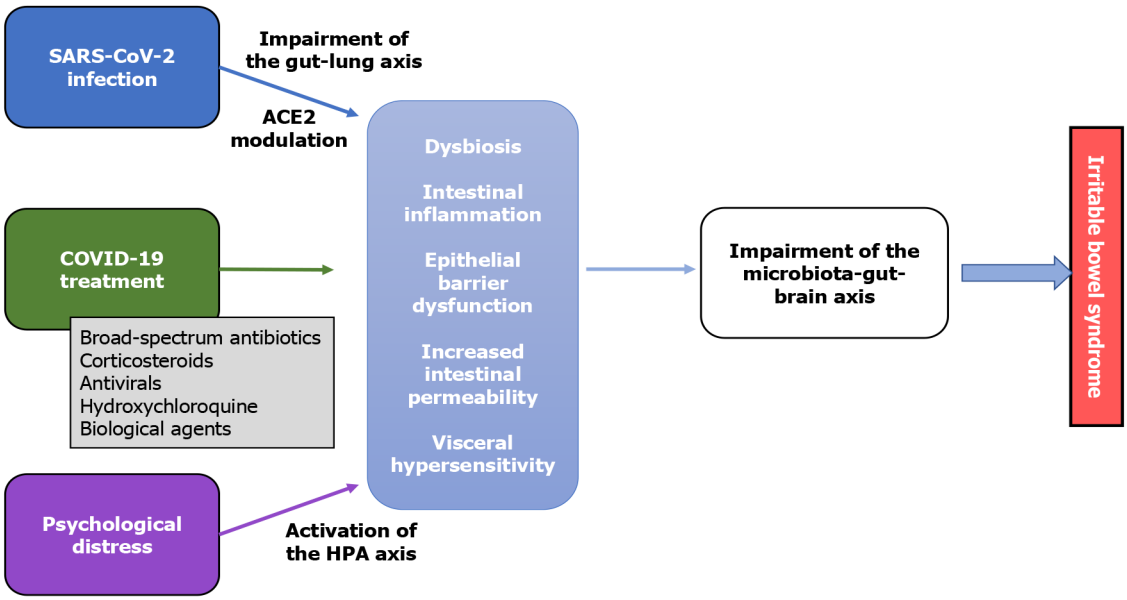

Figure 1 Possible pathophysiology of irritable bowel syndrome in coronavirus disease 2019 patients. ACE2: Peptidase angiotensin receptor 2; COVID-19: Coronavirus disease 2019; HPA: Hypothalamus–pituitary–adrenal; SARS-CoV-2: Severe acute respiratory syndrome coronavirus-2.

of extraintestinal microbes into the gut during COVID-19[50]. Moreover, lung and gut are independent systems which originate from one common embryonic organ, the foregut[56]. The microbiota of these two systems develop almost simultaneously after birth and is influenced by common factors, such as diet[57]. Overall, it is possible that a COVID-19-induced dysregulation of the gut-lung axis may enhance predisposing circumstances for IBS. This is also supported by the increased occurrence of gut disturbances, like inflammatory bowel disease or IBS, in patients with chronic respiratory diseases. Moreover, a pulmonary involvement has been described in approximately 33% of patients with IBS[51].

As previously mentioned, viral enteritis is described as a risk factor for developing PI-IBS. This has been assessed for norovirus infections in particular. Porter *et al*[58] found a significant increase in the incidence of functional gastrointestinal disorders, including constipation, in individuals who experienced a gastroenteritis during a norovirus outbreak, suggesting that dysmotility-related disorders may arise from viral infections[58]. Previously, Marshall *et al*[59] described a significantly increased prevalence of PI-IBS in a small cohort of subjects after a large outbreak of acute gastroenteritis attributed to food-borne norovirus when compared to unexposed individuals (23.6% *vs* 3.4%, $P = 0.014$), with OR of 6.9 (95%CI: 1.0–48.7)[59]. Similarly, an Italian study assessed the incidence of PI-IBS and functional gastrointestinal disorders after a norovirus outbreak. At 12 mo follow up a significant greater proportion of the infected participants (13%, 40 of 186 adults) developed PI-IBS in comparison with unexposed controls (3 of 198 subjects). The mechanisms through which IBS is elicited by norovirus are unknown, but this micro-organism can lead to epithelial barrier dysfunction, increased intestinal permeability, reduction in villous surface area and villous height, and to a mucosal immune response with an increase of cytotoxic intra-epithelial T cells[47]. It is possible that these alterations may trigger a perpetual immune stimulation or a prolonged immune activation toward cross-reacting non-pathogenic antigens, which impairs the gut sensory-motor function[58]. Since SARS-CoV-2 can have an intestinal tropism and induce intestinal flogosis[13,30,31], it can be speculated that a mechanism similar to that of norovirus is involved in the enhancement of PI-IBS.

Other non-infective factors may possibly play a key role in IBS pathophysiology. To date, COVID-19 management has involved a wide range of medications, whose efficacy has yet to be rigorously proven or is still under evaluation, and that can enhance a dysbiotic state. Above all, empiric antimicrobial use is often part of the treatment of respiratory infections, including SARS-CoV-2[60]. It is well known that broad-spectrum antibiotics can cause a rapid and significant drop in taxonomic richness, diversity and evenness, that can persist even for years after the treatment interruption. Beyond taxonomical compositional alterations, the gene expression, protein activity and metabolism of the gut microbiota can be impaired by antibiotics.

Overall, these changes can predispose to intestinal infections, to overgrowth and pathogenic behaviour of resident opportunistic organisms, and to impairment of the immunological equilibrium with systemic and long-term consequences[61]. Alongside with gut dysbiosis, broad-spectrum antibiotics can induce a disruption of the intestinal barrier function by altering the tight junction protein expression and localization, enhancing a pro-inflammatory state by NLRP3 inflammasome activation and promoting autophagy[62]. Hospitalised patients affected by COVID-19 are frequently treated with broad spectrum antibiotics for 5-8 d, mainly to prevent or to treat pathogens causing atypical pneumonia and staphylococci[60]. For instance, azithromycin, which is largely prescribed to COVID-19 patients, can induce a decline in the microbial richness and diversity, as well as changes in microbiota composition, with a shift in the Actinobacteria phylum, a reduction in the relative abundance of Proteobacteria and Verrucomicrobia (including *Akkermansia muciniphila*) and a decrease of the levels of bifidobacteria[63,64]. It is also reported that COVID-19 patients treated with vancomycin and/or ceftriaxone went through significant compositional alteration with reduced diversity of the gut microbiota[30].

Additionally, it is described that the use of corticosteroids, which is considered a treatment option in severe COVID-19 cases, may induce dysbiosis and alter intestinal homeostasis as well[57,65]. This is supported by the influence of steroid hormones on the gut bacterial communities in animal studies. In example, gonadectomy can reduce the microbiota-related sex differences observed between male and female rats. Similarly, hormone replacement to rodent females from the birth can decreased the microbial diversity in adulthood by increasing the Firmicutes:Bacteroidetes ratio[66]. As to systemic glucocorticoid therapy, there is evidence that subcutaneous prednisolone administration can alter the gut microbiota of mice, inducing significant shifts in the relative abundance of bacteria (decrease in Verrucomicobiales and Bacteriodales and increase in Clostridiales) in comparison with controls[67]. Interestingly, it is reported that individuals with glucocorticoid-induced obesity, who received prednisolone for at least three months, have significant decrease in gut microbial diversity in comparison with healthy controls, alongside with increased Firmicutes and Actinobacteria levels, and depletion in Bacteroidetes. Taxonomic analysis revealed a significantly reduced relative abundances of *Bacteroides*, *Bifidobacterium*, and *Eubacterium* in treated patients, whereas *Streptococcus* and *Geobacillus* displayed higher abundances. Also the faecal content of short-chain fatty acids (propionate and butyrate), which are products of carbohydrate fermentation by the gut bacteria, tended to be remarkably lower when compared to healthy control[68].

Other treatment options for the pulmonary phase of COVID-19 include antiviral drugs, mainly the adenosine nucleotide analogue prodrug remdesivir and the protease inhibitor lopinavir in combination with ritonavir[69]. Multicentre randomized controlled trials to assess the efficacy in reducing inpatient mortality, ventilation rate, and duration of hospital stay are still undergoing, although preliminary results are overall not encouraging[70]. While remdesivir seems not to affect the gut microbiota composition, antiretrovirals may somehow have a modulatory activity. The knowledge of the impact of antiretroviral drugs on the microbiome is limited by little evidence and it is predominantly focused on human immunodeficiency virus (HIV) treatment. It is still unclear whether antiretroviral treatment can consistently restore gut health of HIV-infected individuals or not, but it is likely that the initiation and prosecution of these medications can promote dysbiotic states[71,72]. Importantly, there is evidence that this drug class can promote microbiome changes independently from those induced by HIV. Indeed, a decreased alpha diversity is reported among treated patients in comparison with untreated HIV-positive ones. Moreover, protease inhibitors can directly interfere with the *in vitro* adherence of *Candida albicans* to an epithelial cell layer, which can possibly contribute to the reduction of oral candidiasis in HIV treated individuals[73].

Even if current evidence discourages its use in the prevention or treatment of COVID-19, hydroxychloroquine has been largely administered to patients due to its in-vitro capability of inhibiting SARS-CoV-2 by interfering with membrane fusion between host cell and the virus, especially in the early phase of the pandemic[70,74]. Hydroxychloroquine was initially used as an antimalarial, but, subsequently, it has been used as a disease-modifying anti-rheumatic drug to treat rheumatic disorders due to its immunomodulatory properties[75]. The impact of this medication on the human gut microbiota has been assessed by Balmant *et al*[48] in patients affected by systemic lupus erythematosus. They reported that the use of this drug is associated to different degrees of dysbiosis with a dose-dependent effect[48].

Severe SARS-CoV-2 infection is associated to an aberrant immune response with a massive cytokine release, mainly the IL-6 and IL-8. The elevation of inflammatory markers, such as IL-6 and C-reactive protein, has been associated with mortality and severe disease with pulmonary involvement in comparison to moderate disease. Thus, the targeted blockade of systemic inflammation has been proposed as a strategy to treat advanced acute conditions with lung lesions when contrasting the virus alone might not be sufficient. Specifically, Tocilizumab, a monoclonal antibody that inhibits the IL-6 receptor, has been proposed in patients with advanced lung injury[69]. Little evidence about the effect of this biological agent on the intestinal microbiota is available. A study of patients with rheumatoid arthritis reported that biologics, including tocilizumab, significantly reduced the total bacterial count and led to a decrease of *Clostridium coccoides* group, *Bifidobacterium*, and *Lactobacillus plantarum* and *Lactobacillus gasseri* strains after 6 mo[76]. Furthermore, it has been hypothesized that the aetiology of tocilizumab-related intestinal perforation may lie in the compositional or functional microbial changes[8]. Possibly, another IL-6 receptor inhibitor, which is currently being tested for severe COVID-19, may induce similar changes to the intestinal microbiota, but no study with this objective has been performed to date[69].

A further significant imbalance in the commensal bacterial populations may also be caused by polypharmacotherapy (*e.g.* proton-pump inhibitors, laxatives and metformin), which is common especially in elderly comorbid COVID-19 patients, and by the use of commonly prescribed drugs to manage mild COVID-19, as nonsteroidal anti-inflammatory drugs[63].

Finally, another considerable element involved in the IBS onset, exacerbation and relapse, is the activation of the HPA axis consequential to the secretion of the corticotropin-releasing hormone due to acute or chronic stressful conditions. This signalling pathway affects the gut functions by regulating the stimulation of the sympathetic and parasympathetic activity, the release of catecholamines, the mucosal immunity, the intestinal barrier function, the splanchnic blood flow and the composition and growth of the gut microbiota. Immune activation and intestinal micro-inflammation are described in IBS and can increase the intestinal permeability, modulate the peripheral sensitization of mucosal neuronal afferents and the recruitment of "silent" nociceptors involved in the hypersensitivity. Similarly, stress-induced dysbiosis may modulate the neuro-immune-endocrine systems and interfere with the brain-gut axis. Accordingly, the prevalence of at least one psychiatric disorder (mainly depression and anxiety) in patients with IBS ranges from 40% to 60% approximately, and the severity of IBS manifestations is remarkably correlated with the intensity co-morbid psychiatric disturbs. Moreover, early adverse life events and major traumatic experiences are frequently described before the onset of IBS[77,78]. COVID-19 is having a significant impact on mental health worldwide, since various psychological stress-associated factors are linked to the pandemic. More than 40% of patients experiences psychological distress even when the disease is under control during the acute infection phase. A considerable role is also played by anxiety, panic and fear for the isolation environment and the uncertain sequelae following resolution of a new and dangerous disease. Long-term psychological consequences (as anxiety, depression, post-traumatic stress disorder, insomnia, irritability, memory impairment, fatigue, and traumatic memories) are frequently reported among those who suffered from COVID-19, especially hospitalized ones and individuals with previous emotional dysregulation[7,79]. Overall, COVID-19-related psychological disturbances are significant and can definitely contribute to IBS occurrence.

CONCLUSION

The COVID-19 pandemic is a threat to global public health. A wide spectrum of respiratory and systemic symptoms can occur during the acute disease with different degrees of severity, and some of them can persist over time after the recovery. A large body of evidence supports the gastrointestinal involvement of SARS-CoV-2 infection during the acute phase, possibly because the intestinal ACE2 is an additional target of viral infection. Importantly, little is known about the gastrointestinal sequelae; at present, there is no study reporting the occurrence of IBS in individuals recovered from COVID-19. However, a number of considerations may be made regarding the plausible role of COVID-19, its management and global setting in the enhancement of IBS. Specifically, it can be assumed that many factors contributing to promote a dysbiotic state, epithelial barrier impairment, intestinal inflammation and gut dysfunction (like antibiotics and other treatments of the acute phase, gut-lung axis

impairment, disease-related psychological stress, as well as the virus itself) can be involved in this process. Prospective cohort studies are necessary to confirm these hypotheses before clinical significance can be concluded.

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REVIEW

Overview of the microbiota in the gut-liver axis in viral B and C hepatitis

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Abstract

Viral B and C hepatitis are a major current health issue, both diseases having a chronic damaging effect on the liver and its functions. Chronic liver disease can lead to even more severe and life-threatening conditions, such as liver cirrhosis and hepatocellular carcinoma. Recent years have uncovered an important interplay between the liver and the gut microbiome: the gut-liver axis. Hepatitis B and C infections often cause alterations in the gut microbiota by lowering the levels of 'protective' gut microorganisms and, by doing so, hinder the microbiota ability to boost the immune response. Treatments aimed at restoring the gut microbiota balance may provide a valuable addition to current practice therapies and may help limit the chronic changes observed in the liver of hepatitis B and C patients. This review aims to summarize the current knowledge on the anato-functional axis between the gut and liver and to highlight the influence that hepatitis B and C viruses have on the microbiota balance, as well as the influence of treatments aimed at restoring the gut microbiota on infected livers and disease progression.

Key Words: Viral B hepatitis; Viral C hepatitis; Gut-liver axis; Immunomodulation; Lipopolysaccharides; Short-chain fatty acids

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Core Tip: We have provided an overview of the mechanisms involved in the immunomodulation of the gut-liver axis. We highlight the mechanisms by which hepatitis B virus and hepatitis C virus infections influence the microbiota and how in turn these changes affect the liver pathology. We have also looked at the current treatment options and their influence on the intestinal microflora.

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INTRODUCTION

Viral B and C hepatitis are two types of infections with a high rate of morbidity and mortality[1]. Hepatitis B virus (HBV) is a DNA virus belonging to the Hepadna virus, and hepatitis C virus (HCV) is an RNA virus in the Flaviviridae family. These viruses have hepatic tropism, are non-cytopathic with the ability to cause chronic liver inflammation and even liver cirrhosis and hepatocellular carcinoma[2].

Both HBV and HCV may cause similar clinical manifestations. Some patients may be asymptomatic, while others may have mild signs and symptoms from general manifestations (fatigue, fever, loss of appetite) to gastrointestinal symptoms (abdominal pain, nausea, vomiting, jaundice)[3].

The microbiota represents the totality of microbes (bacteria, viruses, fungi, protozoans, and archaea) associated with the human microorganism, while the microbiome consists of all microbes and their genes[4]. The main part of the body colonized by microbes is the gastrointestinal tract, whereas other parts such as skin, airways, vaginal tract, *etc.* are also colonized, but to a lesser extent. Changes in the microbiota are continuous throughout our life and there are many influencing factors, from type of delivery and breastfeeding, to long-term dietary changes, frequent and prolonged antibiotic treatment or other medications, *etc.*[5]. There are six bacterial dominant phyla in the gut microbiota: *Firmicutes* and *Bacteroidetes* (90%), *Proteobacteria, Actinobacteria*, *Fusobacteria* and *Verrucomicrobia*[6]. The intestinal microbiota is a cornerstone in maintaining the homeostasis of the human body. Firstly, this "organ" provides nutrients and energy from ingested food and, secondly, it is able to produce important metabolites that play a role in maintaining the host's metabolism [7].

The liver can be considered the largest immune organ in the body with a high ability to select and activate immune cells in response to metabolic products in the gut or to signals sent by various pathogens[8]. Recent years have seen advances in our understanding of the human microbiome and its interaction with us as hosts. The gut-liver axis is part of these new discoveries, integrating the microbiome modifications and dysbiosis in hepatic pathologies.

Our review will discuss part of the mechanisms by which the microbiome influences host immunity, as well as the gut-liver axis, with an accent on viral hepatitis B and C.

MICROBIOTA AND THE IMMUNE SYSTEM

Through its products, the human microbiota can influence both the local, enteric, and the systemic immune system, dysbiosis being correlated with several autoimmune, metabolic and neurodegenerative diseases (inflammatory bowel disease progression, rheumatoid arthritis, diabetes, asthma and bones homeostasis)[9-15]. This shows that the microbiota is not only involved in intestinal, but also in systemic and organ specific pathologies. This relationship is bidirectional; systemic modifications can trigger intestinal changes, but also intestinal dysbiosis can trigger and maintain organ dysfunctions. Gut-associated lymphoid tissue (GALT) is an important "immunological organ" of the body that belongs to the gut-mucosal immune system. GALT consists of Peyer's patches, intraepithelial lymphocytes, lamina propria lymphocytes (including dendritic cells) and mesenteric lymph nodes. Activation of this system has the ability

to produce various mediators with immunostimulatory or immunosuppressive effect [16].

Some of the products by which the intestinal microflora communicates with the rest of our organism are lipopolysaccharides (LPS), bacterial DNA and RNA, flagellin, short-chain fatty acids (SCFA) such as acetate, propionate and butyrate, tryptophan (Trp) and it's metabolites, teichoic acid and peptidoglycans and secondary bile acids (BA)[9,17]. These bacterial components and products of the bacterial metabolism are recognized by pattern recognition receptors, which particularly include the toll-like receptors (TLR) family. TLRs are expressed on epithelial and immune cells and are capable of recognizing specific bacterial molecules, triggering specific local protective and immunomodulatory (both pro- and anti-inflammatory) responses[18,19]. TLR activation is an essential element of the innate immune systems fight against the HBV and HCV infections[20,21]. Not all of these pathways were studied directly in connection with HBV and HCV. Therefore, more studies are needed to determine the exact relationship between the bacterial products, the immune system and hepatitis.

We will briefly mention some of the most important of the microbial-produced products and their interaction with the immune system (Figure 1).

LPS

In Gram-negative bacteria, LPS are an important pathogen-associated molecular pattern and a well-studied microbial marker in connection with bacterial translocation and host systemic responses[22,23]. The outer membrane of gram-negative bacteria consists of LPS, which possess a hydrophobic endotoxin, called lipid A[24]. This component is recognized by TLR4 and *via* this mechanism it further activates nuclear factor kappa B (NF-κB) and elicits pro-inflammatory effects[25,26]. One type of LPS is *Escherichia coli* (*E. coli*) produced LPS. This stimulates TLR4 receptors and triggers the release of pro-inflammatory cytokines. *E.coli* LPS also increases endotoxin tolerance and decreases the autoimmune activity, protecting against autoimmune diabetes[27]. However, some bacterial species produce LPS molecules with underacylated lipid A that exhibit an immuno-inhibitory effect[28]. These LPS molecules are produced especially by members of the *Bacteroidales* order and instead of stimulating TLR receptors, they silence the TLR4 signaling and the inflammatory process[29]. LPS induces the upregulation of cluster of differentiation 14 protein (CD14) *via* the TLR4 pathway, which decreases the relative epithelial resistance and increases its permeability. Increased intestinal permeability allows for more LPS to reach the general circulation, aiding it in reaching different organs and exhibiting a pro-inflammatory effect[30]. This is also true in cases of dysbiosis with an increase in LPS production that is correlated with an increase in tumor necrosis factor alpha (TNF-α), interleukin (IL) 6 and C-reactive protein levels[31,32]. Intestinal dysbiosis caused an LPS-induced inflammatory response in a mice model, while unaltered host microbiota reduced the inflammatory response to LPS in the liver[33]. LPS-induced monocyte activation has been shown to be increased in patients with HBV or HCV[34].

This underlines the ability of LPS and gut lipid metabolism to modulate both intestinal and organ-specific inflammatory response.

SCFA

In the gut, non-digestible carbohydrates are transformed by the microbiota into SCFA such as acetate, propionate and butyrate[35]. Acetate and propionate are produced mainly by *Bacteroidetes*, while butyrate, the main source of energy for colonocytes, by *Firmicutes*. A small portion of SCFA that is not metabolized can reach the liver through the portal vein, being used as energy substrates for hepatocytes[36,37]. Certain bacteria such as *Butyricimonas* and *Prevotella* have the ability to generate butyrate and propionate, SCFAs with anti-inflammatory effect[38].

SCFA bind to the G-protein coupled free fatty acid receptors (FFA): GPR41 (FFA2) and GPR43 (FFA3)[39,40]. Enteroendocrine and pancreatic β-cells present both GPR41 and GPR43 receptors, while immune cells and adipocytes present mostly GPR41 and peripheral neurons GPR43[41]. This links SCFA production to a multitude of metabolic, neurological and inflammatory mechanisms. Thus, FFA receptors and SCFA production presents therapeutic targets in these diseases[41-43].

In immune cells (leukocytes and neutrophils) SCFA increase the intracellular calcium levels[39,44,45]. This reaction leads to an increased production of reactive oxygen species, as well as an increased neutrophil recruitment and a pro-inflammatory effect[46-48]. GPR41 activation by SCFAs in the gut promotes the function and size of regulatory T cells, protecting against intestinal inflammation[49]. Also, GPR43 was found to be a chemotactic receptor for neutrophils, stimulating their migration towards the source of SCFAs[50,51]. In a mouse model of gout, the intestinal

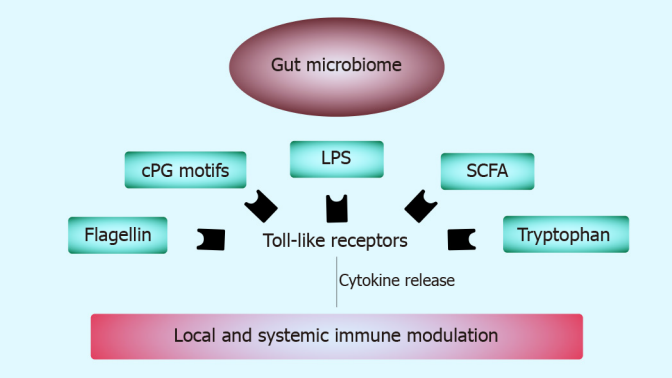

Figure 1 The mechanisms by which the gut microbiome influences the immune system. LPS: Lipopolysaccharides; SCFA: Short-chain fatty acids.

microbiota-produced SCFA determined inflammasome assembly, reactive oxygen species formation and IL-1b production and improved the inflammatory response[52]. Increased SCFA levels determined the production of macrophages and dendritic cells, protecting the lung against allergic inflammation[53]. Also, by activating another G-protein coupled receptor, GPR109A, the microbiota is involved in inflammatory suppression *via* the NF-κB pathway in normal and colon cancer cells[54].

Another SCFA mechanism involved the inhibition of histone deacetylases (HDAC). By non-competitively inhibiting the activity of HDAC 1 and 2, butyrate causes histone hyperacetylation. By this mechanism, butyrate and other SCFAs are thought to serve as a protective factor against colon cancer, dysbiosis being a risk factor for the development of this disease, as well as other chronic inflammatory diseases[55]. HDAC inhibition also promotes macrophage activity and CD8 T cells and improves anti-cancer therapy[56-59]. Furthermore, class 1 HDACs inhibition is proposed as a target in pulmonary inflammation, due to its contribution in the release of pro-inflammatory cytokines[60]. HDAC inhibition promotes effector and regulatory T-cell differentiation and the production of IL-17, interferon-γ (IFN-γ) and IL-10, contributing to an overall anti-inflammatory effect mediated by SCFAs[61,62].

By increasing acetyl-CoA activity and controlling gene expression, SCFA are involved in plasma B cells metabolism, activity, energy production boosting, and differentiation. During an infection, they support B cells antibody production, decreasing the host susceptibility to pathogens[63].

Therefore, SCFA present both a pro- and anti-inflammatory role[61]. There is still the need for more studies to fully understand the implications of SCFA in inflammatory and immune diseases and determine in which conditions they act as pro-inflammatory or as anti-inflammatory factors.

Trp

The microbiota is involved in the transformation of Trp in indole derivatives, serotonin (5-hydroxytryptamine) and kynurenine[64].

Lactobacilli species can metabolize Trp into indole-3-aldehyde, a ligand for the aryl hydrocarbon receptor (AhR) that is involved in intestinal immunity and the production of IL-22[65,66]. There are only a few species such as *Peptostreptococcus russellii* and *Lactobacillus spp.* with the ability to produce AhR ligands[64]. In high fat diets IL-22 can act as an antioxidant and anti-inflammatory agent, protecting the intestinal mucosa and epithelial cells from oxidative and inflammatory stressors[67]. Also, IL-22 is involved in the intestinal mucosa immune response against exterior pathogens[68,69]. However, in patients with inflammatory bowel disease, Il-22 is considered a "two-headed cytokine": it acts as a mucosal producing and healing agent, but in the chronic form of the disease it is also involved in tumorigenesis, promoting tumoral growth[70-72].

The Trp microbiota metabolite AhR regulates the activation and transcription of several other pathways, including IL-6, cytochrome P450 1A1 (CYP1A1), and 1B1 (CYP1B1), vascular endothelial growth factor A, and prostaglandin G/H synthase 2 and also stimulates innate lymphoid cells and intraepithelial lymphocytes development, mediating their anti-inflammatory effects[73,74]. Other bacteria that interfere with Trp metabolism are *E. coli*, *Lactobacilli* and *Clostridium sporogenes*. The first two possess tryptophanase which converts Trp to indole, while the latter decarboxylates Trp and increases tryptamine production[64].

The microbiota influence on Trp provides intestinal anti-inflammatory effects, but it also poses potential research directions regarding systemic inflammation[75,76].

Flagellin

The locomotive bacterial flagella contain flagellin, which is recognized by the host TLR5. Via the TLR pathways, flagellin is involved in several immunological mechanisms, both locally, in the gut, but also systemic, inducing the release of pro-inflammatory molecules[77]. In a study administering purified flagellin in mice, there was a decreased microbial dysbiosis, as well as an amelioration of IL-10 deficiency-induced colitis[78]. This shows that flagellin presenting bacterial species could pose a beneficial effect in chronic inflammatory diseases. However, in patients with inflammatory bowel diseases there have been observed higher concentrations of flagellin, putting into question its supposed protective role[79]. Also, flagellin has been observed to be a potent TLR5/NF-κB activator, promoting inflammation in intestinal epithelial cells[80]. *Via* the same TLR5/NF-κB mechanism, flagellin could also promote the attachment and development of viral molecules, supporting viral infections *via* the intestine[81].

Bacterial CpG motifs

Bacterial DNA contains unmethylated CpG dinucleotides that are recognized by the immune system and produce an immunostimulatory effect[82,83]. These bacterial CpG motifs are recognized by TLR9 receptors and, depending on their localization, they exhibit several effects. Apical TLR9 activation inhibits NF-κB activation, while basolateral receptors stimulate NF-κB activation and the subsequent inflammatory pathways[84].

INFLAMMATION AND B AND C HEPATITIS

Many extrahepatic changes (metabolic, cardiovascular, autoimmune, renal) have been correlated with chronic HCV infection. This statement is supported by a prospective cohort study in which patients with chronic HCV infection (with HCV RNA detected in the serum) had a high risk of death due to liver or non-liver disease (cardiovascular and renal disease) compared to uninfected patients (without serum HCV RNA) or with patients presenting HCV antibodies[85].

Inflammatory cytokines are normally released in response to various stimuli, including viral infection. This limits cellular stress and cell damage[86]. HCV infection is associated with an immune activation status that can further influence the levels of inflammatory markers (Il-6, TNF-α, iNOS, COX-2, IL-1), which are correlated with various extrahepatic diseases[87,88]. In HBV-infected patients there is an increase in Il-8, IL-29 and COX-2. Under normal conditions, adult hepatocytes do not express COX-2, but in chronic inflammatory diseases, the expression of this isoenzyme increases. Furthermore, IL-8 activates the extracellular signal-regulated kinase and c-Jun N-terminal kinase signaling pathways, which are also involved in inflammatory processes[86].

In infected hepatocytes with HCV, the production of type 1 and 3 interferons is blocked by the action of the viral NS3/4A protease. This protease may also influence the innate immune adaptor molecules mitochondrial antiviral signaling proteins with an effect on the intracellular antiviral defense system. In an experimental study on hepatic macrophages the first activated factor in liver macrophages with HCV infection has been shown to be TNF-α that further activates NF-κB and increases IL-1β. Adding to this, the HCV core protein also activates the NLRP3 inflammasome. The hepatic inflammatory environment is ensured by the activity of the NLRP3 inflammasome, phospholipase-C and IL-1β. Thus, NLRP3 inflammasome and IL-1β can be considered as target of treatment in HCV-induced liver disease[89].

THE GUT-LIVER AXIS

The gut microbiome can interact tightly with the liver *via* the so-called gut-liver axis. Blood from the intestine, rich in microbiota-derived molecules, reaches the liver *via* the portal vein. In the liver, these molecules are recognized by TLRs pattern recognition receptors, mediating their effect on the liver tissue[90]. Related to liver pathologies, the gut microbiota is particularly involved in liver fibrosis and cirrhosis, hepatic cancers,

alcoholic and non-alcoholic fatty liver disease, autoimmune hepatitis, primary sclerosing and primary biliary cholangitis as well as viral hepatitis[91-96]. Some of the most studied components that affect liver pathologies are represented by LPS and SCFAs.

LPS produced by the microbiota are scarcely found in the normal liver, being cleared by Kupffer cells and not causing any damage[97]. However, in alcoholic liver disease, because of an increase intestinal permeability, an increased amount of LPS reached the liver[96]. LPS binds to TLR4, causing an excessive release of pro-inflammatory cytokines IL-1 and TNF-α[33,98]. Also, LPS can upregulate the expression of the cluster of differentiation 14 (CD14) receptor on Kupffer cells[99]. This could potentially make the liver more sensitive to LPS toxicity, as CD14 is vital for Kupffer cells LPS activation[100]. Kupffer cells activation produces a pro-inflammatory state, increasing the levels of NF-κB, TNF-α and IL-1. This leads to liver injury and disease progression, dysbiosis favoring the chronic inflammatory state[101].

SCFA such as acetate, propionate and butyrate may have a protective effect on liver diseases progression. High levels of butyrate restore the intestinal microbiota in cases of dysbiosis, reducing the intestinal permeability and thus the levels of endotoxins reaching the liver *via* the portal circulation. This attenuated the histological aspect of steatohepatitis livers, reducing the levels of TNF-α, IL-1, IL-6 and IFN-γ pro-inflammatory cytokines, as well as the expression of TLR4 receptors[102]. In an experimental study by Endo *et al*[103], administering probiotics, aimed at increasing butyrate levels, significantly improved non-alcoholic fatty liver disease progression, reducing the inflammation and oxidative stress. This clearly shows that intestinal-produced metabolites can influence the immune and inflammatory state of the liver. Dysbiosis and an increased intestinal permeability allows for the gut-liver balance to change, causing a pro-inflammatory state of the liver and contributing to disease progression [104,105]. Pathogen-associated molecular patterns (bacterial antigens and products) such as LPS and viral RNAs activate TLR4 on Kupffer cells and other immune cells. Thus, the innate immune response is induced.

The liver is influenced by the intestine through the portal circulation, while the intestine is influenced by the liver through the released mediators and hepatic bile flow. It is known that increased intestinal permeability contributes to systemic inflammation and disease progression[106]. BA and other mediators such as immunoglobulin A (IgA) regulate the gut-liver axis. IgA influences the homeostasis of the intestinal microbiota, preventing bacterial translocation. BA modulate the intestinal barrier and have antimicrobial activity. Several enzymes involved in BA synthesis are regulated by the microbiota. However, some secondary BA (*e.g.*, deoxycholic acid) resulting from intestinal biotransformation produce microbial dysbiosis and increase the intestinal permeability[107].

TGR5 is a G-protein-coupled BA receptor involved in the anti-inflammatory immune response, energy homeostasis, metabolic pathways and in pathologies such as diabetes and obesity[108]. In the intestine, TGR5 is involved in regulating the colonic motility and the intestinal permeability *via* the farnesoid X receptor – cAMP pathway [109,110]. Moreover, TGR5 activation stimulates mucosal proliferation and protects against mucosal injuries[111]. In liver pathologies, the levels of BA are significantly decreased, leading to a reduced activation of TGR5 in the gut[112,113]. In a mouse model with TGR5 silencing, there was a significant reduction in gut epithelial cellularity, with histological abnormalities and distortions and an increased intestinal permeability[114]. BA and TGR5 activation are therefore necessary for a normal functioning of the intestine and the gut-blood barrier. BA administration is beneficial for viral hepatic diseases. In a HBV model, TGR5 agonists administration suppressed the infection[115]. BA and TGR5 agonists pose as potential treatment options for viral hepatitis[116].

Decreased BA quantities in virus hepatitis could be responsible for the increased intestinal permeability and the subsequent increase in LPS and other endotoxins. This in turn favors the progression of the liver pathology, creating a vicious circle where the liver pathology creates an environment that further promotes the liver pathology (Figure 2). Future studies should determine the exact mechanism by which liver diseases influence the intestinal permeability and lead to the production of dysbiosis.

THE GUT MICROBIOTA-VIRAL B AND C HEPATITIS

The presence of the HBV or HCV infection can lead to intestinal dysbiosis[117]. Some of the microbial changes present in patients with HBV and HCV-related liver diseases

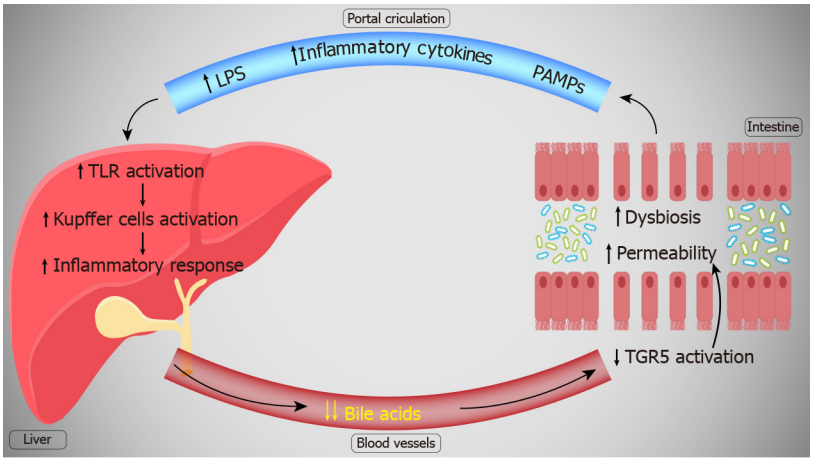

Figure 2 The gut-liver axis in liver diseases. TGR5: G-protein-coupled bile acid receptor; PAMPs: Pathogen-associated molecular patterns; LPS: Lipopolysaccharides; TLR: Toll-like receptor.

are shown in Table 1.

These studies showed significant differences in the composition of the intestinal microbiota between patients with B or C hepatitis with or without cirrhosis present. A healthy gut microbiota means a gut microbiota with great diversity and the ability to react to changes. Thus, B and C viruses can cause changes and can shape the gut microbiota in different directions[122].

Nowadays, the treatment of B and C hepatitis is well established by international guidelines[124-126]. The main question is: does the treatment of B or C hepatitis influence the diversity and abundance of the intestinal microbiota? And if so, are these changes helping in preventing or halting the evolution of the disease? A part of the studies looking into the microbial changes caused by HBV and HCV treatments are presented in Table 2.

Entecavir increases the abundance of the genus *Clostridium sensu stricto 1* which has been associated with large and extra-large HDL particles and also with a decreased risk of cardiovascular disease[131]. Increased lipid content in the liver and steatosis can result in the development of inflammation and, over time, cirrhosis, and can also increase oxidative stress[132]. Genus *Intestinibacter* along with genus *Escherichia, Shigella* can be considered as a major contributor to NAFLD progression. Increases in the abundance of *Intestinibacter* have been correlated with severe intestinal disorders in humans and are recognized as a biomarker of the onset of Crohn's disease[133].

In a study by Pérez-Matute *et al*[129], it was shown that the use of direct antiviral agents in patients with chronic HCV infection could only restore the intestinal bacterial changes in those patients with a lower degree of fibrosis (F0-1). The data highlight a strong relationship between the liver and the intestine and suggest that mild intestinal changes caused by liver damage could possibly be counteracted with the appropriate drugs.

Blautia, Coprococcus, Dorea, Lachnospira, Oribacterium, Roseburia and *L-Ruminococcus* were detected in the human intestine as the main genera belonging to the *Lachnospiraceae* family[134]. *Lachnospiraceae* is considered a "good" family of bacteria, having a beneficial role in host homeostasis. The bacteria belonging to this family can convert carbohydrates into SCFA in the gut[135]. Decreasing the abundance of *Lachnospiraceae* leads to decreased SCFA production and thus increases the pH of the colon. This change increases the production of ammonia and its absorption in the intestine[136].

Direct-acting antivirals (DAA) treatment in cirrhotic patients appears to have a positive impact on changes in the intestinal microbiota, as well as fibrosis and inflammation, but without a positive impact on the function of the intestinal barrier. DAA has greatly reduced the abundance of *Enterobacteriaceae, Staphylococcus*, and *Veillonellaceae*[130]. The abundance of the *Enterobacteriaceae* family, belonging to the *Proteobacteria* phylum, depends on the amount of oxygen that crosses the intestinal barrier. The abundance of *Enterobacteriaceae* is elevated after the oxygen level increases and can aggravate intestinal inflammation. Members of this family cannot degrade complex carbohydrates (as *Clostridia* and *Bacteroidia* do); they are only involved in the passive transport of oligosaccharides. This disadvantage may explain the lower abundance of *Enterobacteriaceae* compared to *Clostridia* and *Bacteroidia* in the healthy distal intestine [137]. *Veillonellaceae* belonging to *Firmicutes* phylum, is one of the main microbial taxa

Table 1 Microbiota changes in different studies regarding hepatic B and C virus

	Changes of gut microbiota in patients *vs* healthy subjects	Ref.
Type of HBV infection		
Chronic HBV infection	↓ *Bacteroidetes* and *Firmicutes*; ↑ *Proteobacteria* and *Actinobacteria*	Chen *et al* [117]
	↑ *Bifidobacterium dentium*; ↓ *Bifidobacterium catenulatum* and *longum*	Xu *et al* [118]
	↑ *Veillonellaceae*; ↓ *Lachnospiraceae, Rikenellaceae, Ruminococcaceae*	Wang *et al* [119]
HBV liver cirrhosis	↓↓↓ *Bacteroidetes* and *Firmicutes*; ↑↑↑ *Proteobacteria* and *Actinobacteria*	Chen *et al* [117]
Decompensated HBV cirrhosis	↓ *Bifidobacteria/Enterobacteriaceae ratio*; ↑ *Enterobacteriaceae*; ↓ *Firmicutes* (*F.prausnitzii, Clostridium clusters XI* and *XIVab, Bifidobacterium*); ↓ *Bacteroidetes*	Lu *et al* [120]
HBV related hepatocellular carcinoma	↓ *Proteobacteria*; ↑ *Prevotella, Phascolarctobacterium, Anaerotruncus*; ↑ *Proteus, Veillonella, Prevotella 2, Barnesiella* and *Ruminococcaceae spp.*	Liu *et al* [121]
Type of HCV infection		
Chronic HCV infection without cirrhosis	↑ *Veillonella spp., Lactobacillus spp., Streptococcus spp.* and *Alloprevotella spp.*; ↓ *Bilophila spp., Clostridium IV spp., Clostridium XIVb spp., Mitsuokella spp.* and *Vampirovibrio spp.*; No changes: *Akkermansia spp., Bifidobacterium spp., Escherichia/Shigella spp., Haemophilus spp., Micrococcus spp.* and *Weissella spp.*	Heidrich *et al*[122]
Chronic HCV infection with cirrhosis	↑↑↑ *Veillonella spp., Lactobacillus spp., Streptococcus spp.* and *Alloprevotella spp.*; ↓↓↓ *Bilophila spp., Clostridium IV spp., Clostridium XIVb spp., Mitsuokella spp.* and *Vampirovibrio spp.*; ↑↑↑ *Akkermansia spp., Bifidobacterium spp., Escherichia/Shigella spp., Haemophilus spp., Micrococcus spp.* and *Weissella spp.*	Heidrich *et al*[122]
Stage 4 HCV infection (cirrhosis)	↓ *Firmicutes*; ↑ *Prevotella, Faecalibacterium* (*F. prausnitzii*); ↑ *Acinetobacter*; ↑ *Veillonella*	Aly *et al* [123]

HBV: Hepatitis B virus; HCV: Hepatitis C virus.

Table 2 Microbial changes as a result of several treatments in viral B and C hepatitis

Drug	Type of study	Changes in gut microbiota	Ref.
Entecavir	Experimental (mice)	↑ *Lachnospiraceae, Akkermansia, Alistipes, Escherichia, Shigella, Oscillibacter, Bilophila*	Li *et al*[127]
	Clinical	↑ *Clostridium sensu stricto 1, Erysipelotrichaceae UCG-007, Intestinibacter*; ↓ *Streptococcus, Atopobium*, and *Murdochiella*	Lu *et al*[128]
Direct antiviral agents in patients with HCV infection	Clinical	↑ *Phylum Firmicutes, genera Lachnospira*	Pérez-Matute *et al*[129]
Direct antiviral agents in patients with HCV-related liver cirrhosis	Clinical	↓ *Enterobacteriaceae, Staphylococcus* and *Veillonellaceae*	Ponziani *et al* [130]

HCV: Hepatitis C virus.

associated with the severity of fibrosis in non-obese patients. This family has the ability to produce propionate, one of the most important SCFAs and has been associated with chronic liver disease[138]. The LPS and SCFA metabolites produced by intestinal *Veillonella* stimulate the release of cytokines (Il-6, IL-10, TNF-α) in human peripheral blood mononuclear cells and thus have a negative impact on liver pathology and host inflammation[139].

GUT MICROBIOTA-TARGET OF TREATMENT

Although standard therapy for B and C viral hepatitis is well established and presented in clinical guidelines, many dietary supplements, including pre-, pro-, and symbiotic agents, are being studied to reduce the toxicity of standard therapy (side effects) or to increase their effect. Also, fecal microbiota transplantation (FMT) is one of the methods that can manipulate the composition of the intestinal microbiota. It has

the ability to strengthen the intestinal barrier, reduce intestinal permeability and also improve host immunity[140]. There are various routes of administration for FMT: nasogastric tube, upper endoscopy or colonoscopy, retention enema, *etc*. The route of administration depends on the characteristics of the disease. For example, good results have been obtained after duodenal administration in metabolic disease[141].

There are only a few studies that support the effect of certain probiotics in viral B or C hepatitis.

Oo *et al*[142] studied the long-term (36-mo) effect of probiotic heat-treated strain Enterococcus faecalis FK-23 in patients with HCV infection. This probiotic may change the microbiota in these patients and may have an important role of decreased ALT in serum.

In patients with HBV-induced liver cirrhosis, the role of a probiotic (*Clostridium butyricum* combined with *Bifidobacterium infantis*) has been studied in the treatment of minimal hepatic encephalopathy. The results claim that the probiotic modulates the intestinal barrier and thus can lower the level of ammonia and can improve cognition [143].

CONCLUSION

Most of the microbiota-derived components elicit an immunomodulatory effect, both pro- and anti-inflammatory. Alteration of the host microbiome produces an unbalance of these factors, leading to negative effects both locally in the intestine, as well as at distance in other organs. Therefore, we can conclude that by its factors, the host microbiota is an important determinant in the hosts immune response modulation. Future experimental and clinical studies are needed to determine the exact mechanisms of these changes, as well as the exact conditions in which the microbiota can serve as a protective factor.

Currently, the intestinal microbiota is a target of treatment for various diseases in humans. Future studies should focus on the effects and efficacy of treatments aimed at restoring the gut microbial environment (prebiotics, probiotics, symbiotics, fecal transplant) and their exact relationship with liver pathologies. By understanding the natural communication pathways between the liver and the gut, in both health and disease, we could potentially formulate better therapies aimed at reducing the effects of the chronic inflammatory response on the progression of liver diseases.

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REVIEW

Hepatocellular carcinoma locoregional therapies: Outcomes and future horizons

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Abstract

Hepatocellular carcinoma (HCC) is the most common primary cancer of the liver and has an overall five-year survival rate of less than twenty percent. For patients with unresectable disease, evolving liver-directed locoregional therapies provide efficacious treatment across the spectrum of disease stages and *via* a variety of catheter-directed and percutaneous techniques. Goals of locoregional therapies in HCC may include curative intent in early-stage disease, bridging or downstaging to surgical resection or transplantation for early or intermediate-stage disease, and local disease control and palliation in advanced-stage disease. This review explores the outcomes of chemoembolization, bland embolization, radioembolization, and percutaneous ablative therapies. Attention is also given to prognostic factors related to each of the respective techniques, as well as future directions of locoregional therapies for HCC.

Key Words: Hepatocellular carcinoma; Bland embolization; Chemoembolization; Radioembolization; Transarterial embolization; Thermal ablation; Locoregional therapy

Core Tip: This article reviews prognostic factors and outcomes of current locoregional

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therapies for hepatocellular carcinoma, as well as future directions and promising new techniques. Therapies including transarterial bland embolization, chemoembolization, and radioembolization, as well as percutaneous ablation are reviewed. Prognostic considerations vary by indication but generally follow baseline disease staging and tumor quantification. Outcomes data reveal survival benefits in appropriately selected patients. New advances in precision medicine, combination therapy, and immunotherapy are being investigated.

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the fifth most common cancer globally[1] and the most common primary liver malignancy[2,3], comprising over 90% of liver tumors[4,5]. The overall prognosis of HCC involves a complex interplay of baseline clinical staging, underlying liver function, and demographic factors[6,7]. Nonetheless, the 5-year relative survival rate for primary liver cancer is estimated to be 19.6%[8], with a mean survival being reported between 6-20 mo[5]. The unfavorable prognosis of HCC highlights the importance of treatment innovation and improvement. Surgical therapy has been the traditional definitive management in eligible patients; however, fewer than 20% of HCC patients are candidates for surgical resection based on a variety of tumor and disease characteristics. For the remainder of HCC patients, liver-directed locoregional therapies form the mainstay of treatment.

Locoregional therapies play a vital role in HCC therapy across a vast range of disease stages[9]. Image-guided techniques with locoregional delivery of chemotherapeutic, radiotherapeutic, or ablative therapy are flourishing[10,11]. Minimally-invasive approaches, such as transarterial chemoembolization (TACE), transarterial embolization (TAE), transarterial radioembolization (TARE), and ablation may be indicated based on patient clinical status and tumor characteristics. Treatment goals may include bridging to or downstaging for transplant eligibility, inducing parenchymal hypertrophy to enhance function following resection, disease control and palliation, and, in some instances, cure[10]. In general, locoregional liver-directed treatments provide less morbidity than traditional surgical options while also improving outcomes compared to traditional systemic therapies[12]. This paper reviews prognostic factors and outcomes of locoregional therapies for HCC. We discuss how prognostic factors overlay the clinical staging systems most commonly used, the existent data regarding survival and treatment response, and future directions of locoregional HCC therapy.

TACE

TACE relies on a combination of targeted chemotherapeutic and embolic agents[13]. Transarterial therapies make use of a mismatch in blood flow between healthy liver parenchymal tissue and hepatocellular tumors. Unlike normal liver parenchyma, which derives most of its blood supply from the portal venous system, hepatocellular tumors are primarily perfused *via* the hepatic arterial system[14]. Thus, normal tissue is preferentially spared when therapies are targeted at tumor tissue through the hepatic arterial tree. Conventional TACE (cTACE) utilizes hepatic arterial administration of a chemotherapeutic agent emulsed with lipiodol oil to increase chemotherapeutic concentration and decrease pharmacologic washout. Chemotherapeutic and embolic agent administration *via* drug-eluting beads (DEB-TACE) has been shown to provide less systemic chemotherapy uptake, an increased ischemic effect, and a more homogenous drug distribution due to decreased variability in delivery technique[15-19]. Use of small drug-eluting microspheres (DEM-TACE) and balloon occlusion

catheters (B-TACE) represent newer approaches to chemoembolization, albeit with less comparative data in HCC treatment at this stage[20-22]. A summary of these TACE approaches, as well as approaches, clinical strengths, and risks of the other locoregional therapies discussed here can be seen in Table 1.

Prognostic factors

The staging of HCC is particularly complex due to the varying presence of accompanying liver dysfunction. Prognostic factors for HCC patients undergoing non-surgical treatment have coalesced into several existing clinical staging systems to predict survival and adverse events. Examples include the Okuda staging system, Cancer of the Liver Italian Program staging system, Hong Kong Liver Cancer staging system, and Barcelona Clinic Liver Cancer (BCLC) classification scheme[23-25]. Other clinical indices which must be considered for prognosis include Albumin-Bilirubin and Model for End-stage Liver Disease[26]. Overall survival (OS) in HCC patients is most strongly related to performance status, tumor burden, hepatic reserve, and extrahepatic spread[23,27,28].

The most widely used prognostic tool in HCC is the BCLC[29,30], which has garnered international consensus endorsement for patient treatment stratification[31]. A treatment schematic for HCC based on BCLC classification is shown in Figure 1. The BCLC staging system matches liver dysfunction, tumor burden, and performance status to a recommended therapy[27,32]. Specifically, the BCLC utilizes Child-Pugh score and Eastern Cooperative Oncology Group (ECOG) status in addition to indicators of tumor burden. BCLC stratifies patients into five stages, categorized from stage 0 for "very early detection" to stage D for the most advanced disease cases.

Patients in BCLC stage 0 and BCLC stage A should generally undergo surgical resection if they are otherwise strong surgical candidates. In certain circumstances, TACE may be used as a bridge to surgery or as primary therapy when patients in these stages are non-candidates for surgery or ablation[12,33]. TACE is a first-line therapy recommendation for intermediate, unresectable HCC (BCLC stage B)[34]. Advanced disease (BCLC stage C) patients typically require systemic therapy, traditionally in the form of sorafenib or Lenvatinib[35]. More recently, combination atezolizumab and bevacizumab has gained endorsement as first-line therapy in the American Society of Clinical Oncology's 2020 clinical practice guideline on systemic therapy[36]. When local disease control is needed for advanced disease, TACE may be indicated for use alone or in combination with systemic therapy. A current phase III study is evaluating TACE in combination with Lenvatinib and Pembrolizumab for advanced, non-metastatic disease[37]. In addition to being useful in stratifying patients to appropriate treatment, BCLC class is a useful tool in prognosticating survival following TACE, with many studies stratifying survival based on these categories.

Despite the prevalent adoption of BCLC as the gold standard staging and management decision tool for HCC, other staging tools have been investigated and are utilized in certain situations. The AFP, BCLC, Child-Pugh, and Response (ABCR) score also predict retreatment success. Specifically, ABCR uses a baseline AFP over 200, more advanced BCLC stage, increase in Child-Pugh score of at least 2 from baseline, and absence of radiologic response to create a score range of -3 to +6, correlating with survival post-TACE retreatment. A score greater than or equal to 4 prior to a second TACE treatment prognosticates poor outcomes.

Prognostic factors may also indicate risks related to post-procedural complications. Post-embolization syndrome (PES) consists of post-procedural fever in the absence of infection, transaminitis, right upper quadrant pain, and nausea or vomiting. PES is a risk common to each of the transarterial therapies. Up to 80% of patients may experience a component of PES following TACE, however rates of serious clinical sequela of PES are much lower[38]. Most cases of PES resolve within 72 h. Features predictive of an increased risk of PES following TACE are tumor > 5 cm, multiple tumors, and technical considerations relating to procedure performance[39]. Other complications of TACE include acute hepatic failure, abscess, biloma, iatrogenic dissection, and acute cholecystitis. These more serious complications are reported at a combined rate of approximately 5% of cases[40,41].

Outcomes

In addition to typical outcomes measures in oncology - such as OS, progression-free survival (PFS), and time-to-progression (TTP) - tumor response outcomes are especially important in HCC due to the complexity of such patients who often undergo multiple therapies that can confound long-term outcomes. The most widely used tool for measuring tumoral response to treatment in HCC is the 2010 modified Response Evaluation Criteria in Solid Tumors (mRECIST)[42,43]. The mRECIST

Table 1 Locoregional therapy techniques, benefits, and risks

Locoregional modality	Techniques	Clinical advantages	Clinical risks
TACE	Drug-eluting beads or conventional delivery	Provides both local embolic and chemotherapeutic effect	PES, biloma, liver abscess, liver failure
TAE	Particulate or other embolic agents	Avoids radio and chemotoxicity; less expensive than other embolotherapies	PES, biloma, liver abscess, liver failure
TARE	Y^{90} microspheres	May be used in early disease with curative intent; intermediate disease can be used to increase FLV to qualify for curative intent surgery; best QoL scores of all options	PRS, RILD, radiation-induced pneumonitis, biloma, liver abscess, liver failure
Ablation	Radiofrequency current, microwaves, or cryoablation	Efficacious as monotherapy for early-stage disease; less morbidity than transarterial therapies	PAS, iatrogenic injury, bleeding

TACE: Transarterial chemoembolization; PES: Post-embolization syndrome; TAE: Transarterial embolization; TARE: Transarterial radioembolization; FLV: Functional liver volume; QoL: Quality of life; PRS: Post-radioembolization syndrome; RILD: Radiation-induced liver disease; PAS: Post-ablation syndrome.

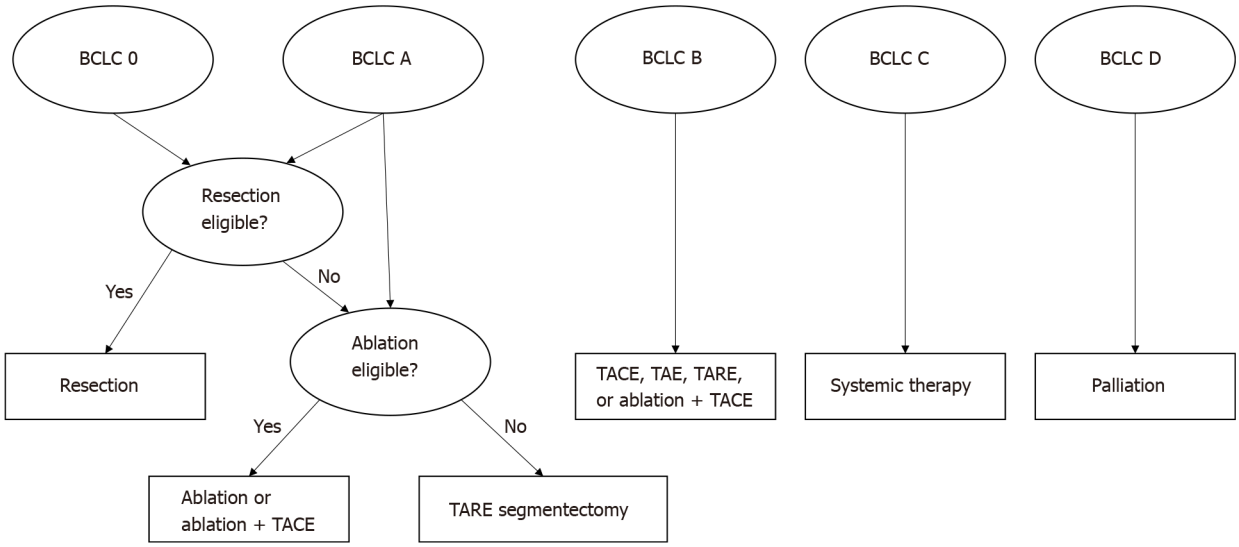

Figure 1 Hepatocellular carcinoma treatment algorithm based on Barcelona Clinic Liver Cancer-staging[13]. BCLC: Barcelona Clinic Liver Cancer; TARE: Transarterial radioembolization; TACE: Transarterial chemoembolization; TAE: Transarterial embolization.

treatment response tool builds on the traditional RECIST model of evaluating reduction in tumor size; however, because locoregional therapies induce devascularization and necrosis—and not always a reduction in size[44] - the American Association for the Study of Liver Diseases proposed mRECIST and the utilization of arterial enhancement measurements[45]. The effectiveness of these tools has been validated by a meta-analysis from Vincenzi *et al*[46]. More recently, three-dimensional imaging techniques have brought about more quantitative versions of these clinical response tools, namely volumetric RECIST and quantitative EASL (qEASL), which are being evaluated for efficacy and specific indications[47]. However, mRECIST remains the most widely used treatment response tool in practice.

Outcomes for both cTACE and DEB-TACE have consistently proven superior to conservative therapy[48,49]. Thus, as previously mentioned, TACE is to be considered for patients with advanced, unresectable disease who may not tolerate side effects of systemic therapy and who have acceptable hepatic function. This becomes especially important because systemic sorafenib has considerable toxicity, including diarrhea, weight loss, dermatitis, and hypophosphatemia[50]. Combination therapy using TACE with systemic sorafenib for both advanced and intermediate disease has been investigated. TACE induces ischemia which leads to the production of neoplastic angiogenic growth factors. The anti-angiogenic actions of sorafenib block these angiogenic factors. The GIDEON study[51] demonstrated through global observational data that patients given TACE concomitantly with sorafenib achieved better OS (21.6 mo) compared to

patients treated with sequential TACE then sorafenib (12.7 mo) and compared to patients treated with only sorafenib (9.7 mo). The concomitant treatment group's survival superiority was present across all BCLC stages. Importantly, the study calls attention to the need for further standardization of TACE technique as many centers reported logistical differences in treatment plans. The phase II SPACE trial[52] randomized intermediate stage HCC patients to DEB-TACE with sorafenib *vs* DEB-TACE with placebo treatment arms. Time to progression was similar in both treatment groups, and both options demonstrated adequate safety profiles. More recently, the phase II TACTICS trial showed a significant difference in PFS of TACE and sorafenib *vs* TACE alone (25.2 mo and 13.5 mo, respectively)[53]. Combination TACE and sorafenib also displayed significantly prolonged TTP.

TACE is also implicated in strategies for early-stage patients who need adjunctive procedures prior to surgery. In combination with portal vein embolization (PVE), TACE may induce contralateral liver hypertrophy to allow for tumor resection in patients with inadequate predicted future liver remnant (FLR). The utility of TACE in this setting is to decrease the risk of tumor progression during the period of time it takes PVE to induce FLR hypertrophy. A systematic review and meta-analysis concluded that TACE combined with PVE provided higher OS than PVE, portal vein ligation (PVL), or radioembolization of the portal vein alone[54]. Of patients receiving both TACE and PVE, 90% went on to receive resection. These strategies may be further explored to increase liver resection eligibility rates in the future. Bridging or downstaging patients for liver transplantation is another use for TACE in early-stage patients[5].

Several prospective studies comparing cTACE *vs* DEB-TACE have found no significant difference in OS, including one meta-analysis examining results from four randomized clinical trials and eight observational studies[48,49,55]. Beyond survival, the PRECISION-V trial demonstrated that in a subgroup of advanced HCC patients with Child-Pugh B, ECOG 1, bilobar, and recurrent disease, patients receiving DEB-TACE had higher rates of complete response, objective response, and disease control at 6 mo compared to patients receiving cTACE[48]. Concerning safety endpoints, DEB-TACE was originally theorized to provide fewer adverse events and a lower risk of post-embolization syndrome characteristics; however, the PRECISION-V trial found comparable 30-day adverse event incidence between the two groups[48]. Comparable safety profiles between cTACE and DEB-TACE were upheld through meta-analysis as well[55]. Observationally, DEB-TACE has displayed higher localized biliary injury rates and global hepatic damage[56]. In another randomized trial, Golfieri *et al*[49] found that DEB-TACE patients suffered less post-operative pain. The overall comparative safety and efficacy of cTACE *vs* DEB-TACE needs further exploration and likely has significant situational considerations which must be applied. Other areas of TACE outcomes that warrant further investigation include more rigorous standardization of cTACE protocols[57], the efficacy and considerations of TACE in portal vein thrombosis, and TACE for larger (> 5 cm) or multifocal lesions[58]. Primary outcomes for TACE, as well as the other forms of locoregional therapy discussed here, can be viewed in Table 2.

TAE

TAE is commonly referred to as "bland" embolization because the embolic particles lack additional chemical or radiation components. With this therapy, endovascular arterial occlusion induces hypoxia and subsequent death of tumor cells[59]. As with other transarterial therapies, optimal vascular catheter placement results in a maximally selective effect on tumor cells. Care must be taken to avoid arterial-venous shunts which could cause pulmonary arterial embolization with smaller particles. Embolic agents used have historically included gel foam, polyvinyl alcohol, and various drug-eluting beads[45]. The lack of chemotoxicity and radiotoxicity associated with this therapy means that tumoral response is primarily derived from a hypoxic cell death mechanism. It also means that the therapy may hold advantages in certain patients who have an especially high need to spare healthy liver parenchyma.

Prognostic factors

BCLC class B patients receive the most disease-control benefit from TAE, followed by class C patients[48]. Additionally, BCLC class A patients may undergo TAE to maintain eligibility for transplantation per the Milan criteria, a prognostic tool shown to improve 4-year OS in liver transplant when used for patient selection[60]. The Milan

Table 2 Summary of primary outcomes of locoregional therapies for hepatocellular carcinoma

Locoregional technique	Primary outcomes
TACE	TACE provides a survival benefit compared to supportive care in unresectable disease[34]. Concomitant TACE and sorafenib is superior to standalone therapy for unresectable disease[51-53]. Comparisons of DEB-TACE versus cTACE have yet to reveal significant differences in OS and short and long-term complication rates. Further studies are needed for considerations in more specific circumstances[48,49]. When combined with PVE, TACE provides more robust FLR increase and results in better survival compared to monotherapy strategies to enhance FLR[54].
TAE	TAE provides a survival benefit compared to supportive care in unresectable disease[34]. Early data of chemoembolization has shown little survival benefit over TAE, but superior proximate outcomes such as TTP and tumor response compared to TAE[63,64].
TARE	TARE shows similar complication and survival rates to TACE, while producing higher QoL scores and longer TTP[77,78]. TARE segmentectomy for early-stage disease (tumors < 3 cm) results in a 5-year survival of 75%, which is comparable to curative intent therapies such as transplantation and surgical resection[81]. TARE lobectomy provides a significant increase in FLR and is a safe mechanism to treat tumor while inducing contralateral hypertrophy[82-86].
Ablation	In early-stage patients, standalone percutaneous ablation produces comparable survival outcomes to surgical resection[113-116]. RFA and MWA techniques show similar outcomes in early-stage disease (tumor < 3 cm)[108,119]. Combination therapy using TACE and ablation (particularly MWA) provide the best outcomes for large tumors (tumor 3-5 cm)[120].

TACE: Transarterial chemoembolization; DEB-TACE: Drug-eluting bead chemoembolization; cTACE: Conventional transarterial chemoembolization; OS: Overall survival; PVE: Portal vein embolization; FLR: Future liver remnant; TAE: Transarterial embolization; TTP: Time-to-progression; TARE: Transarterial embolization; QoL: Quality of life; RFA: Radiofrequency ablation; MWA: Microwave ablation.

criteria consists of one lesion less than 5 cm or up to three lesions less than 3 cm, no evidence of extrahepatic manifestation, and no evidence of vascular invasion. Similarly, TAE is an option for downstaging of BCLC class B patients for transplantation. TAE is contraindicated in patients with severe comorbidities or with poorly preserved liver function as evidenced by Child-Pugh scores of B8 or higher, untreated esophageal varices, elevated markers of liver function, creatinine clearance < 30 mL/min, and high tumor burden[61].

Prognostically, a key advantage of TAE relative to other transarterial options is its gentler impact on short-term adverse events, possibly due to the avoidance of chemotherapy toxicity. As with other embolotherapies, bland embolization poses risk for PES. Agrawal *et al*[62] reported a higher incidence of PES among patients undergoing TACE (74.7%) compared to TAE (68.7%). PES following TACE resulted in a significantly longer hospital stay than PES following TAE (1.47 d *vs* 1.12 d). This observational study further identified that, in addition to the PES risk factors mentioned in the previous section, more patients who were female or who had alcohol-related HCC developed PES.

Outcomes

Like other embolotherapies, TAE offers a survival benefit compared to conservative treatment. Llovet *et al*[34] found that compared to best supportive care, repeated administration of either TACE or TAE showed a survival benefit in patients with unresectable HCC. While TACE provided even higher survival probabilities than TAE in the study, both therapies performed better than conservative treatment. Comparison of bland embolization and chemoembolization is an ongoing focus of research. Despite some data suggesting TACE's superiority to TAE, multiple studies investigating bland embolization compared to either cTACE or DEB-TACE have failed to demonstrate significant differences in OS between the two[63-65]. Importantly, much of the early data accumulated on the comparative efficacy of TACE and TAE was collected during a period of evolving indications and chemotherapeutic protocols for TACE. Furthermore, the development of DEB-TACE may continue to influence overall comparisons of bland embolization and chemoembolization.

Some studies have shown benefits to TACE compared to TAE in more proximate outcomes such as TTP, tumor recurrence, and local response. For example, a trial by Meyer *et al*[65] found insignificant differences in median OS and PFS among TAE and cTACE, but found a significantly greater mRECIST treatment response in cTACE *vs* TAE (47.3% *vs* 67.4% respectively). However, because several studies have shown TAE performing comparably to TACE in terms of survival, and because TAE lacks utilization of chemotherapy particles, TAE may be better tolerated in HCC patients with borderline liver function[45].

TARE

TARE, also referred to as selective internal radiotherapy (SIRT) and Y^{90}, uses a radioisotope form of yttrium to selectively target tumor cells *via* the hepatic arterial tree[66]. The Y^{90} radioisotope is delivered using microspheres and, once reaching target tissue, undergoes beta decay to locally irradiate the tumor in a continuous, low-dose fashion over a fourteen-day period. TARE is considered a two-stage treatment process because a planning arteriography must be performed one to two weeks before the radiation-delivering procedure[67]. This planning stage helps to differentiate tumor and hepatic arterial supply, isolate the future path of radiation delivery *via* embolization of extrahepatic vessels at risk of nontarget microsphere delivery, and identify the degree of hepatopulmonary shunting[68]. Technetium-99m labeled macroaggregated albumin is combined with single photon emission computed tomography imaging technology to provide imaging for this stage[69]. After TARE, there is a longer wait time compared to other embolization techniques until the treatment effect is fully realized, with therapy response imaging taking place 3-6 mo following the procedure[70].

Prognostic factors

Characteristics most predictive of post-TARE prognosis are extrahepatic disease, baseline BCLC stage, ECOG performance status, and tumor burden[71]. In a multicenter study, Sangro *et al*[72] analyzed a cohort of 325 patients undergoing TARE. Median OS was strongly influenced by BCLC staging (BCLC A 24.4 mo; BCLC B 16.9 mo; BCLC C 10.0 mo). Other significant predictors of superior survival following TARE were ECOG performance status (ECOG 0), Child-Pugh class (A), absence of ascites, baseline total bilirubin (< 1.5), number of tumor nodules (< 5), alpha-fetoprotein level (< 400), patent portal vein, single lobe disease, and absence of extrahepatic disease.

Understanding the influence of the pre-procedural disease stage on post-TARE survival is important because TARE maintains indications across the spectrum of HCC severity. Patients with advanced disease may benefit from the local tumor control and palliative effects of TARE. TARE is an acceptable treatment alternative to TACE for first-line therapy for BCLC class B patients[73]. Early-stage patients in BCLC classes 0 and A may benefit from TARE radiation segmentectomy. Early-stage patients may also benefit from TARE lobectomy in an attempt to either downstage for transplantation or induce contralateral hypertrophy for future resection.

Common complications of TARE are well characterized. Radiation-induced liver disease (RILD) is an adverse event unique to TARE among the locoregional therapies. RILD involves an extensive array of local vascular, fibroblastic, and parenchymal change[74]. Risk of RILD may be increased by gemcitabine, which must be held for four weeks prior to the procedure[75]. Padia *et al*[76] report the overall risk of RILD following TARE to be 1%-4%. They also report rates of other common adverse events, including GI ulcers (0%-5%), PES requiring extended hospitalization or readmission (1%-2%), iatrogenic dissection (1%), and death within 30 d (2%). There was a less than 1% reported rate for radiation-induced skin-injury, radiation pneumonitis, radiation-induced pulmonary fibrosis, biloma requiring drainage, and abscess. A hepatopulmonary fraction above 20% predicts an increased likelihood of radiation pneumonitis. Relative contraindications to TARE include an elevated baseline bilirubin level (> 2 mg/dL), an elevated hepatopulmonary fraction (> 20%), Child-Pugh class C, ECOG score over 2, significant transaminitis (ALT or AST > 5x upper limit of normal), and total tumor burden over 70% of the liver or total tumor burden over 50% with a high number of nodules[12]. Of these variables, elevated baseline bilirubin and increased tumor burden have been shown to decrease OS[18].

Outcomes

TARE appears to have comparable complication and survival rates to TACE. The SIRTACE trial compared TARE *vs* TACE in unresectable HCC and found that a single TARE session was as safe and produced a better quality of life (QoL) change than multiple TACE sessions[77]. More recently, the PREMIERE trial revealed a significantly longer median TTP for patients receiving TARE (> 26 mo) compared to cTACE (6.8 mo). A smaller randomized trial compared QoL measures between TACE and TARE, finding that patients treated with TARE had improvements in QoL despite being treated for more severe disease than the TACE cohort. In contrast, the TACE cohort had worsened QoL post-procedurally[78]. Both TACE and TARE are being investigated to identify the optimal transarterial therapy for downstaging tumors for

transplantation eligibility. Lewandowski *et al*[70] compared triple-drug cTACE to TARE in their ability to downstage tumor size from UNOS T3 to UNOS T2 to achieve eligibility. TARE significantly outperformed cTACE in rates of T2 achievement, event-free survival, and OS.

In addition to sustaining or achieving transplantation eligibility, TARE is also useful in early-stage disease for its ability to act as primary therapy in certain circumstances. TARE's effectiveness in early-stage disease is in part due to its evolution into more selective indications for earlier tumors *via* radiation segmentectomy[79]. First described in 2011[80], TARE segmentectomy is an alternative option in non-surgical candidates whose tumor anatomy discourages ablative techniques due to nearby high-risk structures[33]. For example, in a retrospective study, Lewandowski *et al*[81] analyzed 70 patients with similar inclusion criteria with the additional exclusion of patients who received secondary surgery. Median OS in this cohort was found to be 6.7 years with a median TTP of 2.4 years. The cohort had comparable five-year OS (75%) and response rates to other curative-intent treatments like ablation, resection, and transplantation.

Similar to segmentectomy, radiation lobectomy is a relatively novel application of TARE. Its primary use is to treat the tumor-occupied lobe while inducing hypertrophy of the contralateral lobe, thus increasing the FLR in patients who were deemed unresectable due to low FLR[12]. Scarring of the treated lobe slowly creates a shunting of blood to the contralateral portal vein and, over time, induces hypertrophy of that lobe[82]. Multiple observational studies show that TARE lobectomy increases the FLR by an approximate average of 30% from baseline[83-86]. In contrast to TACE treatment to increase FLR, TARE lobectomy does not require concomitant PVE. A comparison of standalone PVE and TARE lobectomy by Garlipp *et al*[84] has proven that, while both display significant increases in FLR, PVE does it more effectively at the 6-wk mark (61.5% *vs* 29%). Issues have been raised with this measurement, however, as PVE has been shown to increase FLR quicker than radiation lobectomy[86], and as some evidence suggests that PVE may actually induce mild growth of existing tumor tissue [87]. The safety of using radiation lobectomy as a strategy to qualify for and subsequently undergo resection was demonstrated in a prospective cohort studied by Gabr *et al*[88]. Among 25 patients receiving major hepatic resection and 6 patients receiving partial hepatectomy, a range of perioperative outcomes following resection were comparable to resection-only outcomes. Survival rates at one and three years was reported at 96% and 86%, respectively.

TARE is also being compared to sorafenib as monotherapy in advanced disease. The phase III SARAH trial[89] randomized 467 patients with intermediate-stage, unresectable HCC to either sorafenib or TARE. Median OS and median PFS were comparable; however, TARE showed significantly fewer treatment-related adverse events, higher QoL scores, and a higher treatment response rate than sorafenib. The SIRveNIB trial[90] was another phase III study that failed to show significant differences in survival between TARE and sorafenib, but which also demonstrated the improved toxicity profile of TARE. Further trials are needed to better power subgroup analyses of TARE *vs* sorafenib and define specific patients who may see the improved tumor response of TARE translated into improved survival over sorafenib[91]. TARE is also useful in advanced disease because it has relatively less embolic activity compared to other transarterial therapies. This becomes useful in the setting of portal vein thrombus (PVT) of unresectable HCC patients. A retrospective study of HCC patients with PVT compared OS between those treated with TARE and those treated with sorafenib. TARE led to significantly longer median OS (26.2 mo) than sorafenib (8.7 mo)[92].

TARE and sorafenib combination therapy for patients ineligible for TACE but with BCLC classes B and C disease was investigated in the SORAMIC trial[93]. Again, no significant differences in median OS were found; however, survival benefit was found with combination therapy among patients without cirrhosis, with cirrhosis of nonalcoholic etiology, and in patients younger than 65 years of age. The phase III STOP-HCC trial is a larger study. It is expected to evaluate further what specific subset of patients may benefit most from combination therapy with TARE and systemic therapy. It is expected to be complete in September 2022[94]. Further, with the development of additional effective systemic therapies for HCC including immuno-therapy and most recently, atezolizumab/bevacizumab, the combination of TARE with these agents is a potential area of synergy and an active area of clinical investigation[95,96].

ABLATION

Generally, ablation is recommended for early-stage, small tumors (up to 3 cm) in patients who would otherwise qualify for resection but are considered unsuitable candidates for surgery[73]. Percutaneous ablative techniques were originally centered around ethanol injection (PEI), however this has fallen out of favor when a patient is a strong candidate for more contemporary ablative techniques[10]. Today, commonly used ablative techniques in the setting of HCC include microwave ablation (MWA), radiofrequency ablation (RFA), and cryoablation (CA). RFA utilizes a radiofrequency electrode to deliver an alternating electric current (460-500kHz) to the target lesion. Frictional heating, necrosis, and cell death ensue. MWA utilizes a common final cell death pathway involving local heating and eventual cell death, however MWA heats tissue *via* an oscillating microwave field (915/2450 MHz). The properties of microwaves result in reduced heat sink effect compared to RFA. RFA has the ability to decrease unwanted energy delivery to nearby structures compared to MWA[97]. However, due to less heat-sink, MWA may perform better near large vessels, in patients with comparatively larger tumors (between 3 cm and 5 cm), and in patients with multiple nodule disease[98]. CA relies on argon and helium gasses to rapidly alternate freezing and thawing of local tissue and vascular structures[99]. CA is not as commonly used due to the complication profile[100]. Laser ablation and irreversible electroporation are two examples of newer therapies still under investigation[10].

Prognostic factors

Prognostic factors for ablative therapy follow general prognostic patterns for HCC. Across multiple studies examining prognostic factors of RFA, survival has been consistently and independently predicted by Child-Pugh classification, tumor size, and tumor number[101-104]. Long-term survival following MWA is predicted by similar factors. Three-year PFS following MWA can range from 27% to 91.7%, with heavy influence from the above clinical characteristics[105]. Prognostic factors for combination therapy of MWA with TACE were well characterized by Ni *et al*[106] Predictably, adjusted prognostic factors associated with better OS rates of MWA with TACE combination therapy were earlier BCLC stage, smaller tumor size, lack of portal vein thrombus, MWA therapy times, and targeted drug usage.

Complication risk of biloma following percutaneous ablation may be predicted by comparatively large lesions situated closer to major bile ducts or near the hilum[107]. Additional characteristics prognostic of increased complication risk include tumor volume, ablated tissue volume, multiple tumors, and Child-Pugh class B or above [108]. In general, however, ablation carries less morbidity than other curative therapies due to its less invasive nature, coagulative properties related to heating tissue, liver preservation, and shorter hospital stay[109,110]. Bertot *et al*[111] found a pooled major complication rate of 3.29% for RFA, PEI, and MWA across 34 randomized trials and observational studies.

A complication unique to ablation among the locoregional therapies is post-ablation syndrome (PAS). PAS is a transient, flu-like illness which may occur about three days following ablation and lasts an average of five days. PAS occurs in roughly 25%-35% of patients undergoing ablation and is correlated with the volume of liver tissue treated. Pre-procedural tumor volume and post-procedural rise in AST are predictive of an increased likelihood of PAS[112].

Outcomes

Studies have demonstrated comparable survival *via* ablation *vs* surgery in early-stage HCC, despite ablative patients usually having poorer baseline hepatic function. In 2006, percutaneous thermal ablation was compared to resection in a randomized trial of 105 patients[113]. Results showed nonsignificant differences in complete tumor elimination rates, time to first recurrence, and disease-free and OS rates at 1, 2, and 3-year follow-ups. More recently, Fang *et al*[114] showed through a randomized trial of 120 patients that RFA had a similar complete remission rate (95%) to surgical resection (96.7%) and similar disease-free and OS rates at years 1, 2, and 3 follow-ups. In addition, RFA demonstrated significantly better hepatic function at day-7 post-treatment and fewer post-operative complications. The trend of comparable ablative survivability does not always extend to patients meeting Milan criteria. A trial that randomized HCC patients meeting Milan criteria to either RFA or surgical resection found significantly lower OS and recurrence-free survival along with higher overall recurrence among RFA patients[115]. As shown by the STORM trial, curative-intent ablation is best as a standalone therapy, without the addition of adjuvant sorafenib

following the procedure[116]. CA has largely fallen out of favor due to the severity of complications despite similar performance to other ablative techniques. A meta-analysis including a total of 433 total HCC patients revealed significantly fewer complications and less local tumor recurrence in RFA compared to CA[117].

As mentioned previously, the two ablative techniques most common in current practice are RFA and MWA, which are a source of ongoing outcomes comparison[118] and which feature similar curative-intent indications in early-stage disease (up to three tumor nodules smaller than 3 cm with the absence of extrahepatic disease) and similar complication rates[108]. A meta-analysis looking at seven studies comparing RFA and MWA found comparable rates of complete response, local recurrence, major complications, and 3-year survival[119].

HCC tumors between 3-5 cm fall outside the purview of curative-intent ablation but may still be addressed by combination therapy with TACE and ablation[9]. Comparison of the best combination therapies for specific indications in this population is ongoing. For example, Sheta *et al*[120] compared cTACE alone, combined MWA with cTACE, and combined RFA with cTACE in a clinical trial of 50 patients with nonresectable, single-lesion HCC greater than 4 cm. They found the highest success rates in the combined MWA with the cTACE group and the lowest success rates in the cTACE alone group. Whereas combination therapy of TACE with MWA may be indicated for 3-5 cm HCC, combination therapy of TACE with RFA may serve a role in the treatment of early-stage HCC. Kim *et al*[121] found that combined cTACE with RFA provided decreased local tumor progression and better PFS at 1, 3, and 5-year follow-ups. OS at follow-up intervals, however, was similar.

FUTURE DIRECTIONS

The future of HCC therapy will likely rely on a combination of the current proven standards of care and several other promising areas of innovation. One such promising area of growing evidence for HCC is immunotherapy. In addition to its use as a second-line monotherapy agent, immunotherapy may augment the effects of sorafenib and locoregional therapy in HCC. Locoregional therapies produce an immune response that can be augmented *via* immune checkpoint inhibition. Given that prognosis in HCC is correlated with T-cell tumoral infiltration[122], potentiation of both tumoral and locoregional therapy-induced T-cell response could improve outcomes. In an early-phase trial, Duffy *et al*[123] safely treated HCC with tremelimumab (anti-CTLA4) combined with ablation and showed that the post-procedural immune response could be enhanced. The effects of combined anti-PD1 inhibitors and TARE or TACE are also being evaluated[124]. The ongoing phase II DEMAND trial evaluates first-line combination therapy of systemic anti-angiogenic and immunotherapy while reserving TACE as second-line therapy[125].

As with immunotherapy, precision medicine has potential to create a paradigm shift in the way HCC patients are treated. By relying on big data and genomics to personalize clinical care, precision medicine will allow further customization of HCC treatment plans across the spectrum of therapeutic modalities, based on an individual's genetic mutations, local tumor environment, and further stratification of many clinical factors already in use today. Precision medicine will likely feature small molecule inhibitors, epigenetic regulators, and monoclonal antibodies specific to an individual's disease. A number of these agents are currently being evaluated for both safety and efficacy in advanced disease[7]. The success of these agents in treating HCC is reliant upon accurate characterization of multiple carcinogenic molecular pathways - including mutations to TERT, Wnt/ß-catenin, P53, Akt/mTOR, VEGFR, and EGFR genetic pathways. Ideally, molecular therapy will target multiple genetic pathways within the same patient and will be combined with other therapies such as locoregional treatment to optimize OS.

As treatments such as immunotherapy and molecular therapy evolve and become integrated with current standards of care, further prognostic sophistication is a priority for immediate improvement of care. This growth is already underway. In some cases, pre-procedural evaluation of inflammatory markers may provide prognostic information for HCC patients treated with locoregional therapy. A meta-analysis of 22 studies showed poorer OS following TACE in HCC patients with higher C-reactive protein levels, neutrophil to lymphocyte ratio, and platelet to lymphocyte ratio[126]. This is in line with the general understanding of inflammation being tumor-protective. More research is needed to routinely integrate inflammatory markers into the larger prognostic landscape of clinical staging systems for locoregional HCC

treatments.

With the numerous advancements within HCC treatment, outcomes research must continue to be robust. With the need for increased outcomes research related to locoregional therapies comes a call for increased reporting of randomized controlled trial data. Grégory *et al*[127] found that nearly two-thirds of RCTs conducted regarding HCC treatment with TACE did not yield public results. This highlights the importance of increased data reporting as evidence and indications behind various locoregional therapies for HCC continue to mature. For the care of HCC patients to continue to improve, and for future directions of care such as personalized medicine and immunotherapy to flourish, high-quality outcomes data must be generated and distributed throughout the field.

CONCLUSION

HCC is the most common primary liver malignancy[2] and carries a 5-year survival rate under 20%. Organ transplant availability and eligibility is limited, and fewer than 20% of HCC patients are candidates for surgical resection. For the remainder of patients with HCC, liver-directed, locoregional therapies serve a growing purpose across a spectrum of disease stages. Transarterial and ablative procedures are involved in treatment for curative-intent, disease control, bridging to surgery, downstaging for future treatment, and palliation. In addition to bland embolization, TAE techniques with locoregional delivery of radioactive or chemotherapeutic microspheres offer survival benefits in appropriately selected patients. Microwave and radiofrequency ablative techniques offer comparatively less morbidity and curative results in select early-stage patients. Multiple indications exist for various locoregional therapies in the adjunctive realm of transplantation, resection, and systemic therapy. Prognostic considerations for locoregional therapies vary by indication but generally follow baseline disease staging and tumor quantification. Outcomes data reveal that locoregional therapies provide survival benefits in appropriately selected patients. New advances in precision medicine, combination therapy, and immunotherapy are being investigated and have potential to augment available treatment strategies.

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MINIREVIEWS

Recent advances in artificial intelligence for pancreatic ductal adenocarcinoma

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Abstract

Pancreatic ductal adenocarcinoma (PDAC) remains the most lethal type of cancer. The 5-year survival rate for patients with early-stage diagnosis can be as high as 20%, suggesting that early diagnosis plays a pivotal role in the prognostic improvement of PDAC cases. In the medical field, the broad availability of biomedical data has led to the advent of the "big data" era. To overcome this deadly disease, how to fully exploit big data is a new challenge in the era of precision medicine. Artificial intelligence (AI) is the ability of a machine to learn and display intelligence to solve problems. AI can help to transform big data into clinically actionable insights more efficiently, reduce inevitable errors to improve diagnostic accuracy, and make real-time predictions. AI-based omics analyses will become the next alterative approach to overcome this poor-prognostic disease by discovering biomarkers for early detection, providing molecular/genomic subtyping, offering treatment guidance, and predicting recurrence and survival. Advances in AI may therefore improve PDAC survival outcomes in the near future. The present review mainly focuses on recent advances of AI in PDAC for clinicians. We believe that breakthroughs will soon emerge to fight this deadly disease using AI-navigated precision medicine.

Key Words: Pancreatic cancer; Pancreatic ductal adenocarcinoma; Artificial intelligence; Machine learning; Precision medicine

Core Tip: Pancreatic ductal adenocarcinoma (PDAC) remains the most lethal type of cancer. Artificial intelligence (AI) is the ability of a machine to learn and display

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intelligence to solve problems. AI can help to transform big data into clinically actionable insights more efficiently, reduce inevitable errors to improve diagnostic accuracy, and make real-time predictions. AI-based omics analyses should be the next alternative approach to improve survival outcomes in PDAC by discovering biomarkers for early detection, molecular/genomic subtyping, treatment guidance, and predicting recurrence and survival. The present review mainly focuses on recent advances of AI in PDAC for clinicians.

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INTRODUCTION

Pancreatic ductal adenocarcinoma (PDAC) stands the most life-threatenning type of cancer[1]. The recent 5-year survival rate for PDAC in all stages is 8.5% according to American Cancer Society statistics 2017. In patients with early-stage diagnosis, the 5-year survival rate for can be as high as 20%. During the past ten years, median overall survival (OS) has improved from 22.1 mo to 35 mo in resectable PDAC, considerably owing to improvements in adjuvant therapies[2-5]. These findings suggest that early diagnosis plays a pivotal role in the prognostic improvement of PDAC cases. Furthermore, the high recurrence rate, even in patients who have undergone curative resection, and chemoresistance to the current systemic chemotherapies (FOLFIRINOX: 5-fluorouracil, folinic acid, irinotecan, and oxaliplatin; and GnP: Gemcitabine plus nab-paclitaxel)[6,7] are major issues. Based on recent advances in genetic analysis, PDACs have been divided into several molecular subtypes[8-11], which is a prelude of precision medicine. Genetic and molecular profiling researches have revealed that up to 25% (range 12%-25%) of PDACs maintained actionable molecular alterations. Actually, matching to relevant molecular-specific treatments improves the OS compared to that of those without actionable mutations or those who do not receive molecular-specific therapy[12]. The comprehensive biomedical data has led to the dawn of the "big data" era in the medical field[13].

To overcome this deadly disease, how to well utilize big data is a next step for physicians and researchers physicians in the era of precision medicine. The main issue for physicians has shifted from gathering data to competently analyzing it. Artificial intelligence (AI) is the ability of a machine to learn and display intelligence to solve problems (Figure 1)[14]. An artificial neural network (ANN) can imitate the human neural meridian system. It is divided into three parts: Input layer, hidden layer, and output layer. "Deep learning" refers to an ANN with multiple hidden layers. Machine learning helps researchers spend less time on data processing. The processess for employing machine learning generally contain the following: Gathering the basic data, separating the data into an experimental group and a verification group, buildinging a screening and processing model, inputting the experimental group data into the model, accounting the output results, and confirming the model's workability using the verification group. The verification group can be employed to examine the sensitivity and specificity of the experimental group, while the experimental group can fabricate more intelligent model. An overview of types in AI is provided in the Supplementary material. Chen *et al*[15] developed a survival prediction model of non-small cell lung cancer patients through the use of ANN. AI has also been applied to tackle and manage the recent coronavirus disease 2019 crisis in many areas, including screening, diagnosis, severity stratification, mortality prediction, and epidemiology controls[16].

In the age of precision medicine, AI can support to convert big data into clinically actionable perception more conveniently, reduce the inevitable errors to improve diagnostic accuracy, and make real-time predictions[17,18]. Due to latest breakthroughs, the demand of AI in cancer treatment has been swiftly increasing, including for PDACs[19-21]. Recent studies have demonstrated considerable potential for AI application in PDAC care (Table 1). Advances in AI for PDACs may thus be the

Table 1 Comprehensive list of artificial intelligence-based investigations in pancreatic ductal adenocarcinoma

Ref.	Modality	Type of algorithm	Sensitivity (%)	Specificity (%)	ROC-AUC (or accuracy %)
PDAC risk prediction					
Boursi *et al*[25], 2021	7 clinical variables	Logistic regression	66.53	54.91	0.71
Appelbaum *et al*[29], 2021	18 risk factors	Logistic regression	NA	NA	0.71
Muhammad *et al*[30], 2018	Personal health data (18 features)	ANN	80.7	80.7	0.85
Hsieh *et al*[28], 2018	ICD-9 code	Logistic regression	NA	NA	0.727
Boursi *et al*[26], 2017	10 clinical variables	Logistic regression	44.7	94	0.82
Cai *et al*[27], 2011	5 clinical variables	Logistic regression	NA	NA	0.72
Early diagnosis of PDAC					
Zhang *et al*[34], 2020	Nine-gene signature	Support vector machine	98.65	100	93.3
Zhang *et al*[83], 2020	CT	DCNN	83.76	91.79	0.9455
Si *et al*[42], 2021	CT	Fully end-to-end deep learning	86.8	69.5	0.871
Liu *et al*[54], 2020	CT	CNN	79 (United States)	97.6 (United States)	0.920 (United States)
Ma *et al*[84], 2020	CT	CNN	98.2	91.6	95
Chu *et al*[85], 2019	CT	Deep learning (details are NA)	94.1	98.5	NA
Liu *et al*[53], 2019	CT	CNN	NA	NA	0.9632
Tonozuka *et al*[86], 2021	EUS	CNN	90.2	74.9	0.924
Ozkan *et al*[87], 2016	EUS	ANN	83.3	93.3	87.5
Săftoiu *et al*[88], 2015	EUS	ANN	94.64	94.44	NA
Zhu *et al*[63], 2013	EUS	Support vector machine	92.52	93.03	NA
Zhang *et al*[62], 2010	EUS	Support vector machine	94.32	99.45	NA
Das *et al*[61], 2008	EUS	ANN	93	92	0.93
Săftoiu *et al*[89] 2008	EUS elastography	NN	91.4	87.9	89.7
Norton *et al*[60], 2001	EUS	NN	73	NA	83
Alizadeh Savareh *et al*[40], 2020	Circulating microRNA signatures	PSO + ANN + NCA	93	92	93
Urman *et al*[90], 2020	Bile juice	NN	88	100	0.98
Pancreatic fistula after pancreaticoduodenectomy					
Kambakamba *et al*[71], 2020	CT	*k*-NN, random forest classifier, etc	96	98	0.95
Mu *et al*[72], 2020	CT	CNN	86.7	87.3	0.89
Pathological tumor response to neoadjuvant chemotherapy					
Watson *et al*[80], 2020	CT and CA19-9	CNN	NA	NA	0.785
Survival model					
Zhang *et al*[77], 2020	CT	CNN	NA	NA	11.81% in IPA
Alizadeh Savareh *et al*[40], 2020	Circulating microRNA signatures	PSO + ANN + NCA	NA	NA	NA
Kaissis *et al*[66], 2019	MRI	Random forest	87	80	0.90
Walczak *et al*[79], 2017	14 clinical variables	ANN	91	38	0.6576

Molecular subtype					
Kaissis *et al*[68], 2020	CT	Random forest	84	92	0.93
Tumor subtype (QM *vs* non-QM)					
Kaissis *et al*[67], 2019	MRI	Gradient boosting decision tree	90	92	0.93
Molecular subtype (KRT81 positive *vs* negative)					
Microsatellite instability status					
Li *et al*[19], 2020	PreMSIm (15-gene signature)	*k*-NN	85	97	95

AI: Artificial intelligence; PDAC: Pancreatic ductal adenocarcinoma; NA: Not available; ROC-AUC: Area under the receiver operating characteristic curve; ICD-9: International Classification of Diseases 9th Revision; ANN: Artificial neural network; CT: Computed tomography; DCNN: Deep convolutional neural network; EUS: Endoscopic ultrasound; NN: Neural network; CA19-9: Carbohydrate antigen 19-9; IPA: Index of prediction accuracy; MRI: Magnetic resonance imaging; QM: Quasi-mesenchymal; PSO: Particle swarm optimization; NCA: Neighborhood components analysis; *k*-NN: *k*-Nearest neighbor.

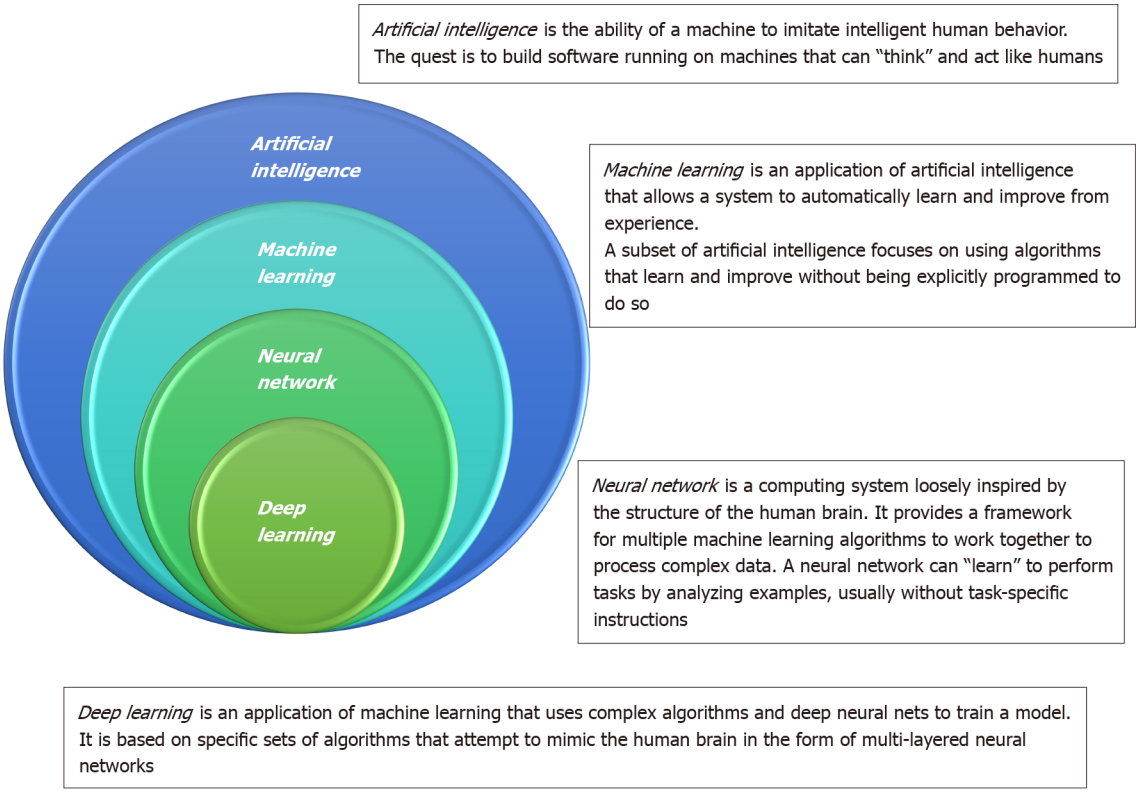

Figure 1 Differences among artificial intelligence, machine learning, neural network, and deep learning.

alternative stream to improve survival outcomes for this deadly disease. The present review mainly focuses on recent advances of AI in PDAC care for clinicians.

PDAC RISK PREDICTION BY AI

The radiographic traits of unoperability and the appearance of symptoms of PDAC occur concurrently[22]. At the time of diagnosis, only a small part of patients (< 15%) have surgically resectable state[22]. In addition, identification of individuals at high risk for PDAC or with early stage is hard due to the absence of trusty screening tools, the lack of clinically relevant biomarkers, and low prevalence[22]. No established screening strategy has been introduced for sporadic PDAC. It is estimated that symptoms manifest about 6 mo after PDAC becomes unresectable[22]. Identifying individuals at high risk but asymptomatic is crucial for finding PDAC while it is still

resectable.

Approximately 50% of all patients with PDAC develop diabetes mellitus prior to their diagnosis[23,24]. Screening patients with new-onset diabetes may enable earlier diagnosis of PDAC. In pre-diabetic and new-onset diabetic patients, an AI-based prediction model of PDAC risk has been developed[25,26]. In a pre-diabetic study, 245 of 138232 patients with impaired fasting glucose were thereafter diagnosed as having PDAC within 3 years of impaired fasting glucose detection. The AI (logistic regression model)-based prediction model consisted of age, body mass index, PPIs, total cholesterol, low-density lipoprotein, alanine transaminase, and alkaline phosphatase [25]. This model achieved an area under the curve (AUC) of 0.71. Furthermore, by analyzing 109,385 new-onset diabetic patients including 390 PDAC cases, a multivariable prediction (logistic regression) model that included age, smoking, body mass index, change in body mass index, usage of proton pump inhibitors and anti-diabetic medications (insulin, oral hypoglycemic except metformin, and metformin), as well as levels of hemoglobin, hemoglobin A1C, creatinine, cholesterol, and alkaline phosphatase, was established (AUC, 0.82)[26]. Among these diabetic patients, 390 (0.4%) were diagnosed with PDAC within 3 years. If the predicted risk threshold for definitive PDAC screening was set at 1% over 3 years, only 6.19% of the new-onset diabetes cases would undergo definitive screening, which could identify PDAC cases with 94.0% specificity, 44.7% sensitivity, and a positive predictive value of 2.6%[26].

Cai *et al*[27] established a PDAC risk prediction model by analyzing 138 chronic pancreatitis patients with focal mass lesions. A scoring method based logistic regression was employed to build the prediction model, which included five variables: sex, mass number, mass location, bilirubin, and carbohydrate antigen 19-9 (CA19-9) (AUC, 0.72). Hsieh *et al*[28] predicted PDAC in patients with type 2 diabetes using ICD-9 code data by logistic regression and ANN models. The AUCs achieved by these models were 0.72[27] and 0.73[28], respectively.

Appelbaum *et al*[29] used a logistic regression model and developed a prediction model of PDAC using electronic health record data. A total of 18 risk factors (*i.e.*, age, gender, race, abdominal pain, angina pectoris, asthma, atherosclerotic heart disease, calculus gallbladder, chest pain, chronic pancreatitis, coronary heart disease, diabetes mellitus, emphysema, essential hypertension, family history pancreatic cancer, jaundice, stroke, and ulcer) were used to weigh the risk factors, and their prediction model displayed an AUC of 0.71. Their risk model based on patients' prior diagnoses derived from electronic health record data would predict PDAC 6-12 mo before an eventual diagnosis date. Such a risk score could be employed as an initial screening prior to additional biomarkers or genetic testing, to pick out individuals from the general population for closer surveillance.

Muhammad *et al*[30] used the ANN model to focus on the early prediction and stratification of PDAC risk based on personal health data (800114 answers in the National Health Interview Survey and Pancreatic, Lung, Colorectal, and Ovarian cancer datasets, including 898 cases diagnosed with pancreatic cancer) before symptoms appear. The prediction model using 18 personal health features produced a specificity of 80.7%, a sensitivity of 80.7%, and an AUC of 0.85 to predict PDAC[30]. Furthermore, the model based solely on personal health data was able to divide individuals into low, medium, and high cancer risk. Identification of high-risk individuals who would benefit from tailored screening may increase the probability of detecting early PDAC. While logistic regression was used to develop risk prediction models in many previous studies, Muhammad *et al*[30] employed an ANN model based on personal health big data and produced the highest AUC in the prediction model of PDAC risk.

Such prediction models using AI will be beneficial for clinicians to estimate the PDAC risk of their patients easily after inputting their data. These models can be combined into an electronic medical record system or be available on portable devices such as tablets and mobile phones. They may also be useful for primary care physicians to stratify individuals into various risk categories. By such PDAC risk stratification, higher-risk individuals can be referred to a diagnostic department for more intensive and tailored assessments. More data and testing will be required to refine the performance of the AI-based prediction model of PDAC in order to facilitate its application in the clinical setting. An AI-based prediction model using clinical variables is non-invasive, cost-effective, and easy for early diagnosis of PDAC. Using AI to recognize signs in early PDAC and precancerous lesions is one of the key strategies to improving survival.

DETECTION OF EARLY PDAC BY BIOMARKERS USING AI

It is highly desirable to identify an effective PDAC diagnostic biomarker. Currently, the most widely employed biomarker for early PDAC detection is CA19-9, however it is not an perfect because of its comparatively low level of specificity and sensitivity (70% with a 5% error rate, for diagnosis of PDAC)[31,32]. Several molecular elements such as CA19-9, CEA, DUPAN, and Span-1 have been employed as biomarkers for diagnosis of pancreatic tumors[33], but none of them are sufficiently specific and sensitive to clearly distinguish cancer from healthy or benign diseases. Therefore, a solid tool with sufficient specificity and sensitivity is required to enable early PDAC diagnosis. Zhang *et al*[34] designed a novel AI (support vector machine) method based on relative gene expression ranking within tissue samples using the microarray gene expression data and RNA-seq data collected from two databases, GEO and TCGA. Zhang *et al*[34] then identified a qualitative diagnostic signature comprising 9 gene pairs (16 genes), that could distinguish PDAC (using expression profiles from PDAC and adjacent normal tissues) patients from non-PDAC (pancreatitis and normal tissues) and was a useful biomarker for early detection of PDAC. Seven genes in the nine-gene-pair signature, namely CTSE, HOXB7, LAMC2, ONECUT1, RRM2, SERPINB5, and UBE2C, had previously been known to be associated with PDAC. Thus, AI-based tissue biomarker analysis identified a multiple-gene expression signature for detection of early PDAC.

MicroRNAs (miRNAs) have been proposed as promising biomarkers for diagnosis of PDAC[35]. miRNAs are a group of short non-coding RNA molecules with 19-25 nucleotides that have been considered as candidate biomarkers for early cancer diagnosis and precise prognosis[36]. Recently, miRNA-used liquid biopsy has become a promising approach for early detection of cancers. Several miRNAs in plasma of PDAC patients are abundantly expressed, supporting that circulating miRNAs could be helpful for PDAC detection[37]. Ganepola *et al*[38] employed three circulating miRNAs (miR-22, miR-642b-3p, and miR-885-5) for detection of PDAC, and the AUC value was 0.97 for discrimination of the PDAC cases. Liu *et al*[39] utilized a serum panel including miR-20a, miR-21, miR-24, miR-25, miR-99a, miR-185, and miR-191 for diagnosis of PDAC at different stages, and the AUC value was 0.99. Alizadeh Savareh *et al*[40] assessed the value of top miRNAs using a machine learning method (particle swarm optimization + ANN + neighborhood components analysis) to assist early diagnosis of PDAC. They identified a number of serum miRNAs that were significantly differentially expressed in 671 microarray PDAC expression profiles, using bioinformatics techniques Their final model comprised the most promising miRNAs (miR-92a-2-5p, miR-125b-3p, miR-532e5p, miR-663a, and miR-1469) with the high performance (accuracy, 0.93; sensitivity, 0.93; and specificity, 0.92) in differentiation of PDAC from controls.

Early detection of PDAC using tissue and/or blood biomarkers in conjunction with AI is an alternative approach to improving survival.

DETECTION OF EARLY PDAC BY RADIOMICS

Nowadays, computed tomography (CT), magnetic resonance imaging (MRI), and ultrasound, endoscopic ultrasound are the most popular imaging modalities for PDAC detection. However, these modalities are often employed in patients with symptoms, which results in delayed detection of PDACs in most cases. A promising application of AI technology is in the earlier detection of PDAC from radiological findings. CT is the most frequently used modality for the initial assessment of suspected PDAC, and its sensitivity of detection ranges from 76%-96%[41]. CT imaging can collect information about tumor location, size, and morphology. The pancreas is considerably different in size, shape, and location among the individuals and possesses only a very small part of the entire CT image, or about 1.3% of each CT image in a CT dataset[42]. Furthermore, a tumor shows high similarity to the surrounding tissues. Therefore, visual diagnosis demands doctors with enough clinical experience, because the quality of CT images varies between different CT scanners and operators, and pathological texture features are hard to distinguish. Actually, 19% of patients with pancreatic cancer who underwent a review of submitted outside imaging and repeat imaging at a tertiary referral center received major changes in diagnosis and/or disease stage[43].

The features of early PDAC can be delicate and retrospectively ascertained up to 34 mo before the diagnosis of PDAC[44]. In a tertiary medical center, 7.1% of PDACs were missed even by radiologist assessment. This fact emphasizes the limitations in

the conventional CT approach for PDAC. Prognostic outcome in patients with PDACs considerably deteriorates when tumor size exceeds 2 cm[45], however tumors smaller than 2 cm are frequently invisible on CT images and so about 40% of small PDACs are missed[46]. An AI-based diagnostic tool might minimize such oversight. Therefore, there is a growing need to develop AI-based algorithms for accurate pancreatic tumor detection. Although deep learning has been investigated for the diagnosis of pancreatic cystic neoplasms[47], neuroendocrine tumors[48] and segmentation of the pancreas[49-52], the usefulness of AI in the detection of PDAC has not yet been widely explored. AI can analyze thousands of images on a pixel-by-pixel level and is not susceptible to mistakes due to human error. Another strength of AI is automatic diagnosis, which takes no more than approximately 20 s per case from inputting the original CT image to obtaining a diagnosis.

Liu *et al*[53] reported that the AUC of an AI [convolutional neural network (CNN)] platform for CT-assisted diagnosis of PDAC was 0.963. Furthermore, the time of the CT-assisted diagnosis was 20 s/case, which is remarkably shorter than the duration required for diagnosis by radiologists, indicating AI has good clinical feasibility. In a deep learning (fully end-to-end deep learning) model for diagnosing pancreatic tumors, Si *et al*[42] reported that the average test time per case was 18.6 s, compared with at least 8 min for manual reviewing. Thus, the AI diagnosis system was more efficient than the conventional diagnostic approach.

Liu *et al*[54] showed that CNN-based analysis could precisely discriminate cases with and without PDAC in portal venous CT. The CNN-based analysis achieved an accuracy approaching 99% and missed fewer tumors than did radiologists. In this study, CNN-based analysis provided higher sensitivity compared to radiologists (0.983 *vs* 0.929, respectively)[54]. CNN missed three (1.7%) of 176 PDACs (1.1-1.2 cm). Radiologists missed 12 (7%) of 168 PDACs (1.0-3.3 cm), of which 11 (92%) were correctly classified by CNN. The sensitivity of CNN for tumors smaller than 2 cm was 92.1% in local test sets and 63.1% in an external (US) test set. Although the latter sensitivity for tumors smaller than 2 cm initially seemed unsatisfactory, DeWitt *et al* [46] reported that the sensitivity of CT by radiologist assessment was 53% for PDACs smaller than 2.5 cm[46]. Consequently, the sensitivity of the CNN-based analysis was equivalent to radiologist assessment. The lower sensitivity of the CNN in the external test set compared with local test sets might be attributed to differences in patients' ethnicity and race, and protocols or scanners, between the training and external test sets, which could present greater challenges for small tumors. An important factor that affects the imaging features of the pancreas is fat content. Higher fat content decreases the density of the pancreas on CT images, and several studies reported marked differences in pancreatic fat content between ethnicities and races[55,56].

Radiologists were given with important clinical information from the clinicians when they assessed the CT images, whereas the CNN was provided with no information except CT images. Therefore, the major utility of the CNN was to support radiologists in judging whether a lesion or suspicious area in the pancreas harbored pancreatic cancer. For example, patients present with obstructive jaundice which is a typical sign of pancreatic cancer in the pancreatic head. Nevertheless, the CT findings are negative or equivocal. In such a situation, occult pancreatic cancer should be highly suspected even if no apparent mass is noted on CT image, given that about 40% of PDACs smaller than 2 cm are missed on CT image due to undefined borders with surrounding tissue[46,57].

With the wide application of endoscopic ultrasonography (EUS) and EUS-fine needle aspiration (FNA) have become the important diagnostic modalities for PDAC; these modalities provide diagnostic accuracies up to 85%, which are remarkably greater than the 50% accuracy in CT-assisted diagnosis. The sensitivity of diagnosis of pancreatic tumors 3 cm in diameter was reported to be 93% for EUS, which was greater than that of CT (53%) and MRI (67%)[58]. A meta-analysis revealed that CT and EUS were comparable in determining the resectability of PDAC, with high sensitivity and specificity[59].

However, based on EUS for early diagnosis of PDAC, the experience and subjective factors affect on the accuracy, especially in the presence of chronic pancreatitis. Additionally, the availability of the EUS-FNA is restricted in community hospitals. Even when the EUS-FNA is utilized, the diagnosis can be also influenced by the operator's experience and the location of the needle insertion. In 2001, Norton *et al*[60] reported the usefulness of neural network analysis of EUS images to distinguish between PDAC and chronic pancreatitis using 4 different image parameters. Although they provided a high sensitivity, the specificity was only 50%. In 2008, Das *et al*[61] applied techniques of digital image analysis to EUS images of the pancreas to develop a classification model that could differentiate PDAC from non-neoplastic tissue using

ANN. The model accurately classified PDAC, with an AUC of 0.93 and a 93% sensitivity rate[61]. Digital analysis of EUS images is useful in differentiating PDAC from normal tissue and chronic inflammation. Given the possibility of real-time application, digital image analysis may become a helpful diagnostic modality in pancreatic diseases and may sometimes evade EUS-guided FNA. In another study, Zhang *et al*[62] differentiated between PDAC and normal tissue on EUS images. Regions of interest were selected from 216 images obtained from 153 cancer and 63 non-cancer patients, and a 97.98% sensitivity rate was obtained from the 29 features that were identified[62]. Zhu *et al*[63] conducted a computer-aided diagnosis utilizing EUS images of 262 PDAC and 126 chronic pancreatitis patients, from which 105 features were extracted. Sixteen of these features were selected for classification by a support vector machine and a 94% sensitivity rate was obtained[63].

EUS imaging is a common imaging method for diagnosing PDAC, and is often applied with FNA in distinguishing benign and malignant tumors. However, FNA is not available in all health centers. AI-assisted diagnosis *via* EUS images should guide physicians toward more accurate and easier diagnosis.

Collectively, AI can supplement radiologists to reduce miss rates, rather than replace them. The AI stands as a diagnostic tool to assist clinicians and radiologists in diagnosing PDAC. The application of AI in the diagnosis of PDAC has made substantial advances and is certainly improving.

AI IN MOLECULAR/GENETIC SUBTYPE CLASSIFICATION

Recent advances in biotechnology enable us to execute comprehensive genomic, transcriptomic, proteomic, and metabolomic analyses rapidly and cheaply. Such inclusive gene expression studies have uncovered subtypes of PDAC with biological and prognostic relevance. Collisson *et al*[9] proposed the categorization of PDACs into three subtypes: classical, quasi-mesenchymal (QM), and exocrine-like. The prognostic outcome of PDAC patients following operation and conventional medical treatment was notably better in the classical subtype than in patients with the QM subtype; patientss with the exocrine-like subtype displayed intermediate prognostic outcome between the two other subtypes[9]. Muckenhuber *et al*[64] subsequently reported that the most of PDAC can be categorized into two distinct subtypes based on transcriptome profiling and on immunohistochemical staining of cytokeratin-81 (KRT81) and hepatocyte nuclear factor-1A (HNF1a). The epithelial KRT81-/HNF1a- (double-negative) subtype (the so-called classical subtype) showed better survival and response to chemotherapy, notably to the FOLFIRINOX regimen, but not to a gemcitabine-based regimen. On the other hand, the epithelial KRT81+/HNF1a- subtype (the so-called QM subtype) has worse OS. But, the QM subtype displays a better response to the gemcitabine-based regimen compared to the non-QM subtype [65]. These features encourage precision medicine based on individual molecular features.

Recent developments in AI using medical image analysis such as radiomics reveal promising models of molecular phenotyping from imaging data. The radiomics approach can perform whole-tumor analytics without invasiveness. Kaissis *et al*[66,67] have reported on machine learning algorithms to preoperatively predict molecular subtypes and survival risk in PDAC patients from MRI. However, the restricted availability of MRI data, overall decreased image quality, and the less-quantitative and unstandardized nature of MRI render obstacles to algorithm development and generalization. To reinfoce clinical application, Kaissis *et al*[68] extended their previous results to CT by training and validating an algorithm (random forest) capable of discriminating between the QM and the non-QM subtypes of PDAC. The advantages of CT comprise broad availability, fewer motion artifacts, and high standardization. Their retrospective study assessed baseline CT from 207 PDAC cases. By immunohistochemical staining for KRT81 and HNF1a, the molecular subtype was determined as QM *vs* non-QM. The random forest algorithm was used to predict the molecular subtype from the radiomic features. Then, the algorithm was applied to an independent cohort of histopathologically unclassifiable tumors. The classification algorithm achieved sensitivity, specificity, and AUC of 0.84, 0.92, and 0.93, respectively. The median OS for predicted QM and non-QM tumors was 16.1 and 20.9 mo, respectively. The application of the algorithm to histopathologically unclassifiable tumors showed two groups with remarkably different survival (8.9 and 39.8 mo). Thus, the machine learning-based analysis of CT imaging provided the possibility of the prediction of molecular subtypes that is clinically relevant for prognostic outcome,

permitting pre-operative stratification for precision medicine. This approach is encouraged by the fact that histopathological approachs are by default a significant underrepresentation of the tumor, since they are derived from a small sub-section of the tissue, and regions of differing molecular subtype are likely to coexist within the same tumor[69]. On the other hand, the radiomic approach enables whole-tumor assessment, providing better information required for precision therapy.

Microsatellite instability (MSI) is a genomic property of cancers with defective DNA mismatch repair. Notably, MSI has been recognized as a biomarker for the favorable immune checkpoint blockade therapy response in cancer[70]. Most standard methods for examing MSI are based on DNA sequencing data and a few are based on mRNA expression data. Using RNA-Seq pan-cancer datasets for three cancer cohorts (colon, endometrial, and gastric cancers) from TCGA program, Li *et al*[19] established an algorithm called PreMSIm (Predicting MSI from mRNA) to predict MSI in cancer from the expression profiling of a 15-gene panel. A benefit of mRNA-based over DNA-based MSI prediction algorithms is that mRNA data are closer to protein and phenotype than DNA data. Pathway analysis revealed that these genes were mainly involved in DNA damage repair (MLH1 and MSH4), gene expression (MLH1, HENMT1, and RPL22L1), cell cycle regulation (MLH1, MSH4, and HENMT1), and metabolism (NHLRC1 and RPL22L1). Gene ontology analysis showed that these genes were involved in the biological processes of DNA repair (MLH1, MSH4, and RTF2), gene expression regulation (NHLRC1 and HENMT1), cell cycle (MLH1, MSH4, RPL22L1, and RTF2), biogenesis (DDX27, EPM2AIP1, NHLRC1, and RNLS), metabolic process (HENMT1, LYG1, NHLRC1, and SMAP1), and cell and organism development (SMAP1, SHROOM4, and TTC30A). The PreMSIm algorithm provided high performance in predicting MSI using both RNA-Seq and microarray gene expression datasets[19]. Furthermore, PreMSIm showed superior or comparable performance *vs* other DNA- or mRNA-based methods. Li *et al*[19] comment that PreMSIm can be an alternative approach for identifying MSI. The introduction of machine learning algorithms such as this as a clinical decision support tool should be beneficial to predict molecular/genetic signatures that may help to stratify patients in clinical routines.

AI IN RISK ASSESSMENT FOR PANCREATIC SURGERY

In the pancreatic fields, the availability of AI in surgery is still very limited. An AI-based risk prediction model of postoperative complication has been reported[71,72]. Postoperative pancreatic fistula (POPF) is a serious complication after pancreatoduodenectomy (PD). The fistula risk score (FRS), which consists of four variables — soft pancreas, small main pancreatic duct, high-risk pathology (PDAC or chronic pancreatitis), and massive intraoperative blood loss — is useful to predict clinically relevant POPF development after PD[73,74]. However, the score contains subjective factors related to surgeons. Therefore, an accurate and easy-to-use preoperative index is desired. Kambakamba *et al*[71] examined whether quantitative analysis of plain CT with five types of machine learning algorithms (*k*-nearest neighbors, sequential minimum optimization, multilayer perceptron, random forest, and C5.0) could predict clinically relevant POPF in 110 patients from a single institution, and found that machine learning-based CT analysis provided an magnificent AUC of 0.95 in predicting clinically relevant POPF[71]. Mu *et al*[72] tried to predict clinically relevant POPF after PD using a deep learning (CNN model) score derived from preoperative CT. The deep learning score offered significantly greater predictability compared to FRS in training (0.85 *vs* 0.78 in AUC, respectively), validation (0.81 *vs* 0.76 in AUC, respectively) and test (0.89 *vs* 0.73 in AUC, respectively) cohorts. In particular, in patients of intermediate risk (FRS 3-6), the deep learning score achieved remarkably higher accuracy compared to FRS (test: 92.1% *vs* 65.8%, respectively). Interestingly, the deep learning score was independently associated with pancreatic fibrosis, diameter of main pancreatic duct and remnant volume in multivariate linear regression analysis. The automated scores reflected histomorphological features related to pancreatic duct, parenchymal fibrosis, and remnant pancreatic tissue volume. Thus, an AI model using preoperative CT represents a novel tool to predict clinically relevant POPF after PD, especially at intermediate risk levels. Such an AI system helps surgeons to optimize preoperative strategy.

AI IN SURVIVAL PREDICTION AND RESPONSE TO CHEMOTHERAPY

The potential of radiomics in prediction of clinically relevant conditions, such as expected OS or response to a specific therapy, has been reported in recent studies[75,76]: For instance, CT-derived radiomic features were useful to predict local disease control and OS in PDAC[75,76]. Entropy-related and cluster tendency features were described as predictive of OS in PDAC[76]. Zhang *et al*[77] proposed that a CNN-based survival model outperforms a Cox proportional hazard model-based radiomics pipeline in PDAC prognosis. This model provides a better fit for survival patterns based on CT images and overcomes the limitations of conventional survival models. Kaissis *et al*[66] reported that a machine learning algorithm (random forest) using MRI achieved 87% sensitivity, 80% specificity, and AUC 0.9 for the prediction of above- *vs* below-median OS in the independent validation cohort. Alizadeh Savareh *et al*[40] have identified several circulating miRNAs as a diagnosis model in PDAC patients by analyzing microarray miRNA expression profiles from the Gene Expression Omnibus database. Three (hsa-mir-1469, hsa-mir-663a and hsa-mir-532) of five miRNAs with a high rank in the final model were comprehensively associated with the OS of patients with PDAC based on their up- or down-regulated expression patterns[40].

Late diagnosis of PDAC can cause to lose the chance of surgical treatment and lead to a high mortality rate[78]. On the other hand, surgical treatments for PDAC can have a high morbidity and mortality rate. Therefore, the clinicians must weigh the potential survival advantage of the invasive treatment, the complications due to invasive treatment, and the impacts on the patient's quality of life with and without treatment.

Walczak and Velanovich[79] established ANN models that could accurately predict the 7-mo survival of PDAC patients using 14 clinical variables including eight SF-36 domain values, both with and without surgical resection, at 91% sensitivity and 38% specificity. The ANN model to predict 7-mo survival consisted of age, sex, the eight domains of quality of life measurements from the SF-36, the stage of the cancer, whether or not a resection had taken place, if any adjuvant therapy had been given, and time in months since diagnosis. The quality of life domains from the SF-36 are bodily pain, vitality, physical functioning, social functioning, role-physical, role-emotional, general health, and mental health. Such an ANN model for predicting the survival of PDAC patients helps physicians and patients to reduce anticipated regret from treatment decisions including observation. This information may be useful for patients and surgeons in determining invasive treatment plans to minimize regret and improve the patients' quality of life.

Neoadjuvant therapy may provide improved survival of PDAC patients; but, determining the efficacy is difficult. Watson *et al*[80] hypothesized that a deep learning (CNN) model could predict the tumor response to neoadjuvant therapy using CT and CA19-9. A total of 81 cases were divided between partial responder (333 images) and non-responder (443 images). The model using only the deep learning model had an AUC of 0.738, whereas a hybrid model incorporating a decrease in CA19-9 of 10% in addition to the deep learning model had an AUC of 0.785. CA19-9 reduction alone was not an effective predictor of the response to neoadjuvant therapy, with an AUC of 0.564. A deep learning model can predict the pathological response to neoadjuvant therapy for PDAC patients, and the model is amended with the incorporation of decreases in serum CA19-9. Abraham *et al*[81] investigated the clinical relevance of a machine learning-derived signature in predicting the responses from first-line oxaliplatin-based chemotherapy in PDAC and advanced colorectal cancer. The machine learning-derived signature was effective for metastatic colorectal cancer, but not for PDAC. AI has already been applied to match biological information with chemical properties of specific drugs to predict the response to these specific agents in cancers[82].

In the near future, the combined analysis of clinical variables, less-invasive biological samples, and radiological features through machine learning should be able to simulate responses to chemotherapy and patient survival. On the other hand, radiological features and biological tissue can be variable in response to the treatment including chemotherapy and radiotherapy. In particular, PDAC has a high potential for acquired drug resistance. During sequential treatment, good communication and the accumulation of knowledge from various fields such as gastroenterology, radiology, oncology, computer science, and pathology must will be required to fight this deadly disease.

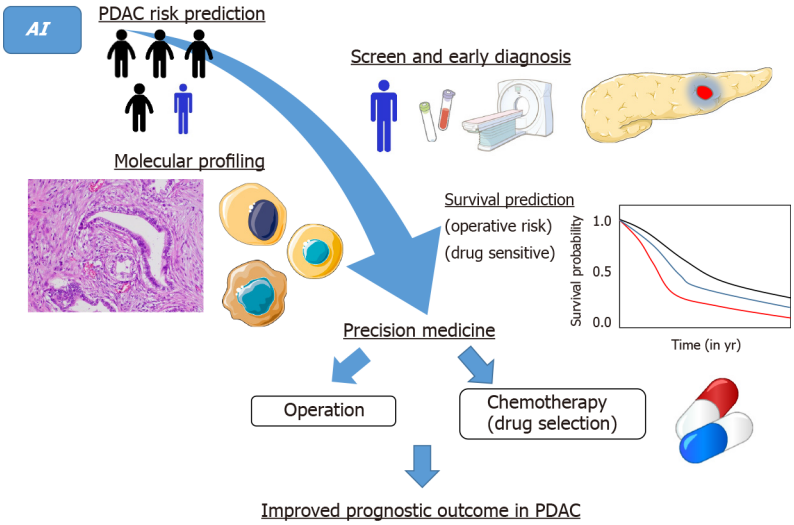

Figure 2 Future perspectives in the management of pancreatic ductal adenocarcinoma by artificial intelligence. AI: Artificial intelligence; PDAC: Pancreatic ductal adenocarcinoma.

LIMITATIONS AND FUTURE PERSPECTIVES

A major limitation is the lack of adequate standardization. Universal and uniform protocols for data collection, data quality, storage, processing, reproduction, and analysis must be established. For instance, ANNs can be trained to appropriately categorize histologic slides of pancreatic biopsies. However, the trained ANNs may underperform, or not perform at all, when the prepared slides are fixed and stained in a different manner. Development of universal and uniform protocols during data and sample processing will be required for medical AI to be feasible. Further improvement of the technology is also essential for medical AI in clinical practice.

AI in the medical field should become an indispensable tool to reduce human error. Because of human limitations, we cannot achieve zero errors. Furthermore, it is time-consuming to train professional radiologists, gastroenterologists, oncologists, and pathologists. Combined work by experts from multiple fields will be needed to establish feasible medical AI systems in clinical practice. With further research, AI must have a great impact on the diagnosis and treatment of PDAC in near future. Ultimately, a sequential approach involving risk prediction, diagnosis, treatment, and survival prediction using IA will realize timely and consecutive precision medicine and lead to improved prognosis in PDAC (Figure 2). Dr. William Osler stated: "Medicine is a science of uncertainty and an art of probability." This is still true in medical AI, which is also "a science of uncertainty and an art of probability." However, the degree of uncertainty and probability will consistently shrink with the advance of AI technology and cooperation among various experts. While AI applications in PDAC are still in the early stage of development, further research must lead to great advances in screening, early diagnosis, and treatment.

CONCLUSION

Here we summarize the current advances of AI in PDAC. AI-based omics analyses are likely to be the next alternative approach to overcome this poor-prognostic disease by the discovery of biomarkers for early detection, molecular/genomic subtyping, and treatment guidance, and by the improved prediction of recurrence and survival. How to entirely utilize "big data" is a new challenge for physicians and researchers in the era of precision medicine. On the other hand, AI will not entirely act for doctors — human beings and machines working harmoniously together is the ideal state that results in excellent performance. Although AI data reveal that the diagnostic accuracy of deep learning models is better than that of radiologists, the aim in this field is to develop a helpful tool to aid radiologists in making effective and accurate diagnoses, not to be a replacement for doctors. To facilitate AI-based omics analyses, multidisciplinary collaboration between physicians, basic scientists, radiologists, statisticians, and engineers is mandatory. To further validate the clinical relevance of AI systems, next

step is to conduct a prospective study based on multicenter clinical data. We believe that breakthroughs will soon emerge to fight this deadly disease using AI-navigated precision medicine.

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MINIREVIEWS

Hepatitis B: Who should be treated?-managing patients with chronic hepatitis B during the immune-tolerant and immunoactive phases

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Abstract

New hepatitis B virus (HBV) infections are decreasing owing to improved antiviral therapy and increased HBV vaccination worldwide; however, the number of HBV infections remains a major cause of liver carcinogenesis. HBV triggers cytotoxic immunity to eliminate HBV-infected cells. Therefore, the HBV pathophysiology changes in persistently infected individuals depending on host immune responses and HBV DNA proliferation state. To prevent liver cirrhosis and carcinogenesis caused by HBV, it is important to treat HBV infection at an early stage. Active treatment is recommended for the immunoactive hepatitis B surface-antigen-positive and -negative phase, but not during the immune-inactive phase or immune-tolerant phase; instead, follow-up is recommended. However, these patients should be monitored through regular blood tests to accurately diagnose the immune-inactive or -tolerant phases. The treatment regimen should be determined based on the age, sex, family history of liver cancer, and liver fibrosis status of patients. Early treatment is often recommended due to various problems during the immune-tolerant phase. This review compares the four major international practice guidelines, including those from the Japanese Society of Hepatology, and discusses strategies for chronic hepatitis B treatment during the immune-tolerant, immune-inactive, and resolved phases. Finally, recommended hepatitis B antiviral therapy and follow-up protocols are discussed.

Key Words: Hepatitis B; Immune tolerance; Immune-inactive; Anti-viral therapy; Hepatocellular carcinoma; Cirrhosis

Core Tip: Hepatitis B virus (HBV) is a global health problem that causes acute and chronic infections and often leads to liver cirrhosis and hepatocellular carcinoma.

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Treatment of HBV is recommended for patients in the immunoactive hepatitis B surface-antigen-positive and -negative phases. Follow-up is recommended only for patients in the immune-inactive phase and the immune-tolerant phase, but opinion on this recommendation remain divided. This review discusses the major international guidelines for the treatment of chronic hepatitis B and highlights the importance of clinical factors for making decisions regarding the management of patients with HBV infection.

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INTRODUCTION

Chronic hepatitis B (CHB) is the leading cause of hepatocellular carcinoma (HCC), and it is estimated that 240 million individuals are persistently infected by the hepatitis B virus (HBV)[1-4]. The prevalence of hepatitis B surface-antigen (HBsAg) is approximately 3%-4% worldwide; in addition, more than 880000 individuals die each year from HBV-derived liver damage[1-4]. The high prevalence of hepatitis B in Asia and Africa is accompanied by high mortality, and infections in the Western Pacific region account for approximately 50% of chronic HBV infections worldwide[1,5,6]. Therefore, prevention, diagnosis, evaluation, indication of treatment, and management of co-infected patients are important in these areas. HBV may exploit the immature neonatal immune system to establish a persistent infection. Following vertical or perinatal transmission, 90% of neonates develop CHB, whereas children infected between 1 and 5 years of age have a 30% chance of developing chronic infection. Studies on the natural history of HBV infection have shown that the timing of hepatitis B e-antigen (HBeAg) seroconversion varies from childhood to adulthood, with HBeAg seroconversion occurring primarily between 15 and 35 years of age[7-9]. Natural HBeAg seroconversion has been reported to be 4.6%, 7.1%, and 28% for those under 6, between 6 and 12, and above 12 years of age, respectively[9]. Most patients with HBV can resolve their infections, but approximately 10% continue to have viral activity, progress to cirrhosis at an annual rate of 2% and develop HCC or liver failure[5,10-14]. Since its discovery in 1965, the gene structure and replication mechanism of HBV, its infection route, natural course, and pathophysiology have been clarified, and treatment methods have been advancing continuously. The timeline of HBV infection is complex and comprises various overlapping immune phases[2,3,10,13].

The European Association for the Study of Liver Disease (EASL), American Association for the Study of Liver Disease (AASLD), Asian-Pacific Association for the Study of the Liver (APASL), and the Japanese Society of Hepatology (JSG) have defined HBsAg loss as a CHB treatment goal[15-18]. HBsAg loss is associated with improved clinical outcomes such as prevention of HCC and survival[19]. However, this endpoint is difficult to achieve with the existing antiviral therapies that include long-term nucleos(t)ide analog (NA) and pegylated interferon (Peg-IFN) therapies[]. A recent study reported that a higher portion of patients showed HBsAg loss in response to combined treatment with tenofovir disoproxil fumarate (TDF) and Peg-IFN α-2a for 48 wk than to TDF or Peg-IFN α-2a monotherapy[20]. Therefore, NA and Peg-IFN combination therapies are being constantly used and developed to prevent cirrhosis and HCC[20-22]. Several factors, such as the immune phase, genotype, race, degree of liver fibrosis, HCC family history[23-25], age, and sex should be considered to determine the most efficient treatment for CHB. Moreover, the appropriate drug type and timing of drug administration are crucial[19,26]. CHB immune phases can be divided into five categories, including the immunotolerant phase, immunoactive HBeAg-positive phase, immune-inactive phase, immunoactive HBeAg-negative phase, and resolved CHB phase; each phase is identified according to immunological features, virology, biochemistry, and histology specific to the infection[3,5,7,13]. Among these phases, phases 1, 3, and 5 are often not indicated for treatment, and instead, follow-up is often recommended[15-18]. In any case, it remains uncertain

whether follow-up is sufficient, especially for the immunotolerant phase.

CURRENT TREATMENT CONSIDERATIONS FOR HEPATITIS B PATIENTS

Indicators for initiating HBV treatment

The CHB treatment goals are to reduce liver disease mortality, improve survival, and enhance quality of life by preventing liver disease progression from fibrosis to cirrhosis and HCC. All global guidelines recommend initiating treatment based on the presence of HBV DNA and serum alanine aminotransferase (ALT) levels. Patients in either the immunoactive HBeAg-positive or -negative phase are likely to progress to cirrhosis and liver carcinogenesis. Considering the risk of developing cirrhosis and HCC associated with them, these phases are used as the main characteristics for initiating antiviral therapy[15-18]. The following host indicators are also considered prior to treatment: Sex (male), age > 40 years, family history of HCC, place of birth (sub-Saharan Africa or Asia), presence of virus- or disease-related cirrhosis, HBV-DNA > 2000 IU/mL and elevated ALT levels, presence of HBeAg, and genotype C, which causes delayed HBeAg seroconversion[23-28]. In patients treated using NA, HBsAg and HB core-related antigen (HBcrAg) levels are associated with HCC carcinogenesis, even at low levels of HBV DNA levels[29-32]. Recently, modified PAGE-B scores, which are determined based on patient age, sex, baseline platelet count, and serum albumin levels were shown to predict HCC in patients receiving NA treatment[33].

The AASLD guidelines propose different HBV-DNA levels for antiviral therapy, depending on the HBeAg status[17]. According to the APASL guidelines, the HBV DNA levels considered for starting treatment depend on whether the patients are HBeAg-positive or -negative, regardless of the ALT levels[16]. In contrast, the EASL guidelines state that treatment determination should be based upon HBV-DNA and ALT levels, regardless of HBeAg status[15]. Furthermore, according to the JSG guidelines, an ALT level value of 31 or higher, which exceeds normal values in Japan, and HBV DNA levels ≥ 2000 IU/mL are indicated for antiviral treatment, regardless of the HBeAg status[18]. For HBV cirrhosis, antiviral treatment is recommended by all the global guidelines[15-18] (Table 1).

Treatment indications for patients in the immune-tolerant phase

The first phase of CHB, the immune-tolerant phase, is characterized by minimal or no necroinflammatory activity, during which the risk of disease progression is minimal[8, 34]; moreover, 90% of patients infected in early childhood undergo seroconversion from HBeAg-positive to -negative at a young age, and the disease stabilizes in most cases[7-9]. Thus, most clinical practice guidelines do not recommend antiviral therapy for these patients, and follow-up is recommended[15-18]. Among these, the 2017 EASL guidelines[15] set the immune-tolerant phase as HBeAg-positive chronic HBV infection, and the treatment indications for the immune-tolerant phase are expanded compared to other guidelines. Furthermore, serum HBV DNA and HBsAg levels are associated with increased HCC risk and disease progression at serum ALT levels with or without HBeAg[26,27]. Early HBV treatment decreases mortality, improve transplantation outcomes, and decreases the risk of HCC[19]. Therefore, therapeutic intervention should be considered in cases of immune tolerance.

The HBV immune-tolerant phase exhibits high HBsAg and HBeAg titers, indicating high viral replication, and either normal or minimally elevated serum ALT levels. In this case, HBV proliferation is active but 'tolerated' as the host immune system does not recognize the viral antigen.

Table 2 presents the definition of immune tolerance and treatment guidelines[15-18]. The ALT cutoff value during the immune tolerance phase depends on the global guidelines. The AASLD guidelines indicate cutoff values of 35 IU/L for men and 25 IU/L for women. The APASL and EASL guidelines set the cutoff at 40 IU/L, whereas the JSG guidelines recommend no treatment for ALT < 30 IU/L. High HBV DNA level cutoffs are important to distinguish immune-tolerant CHB from other phases. The HBV DNA criteria during the immune-tolerant phase differs between the EASL (≥ 2000 IU/mL), and the AASLD and APASL (≥ 20000 IU/mL for both). Moreover, the age limit for considering a liver biopsy or treatment in the immune-tolerant phase also depends on the guidelines: > 40 years old (both AASLD and JSG), > 35 years old (APASL), or > 30 years old (EASL). Furthermore, antiviral therapy is considered when ALT levels increase during monitoring. According to the AASLD guidelines, treatment is indicated for patients with liver fibrosis stage F2 or higher, especially for patients over 40 years of age. The EASL guidelines indicate treatment for patients with liver

Table 1 Summary of treatment criteria for chronic hepatitis B

	HBeAg+; HBV DNA (IU/mL)	HBeAg+; ALT (IU/L)	HBeAg–; HBV DNA (IU/mL)	HBeAg–; ALT (IU/L)	Cirrhosis
EASL[15]	≥ 2000	> ULN and/or at least moderate liver necro-inflammation or fibrosis	≥ 2000	> × ULN or significant histological disease	HBV-DNA detectable
	≥ 20000	> 2 × ULN or irrespective of fibrosis	≥ 20,000	> 2 × ULN irrespective of fibrosis	
APASL[16]	≥ 20000	> 2 × ULN or significant histological disease	≥ 2000	> 2 × ULN or significant histological disease	HBV-DNA detectable
AASLD[17]	> 20000	> 2 × ULN or significant histological disease	≥ 2000	> 2 × ULN or significant histological disease	HBV-DNA detectable
JSG[18]	≥ 2000	> ULN	≥ 2000	> ULN	HBV-DNA detectable

AASLD: American Association for the Study of Liver Disease; ALT: Alanine aminotransferase; APASL: Asian-Pacific Association for the Study of the Liver; EASL: European Association for the Study of Liver Disease; HBeAg: Hepatitis B e-antigen; HBV: Hepatitis B virus; JSG: Japanese Society of Hepatology; ULN: Upper limit of normal.

Table 2 Treatment indications for patients with hepatitis B e-antigen positive, alanine aminotransferase < upper limit of normal for chronic hepatitis B

	Monitor criteria	Consideration for anti-viral therapy
EASL[15]	Normal ALT (< 40 IU/L) and high HBV DNA (≥ 2000 IU/mL) levels. Monitor ALT and HBV DNA levels every 3-6 mo	Age > 30 yr, family history of HCC or cirrhosis and extrahepatic manifestations. Consider liver biopsy or non-invasive test if: ALT level is elevated; excluding other causes
APASL[16]	Normal ALT (< 40 IU/L) and high HBV DNA (≥ 20000 IU/mL) levels Monitor ALT and HBV DNA levels every 3 mo	Age > 35 yr, liver biopsy showing F2/A2, significant fibrosis by non-invasive tests, stiffness ≥ 8 kPa, persistently elevated ALT, family history of HCC/cirrhosis
AASLD[17]	Normal ALT [< 35 IU/L (male), < 25 IU/L (female)] and high HBV DNA (≥ 20000 IU/mL) levels. Monitor ALT and HBV DNA levels every 3-6 mo	Liver biopsy or non-invasive test shows ≥ F2 or F3, persistently elevated ALT level; exclude other causes, especially age > 40 yr
JSG[18]	Normal ALT (≤ 30 IU/L) level	Consider liver biopsy or non-invasive test if Age > 40 yr, high HBV DNA or platelet counts < 15×10^4/uL, family history of HCC

A2: Activity score 2; AASLD: American Association for the Study of Liver Disease; ALT: Alanine aminotransferase; APASL: Asian-Pacific Association for the Study of the Liver; EASL: European Association for the Study of Liver Disease; F2/3: Fibrosis score 2/3; HBeAg: Hepatitis B e-antigen; HBV: Hepatitis B virus; HCC: Hepatocellular carcinoma; JSG: Japanese Society of Hepatology.

fibrosis stage F2/A2 or higher (as determined using elastography and/or liver biopsy), over 30 years old, and who have liver stiffness of ≥ 12 kPa. The APASL guidelines simply recommend that patients with F2/A2 or a higher score be considered for treatment. According to the JSG guidelines, determination of liver fibrosis (by liver biopsy or a non-invasive procedure) is recommended if the ALT level is intermittently elevated, HBV DNA is high, platelet counts are less than 15×10^4/μL, and the patient is older than 40 years of age.

To summarize the global guidelines: Age, family history of developing HCC or cirrhosis, and liver fibrosis are important factors for deciding the best therapeutic strategy during the immune-tolerant phase.

Arguments against treatment during the immune-tolerant phase

There are various opinions regarding the need to treat patients in the HBV immune-tolerant phase. This phase is characterized by high viral replication, the presence of HBeAg, and normal or minimally elevated serum ALT and/or aspartate aminotransferase levels[3,5,10,13]. The immune-tolerant phase is usually identified in patients below 30 years of age. Very mild non-specific hepatitis has also been reported in patients in immune-tolerant phase who are between the ages of 10 and 12 years, although the 5 years progression of liver damage is minimal among patients who remain in the immune-tolerant phase[8,34].

Immune-active HBeAg-positive CHB that occurs in adolescence is associated with continued hepatitis activity. Some patients develop fibrosis or cirrhosis during the HBeAg-positive phase, but most show decreased serum HBV DNA levels and HBeAg seroconversion, resulting in an immunoactive HBeAg-negative CHB phase. This phase is characterized by persistent normal serum ALT levels and low HBV DNA load. Some patients show spontaneous HBeAg antibody positivity and may not require antiviral therapy. Patients who spontaneously show HBeAg seroconversion before the age of 30 years usually have a good prognosis[35,36]. In fact, approximately 90% of patients will either be cured, or the hepatitis will not progress into adulthood. For instance, Tada *et al*[35] examined 408 HBV carriers who did not receive NA and found that individuals under the age of 40 with HBeAg seroconversion had a better prognosis than those without HBeAg seroconversion, even when matched by sex and age.

Furthermore, the HBeAg seroconversion rate is very low even if antiviral treatment is administered during the immune-tolerant phase[37-39]. Patients in the immune-tolerant phase requires continuous NA treatment due to high HBsAg levels; moreover, the HBeAg seroconversion rate is low, and serum clearance of HBsAg is rarely achieved[19,39].

In another example, Chan *et al*[39] administered TDF or a combination of TDF and entecavir (ETV) to 126 HBeAg-positive patients with normal ALT levels, who were close to the immune-tolerant phase and found that only 5% of patients receiving treatment showed HBeAg seroconversion. Although none of these cases were in the immune-tolerant phase, other reports have shown that HBeAg seroconversion is high in cases with elevated ALT levels[20]. In general, the higher the ALT level, the better the seroconversion. As ALT levels are naturally low during the immune-tolerant phase, HBeAg seroconversion is not expected.

Several patients in the HBV immune-tolerant phase are young and the NA treatment period is extensive. Treatment is also complicated by several factors, including the development of viral resistance, treatment cost, and long-term safety issues. Andreani *et al*[8] also showed that patients with normal ALT levels and high HBV DNA (10^7 copies/mL) do not require liver biopsies as liver tissue-related changes are minimal during the immune-tolerant phase. In conclusion, there is little evidence to support that treatment alters the clinical outcome in these patients, though some guidelines recommend follow-up[40].

Recommendations for treating patients in the HBV immune-tolerant phase

Earlier, most clinical practice guidelines did not recommend antiviral therapy for patients in the immune-tolerant phase. However, it was recently reported that antiviral treatment during the immune-tolerant phase reduces the risk of HCC, liver transplantation, and death. Thus, some evidence supports antiviral therapy for patients in the immune-tolerant phase[41]. Studies have reported that increase in HBV DNA levels is a risk factor for cirrhosis and HCC[26-28]; moreover, HBV infection itself may lead to HCC. Patients infected with HBV treated with antiviral NA therapy can achieve remarkable viral suppression[39,42]. Further, combined Peg-IFN and NA therapy is more effective in children who are in immune-tolerant phase than in adults [43-45]. A randomized control study evaluated the usefulness of IFN with lamivudine combined therapy for naive HBV infection for patients in immune-tolerant phase, aged 1–16 years, and the results showed reduced HBV DNA load, improved HBeAg seroconversion, and improved rate of HBsAg loss[45]. Management of hepatitis B in children has also been recently reported[46,47]. In fact, hepatitis B vaccination for infants and young children has markedly reduced HBV infections in the vaccinated younger generation, but a significant number of children are still infected with HBV. Most HBV infections in children are in a phase of immune tolerance, and therefore, many are followed up. Most chronically HBV-infected children have mild disease, but a small number may develop undetected fibrosis, cirrhosis, or HCC[47]. There are insufficient data to identify high-risk groups for HCC among children. In fact, children with HBV-associated HCC do not have cirrhosis and have normal alfa-fetoprotein levels, which should be considered for the duration of follow-up and indications for treatment[48].

In recent years, advances in understanding the immunopathogenicity of CHB have questioned whether treatment should be administered at an early stage of CHB regardless of ALT level or severity of liver disease. The immune-tolerant phase is not associated with immunological tolerance, and the results question whether good follow-up as benign is good[49].

Antiviral therapy may also suppress the risk of disease spread from patients with hepatitis B to other individuals. Indeed, antiviral therapy reduces the risk of horizontal transfer of HBV infection from immunotolerant patients with very high viral load, as

well as vertical HBV transmission by mothers with high viral load. Cases without hepatitis B immune globulin and a vaccine occur almost exclusively in HBeAg-positive women with high HBV DNA levels (> 200000 IU/mL) and HBsAg levels exceeding 4–4.5 logs/mL. Mother-to-child HBV transmission rates (MTCTs) were 0% in HBsAg-positive pregnant patients showing high HBV DNA levels (> 200000 IU/mL), who were treated with TDF at 28 gestational weeks[50]. In a meta-analysis based on 595 articles, the administration of antiviral drugs during pregnancy, especially TDF, was deemed safe in helping prevent MTCTs[51,52]. In addition to TDF, tenofovir alafenamide fumarate is administered to pregnant women at 24–35 wk gestation, and the safety of the pregnant women and infants as well as the prevention of MTCT were reported recently[53].

The WHO advocates administration of TDF to infected pregnant women with high HBV load (≥ 5.3 log 10 IU/mL; or ≥ 200000 IU/mL) from the 28th week of pregnancy till delivery. This treatment regimen is recommended to prevent MTCT. The WHO also suggests three hepatitis B vaccinations for newborns, including one dose at birth [54].

Standard follow-up of the immune-tolerant phase may not identify the transition to the immunoactive phase. Thus, evaluating the progression of liver fibrosis and liver carcinogenesis during follow-up is important.

Challenges associated with immune-tolerant phase-diagnosis, liver fibrosis, and liver carcinogenesis

Liver damage caused by HBV causes hepatocellular regeneration associated with chronic necrotizing inflammation, which leads to HCC. The onset of HCC in HBV may arise from immunopathogenic factors[11,55]. In addition, adult serum ALT levels and HBV DNA levels are associated with liver carcinogenesis. Active HBV DNA replication is strongly associated with HCC development and cirrhosis, regardless of ALT levels[26-28,56]. The cumulative incidences of HCC and liver-related diseases in patients who have been in the immune-tolerant phase for over 10 years is 2.7% and 12.7%, respectively[41]. Lee *et al*[57] examined the cumulative HCC risk over a 10-year period in immunologically active patients who achieved a virological response with antivirals in comparison with that in untreated patients in immune-tolerant phase. Surprisingly, the HCC risk was similar between these two groups of patients. In this study, the immune-tolerant phase was diagnosed by regular blood chemistry tests and serum HBV DNA tests every 3–6 mo. Additionally, liver stiffness measured by transient elastography was used to strictly determine whether patients were in the immune-tolerant phase. Thus, this report indicates that HCC carcinogenesis is as high during the immune tolerance period as in the immunologically active phase.

To clarify these points, it is important to determine whether the subject is truly in the immune-tolerant phase. One study found significant fibrosis in 60% of patients with a high viral load and normal or slightly elevated serum ALT for at least 12 mo [58]. Another study found that 37% of HBeAg-positive patients aged 35 years or above, with ALT greater than 0.5 × ULN, had progressive fibrosis, as assessed by transient elastography[59]. Therefore, patients with normal ALT levels and high HBV DNA levels are more likely to be immune-tolerant, subject to the status of their liver fibrosis status[60].

Patients with ALT and HBV DNA greater than 10,000 copies/mL should be carefully evaluated and monitored, even if they appear normal. Necrotic inflammation of the liver and/or fibrosis is observed or progresses unnoticed in certain cases, even in patients with persistently normal ALT levels[56,59-61]. Serum ALT levels cannot be used as a surrogate marker for hepatocyte damage to assess the severity of hepatitis activity.

For this reason, the 2017 EASL guidelines renamed this phase to "HBeAg-positive chronic HBV infection" instead of the "immune tolerance phase," to avoid confusion and the need for early treatment[15]. The AASLD guidelines suggest that ALT levels should be tested every 6 mo at least, even if the patient is in the "immune-tolerant phase," to monitor the potential for progression to "immunoactive or immune-inactive phase"[17]. Various global guidelines recommend regular evaluation of ALT level and HBV DNA load every 3–6 mo in patients who are in the immune-tolerant phase (Table 3). A previous study showed that 4.6% children (up to 12 years of age) progress from the immune tolerance to the immunoclearance phase; therefore, it may be helpful to evaluate ALT levels and HBV DNA load once every 6–12 mo in such children[9].

To diagnose the immune tolerance period accurately, constant monitoring of HBsAg levels, HBeAg levels, and HBV DNA, and ALT levels is required. Chan *et al*[62] performed transient elastography on 161 patients with HBV infection and determined liver stiffness cutoffs associated with normal and elevated ALT levels (> 1–5-fold

Table 3 Treatment indications for patients in the hepatitis B e-antigen-negative immune-inactive phase

	Monitor criteria	Consideration for anti-viral therapy
EASL[15]	Normal ALT (< 40 IU/L) and HBV DNA (< 2000 IU/mL) levels. Monitor ALT and HBV DNA levels (< 2000 IU/mL)every 6-12 mo, (≥ 2000 IU/mL)every 3-6 mo	Age > 30 yr, family history of HCC or cirrhosis and extrahepatic manifestations
APASL[16]	Normal ALT (< 40 IU/L) and HBV DNA (< 2000 IU/mL) levels. Monitor ALT and HBV DNA levels every 3-6 mo	Age > 35 yr, liver biopsy showing F2 or A2, significant fibrosis by non-invasive tests, stiffness ≥ 8 kPa, persistently elevated ALT, family history of HCC/cirrhosis
AASLD[17]	Normal ALT [< 35 IU/L (male), < 25 IU/L (female)] and HBV DNA (< 2000 IU/mL) levels. Monitor ALT and HBV DNA levels every 3 mo for 1 yr, then every 6 mo	Liver biopsy or non-invasive test shows ≥ F2 or F3, persistently elevated ALT level; exclude other causes, especially age > 40 yr
JSG[18]	Normal ALT level (≤ 30 IU/L) and HBV DNA (< 2000 IU/mL) levels	Consider liver biopsy or non-invasive test if age > 40 yr, high HBV DNA or platelet counts < 15 × 10^4/uL, family history of HCC

A2: Activity score 2; AASLD: American Association for the Study of Liver Disease; ALT: Alanine aminotransferase; APASL: Asian-Pacific Association for the Study of the Liver; EASL: European Association for the Study of Liver Disease; F2/3: Fibrosis score 2/3; HBeAg: Hepatitis B e-antigen; HBV: Hepatitis B virus; HCC: Hepatocellular carcinoma; JSG: Japanese Society of Hepatology.

ULN). These patients were divided into reassurance, observation, liver biopsy, and treatment consideration groups; notably, 58% and 62% of patients with normal and elevated ALT levels (> 1–5 times ULN), respectively, did not require a liver biopsy. In addition to measuring HBsAg, ALT levels, HBV DNA load, and liver fibrosis should be constantly monitored to diagnose true immune tolerance. Recently, non-invasive diagnostic methods for diagnosing fibrosis, such as transient elastography, and markers for liver fibrosis have been developed[63,64]. These methods must be used to accurately diagnose HBV and develop future policies for clinical management of patients with HBV infection.

Antiviral treatment during the immune-inactive phase

The third phase of CHB, which is the immune-inactive phase, is characterized by low HBV DNA load (usually < 2000 IU/mL) and ALT levels within the normal range. HBeAg seroconversion often reduces hepatitis symptoms, however, in 20%–30% of these patients, HBV re-proliferates, hepatitis relapses, and the third immunoactive HBeAg phase transitions into the immune-negative phase. When HBV does not repopulate, it leads to a decrease in HBV DNA load as well as reduction in HBsAg; then, CHB progresses to the HBsAg-negative resolved phase[2,5,7,13]. JSG guidelines define the immune-inactive phase (phase 3) when the patient is HBeAg negative at least three times over 1-year follow-up period, with HBV DNA levels < 2000 IU/mL, and ALT ≤ 30 IU/L. This guideline is based on the fact that histologically favorable liver disease is rare when HBeAg is negative three or more times during follow-up for 1 year or more. In this case, HBV DNA is usually < 2000 IU/mL and ALT is typically < 40 IU/L. Although liver biopsy is not required at this time, lifelong monitoring is indeed required. However, even under these conditions, patients with advanced fibrosis are at increased risk of liver carcinogenesis. Thus, treatment should be considered if liver fibrosis is suspected as recommended *per* the AASLD, EASL, and APASL guidelines. During this phase, antiviral treatment is recommended if the patient has a family history of HCC or cirrhosis or if significant histological findings are noted on the liver biopsy. Thus, in this phase, age, family history of cirrhosis and HCC, and fibrosis progression are important factors for consideration.

Loss of HBsAg is an ideal endpoint and antiviral therapy during the immune-inactive phase may promote HBsAg clearance and lead to low HBsAg levels. The annual incidence of HCC and liver-related deaths among patients in this phase are higher and this trend may be reduced by antiviral treatment. However, treatment should be carefully considered, as chances of HBsAg loss can still increase naturally during the immune stage compared to other stages. It is also believed that antiviral treatment is not necessary and should only be considered in exceptional circumstance. If antiviral treatment is provided, the administration time during the immune-inactive period will be shorter than that during the immune tolerance period.

Need for treatment in the resolved phase of CHB

In the resolved phase of CHB, the patient tests negative for HBsAg, HBV DNA is not detected, ALT level is normalized, and liver inflammation disappears. This condition

is the goal of hepatitis B treatment and no longer requires NA therapy. However, in cases where treatment is initiated with NA(s), AASLD guidelines recommend that patients consider discontinuing treatment when HBsAg is negative, and cirrhosis is absent[20]. The EASL guidelines may consider long-term (*i.e.*, 3 years or longer) NA treatment after the loss of HBsAg, with or without HBsAg positivity. However, the EASL guidelines do not consider cirrhosis at this stage[15]. The APASL guidelines recommend treatment discontinuation if HBsAg level decreases, antibody reversal is observed, and HBV DNA is not detected for at least two years at three separate follow-up visits every six months. Nevertheless, it is recommended that lifelong NA therapy be continued for patients with cirrhosis[16]. Although the JSG guidelines also set criteria for discontinuing NA therapy based on HBsAg levels and HBcrAg[18], treatment discontinuation in the resolved CHB phase is not specified. However, for liver cirrhosis, relapse after discontinuation of NA treatment has a risk of inducing liver failure; therefore, treatment is generally continued lifelong.

If HBsAg is absent, treatment discontinuation remains an option; however, this is not recommended since results on the long-term prognosis of patients with discontinued treatment are currently unavailable. Thus, further therapy is not required if the HBsAg test yields negative results; however, indefinite treatment is recommended for patients with liver cirrhosis.

CONCLUSION

In summary, hepatitis B should be followed up or treated according to the hepatitis B disease course. Toward that end, the treatment selection, as well as mode and timing of drug administration are important for improving HBV prognosis. It is necessary to determine whether patients are in the immune-tolerant or immune-inactive phase to recommend appropriate follow-up and assess their need for antiviral therapy. Furthermore, a major consideration during the course of HBV treatment is to determine the true extent of immune inactivity and immune resistance, for which it is necessary to devise non-invasive evaluation of continuous ALT and HBV DNA level changes, and liver fibrosis. In addition, it is important to consider clinically relevant factors such as age, sex, and genotype during the treatment decision-making process. The option of early treatment also needs to be discussed during the immune tolerance stage. As these factors are not yet clarified in the global guidelines, future research is warranted to elucidate treatment options and prognosis according to the cirrhosis and HCC risk profiles of HBV-infected patients.

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ORIGINAL ARTICLE

Basic Study

Liver injury changes the biological characters of serum small extracellular vesicles and reprograms hepatic macrophages in mice

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Author contributions: Ji JL and Ji YH conceived the study and interpreted the data; Lv XF performed a systematic review and statistical analysis; Zhang AQ performed the Western blot analysis; Liu WQ performed qRT-PCR; Zhao M isolated the serum sEVs and performed the characterization; Li J, He L and Lu P performed the mouse model experiments; Cheng L performed the mouse model and the primary mouse hepatic macrophage-related experiments; Sun YF performed the histopathologic examination and the sEV characterization; Qin G performed the liver function test; Ji JL and Lv XF prepared the figures and drafted the manuscript; All authors read and approved the final version of the manuscript.

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Abstract

BACKGROUND

Serum small extracellular vesicles (sEVs) and their small RNA (sRNA) cargoes could be promising biomarkers for the diagnosis of liver injury. However, the dynamic changes in serum sEVs and their sRNA components during liver injury have not been well characterized. Given that hepatic macrophages can quickly

clear intravenously injected sEVs, the effect of liver injury-related serum sEVs on hepatic macrophages deserves to be explored.

AIM

To identify the characteristics of serum sEVs and the sRNAs during liver injury and explore their effects on hepatic macrophages.

METHODS

To identify serum sEV biomarkers for liver injury, we established a CCL_4-induced mouse liver injury model in C57BL/6 mice to simulate acute liver injury (ALI), chronic liver injury (CLI) and recovery. Serum sEVs were obtained and characterized by transmission electron microscopy and nanoparticle tracking analysis. Serum sEV sRNAs were profiled by sRNA sequencing. Differentially expressed microRNAs (miRNAs) were compared to mouse liver-enriched miRNAs and previously reported circulating miRNAs related to human liver diseases. The biological significance was evaluated by Ingenuity Pathway Analysis of altered sEV miRNAs and conditioned cultures of ALI serum sEVs with primary hepatic macrophages.

RESULTS

We found that both ALI and CLI changed the concentration and morphology of serum sEVs. The proportion of serum sEV miRNAs increased upon liver injury, with the liver as the primary contributor. The altered serum sEV miRNAs based on mouse studies were consistent with human liver disease-related circulating miRNAs. We established serum sEV miRNA signatures for ALI and CLI and a panel of miRNAs (miR-122-5p, miR-192-5p, and miR-22-3p) as a common marker for liver injury. The differential serum sEV miRNAs in ALI contributed mainly to liver steatosis and inflammation, while those in CLI contributed primarily to hepatocellular carcinoma and hyperplasia. ALI serum sEVs decreased both CD86 and CD206 expression in monocyte-derived macrophages but increased CD206 expression in resident macrophages *in vitro*.

CONCLUSION

Serum sEVs acquired different concentrations, sizes, morphologies and sRNA contents upon liver injury and could change the phenotype of liver macrophages. Serum sEVs therefore have good diagnostic and therapeutic potential for liver injury.

Key Words: MicroRNA; Small RNA sequencing; Biomarker; Monocyte-derived macrophage; Resident macrophage

Core Tip: The liver injury changed the concentration, morphology and small RNA contents of serum small extracellular vesicles (sEVs). Altered serum sEV microRNAs (miRNAs) based on mouse studies were highly consistent with the circulating miRNAs reported in human liver diseases. Serum sEV miRNA signatures for acute liver injury and chronic liver injury and a panel of miRNAs that can be used as a common marker for liver injury were established. Acute liver injury serum sEVs depolarized monocyte-derived macrophages and educated resident liver macrophages to transform into M2-like cells. Serum sEVs have good diagnostic and therapeutic potential for liver injury.

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INTRODUCTION

Because of its strategic location and biological functions, the liver is particularly susceptible to various pathogenic factors, including drugs, alcohol and viruses. The initial insult induces acute liver injury (ALI) or even liver failure. Repeated or persistent insults will cause chronic liver injury (CLI), resulting in liver fibrosis and finally fatal cirrhosis[1,2]. Therefore, it is important to identify individuals with liver injury. However, liver injury does not always cause noticeable signs and symptoms. Aside from the widely used blood liver function tests on serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST), which are not always restricted to liver injury[3], there is still a need to explore specific and sensitive biomarkers. With the rapid progress in medical research, it is now possible and necessary to search for new biomarkers from serum small extracellular vesicles (sEVs).

Recently, serum sEVs have attracted tremendous interest due to their essential roles in intercellular communication and to their diagnostic and therapeutic potential[4]. The term sEVs refers to extracellular vesicles released by cells that are of relatively small size (< 200 nm) and were previously regarded as exosomes[5]. The cargoes carried by sEVs represent a snapshot of the parental cells at the time of release and change depending on the physiological and pathological states[6,7]. In the liver, sEVs are released from both hepatocytes and nonparenchymal cells into the extracellular space and circulation. Several studies have reported that circulating sEV RNAs or proteins are abnormally expressed in the contexts of drug-induced liver injury (DILI), steatohepatitis, viral hepatitis and hepatocellular carcinoma (HCC)[6,8].

MicroRNAs (miRNAs) are 22-24 nt small noncoding RNAs involved in posttranscriptional regulation and various biological processes[9]. Tissue-specific distribution is a key feature of miRNAs, making miRNAs good candidates as biomarkers or therapeutic targets for particular types of tissue injury[10-12]. Serum miRNAs have been studied in a variety of liver diseases[13]. However, compared to serum miRNAs, serum sEV miRNAs are well protected from RNA enzymes. Thus, serum sEV can serve as a more reliable miRNA pool[14]. We hypothesized that serum sEVs and their miRNA cargoes might reflect liver damage upon injury and could be promising biomarkers.

In the present study, we tried to determine the effects of liver injury on serum sEVs and the small RNAs (sRNAs) they transport; we were also interested in determining if there is any difference between acute and chronic injury. A study in this regard will aid in identification of potential serum sEV miRNA biomarkers. The dynamic changes in the number and morphology of serum sEVs and the sRNA components of serum sEVs were examined. The profiles of deregulated serum sEV miRNAs were obtained and compared to those of mouse liver enriched miRNAs and previously reported circulating miRNAs related to human liver diseases (HLD). To further evaluate the biological significance of serum sEVs upon liver injury, conditioned cultures of ALI serum sEVs and primary hepatic macrophages were carried out.

MATERIALS AND METHODS

Animal studies

Male C57BL/6 mice (8 wk old) were purchased from the Shanghai Medical Laboratory Animal Center (Shanghai, China) and housed in the animal facility of Nantong University with temperature 25 ± 2 °C and 12 h light/dark cycle controls. All experimental protocols were approved by the Animal Ethics Committee of Nantong University. The animal care and experiments were performed in accordance with the relevant guidelines and regulations. For ALI, mice were treated with a single dose of CCL_4 (0.5 mL/kg intraperitoneal injection) dissolved in olive oil (1:9). The mice were sacrificed at 2 d or 7 d. For CLI, mice were treated with CCL_4 (0.5 mL/kg) or vehicle twice a week for 8 wk[15]. The mice were sacrificed 48 h after the last injection at 8 wk or at 12 wk (Figure 1A). Mice treated with the same volume of olive oil served as the controls for the ALI and CLI models, and 10-12 mice were used in each group. Blood or livers were collected from each group for further analyses.

Liver function test and histopathologic examination

Blood was collected by left ventricular puncture from mice and was left undisturbed for 1 h at 37 °C and 2 h at 4 °C. Afterward, the samples were centrifuged at 1000 × *g* for 10 min at 4 °C; the clear upper fractions were aliquoted and stored at -80 °C. Serum ALT and AST levels were measured on an ADVIA 1800 autoanalyzer (Siemens

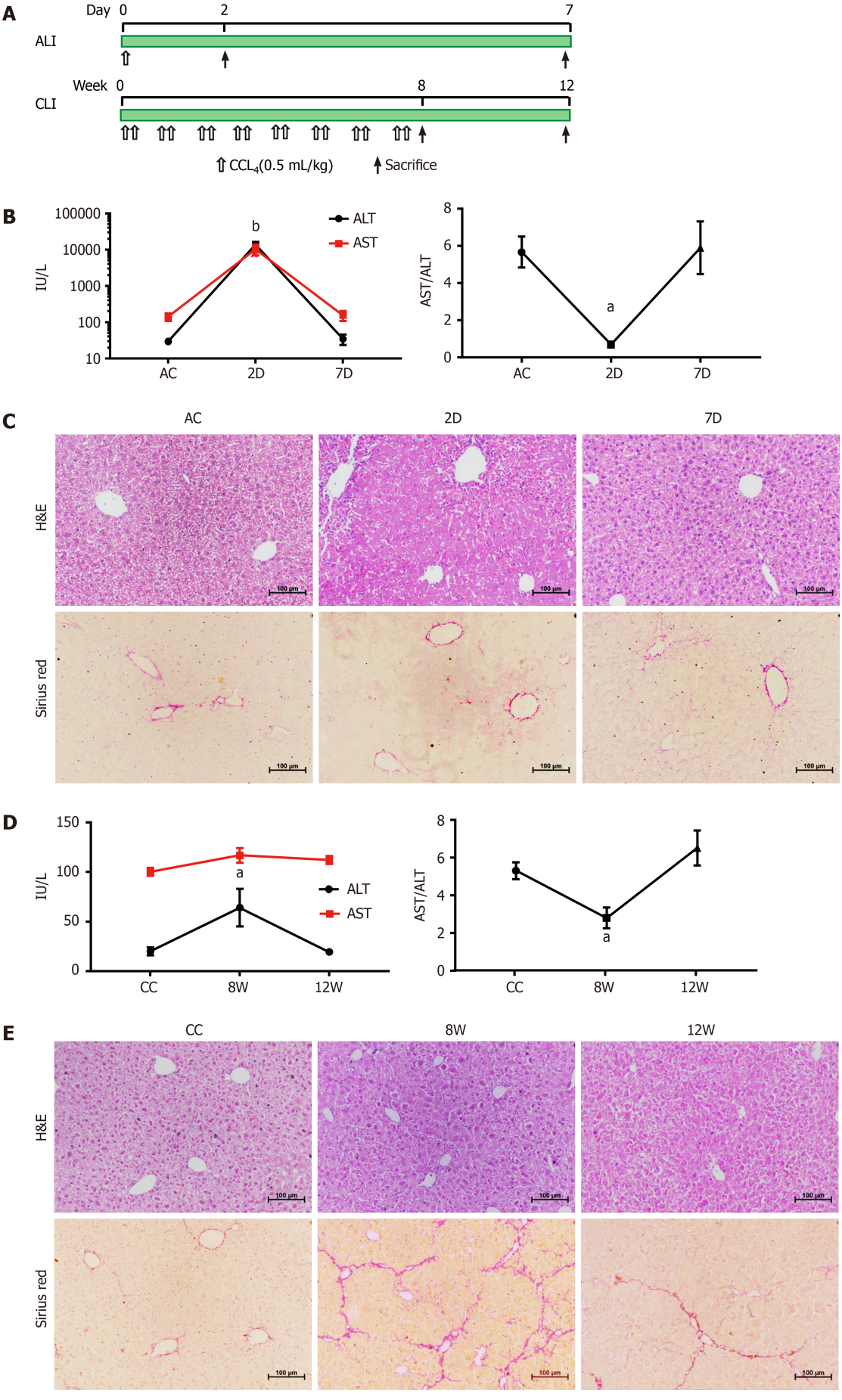


Figure 1 Establishment and validation of CCL_4-induced acute liver injury and chronic liver injury in mice. A: Workflow for the establishment of

the acute liver injury (ALI) and chronic liver injury (CLI) mouse models; B: Serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels in ALI mice. Compared with the ALI control (AC) group, $^{a}P < 0.05$, $^{b}P < 0.01$; C: Hematoxylin-eosin (H&E) and Sirius Red staining of liver sections from AC, 2 d and 7 d mice; D: Serum ALT and AST levels in CLI mice. Compared with the CC group, $^{a}P < 0.05$; E: H&E and Sirius Red staining of liver sections from CLI control (CC), 8 wk and 12 wk mice. Scale bar = 100 μm. D: Day; W: Week.

Healthcare Diagnostics, Deerfield, IL, United States). The livers were preserved in 4% paraformaldehyde, paraffin-embedded and sectioned. The liver tissue sections were stained with hematoxylin and eosin (Beyotime Biotechnology, Shanghai, China) for routine histology and 0.1% Sirius Red (Sigma-Aldrich, St. Louis, MO, United States) for collagen evaluation.

Mouse serum sEV isolation and characterization

Exosome-enriched serum sEV fractions were precipitated using ultracentrifugation and an ExoQuick precipitation kit (System Biosciences Inc., Mountain View, CA, United States)[16]. The sizes and particle concentrations of the isolated serum sEVs were measured by nanoparticle tracking analysis (NTA, NanoSight NS300, Malvern, United Kingdom). Serum sEVs were visualized using transmission electron microscopy (TEM, HT7700, Hitachi Ltd., Tokyo, Japan). The expression of exosomal protein markers was determined by Western blot analysis. The details are provided in the Supplementary material, Supporting Information.

sRNA library construction and deep sequencing

Serum sEV sRNA sequencing (RNA-seq) was conducted by BMK Biotech Co., Ltd. (Beijing, China) with biological replicates for each group.

Sequencing data analysis and bioinformatic analysis

The raw data were processed as described previously[16]. The trimmed sequencing reads were deposited in the European Nucleotide Archive (https://www.ebi.ac.uk/arrayexpress/experiments/E-MTAB-9462). Bioinformatic analysis of the differentially expressed serum sEV miRNAs was performed using Ingenuity Pathways Analysis (Qiagen, Valencia, CA, United States). The significance of enrichment for genes with particular biologically relevant functions was determined with a one-sided Fisher's exact test.

Systematic review of abnormally expressed circulating miRNAs in HLDs

The detailed procedure is provided in the Supplementary material, Supporting Information.

Isolation and culture of mouse hepatic macrophages

Primary mouse hepatic macrophages were isolated from male C57BL/6 mouse livers by Percoll (GE Healthcare, Princeton, NJ, United States) density gradient centrifugation. Incubation of liver macrophages with mouse serum sEVs and subsequent multiple-color flow cytometric analysis were carried out. The details are provided in the Supplementary material, Supporting Information.

Statistical analysis

Statistical analyses were performed with GraphPad Prism 7.0 software (GraphPad Software, Inc., La Jolla, CA, United States). Quantitative data were reported as the mean ± standard deviation. Comparisons between groups were made by Student's *t*-test or one-way analysis of variance. All *P* values were two-sided, and statistical significance was accepted for a value less than 0.05. Except for the data from sRNA-seq experiments, which included two biological replicates for each group, the data provided in the present study were from three or more independent experiments.

Additional methods and details are provided in the Supplementary material, Supporting Information.

RESULTS

Establishment and validation of CCL_4-induced ALI and CLI in mice

The mouse CCL_4-induced ALI and recovery model and the mouse CCL_4-induced CLI and recovery model were established and validated (Figure 1).

In acutely injured mice (at 2 d; 2D group), serum ALT and AST levels were increased, and hepatocyte necrosis and inflammatory cell infiltration were observed around the lobular central vein. After 5 d of recovery (at 7 d; 7D group), the elevated ALT and AST levels had returned to the baseline of the ALI control group, and the histological changes were also reversed (Figure 1B and C). For CLI, repeated CCL_4 treatment induced a slight elevation in serum ALT (at 8 wk; 8W group), but the level returned to the baseline of the CLI control (CC) group by 4 wk after cessation of CCL_4 treatment (at 12 wk; 12W group) (Figure 1D). Although the ALT level change in CLI at 8W was not as prominent as those in ALI at 2D, the change was comparable to the ALT level changes reported by other study groups using the same mice CLI model[17, 18]. Damaged hepatocytes and centrilobular contracture were observed in the livers of the CLI mice (8W), with mild inflammatory cell infiltration. Sirius Red staining showed obvious collagen deposition and pseudolobule formation in 8W CLI mice. These morphological changes were alleviated in recovered mice (12W) (Figure 1E).

Identification and characterization of serum sEVs from ALI and CLI mice

The isolated particles were spherical or cup-shaped, as observed by TEM (Figure 2A). Exosomal protein markers, including CD63, CD81 and CD9, were all highly expressed (Figure 2B), as determined by Western blot analysis.

NTA showed that the mean diameter of the particles ranged from 90.2 nm to 127.8 nm. The number of particles was higher in the 2D group but lower in the 7D group than in the control group, and the particle diameters were smaller in the 2D group but larger in the 7D group than in the control group. We also noticed that the size distribution of sEVs widened and that multiple peaks were present in the 7D group (Figure 2C). For CLI mice, the particle concentrations in the 8W and 12W groups tended to be lower than those in the control group, although there were no significant differences. The size distribution of sEVs was expanded with multiple peaks in both the 8W and 12W groups, and the particle diameters were larger in both the 8W and 12W groups (Figure 2D). TEM examination revealed that the multiple peaks reflected the aggregation or fusion of mouse serum sEVs present in 7D, 8W and 12W samples (Figure 2C and D).

These findings suggested that both ALI and CLI changed the number and morphology of mouse serum sEVs, and even when the visible histological changes of the liver had recovered in the 7D and 12W groups, the changes in particle number and morphology of serum sEVs persisted.

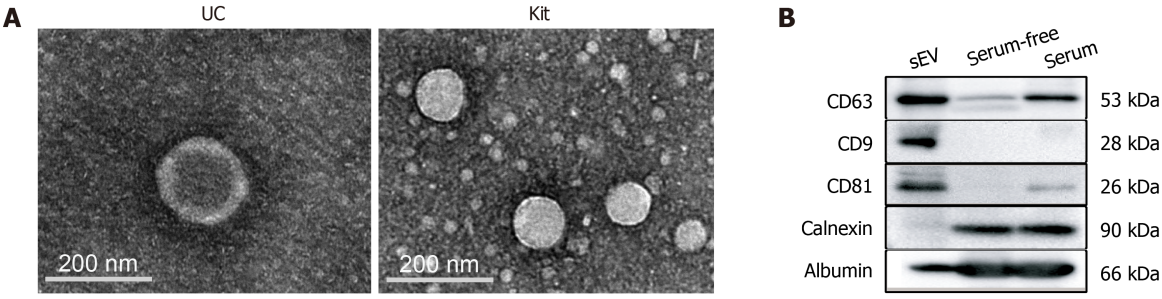
Comparison of the sRNA components in serum sEVs from ALI and CLI mice

Dramatic increases in both total RNA and sRNA levels in serum sEVs were observed upon ALI (2D) (Figure 3A and B). For CLI, there was no significant difference in either sEV RNA or sRNA content among the groups. RNA-seq and annotation revealed that each pair of biological repeats had at least 94.97% common sequences in clean reads (Supplementary Figure 1). In the control groups (ALI control and CC), tRNA was the dominant sRNA species in serum sEVs, followed by rRNA and miRNA (Figure 3C). The most remarkable change in serum sEV sRNAs was the increase in miRNA proportion in both ALI and CLI mice. Compared to the control condition, ALI increased the proportion of miRNAs by more than four-fold, but the proportion returned to baseline by 7 d; the proportion of miRNAs increased by almost three-fold in CLI mice but was partially restored by 12 wk. With the increase in miRNA, the proportion of tRNA decreased (Figure 3D).

miRNA expression profiles of serum sEVs from ALI and CLI mice

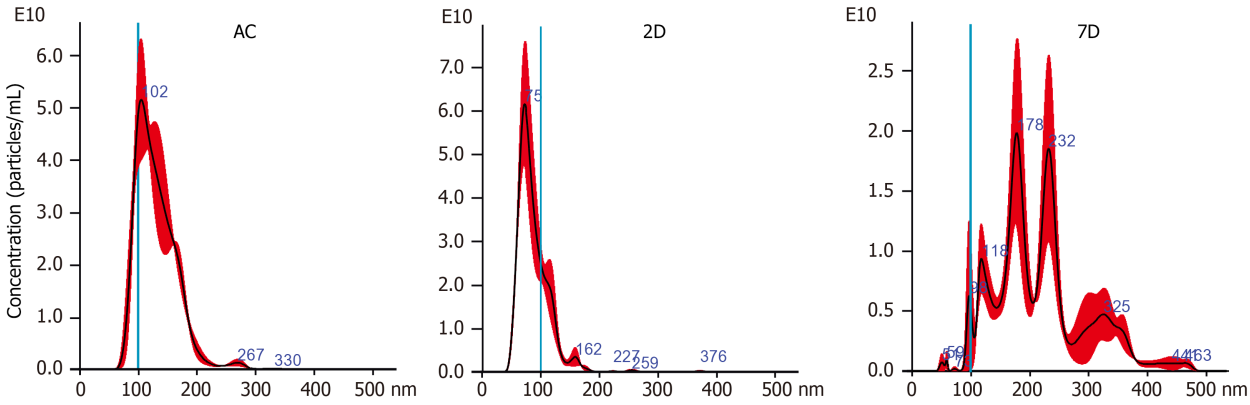
For the ALI and CLI groups, 467 and 488 detectable sEVs (transcripts per million reads ≥ 5.0) miRNAs were obtained, respectively. The biological replications were highly correlated in each group (Figure 4A). The RNA-seq data were further validated by quantitative real-time polymerase chain reaction. Differentially expressed miRNAs with different abundances were selected for validation (Figure 4B).

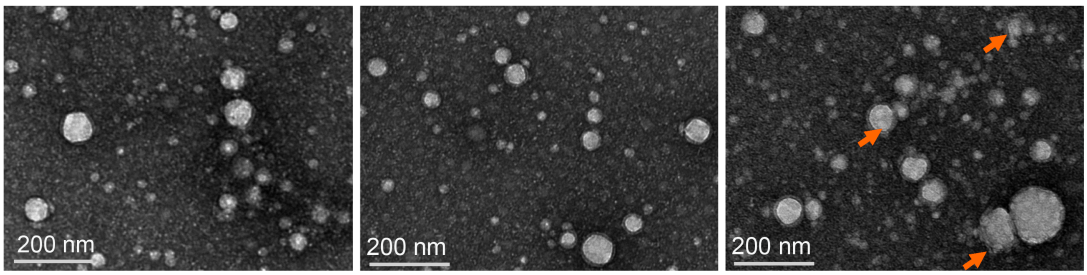
In total, 91 miRNAs were upregulated and 85 miRNAs were downregulated (fold change ≥ 2.0, $P < 0.05$) in the 2D group compared with the ALI control group (Figure 4C and Supplementary Table 1). The levels of most of these miRNAs had recovered to baseline levels in the 7D group, in which only 8 upregulated miRNAs and 11 downregulated miRNAs were detected (fold change ≥ 2.0, $P < 0.05$) (Figure 4C and Supplementary Table 2). The cumulative distribution frequency was calculated by adding each proportion of miRNAs from most to least abundant. The plot showed that the top five upregulated miRNAs (miR-148a-3p, miR-122-5p, miR-192-5p, miR-22-3p and miR-21a-5p) in the 2D group accounted for up to 84.27% of all detectable miRNAs



C

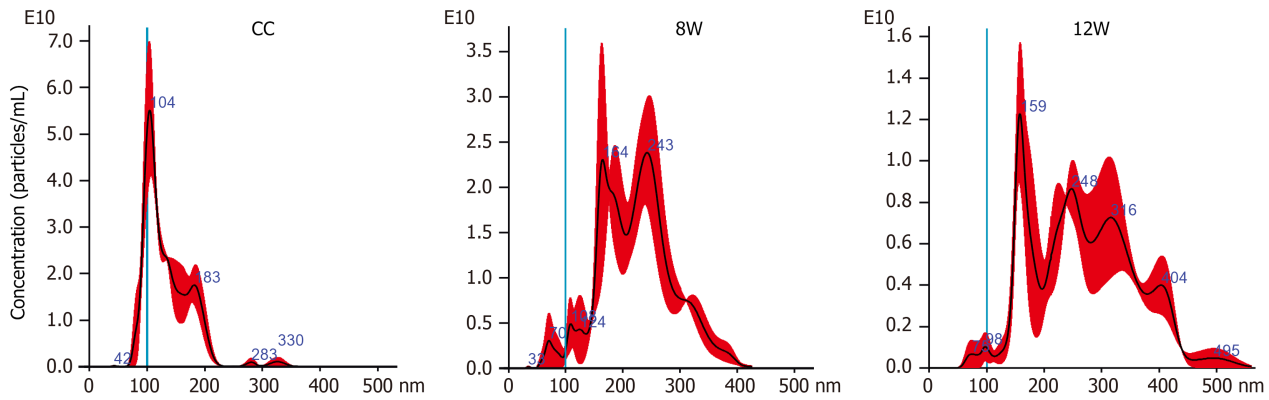
Group	Concentration (particles/mL)	Diameter (nm)
AC	4.80 ± 0.14 × 10^{12}	151.5 ± 11.23
2D	1.86 ± 0.44 × 10^{13a}	106.7 ± 11.98[a]
7D	1.50 ± 0.05 × 10^{12}	252.7 ± 21.34[b]





D

Group	Concentration (particles/mL)	Diameter (nm)
CC	4.91 ± 0.92 × 10^{12}	135.2 ± 2.23
8W	1.83 ± 0.76 × 10^{12}	258.9 ± 19.96[b]
12W	1.47 ± 0.31 × 10^{12}	268.2 ± 6.65[b]



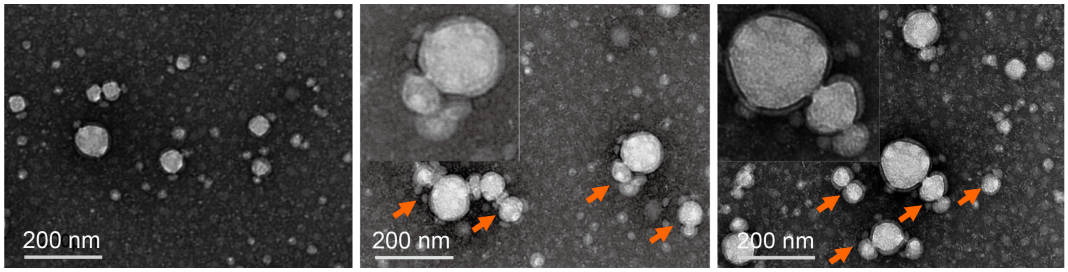


Figure 2 Characterization of isolated mouse serum small extracellular vesicles. A: Transmission electron microscopy (TEM) images of the particles isolated using ultracentrifugation and an ExoQuick precipitation kit; B: Representative Western blotting bands for CD63, CD81, CD9, calnexin and albumin; C: Nanoparticle tracking analysis (NTA) plots for the size distribution and concentration of the isolated particles in each group from ALI mice and the corresponding TEM images. Compared with the ALI control group, $^{a}P < 0.05$, $^{b}P < 0.01$; D: NTA plots for the size distribution and concentration of the isolated particles in each group from CLI mice and the corresponding TEM images. Compared with the CLI control group, $^{b}P < 0.01$. The red arrow indicates aggregated particles; representative particles were amplified and were exemplified in the top left corner. Scale bar = 200 nm. UC: Ultracentrifuge; D: Day; W: Week; AC: Acute liver injury control; CC: Chronic liver injury control; sEVs: Small extracellular vesicles.

(Figure 4C).

Only 13 miRNAs were upregulated and six miRNAs were downregulated (fold change ≥ 2.0, $P < 0.05$) in the 8W group compared with the CC group (Figure 4D and Supplementary Table 3); in addition, 8 miRNAs were upregulated and 3 miRNAs were downregulated (fold change ≥ 2.0, $P < 0.05$) in the 12W group compared with the CC group (Figure 4D and Supplementary Table 4). The cumulative distribution frequency analysis showed that the top three upregulated miRNAs (miR-122-5p, miR-192-5p, and miR-22-3p) in the 8W group constituted up to 43.48% of all detectable miRNAs (Supplementary Figure 2). These findings suggested that both ALI and CLI induced changes in serum sEV miRNA composition. The changes were caused by the differential expression of a small number of miRNAs with high abundance.

The biological significance of these differentially expressed serum sEV miRNAs in liver injury was explored by Ingenuity Pathway Analysis (www.qiagen.com/ingenuity). For the 176 differentially expressed serum sEV miRNAs upon ALI, hepatic steatosis was the most significant hepatotoxic effect, followed by liver inflammation. For the 19 differentially expressed serum sEV miRNAs upon CLI, HCC was the most significant, followed by liver hyperplasia (Figure 4E).

The liver is the main contributor to the differentially expressed serum sEV miRNAs during ALI and CLI

We were interested in determining the contribution of liver cells to the changes in serum sEV miRNAs upon ALI and CLI. First, the liver miRNA expression profile for wild-type male C57BL/6 mice was established using BRB-Array Tool v4.6.0 (https://brb.nci.nih.gov) based on the RNA-seq data from GSE78792 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE78792)[19] (Supplementary Table 5). Differentially expressed serum sEV miRNAs from ALI and CLI mice were compared to the liver miRNA expression profile. Among the top ten most abundant liver miRNAs, seven were also among the top ten increased miRNAs in serum sEVs upon ALI (Table 1) and constituted up to 84.70% of the increased serum sEV miRNAs. For CLI, the levels of three were raised in serum sEVs. These three miRNAs were the top three miRNAs that increased upon CLI (Table 1) and constituted up to 60.56% of the total increased serum sEV miRNAs. These findings suggested the liver as the primary contributor to the upregulated serum sEV miRNAs during ALI and CLI and confirmed that the serum sEV miRNA test could be a reliable and sensitive way to monitor either ALI or CLI.

ALI and CLI signatures based on serum sEV miRNA profiling

To identify serum sEV miRNA signatures for liver injury, we compared the differentially expressed serum sEV miRNAs in various stages of ALI and CLI. Compared to the levels in the vehicle control samples, eight miRNAs were upregulated and two miRNAs were downregulated significantly during the acute injury stage, and these changes were sustained through the chronic phase (fold change ≥ 2.0, $P < 0.05$); thus, they can serve as common liver injury signatures (Figure 5A). In addition, the levels of 166 miRNAs changed significantly during acute injury, while those of nine miRNAs changed dramatically during chronic injury. These miRNAs with high abundance (transcripts per million reads _mean > 1000) have the potential to be ALI or CLI

Table 1 Liver-enriched microRNAs ranked highly among serum small extracellular vesicle microRNAs from acute liver injury and chronic liver injury model mice

GSE78792 liver	Rank	ALI	ALI rank	CLI	CLI rank
miR-192	1	miR-192-5p	3	miR-192-5p	2
miR-22	2	miR-22-3p	4	miR-22-3p	3
miR-30a	3	miR-30a-5p	7	N/A	N/A
miR-148a	4	miR-148a-3p	1	N/A	N/A
miR-21a	5	miR-21a-5p	5	N/A	N/A
miR-26a-2	6	miR-26a-5p	105	N/A	N/A
miR-122	7	miR-122-5p	2	miR-122-5p	1
miR-10a	8	N/A	N/A	N/A	N/A
miR-143	9	miR-143-3p	92	N/A	N/A
miR-27b	10	miR-27b-3p	10	N/A	N/A

N/A: Not applicable; ALI: Acute liver injury; CLI: Chronic liver injury.

signatures. The complete lists of these potential ALI and CLI serum sEV miRNA signatures are provided in Supplementary Table 6. According to their abundance, up to the top 20 miRNAs are listed in Figure 5. For the recovery stage, 18 miRNAs were changed significantly in the ALI group, and 11 miRNAs were changed significantly in the CLI group (Figure 5B). Some of these miRNAs overlapped with differentially expressed serum sEV miRNAs in corresponding acute or chronic injury stages.

Comparison of the potential ALI and CLI serum sEV miRNA signatures to HLD-related circulating miRNAs

To explore the biological significance of the ALI and CLI serum sEV miRNA signatures in HLDs, we performed a systematic review of abnormally expressed circulating miRNAs reported in various HLDs. In total, 299 and 257 studies were identified from PubMed (https://pubmed.ncbi.nlm.nih.gov) and Web of Science (http://apps.webofknowledge.com/) databases, respectively (Figure 6A). Data were retrieved from 14 studies, including drug-induced liver injury[20], chronic hepatitis B [21-25], chronic hepatitis C[21,25-27], nonalcoholic fatty liver disease[28], nonalcoholic steatohepatitis[23], liver cirrhosis[24,29,30] and HCC[21-24,28-33] studies. Details on the 14 articles are summarized in Supplementary Table 7.

In total, 269 nonredundant abnormally expressed circulating miRNAs related to HLDs were extracted, and those that appeared ≥ four times were defined as high-frequency miRNAs (Figure 6A). Of the 30 high-frequency miRNAs (Supplementary Table 7), 23 miRNAs were detected in ALI and CLI serum sEVs (Figure 6B), 14 miRNAs overlapped with ALI and CLI serum sEV signatures, and 12 miRNAs showed the same expression trend. Of the 12 miRNAs, three miRNAs (miR-122-5p, miR-192-5p, and miR-22-3p) were identified as being increased in both ALI and CLI mice and thus have the potential to serve as common signatures for either ALI or CLI. The other 9 miRNAs were identified as ALI signatures (miR-21a-5p, miR-92a-3p, miR-194-5p, miR-17-5p and miR-19b-3p were increased; miR-451a, miR-27a-3p, miR-26a-5p, and miR-223-3p were decreased) and may reflect acute or active liver injury (Figure 6C). In addition, it was noteworthy that the four high-frequency circulating miRNAs reported in HLDs with decreased levels all exhibited decreased levels in serum sEVs upon ALI.

Serum sEVs from liver injury mice induced hepatic macrophage reprogramming

Primary mouse hepatic macrophages were isolated, purified and incubated with SYTO-labeled serum sEVs. After 24 h, green fluorescence was observed in most macrophages in both the control and ALI (2D) serum sEV incubation groups (Figure 7A). These observations indicated that serum sEVs could be taken up by hepatic macrophages. Serum sEVs from ALI mice (2D) accelerated the adhesion of hepatic macrophages (Figure 7B). As determined by quantitative real-time polymerase chain reaction, normal serum sEVs increased M1-like gene (*IL-1B* and *TNFA)*

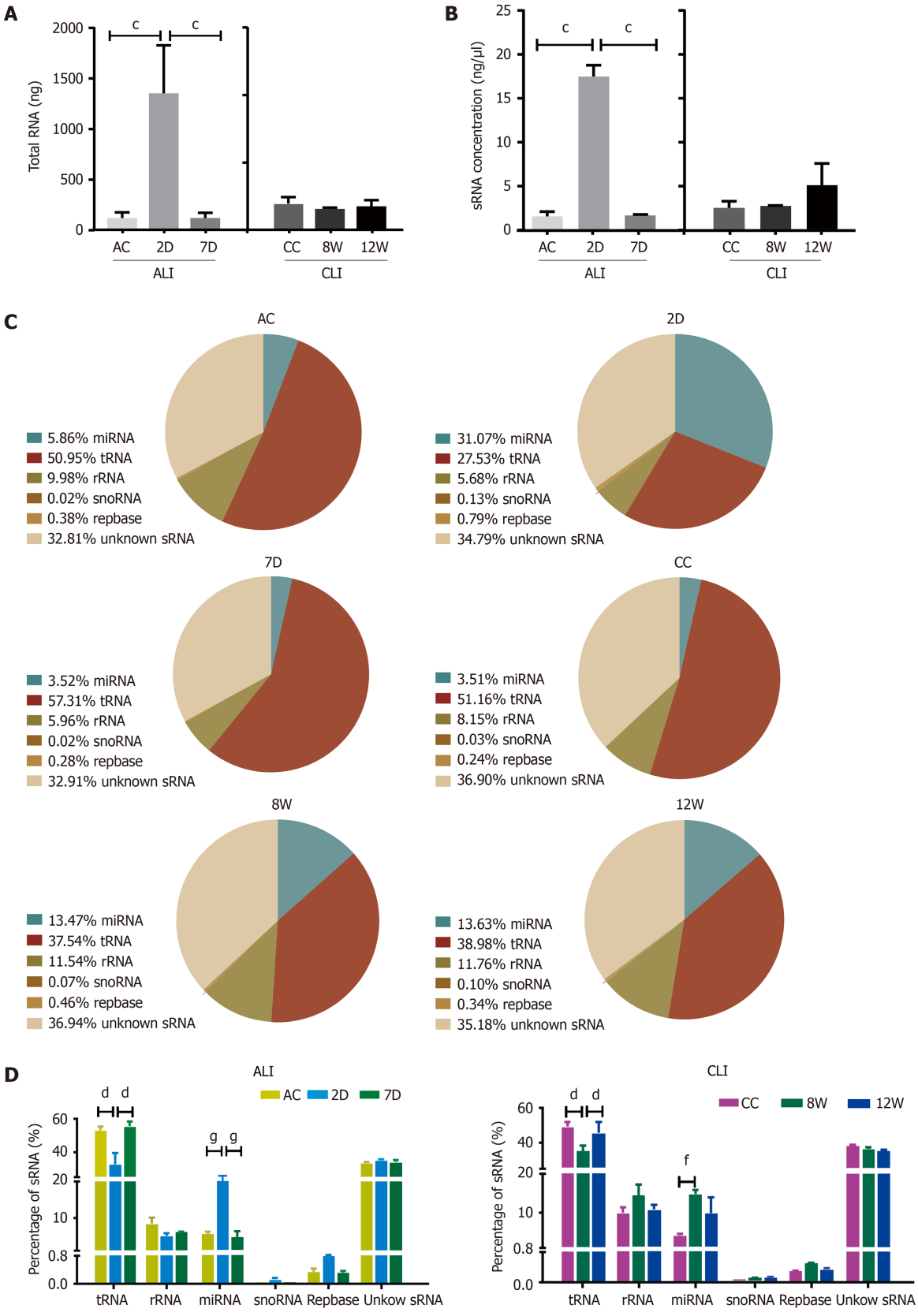
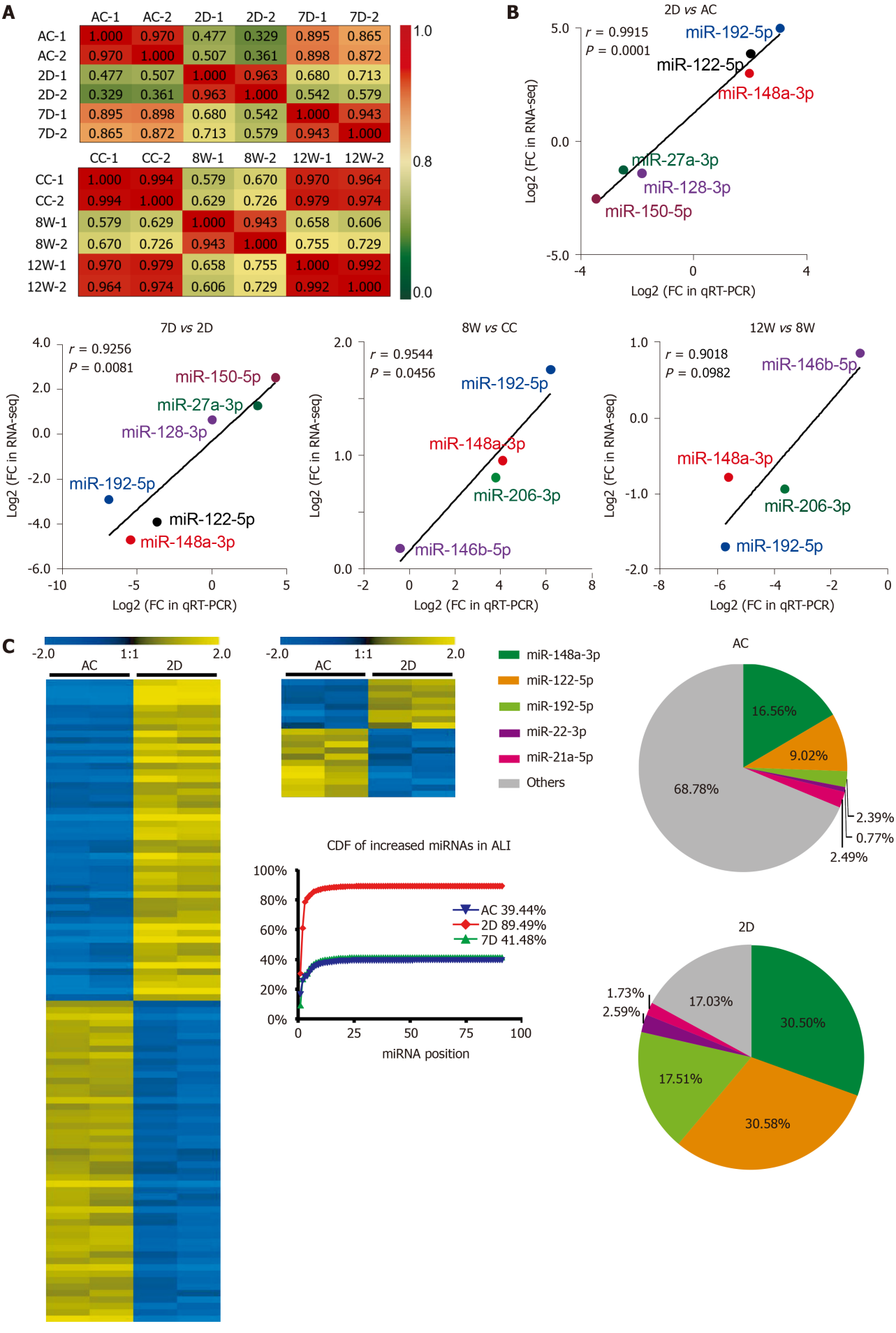


Figure 3 Annotations of small RNA in serum small extracellular vesicles from acute liver injury and chronic liver injury mice. A: Total RNA in serum small extracellular vesicles (sEVs) compared with the 2D group, $^{c}P < 0.001$; B: Small RNA (sRNA) concentrations in serum sEVs compared with the 2 d group, $^{c}P < 0.001$; C: Representative pie charts depicting the annotated sRNA species and their proportions in the different groups; D: Statistical analysis of sRNA species in sEVs. Compared with the 2 d group, $^{d}P < 0.0001$; compared with the 8 wk group, $^{f}P < 0.01$, $^{g}P < 0.0001$. D: Day; W: Week; AC: Acute liver injury control; CC: Chronic liver injury control; ALI: Acute liver injury; CLI: Chronic liver injury; miRNA: MicroRNA; snoRNA: Small nucleolar RNA.


A
AC-1 AC-2 2D-1 2D-2 7D-1 7D-2
AC-1 1.000 0.970 0.477 0.329 0.895 0.865
AC-2 0.970 1.000 0.507 0.361 0.898 0.872
2D-1 0.477 0.507 1.000 0.963 0.680 0.713
2D-2 0.329 0.361 0.963 1.000 0.542 0.579
7D-1 0.895 0.898 0.680 0.542 1.000 0.943
7D-2 0.865 0.872 0.713 0.579 0.943 1.000
CC-1 CC-2 8W-1 8W-2 12W-1 12W-2
CC-1 1.000 0.994 0.579 0.670 0.970 0.964
CC-2 0.994 1.000 0.629 0.726 0.979 0.974
8W-1 0.579 0.629 1.000 0.943 0.658 0.606
8W-2 0.670 0.726 0.943 1.000 0.755 0.729
12W-1 0.970 0.979 0.658 0.755 1.000 0.992
12W-2 0.964 0.974 0.606 0.729 0.992 1.000
1.0
0.8
0.0
B
2D vs AC
r = 0.9915
P = 0.0001
miR-192-5p
miR-122-5p
miR-148a-3p
miR-27a-3p
miR-128-3p
miR-150-5p
Log2 (FC in RNA-seq)
Log2 (FC in qRT-PCR)
7D vs 2D
r = 0.9256
P = 0.0081
miR-150-5p
miR-27a-3p
miR-128-3p
miR-192-5p
miR-122-5p
miR-148a-3p
8W vs CC
r = 0.9544
P = 0.0456
miR-192-5p
miR-148a-3p
miR-206-3p
miR-146b-5p
12W vs 8W
r = 0.9018
P = 0.0982
miR-146b-5p
miR-148a-3p
miR-206-3p
miR-192-5p
C
-2.0 1:1 2.0
AC 2D
miR-148a-3p
miR-122-5p
miR-192-5p
miR-22-3p
miR-21a-5p
Others
CDF of increased miRNAs in ALI
AC 39.44%
2D 89.49%
7D 41.48%
miRNA position
AC
16.56%
9.02%
68.78%
2.39%
0.77%
2.49%
2D
1.73%
2.59%
17.03%
30.50%
17.51%
30.58%

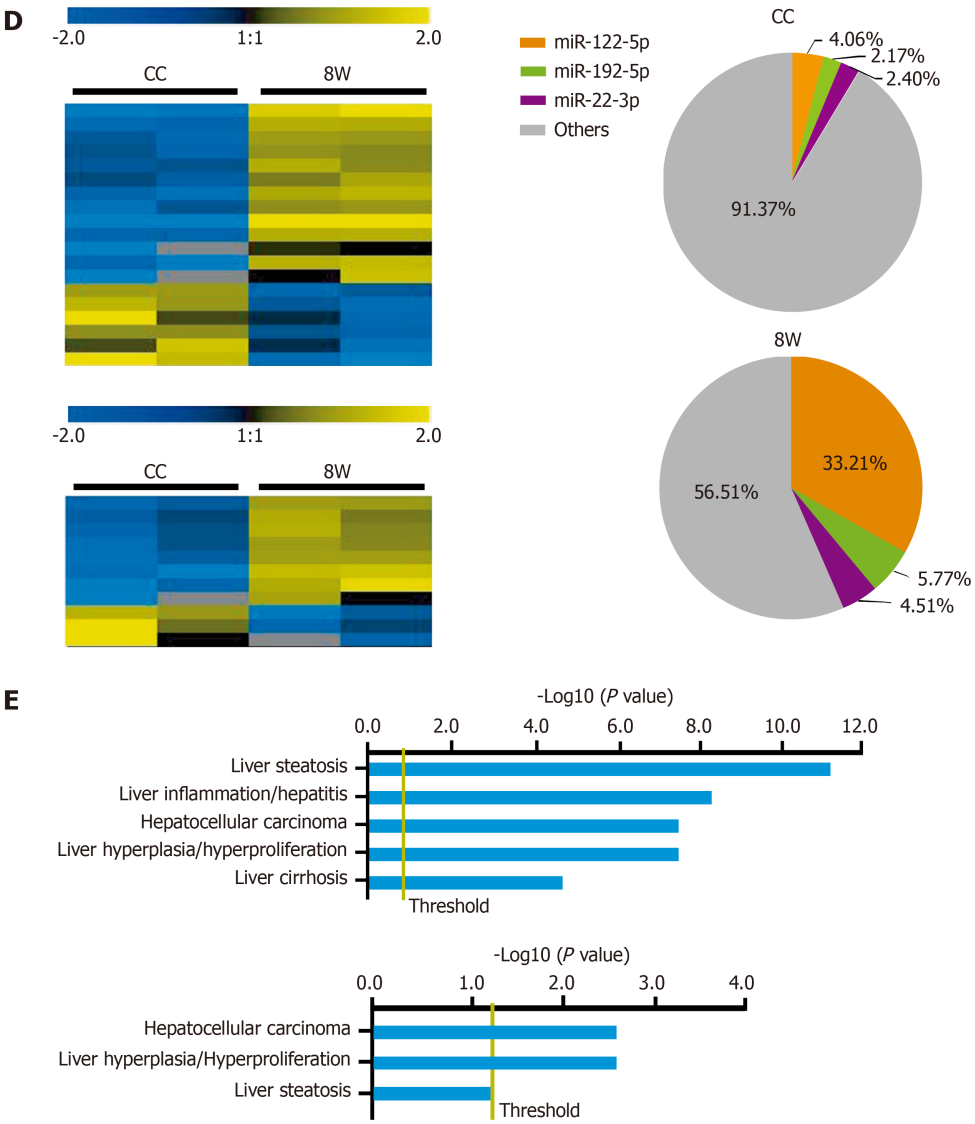


Figure 4 Differentially expressed microRNAs in serum small extracellular vesicles from acute liver injury and chronic liver injury mice and their biological significance. A: Pearson correlation coefficients between samples from acute liver injury (ALI) and chronic liver injury (CLI). The correlation coefficient values are labeled in the heat map; red or green represents high or low correlation, respectively; B: Validation of RNA sequencing data by quantitative real-time polymerase chain reaction for microRNAs (miRNAs) from ALI and CLI; C: Heatmap for the differentially expressed miRNAs in the ALI injury stage (2 d *vs* ALI control) and recovery stage (7 d *vs* ALI control), a cumulative distribution frequency plot for the increased miRNAs in ALI, and pie charts illustrating the proportions of the top five upregulated miRNAs in the ALI control and 2 d groups; D: Heatmap for the differentially expressed miRNAs in the CLI injury stage (8 wk *vs* CLI control) and recovery stage (12 wk *vs* CLI control). The pie charts illustrate the proportions of the top three upregulated miRNAs in the CLI control and 8 wk groups; E: The top hepatotoxicity processes related to the differentially expressed serum sEV miRNAs in ALI (top) and the differentially expressed serum sEV miRNAs in CLI (bottom) cataloged by Ingenuity Pathway Analysis-Tox function analysis. D: Day; W: Week; AC: Acute liver injury control; CC: Chronic liver injury control; ALI: Acute liver injury; CDF: Cumulative distribution frequency; qRT-PCR: Quantitative real-time polymerase chain reaction; FC: Fold change; RNA-seq: RNA sequencing; miRNA: MicroRNA.

expression and decreased the expression of some M2-like genes (*IL-10RB*, *CD163*, *ARG1* and *CD206*); ALI serum sEVs showed similar effects. However, compared to normal serum sEVs, ALI serum sEVs tended to decrease M1-like *IL-1B* but increase M2-like *CD163* gene expression (Figure 7C).

In order to further dissect the effects of ALI serum sEVs on monocyte-derived and resident hepatic macrophage subgroups, multiple-color flow cytometric analyses were performed to assess the expression of M1-like CD86 and M2-like CD206 in $CD11b^{+}F4/80^{Low}$ monocyte-derived and $CD11b^{+}F4/80^{High}$ resident macrophages (Tacke and Zimmermann[34] and our unpublished data). ALI serum sEVs decreased both CD86 and CD206 expression in the $CD11b^{+}F4/80^{Low}$ subgroup but increased CD206 expression in the $CD11b^{+}F4/80^{High}$ subgroup (Figure 7D and E). These findings indicated that ALI serum sEVs might induce depolarization of $CD11b^{+}F4/80^{Low}$ monocyte-derived macrophages but M2 differentiation of $CD11b^{+}F4/80^{High}$ resident macrophages.

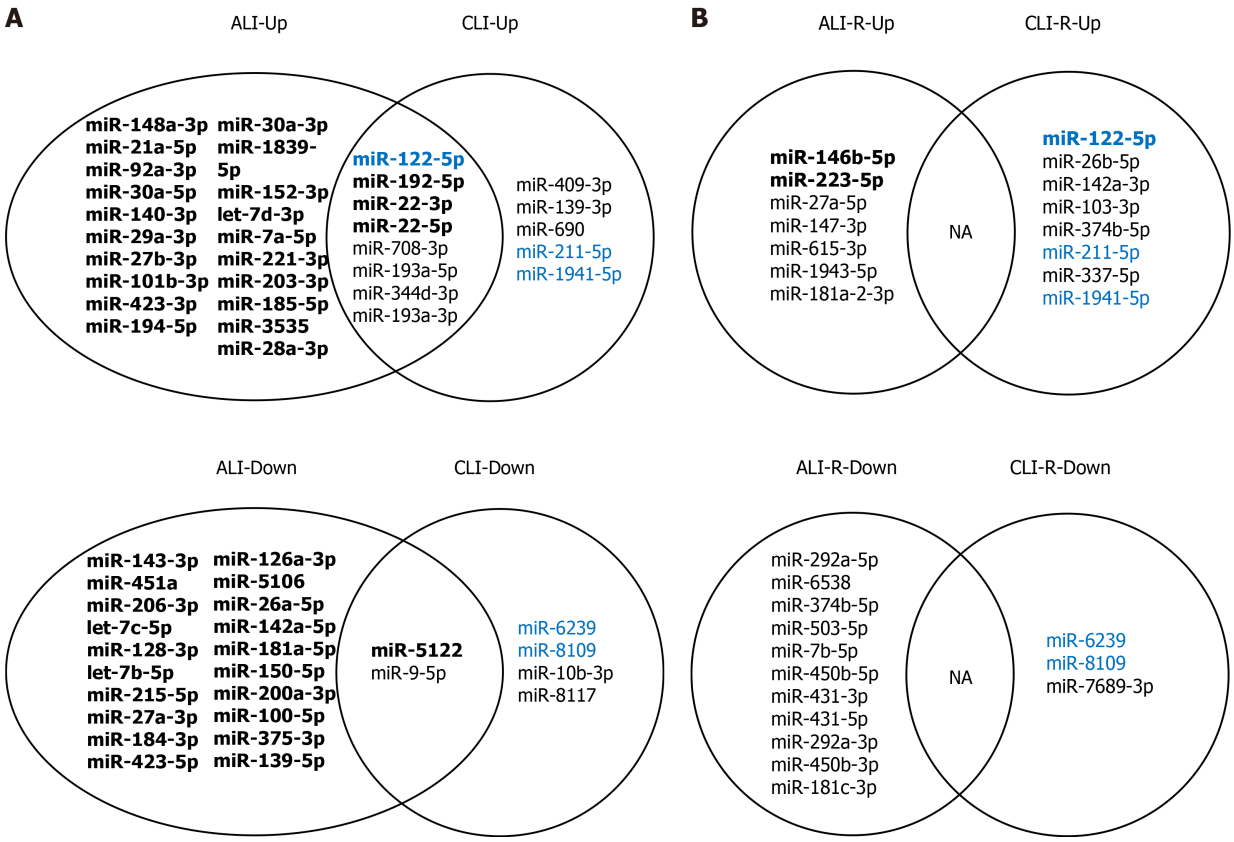


Figure 5 Common and specific microRNAs for acute and chronic liver injury and recovery in mouse models. A: Common and specific microRNAs (miRNAs) for acute and chronic liver injury; B: Common and specific miRNAs for the recovery stages of acute and chronic liver injury. Bold font: Transcripts per million reads Mean > 1000; Blue font: Overlapping microRNAs in injury and recovery; NA: Not applicable; ALI: Acute liver injury; CLI: Chronic liver injury; ALI-R: Acute liver injury recovery; CLI-R: Chronic liver injury recovery.

DISCUSSION

A growing number of studies have suggested the diagnostic value of serum sEV content for liver injury, especially miRNAs[6,8]. To explore potential serum sEV miRNA biomarkers for liver injury, we simulated the complex processes of liver injury and recovery in CCL_4-induced ALI and CLI mouse models. The 2D group and 8W group represented ALI and CLI, respectively, while the 7D group and 12W group represented the recovery stage of ALI and CLI, respectively. The ALT and AST levels elevated in the 2D group and 8W group and returned to the baseline of the control groups (Figure 1B and D). Moreover, the histological changes also reversed in the 7D and 12W recovery groups (Figure 1C and E), which indicated that the ALI and CLI and recovery models were well established.

Unexpectedly, aside from differentially expressed miRNAs, we found that the concentration, size and morphology of serum sEVs might be essential features in liver injury (Figure 2). The number of serum sEVs increased upon ALI, which has been reported in human alcoholic hepatitis and alcoholic liver injury mouse models[35-37]. Furthermore, we found sustained decreases in serum sEV number during the chronic injury stage and the recovery stage for both ALI and CLI. Moreover, serum sEVs became smaller upon ALI but enlarged during ALI recovery and the CLI stage, and the increased size persisted through the CLI recovery stage, at which time there were multiple peaks, as observed by NTA. Interestingly, aggregation of serum sEV particles was observed in samples from the ALI recovery stage, CLI stage and CLI recovery stage by TEM, which explained the multiple peaks and increased particle size found by NTA. We propose that the aggregation of sEVs might reflect membrane damage of extracellular vesicles following liver injury. Thus, changes in serum sEV concentration, size, and morphology are well connected to a particular stage of liver injury and could provide diagnostic clues. In addition, the changes in serum sEVs persisted even when liver function and visible histopathological changes were restored; thus, they could be useful to trace recent liver injury.

Figure 6 Differentially expressed circulating microRNAs in human liver disease and common microRNAs in mice. A: Flow chart of study selection and microRNA (miRNA) screening; B: Heatmap for the expression of high-frequency human liver disease (HLD)-related circulating miRNAs in acute liver

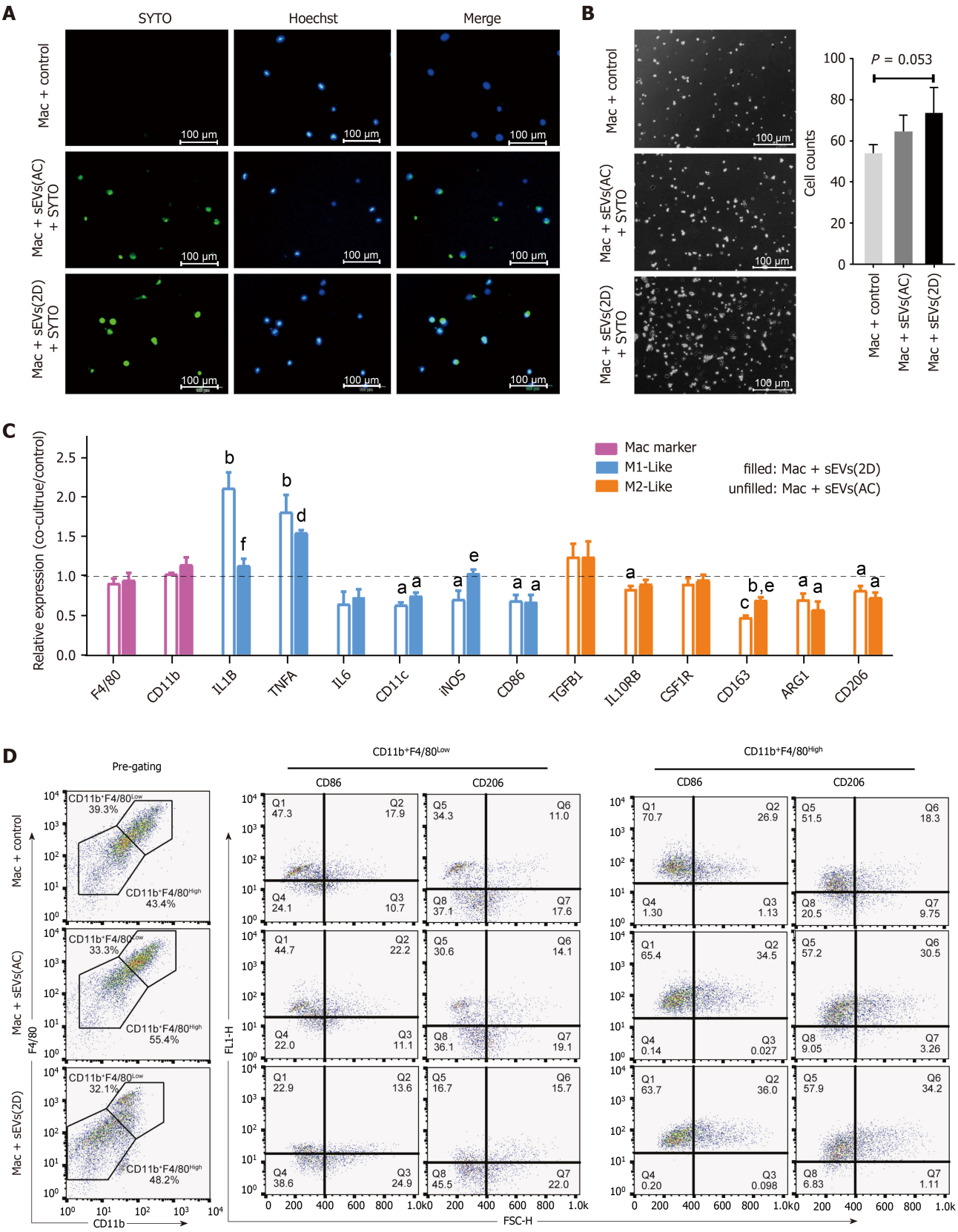
injury (ALI) and chronic liver injury (CLI) serum small extracellular vesicles (sEVs); C: Common miRNAs in the serum sEVs from mice with ALI and CLI and in the circulation in the context of HLDs. Red font: Upregulated miRNAs; Green font: Downregulated miRNAs; HC: Healthy control; AC: Acute liver injury control; CC: Chronic liver injury control; WoS: Web of Science; D: Day; W: Week.

Then, we showed that ALI and CLI altered the sRNA levels and components in serum sEVs. For ALI, both the total RNA and sRNA levels of serum sEVs increased significantly, and the proportion of miRNAs in sRNA also increased. Although there were no significant increases in RNA or sRNA levels for CLI, the proportion of miRNAs increased significantly. Compared to CLI, ALI significantly changed more miRNA species (176 *vs* 19, fold change ≥ 2.0, $P < 0.05$). The increased proportion of miRNAs upon liver injury was mainly attributable to a few highly abundant miRNAs. To traceback the primary source of the increased serum sEV miRNAs, the highly abundant miRNAs detected in serum sEVs from ALI and CLI mice were evaluated in the livers of normal male C57BL/6 mice. The miRNAs with the highest abundance were all liver-enriched miRNAs (Table 1). We propose that the liver is the main contributor to the elevations in miRNAs in serum sEVs for both ALI and CLI. Thus, serum sEVs carry the miRNA messages released from the injured liver, and examining the serum sEV miRNAs could be a reliable way to monitor either ALI or CLI.

By comparing the expression profiles of serum sEV miRNAs in various stages of ALI and CLI, we obtained a list of miRNAs that can be used as common liver injury signatures as well as the miRNA signatures for ALI, CLI and the recovery stages. However, these signatures were obtained from mouse models and need to be validated in human patients. Hence, we carried out a systematic review of previously published studies and obtained 30 miRNAs that were highly correlated with HLD, including drug-induced liver injury, chronic hepatitis B, chronic hepatitis C, nonalcoholic fatty liver disease, nonalcoholic steatohepatitis and liver cirrhosis (Figure 6). Of these 30 miRNAs, 3 miRNAs (miR-122-5p, miR-192-5p and miR-22-3p) were identified as common injury signatures that were increased in both ALI and CLI mice. Although these three miRNAs have been studied separately[24,36,38,39], here, for the first time, they were combined as a universal signature for either ALI or CLI. Nine miRNAs were identified as ALI signatures. Along with the five increased miRNAs (miR-21a-5p, miR-92a-3p, miR-194-5p, miR-17-5p and miR-19b-3p), four miRNAs were decreased (miR-451a, miR-27a-3p, miR-26a-5p and miR-223-3p): the same four miRNAs that are frequently reported to be decreased in HLD (Figure 6). Although the human data were mostly from patients with CLI, the overlapping ALI signatures might reflect active lesions. Based on serum sEV miRNAs, we established common signatures for liver injury and specific signatures for acute/active liver injury.

The biological significance of the alterations in sEV miRNAs upon liver injury was explored by Ingenuity Pathway Analysis. The top hepatotoxicity-related functions of the altered serum sEV miRNAs in ALI were hepatic steatosis and liver inflammation, while the altered miRNAs carried by serum sEVs from CLI were linked primarily to HCC, followed by liver hyperplasia. These findings are consistent with the clinical findings that ALI mainly causes inflammation and that sustained damage increases the risk of carcinoma[40,41]. Hepatic steatosis reflects fatty degeneration that is typically caused by CCL_4[42]. These findings reveal the pathological importance of serum sEVs during the processes of ALI and CLI.

It has been reported that hepatic macrophages can take up serum sEVs and play essential roles in the clearance of intravenously injected sEVs from the systemic circulation[43,44]. We were interested in determining the effects of liver injury-related serum sEVs on hepatic macrophages and whether these effects could aggravate liver damage or play a protective role. We found that *in vitro*, ALI serum sEVs could be taken up by hepatic macrophages and promote macrophage adhesion. Furthermore, ALI serum sEVs tended to decrease M1-like gene expression, such as *IL-1B* and *TNFA* expression, and increase M2-like gene expression, including *CD163* expression. However, the trends were not consistent. Considering that hepatic macrophages are heterogeneous populations composed of two subgroups, including resident macrophages and circulating monocyte-derived macrophages[34], we propose that macrophages of different origins might react inconsistently to ALI serum sEVs. The expression of M1 (CD86) and M2 (CD206) polarization signatures on the two subgroups of hepatic macrophages was examined by flow cytometry. ALI serum sEVs induced depolarization of $CD11b^{+}F4/80^{Low}$ monocyte-derived macrophages but induced M2 differentiation of $CD11b^{+}F4/80^{High}$ resident macrophages. We propose that the changes in serum sEVs upon ALI might alleviate liver damage by depolarizing


A
SYTO
Hoechst
Merge
Mac + control
Mac + sEVs(AC) + SYTO
Mac + sEVs(2D) + SYTO
100 μm
B
Mac + control
Mac + sEVs(AC) + SYTO
Mac + sEVs(2D) + SYTO
100 μm
P = 0.053
Cell counts
100
80
60
40
20
0
Mac + control
Mac + sEVs(AC)
Mac + sEVs(2D)
C
Relative expression (co-cultrue/control)
2.5
2.0
1.5
1.0
0.5
0.0
Mac marker
M1-Like
M2-Like
filled: Mac + sEVs(2D)
unfilled: Mac + sEVs(AC)
F4/80
CD11b
IL1B
TNFA
IL6
CD11c
iNOS
CD86
TGFB1
IL10RB
CSF1R
CD163
ARG1
CD206
D
Pre-gating
CD11b+F4/80Low
CD11b+F4/80High
CD86
CD206
Mac + control
Mac + sEVs(AC)
Mac + sEVs(2D)
F4/80
CD11b
FL1-H
FSC-H
CD11b+F4/80Low 39.3%
CD11b+F4/80High 43.4%
CD11b+F4/80Low 33.3%
CD11b+F4/80High 55.4%
CD11b+F4/80Low 32.1%
CD11b+F4/80High 48.2%
Q1 47.3
Q2 17.9
Q4 24.1
Q3 10.7
Q5 34.3
Q6 11.0
Q8 37.1
Q7 17.6
Q1 44.7
Q2 22.2
Q4 22.0
Q3 11.1
Q5 30.6
Q6 14.1
Q8 36.1
Q7 19.1
Q1 22.9
Q2 13.6
Q4 38.6
Q3 24.9
Q5 16.7
Q6 15.7
Q8 45.5
Q7 22.0
Q1 70.7
Q2 26.9
Q4 1.30
Q3 1.13
Q5 51.5
Q6 18.3
Q8 20.5
Q7 9.75
Q1 65.4
Q2 34.5
Q4 0.14
Q3 0.027
Q5 57.2
Q6 30.5
Q8 9.05
Q7 3.26
Q1 63.7
Q2 36.0
Q4 0.20
Q3 0.098
Q5 57.9
Q6 34.2
Q8 6.83
Q7 1.11

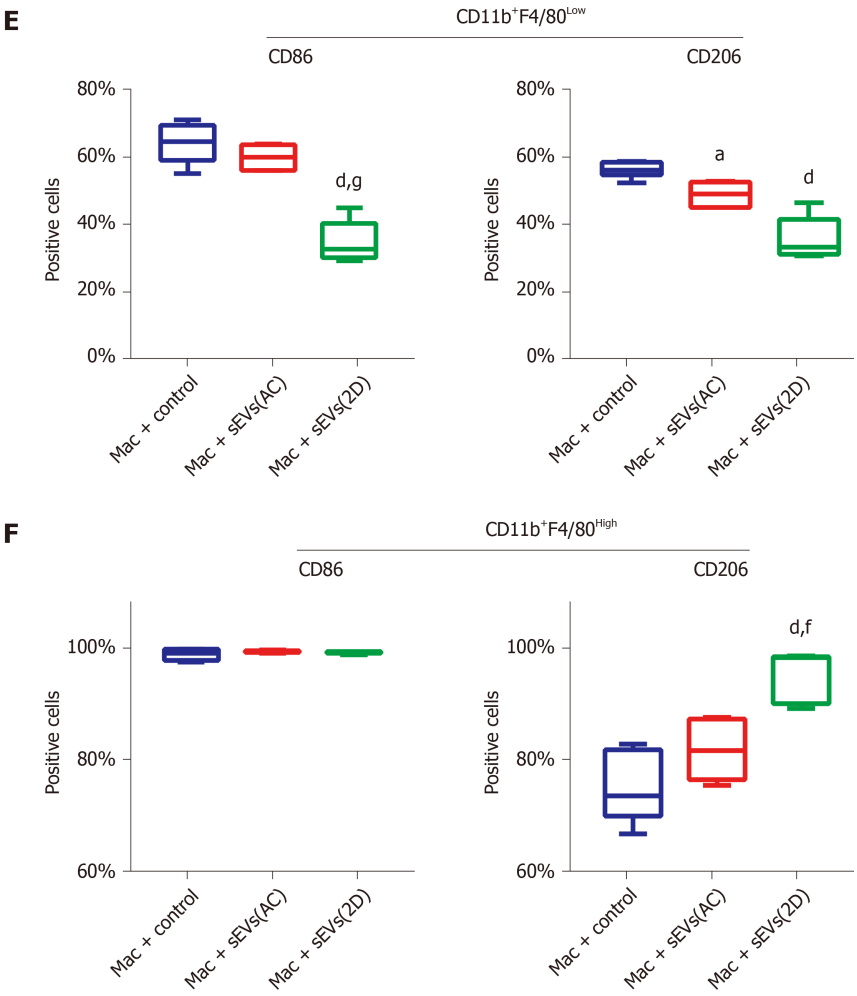


Figure 7 Uptake of serum small extracellular vesicles by hepatic macrophages and subsequent reprogramming. A: Uptake of SYTO-labeled serum small extracellular vesicles (sEVs) from normal (AC) or acute liver injury (ALI) (2D) mice by primary hepatic macrophages; B: Hepatic macrophages were incubated with AC or 2D serum sEVs for 24 h. The number of attached cells per 200 × field is shown; C: Expression of M1- and M2-like cell surface markers and cytokines in hepatic macrophages incubated with mice serum sEVs. The unfilled column represents macrophages incubated with AC sEVs, and the filled column represents macrophages incubated with 2D sEVs. Compared with the untreated control group, $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$, $^{d}P < 0.0001$; compared with the AC sEV treatment group, $^{e}P < 0.05$, $^{f}P < 0.01$; D: Macrophages were defined as $CD11b^{+}F4/80^{Low}$ and $CD11b^{+}F4/80^{High}$ subgroups. The representative images show the percentage of CD86-and CD206-positive cells in each subgroup subjected to the control, AC sEV and 2D sEV treatments; E: CD86- and CD206-positive cells in each subgroup. Compared with the control group, $^{a}P < 0.05$, $^{d}P < 0.0001$; compared with the AC sEV treatment group, $^{f}P < 0.01$, $^{g}P < 0.0001$. Scale bar = 100 μm. Mac: Macrophage; D: Day; AC: Acute liver injury control; sEVs: Small extracellular vesicles.

monocyte-derived macrophages and educating resident hepatic macrophages to transform into M2-like cells.

CONCLUSION

In conclusion, we found that the concentration, size and morphology of serum sEV particles were essential features for liver injury. We established specific serum sEV miRNA signatures for different liver injury stages and created a list of miRNAs that can be used as common liver injury biomarkers. The altered ALI and CLI serum sEV miRNAs were connected to diverse liver pathological processes. ALI serum sEVs reprogrammed hepatic macrophage subgroups differently. Serum sEVs not only have good diagnostic potential but also could be used to ameliorate liver injury. However, the diagnostic and therapeutic potential of these altered serum sEVs upon liver injury deserves further study.

ARTICLE HIGHLIGHTS

Research background

Both acute liver injury (ALI) and chronic liver injury (CLI) do not always cause noticeable signs and symptoms. Serum small extracellular vesicles (sEVs) have attracted tremendous interest due to their essential roles in intercellular communication and their diagnostic and therapeutic potential. The cargoes carried by sEVs represent a snapshot of the parental cells and change depending on the physiological and pathological states.

Research motivation

Serum sEVs and their small RNA (sRNA) cargoes could be promising biomarkers for the diagnosis of liver injury.

Research objectives

The present study aimed to characterize the dynamic changes of serum sEVs and their sRNA components during liver injury and to explore the effect of liver injury-related serum sEVs on hepatic macrophages.

Research methods

Male C57BL/6 mice were treated with CCL_4 to establish a mouse liver injury model for simulating ALI, CLI and recovery. Serum sEVs were obtained and characterized by transmission electron microscopy and nanoparticle tracking analysis. Serum sEV sRNAs were profiled by sRNA sequencing. Differentially expressed microRNAs (miRNAs) were compared to mouse liver-enriched miRNAs and previously reported circulating miRNAs related to human liver diseases. The biological significance was evaluated by Ingenuity Pathway Analysis of the altered sEV miRNAs and conditioned cultures of ALI serum sEVs with primary hepatic macrophages.

Research results

Both ALI and CLI changed the concentration and morphology of serum sEVs. The proportion of serum sEV miRNAs increased upon liver injury, with the liver as the primary contributor. The altered serum sEV miRNAs based on mouse study were consistent with human liver disease-related circulating miRNAs. We established serum sEV miRNA signatures for ALI and CLI and a panel of miRNAs (miR-122-5p, miR-192-5p, and miR-22-3p) as a common marker for liver injury. ALI serum sEVs decreased both CD86 and CD206 expression in monocyte-derived macrophages but increased CD206 expression in resident macrophages *in vitro*.

Research conclusions

Serum sEVs acquired different concentrations, sizes, morphologies and sRNA contents upon diverse liver injured pathological processes. ALI serum sEVs reprogrammed hepatic macrophage subgroups differently.

Research perspectives

Serum sEVs have good diagnostic and therapeutic potential for liver injury.

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ORIGINAL ARTICLE

Basic Study

Genome-wide map of N6-methyladenosine circular RNAs identified in mice model of severe acute pancreatitis

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Abstract

BACKGROUND

Severe acute pancreatitis (SAP) is a deadly inflammatory disease with complex pathogenesis and lack of effective therapeutic options. N6-methyladenosine (m6A) modification of circRNAs plays important roles in physiological and pathological processes. However, the roles of m6A circRNA in the pathological process of SAP remains unknown.

AIM

To identify transcriptome-wide map of m6A circRNAs and to determine their biological significance and potential mechanisms in SAP.

METHODS

The SAP in C57BL/6 mice was induced using 4% sodium taurocholate salt. The transcriptome-wide map of m6A circRNAs was identified by m6A-modified RNA immunoprecipitation sequencing. The biological significance of circRNAs with differentially expressed m6A peaks was evaluated through gene ontology and Kyoto Encyclopedia of Genes and Genomes analysis. The underlying mechanism of m6A circRNAs in SAP was analyzed by constructing of m6A circRNA-microRNA networks. The expression of demethylases was determined by

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quantitative polymerase chain reaction and western blot to deduce the possible mechanism of reversible m^{6}A process in SAP.

RESULTS

Fifty-seven circRNAs with differentially expressed m^{6}A peaks were identified by m^{6}A-modified RNA immunoprecipitation sequencing, of which 32 were upregulated and 25 downregulated. Functional analysis of these m^{6}A circRNAs in SAP found some important pathways involved in the pathogenesis of SAP, such as regulation of autophagy and protein digestion. In m^{6}A circRNA–miRNA networks, several important miRNAs participated in the occurrence and progression of SAP were found to bind to these m^{6}A circRNAs, such as miR-24-3p, miR-26a, miR-92b, miR-216b, miR-324-5p and miR-762. Notably, the total m^{6}A level of circRNAs was reduced, while the demethylase alkylation repair homolog 5 was upregulated in SAP.

CONCLUSION

m^{6}A modification of circRNAs may be involved in the pathogenesis of SAP. Our findings may provide novel insights to explore the possible pathogenetic mechanism of SAP and seek new potential therapeutic targets for SAP.

Key Words: Severe acute pancreatitis; Circular RNAs; N^6-methyladenosine; MeRIP-seq; Epigenetic analysis

Core Tip: We identified a transcriptome-wide map of N^6-methyladenosine (m^{6}A) circRNAs and determined their biological significance and potential mechanisms in severe acute pancreatitis (SAP). The main findings were: (1) Function analysis found that circRNAs with differentially expressed m^{6}A peaks were involved in the key process of SAP; (2) m^{6}A may affect the interplays of circRNAs and microRNAs to participate in the pathogenesis of SAP; and (3) Demethylase alkylation repair homolog 5 may play key roles in dynamic process of m^{6}A to downregulate the total m^{6}A level of circRNAs in SAP. We provided novel insights to explore the possible pathophysiological mechanism of SAP and seek new potential therapeutic targets.

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INTRODUCTION

Acute pancreatitis (AP) is a pancreatic inflammatory disorder that is associated with substantial morbidity and mortality[1]. Approximately 20% of patients with AP develop into severe AP (SAP)[2]. Due to the extensive pancreatic necrosis, subsequent infection, systemic inflammatory response syndrome and multiple organ failure, the mortality of SAP is up to 30%[2,3]. Previous studies have suggested that some important pathological mechanisms, including premature trypsinogen activation in the acinar cells and macrophages, mitochondrial dysfunction, pathological calcium signaling, endoplasmic reticulum (ER) stress, and impaired autophagy, are involved in the initiation and development of SAP[1]. However, the pathophysiology of SAP is complex and remains unclear, especially the level of gene regulation.

CircRNAs were discovered in the 1970s[4] and were identified as single-stranded covalently closed RNA molecules that lack 5′ caps and 3′ tails[5]. Long after, they were thought to be the byproducts of splicing[6]. In recent years, as high-throughput sequencing developed, thousands of circRNAs were found to be expressed in a wide range of mammalian tissues[7,8], including the pancreas[9], and accumulating studies have demonstrated that circRNAs play vital roles in the whole process and prognosis

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of many diseases, including cardiovascular diseases[8], cancer[10], neurodevelopmental processes[11], immune responses and immune diseases[12]. The main mechanisms of circRNAs participated in the initiation and development of diseases include the following functions[6,8,10,12]: interplay with RNA-binding proteins, microRNA (miRNA) sponges, regulating the stability of mRNAs, modulating the transcription of parental gene and the templates for protein synthesis. However, the post-transcription modification of circRNAs remains unclear.

N^6-methyladenosine (m^{6}A) is the most prevalent internal modification of RNA in eukaryotic cells[13]. In 2017, Zhou *et al*[14] reported that the m^{6}A modification is widespread in circRNAs and m^{6}A modifications are read and written by the same complexes in circRNAs and mRNAs. The regulatory role of m^{6}A is mainly performed by three homologous factors, namely so-called "writers", "erasers" and "readers"[13-15]. The writers mainly include methyltransferase-like 3 and 14 proteins (METTL3 and METTL14) and their cofactor WT1-associated protein (WTAP). They form a methyltransferase complex to catalyze the installation of m^{6}A. The erasers, including alkylation repair homolog 5 (ALKBH5) and fat mass and obesity related protein (FTO), can catalyze the oxidative demethylation of N-alkylated nucleic acid bases. The readers are mainly YT521-B homology (YTH) domain containing proteins family, including YTHDC1, YTHDC2, YTHDF1, YTHDF2 and YTHDF3. They can specifically recognize m^{6}A and regulate splicing, localization, degradation and translation of RNAs. Recently, it has been found that the m^{6}A modification of circRNAs plays a key role in innate immunity and tumors though regulating the metabolism and function of circRNAs[15]. In human embryonic stem cells and HeLa cells, m^{6}A circRNAs display cell-type-specific methylation patterns[14]. In colorectal carcinoma, the m^{6}A modification can modulate cytoplasmic export of circNSUN2 and stabilize HMGA2, ultimately enhancing the colorectal liver metastasis[16]. However, the roles of m^{6}A circRNAs in SAP are still unknown.

Here, we investigated the expression profile of m^{6}A circRNAs in SAP through m^{6}A-modified RNA immunoprecipitation sequencing (MeRIP-seq). We evaluated the biological significance of circRNAs with differentially expressed m^{6}A peaks though gene ontology (GO) analysis, Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis, and explored their underlying mechanism by construction of m^{6}A circRNA–miRNA networks. In addition, we determined the expression of demethyltransferase, ALKBH5 and FTO, to deduce the possible mechanism of reversible m^{6}A process in SAP.

MATERIALS AND METHODS

Animals and preparation of SAP model

Male C57BL/6 mice weighing 22-25 g were provided by Chengdu Dashuo Experimental Animal Technology Co. Ltd. All the mice were housed in ventilated plastic cage system and fed with the same food and water for 7 d to adapt to the environment. The entire research protocol was approved by the Institutional Animal Care and Use Committee at the General Hospital of Western Theater Command.

Before the operation, the mice were divided into SAP and control groups randomly (3 mice per group) and fasted for 12 h but had free access to water. Isoflurane (5%) was used to anesthetize mice by induction box prior to surgery. Then, the SAP was induced through 4% sodium taurocholate salt that was slowly retrogradely injected into the biliopancreatic duct with a microinfusion pump. All mice were killed 24 h after the establishment of model, and the blood samples and pancreatic tissues were collected for further analysis.

Pancreatic histological analysis

Pancreatic tissue (0.4 cm × 0.4 cm) was fixed in 4% paraformaldehyde solution. After dehydrating with ethanol, the tissue samples were embedded in paraffin. Then, the samples were cut into about 4-μm-thick sections, and the sections were stained with hematoxylin and eosin. The light microscopy at × 200 magnification was used to examine the slide. The scoring system described previously was used to evaluate the degree of pancreatic injury[17]. The scores were averaged for five different slides that were selected randomly from each pancreas.

Amylase and lipase measurement

The concentrations of lipase and amylase in serum were determined using Lipase Assay kit and Amylase Assay kit (Nanjing Jiancheng Bioengineering Institute,

Nanjing, China) according to the instructions.

RNA isolation and RNA quality control

TRIzol reagent (Invitrogen, Carlsbad, CA, United States) was used to extract total RNA from the homogenized pancreatic tissues of the control and SAP groups. The concentration of extracted RNA was measured at OD260 and 280 by NanoDrop ND-2000 instrument (Thermo Fisher Scientific, Waltham, MA, United States). We assessed the integrity of RNA through denaturing agarose gel electrophoresis. The OD A260/A280 ratio between 1.8 and 2.0 was set as the RNA purity standard.

Library preparation and MeRIP-seq

rRNAs in total RNA were removed using Ribo-Zero rRNA Removal Kits (Illumina, San Diego, CA, United States). The removal efficiency of rRNA by the residual determination of 28S and 18S of rRNA using quantitative polymerase chain reaction (qPCR). The fragmented RNA was incubated with the anti-m^{6}A antibody at 4 °C for 2 h in IPP buffer. Then, the mixture was immunoprecipitated by incubation with protein-A beads (Thermo Fisher Scientific) for 2 h at 4 °C. The bound RNA was eluted from the beads with m^{6}A (Berry & Associates) in IPP buffer and then extracted with TRIzol reagent (Thermo Fisher Scientific). The immunoprecipitated RNA and input RNA were used to construct the library using NEBNext® Ultra™ RNA Library Prep Kit and double-ended 150-bp sequencing of the m^{6}A-IP and input samples was performed on an Illumina HiSeq sequencer (performed by Cloudseq Biotech Inc., Shanghai, China).

Analysis of MeRIP-Seq data

Paired-end reads were harvested from the Illumina HiSeq 4000 sequencer, and were quality controlled by Q30. To obtain high quality clean reads, 3′ adaptor-trimming and low-quality reads were removed by cutadapt software. The clean reads with high quality of the input library were aligned to the mouse reference genome (UCSC MM10) with STAR software. DCC software was used for detecting and identifying the circRNAs. The identified circRNAs were annotated using the circBase database and Circ2Traits database. For all samples, raw junction reads were normalized to the number of total mapped reads and log2 transformed. The read alignments on the genome were visualized using the tool integrative genomics viewer. The adapter-removal reads were aligned to the reference genome using Hisat2 software. The methylated sites in each sample were identified using MACS software. Differentially methylated sites were identified using diffReps software.

GO and KEGG analysis

The parent genes of circRNAs with differential m^{6}A peaks were selected to analyze their potential biological roles through GO and KEGG pathway analysis. GO analysis included three parts, namely, biological process (BP) analysis, molecular function (MF) analysis, and cell component (CC) analysis[18]. GO analysis was performed by R topGO package. Fisher's exact test in Matlab MCR software was applied to calculate the enrichment of each pathway. The bubble plots and column plots were generated using the ggplot2 in R package (https://ggplot2.tidyverse.org).

Construction of circRNA–miRNA networks

circRNA containing miRNA-binding sites can bind to miRNA response elements competitively, further regulating the target mRNAs[19]. The top 10 upregulated and top 10 downregulated circRNAs according to the level of m^{6}A were selected to construct circRNA–miRNA networks. The m^{6}A circRNA–miRNA networks were constructed using TargetScan software and miRanda software and the circRNA–miRNA interactions were visualized by Cytoscape.

Conservation analysis

The top 10 upregulated and top 10 downregulated circRNAs were selected to analyze their homology with human circRNAs. The sequence of human circRNAs was downloaded from circBase database and the sequence of each selected m^{6}A circRNA was blasted against the human circRNAs sequence by the blastn function of Blast software.

Western blotting

The whole pancreatic tissues from SAP and control groups were placed in RIPA lysate buffer with protease inhibitor, phosphatase inhibitor and phenylmethylsulfonyl fluoride inside (Total Protein Extraction Kit; Beijing Solarbio Science and Technology

Inc., Beijing, China), and the tissues were homogenized with homogenizer. The tissue homogenate was centrifuged at 12000 g for 30 min at 4 °C, and the supernatant was collected. After protein concentration was measured by BCA Protein Assay Kit (Beyotime Biotechnology, Jiangsu, China), the supernatant was mixed with loading buffer (Beijing Solarbio Science and Technology), boiled at 100 °C for 10 min for protein denaturation, and stored at -80 °C after separation. The target proteins were separated by SDS-PAGE. The proteins were transferred to polyvinylidene difluoride membrane (0.45 μm, IPVH00010; Millipore, Billerica, MA, United States), blocked in 5% nonfat milk for 1 h at room temperature (22 ± 3 °C), and then incubated with primary antibody, FTO (1:1000, D2V1I; Cell Signaling Technology, Danvers, MA, United States), ALKBH5 (1:2000, 16837-1-AP; Proteintech, Rosemont, IL, United States), GAPDH (1:5000, 10494-1-AP; Proteintech) at 4 °C overnight. The membranes were washed with Tris-buffered saline with Tween-20 (TBST) (Beijing Solarbio Science and Technology) three times and incubated with secondary antibody (1:10000, 15015; Proteintech) at room temperature for 1 h. After being washed three times with TBST, the protein bands were visualized by enhanced chemiluminescence (Immobilon Western Chemilum HRP Substrate; Millipore) in a biological imaging system.

qPCR

The total RNA was extracted from SAP and control groups as described above. qPCR was performed using One Step SYBR® PrimeScript™ RT-PCR kit II (Takara Biotechnology Co., Ltd., Dalian, China) and the primers (ALKBH5: forward 5′-GGCGGTCATCATTCTCAGGAAGAC-3′ and reverse 5′-CTGACAGGCGATCTGAAGCATAGC-3′; FTO: forward 5′-CTCACAGCC TCGGTTTAGTTCCAC-3′ and reverse 5′–CGTCGCCATCGTCTGAGTCATT G-3′; GAPDH: forward 5′-GGTGAAGGTCGGTGTGAACG-3′ and reverse 5′-CTCGCTCCTGGAAGATGGTG-3′) were synthesized by Shanghai Sangon Biotech Co., Ltd.. The outcomes were analyzed by means of $2^{-\Delta\Delta CT}$ through normalizing the quantity of GAPDH.

Data analysis

GraphPad Prism 8 (La Jolla, CA, United States) and SPSS 22.0 (IBM Corp., Armonk, NY, United States) were used for performing statistical analyses. Student's *t* test was used for estimating statistically significance between two groups. The results were evaluated through Spearman's correlation coefficient test. All values are shown as mean ± SE of the mean; $P < 0.05$ was regarded as statistically significant.

RESULTS

Evaluation of mouse model of SAP

Twenty-four hours after treatment with sodium taurocholate salt, the staining of hematoxylin and eosin on the pancreatic tissues from the SAP group showed typical histopathological changes, including pancreatic lobular edema, extensive acinar cell necrosis, focal expansion of the pancreatic interlobular septum and granulocyte infiltration (Figure 1A). By contrast, under light microscopy, the pancreases from the control group had a complete normal structure. Figure 1B showed the corresponding histopathological scores. At the same time, considering that the levels of serum lipase and amylase are as one of the diagnostic criteria of AP[20], we determined their concentrations in serum. As a result, the serum lipase and amylase levels in the SAP group were also markedly higher than those in the control group ($P < 0.05$; Figure 1C and 1D). These results confirmed the successful establishment of the SAP mice model.

Overview of m^{6}A circRNAs in SAP

We used MeRIP-seq to investigate the expression of m^{6}A circRNAs in pancreatic tissues from the control and SAP groups. We had submitted the data to the online repository, which can be found at: https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE173298. Before performing MeRIP-seq, the residual determination of 28S and 18S of rRNA showed that the rRNAs in total RNA were removed effectively (Supplementary Figure 1). In general, a total of 409 m^{6}A circRNAs were identified in all chromosomes (Figure 2A). Among these, 178 were specifically expressed in the SAP group, 107 in the control group, and 124 were shared in both groups (Figure 2B). m^{6}A level in total circRNAs from the SAP group was lower than that from the control group (Figure 2C). Besides, > 80% of circRNAs contained only one m^{6}A peak in both SAP and control groups (Figure 2D).

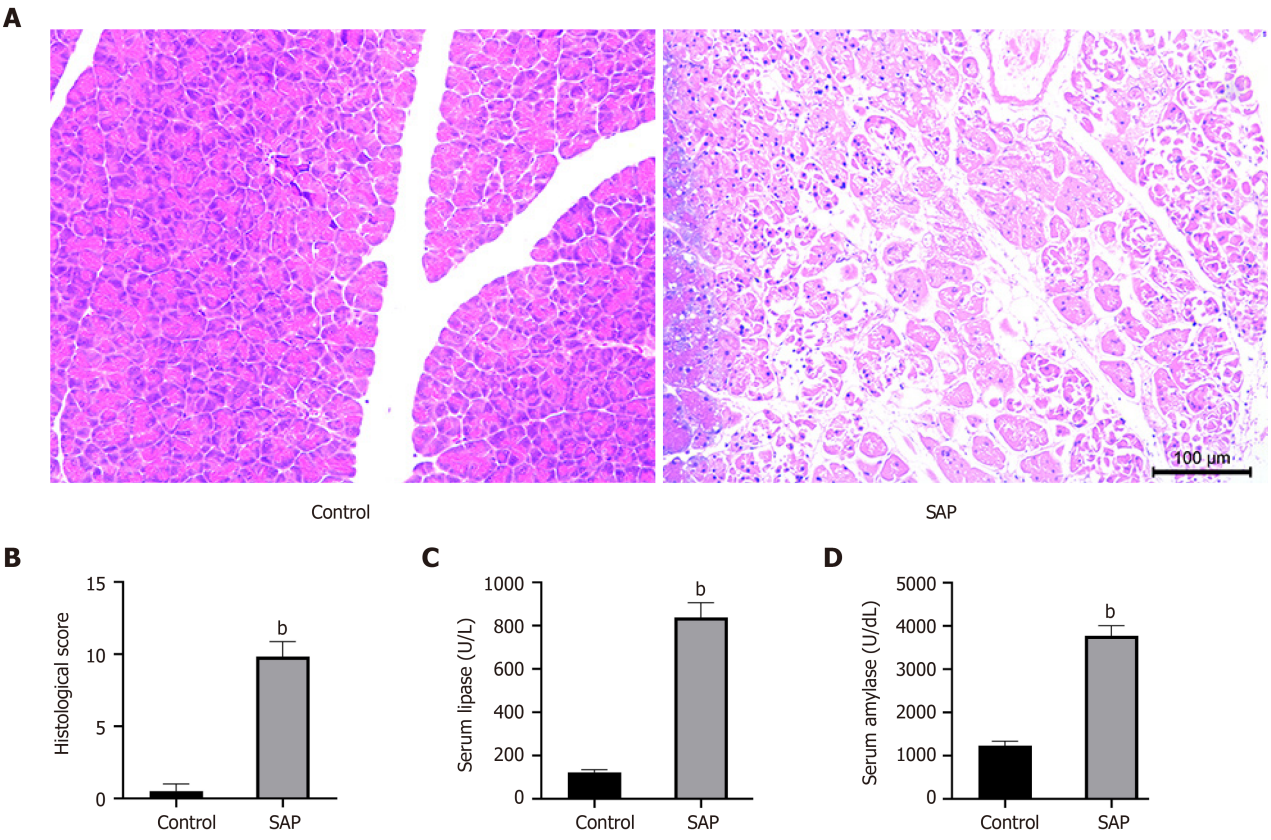

Figure 1 Evaluation of mouse model of severe acute pancreatitis. A: Representative images of pancreatic tissues stained with hematoxylin from control (left) and severe acute pancreatitis (SAP) (right) groups (× 100 magnification); B: Histological score of pancreatic tissues in control and SAP groups; C and D: Levels of serum lipase and amylase, respectively. $^bP < 0.01$ *vs* control group, $n = 3$ per group.

Differential m^{6}A modification of circRNAs in SAP

To understand the biological role of m^{6}A modification of circRNAs in SAP, the circRNAs with differentially expressed (DE) m^{6}A peaks were further analyzed. Significant differential expression was defined as fold-change > 2 and $P < 0.05$. Compared with the control group, 57 circRNAs with DE m^{6}A peaks were identified; 32 were upregulated and 25 downregulated in the SAP group. Table 1 presents the top 10 methylated m^{6}A sites that were up- and downregulated within circRNAs. Figure 3A shows the m^{6}A circRNAs expression profile in the SAP and control groups though hierarchical cluster analysis. The scatter plot exhibits the variation of DE m^{6}A circRNAs between the SAP and control groups (Figure 3B). The volcano plot depicted DE m^{6}A circRNAs between the two groups (Figure 3C).

Distribution of m^{6}A sites in SAP and control groups

We identified 903 m^{6}A peaks distributed on 781 circRNAs and it is reported that circRNAs can be generated from any region of the genome[21]. Therefore, we firstly analyzed the genomic distribution of m^{6}A and non-m^{6}A circRNAs according to their genomic origins to explore their distribution features. As a results, in non-m^{6}A circRNAs, 45.33% were sense overlapping, 21.15% exonic, 26.71% intronic, 4.94% intergenic and a few antisense; in m^{6}A circRNAs, 42.78% were sense overlapping, 30.32% exonic, 21.27% intronic, 3.42% intergenic and a few antisense (Figure 4A). These results indicated that the majority of m^{6}A and non-m^{6}A circRNAs were commonly encoded by sense overlapping sequences and the number of circRNAs that generated from protein-coding genes in m^{6}A circRNAs was more than those in non-m^{6}A circRNAs.

We further analyzed the distribution of circRNAs with DE m^{6}A peaks. The length of DE m^{6}A circRNAs was mainly enriched in 1–10 000 base pairs (Figure 4B). Although the host genes of m^{6}A circRNAs located in all chromosomes, the dysregulated parts mostly located in chromosomes 4, 9 and 11 (Figure 4C). A previous study reported that most circRNAs that derived from protein-coding genes spanned two or three exons [14]. In this study, the majority of circRNAs from protein-coding genes spanned one or two exons (Figure 4D). Similarly, the majority of m^{6}A circRNAs and non-m^{6}A

Table 1 Top 20 differently expressed N6-methyladenosine peaks compared with control group

	PeakStart	PeakEnd	circRNA	Regulation	Fold-change	*P* value
chr15	98658229	98658320	chr15:98656602-98658435-	Up	187.2	3.67392E-09
chr11	74929241	74929540	chr11:74928993-74990215+	Up	172.8	3.92116E-09
chr9	108248361	108248660	chr9:108207543-108263690-	Up	120.034482	3.70238E-08
chr2	153763381	153763760	chr2:153756037-153769786+	Up	106.330434	2.09239E-08
chr18	30281961	30282053	chr18:30276981-30282053+	Up	50.9545454	7.61781E-09
chr19	40346381	40346760	chr19:40314443-40373578-	Up	42.4	0.026929988
chr16	94641481	94641740	chr16:94611419-94694141+	Up	37.6772727	7.9549E-08
chr10	60144412	60144720	chr10:60144413-60144723-	Up	24.9	0.014047401
chr11	44652781	44652825	chr11:44651797-44652825+	Up	23.9	0.007108941
chr7	63895821	63896100	chr7:63891679-63938495-	Up	21.1	0.034908475
chr9	107852341	107852720	chr9:107847268-107860459-	Down	302.6	3.63198E-09
chr8	104143561	104143760	chr8:104143031-104143793+	Down	160.728571	1.15908E-08
chr1	150426881	150427260	chr1:150413021-150442180+	Down	52.095238	1.98141E-08
chr1	13312381	13312680	chr1:13298706-13325802-	Down	51.7	6.86061E-05
chr6	119970581	119970800	chr6:119951703-120038640-	Down	47.4	9.37286E-05
chr11	23271132	23271205	chr11:23261835-23271205+	Down	40.6	0.000442849
chr4	108499346	108499398	chr4:108486454-108508433+	Down	27.79	3.27262E-08
chr9	102619691	102619760	chr9:102618811-102619760-	Down	24.25	4.83147E-07
chr11	32296401	32296600	chr11:32283981-32297161+	Down	24.1	4.96449E-07
chr9	69414201	69414580	chr9:69408311-69432615+	Down	22.6	0.00943617

circRNAs were more commonly encoded by a single or two exons (Figure 4E).

Functional analysis of circRNAs with DE m^{6}A peaks

To explore the function of m^{6}A circRNAs in SAP, GO analysis and KEGG pathway analysis of circRNAs with the DE m^{6}A peaks were performed. Figure 5A presented the top 10 GO terms of circRNAs with upregulated m^{6}A peaks from the three aspects: BP, CC and MF. For BP, the most enriched and meaningful GO terms were cellular component organization, macromolecule metabolic process and regulation of developmental process. For CC, the top three terms were focal adhesion, cell–substrate junction and anchoring junction. For MF, the main represented GO terms were C2H2 zinc finger domain binding and protein binding. The top 10 pathways from KEGG pathway analysis for circRNAs with upregulated m^{6}A peaks were selected and presented in a bubble chart (Figure 5B). Among them, protein digestion and absorption and regulation of autophagy were the major signaling pathways associated with the SAP progression.

The GO terms of circRNAs with downregulated m^{6}A peaks are presented in Figure 5C. For BP, protein-containing complex localization, RNA transport and macromolecule metabolic process were the most enriched and meaningful GO terms. For CC, nucleus, dendrite and dendritic tree were the top three terms. For MF, the main represented GO terms were channel regulator activity, RNA, enzyme and protein binding. As for the KEGG pathway analysis of circRNAs with downregulated m^{6}A peaks, RNA transport was the main pathway (Figure 5D).

Relationship between m^{6}A level and expression of circRNAs in SAP

To explore whether m^{6}A modification could affect the expression of circRNAs, we analyzed the expression of m^{6}A circRNAs. The expression level of these circRNAs with DE m^{6}A peaks did not have significant differences (fold-change < 2 or $P > 0.05$; Supplementary Table 1), indicating that m^{6}A modification of circRNAs did not influence the expression of circRNAs. To verify this result further, we analyzed the cumulative distribution of circRNA expression between the control and SAP groups

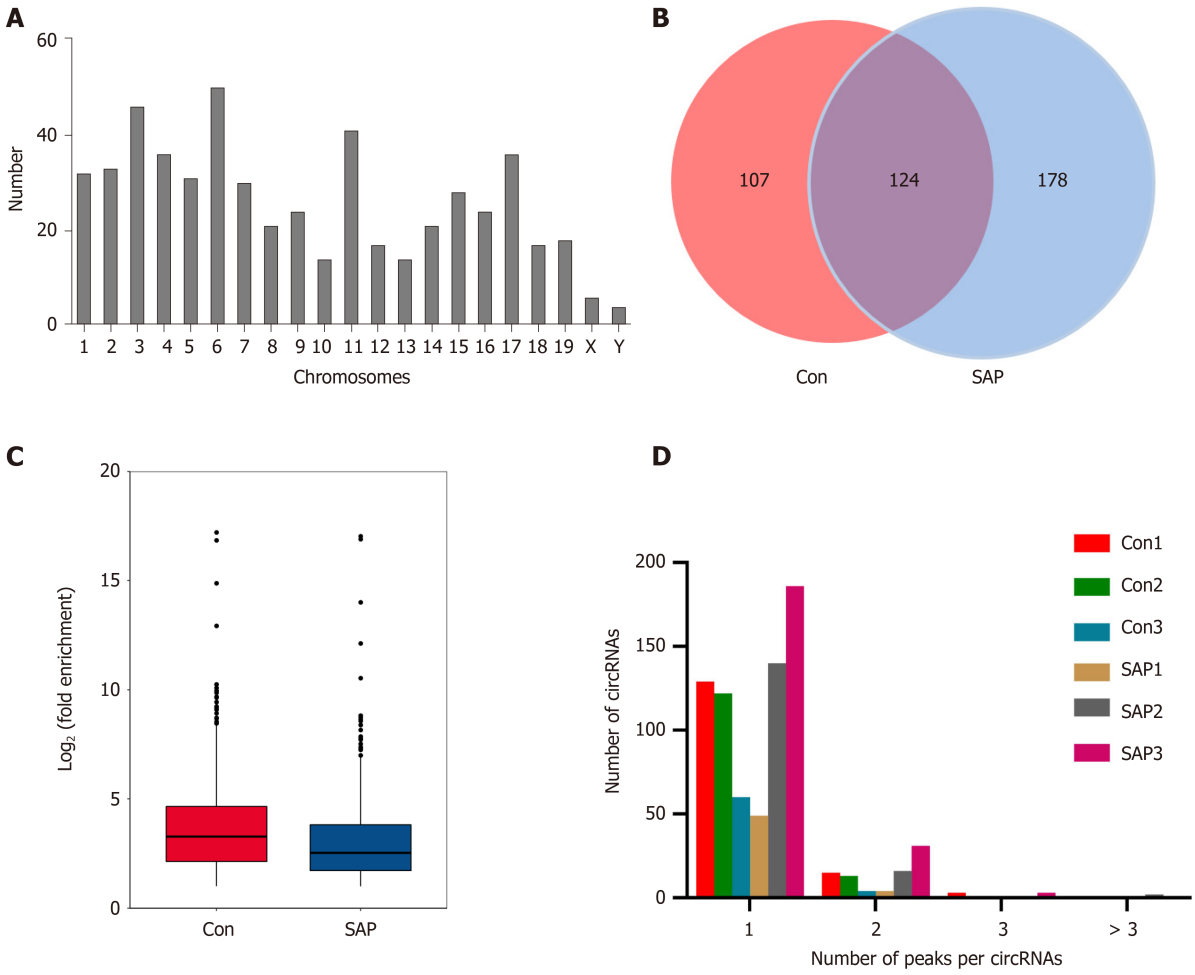

Figure 2 Overview of N^6-methyladenosine circRNAs in severe acute pancreatitis. A: Number of identified N6-methyladenosine (m^{6}A) circRNAs according to distribution on chromosomes; B: Venn diagram exhibiting number of common and specific m^{6}A circRNAs between control and severe acute pancreatitis (SAP) groups; C: Box plot showing level of m^{6}A peaks enrichment in circRNAs in control and SAP groups; D: Number of circRNAs containing variant numbers of m^{6}A peaks.

for m^{6}A and non-m^{6}A circRNAs (Figure 6). This was consistent with the above result.

Construction of m^{6}A circRNA–miRNA networks in SAP

Given the importance of circRNA–miRNA interaction[22] and to further explore the underlying mechanism of these circRNAs with DE m^{6}A peaks, the top 10 upregulated and top 10 downregulated circRNAs according to the level of m^{6}A were selected to construct circRNA–miRNA networks. In this network map, several important miRNAs participated in the occurrence and development of SAP were found to bind to these m^{6}A circRNAs (Figure 7), such as miR-24-3p, miR-26a, miR-92b, miR-216b, miR-324-5p and miR-762. These data suggest that these circRNAs with DE m^{6}A peaks might play a role in the pathological process of SAP.

Conservation analysis of identified m^{6}A circRNAs with human circRNAs

To explore whether the circRNAs with DE m^{6}A peaks identified in mouse SAP may have similar roles in human SAP, we performed the conservation analysis of the sequence of the top 10 upregulated and top 10 downregulated circRNAs preliminarily. Through aligning with the sequence of human circRNAs that downloaded from circBase database, we found that 15/20 of the selected circRNAs that have highly similar sequences to human circRNAs (sequence identity > 80%), as shown in the Table 2. These results suggested that these circRNAs may have similar roles in human SAP.

Expression of demethyltransferase in SAP

Given that the total m^{6}A level of circRNAs was reduced in SAP and to explore how the m^{6}A level was regulated in SAP, we detected the protein and mRNA expression of two

Table 2 The conservation analysis of the sequence between the selected circRNAs and human circRNAs

	Human circRNA				
Mouse circRNA	Human circRNA	Hg19 location	Transcript	Parent gene	Sequence identity, %
chr15:98656602-98658435-	hsa_circ_0026065	chr12:49223538-49245957-	NM_004818	*DDX23*	88.75
chr11:74928993-74990215+	hsa_circ_0041387	chr17:2139785-2203958-	NM_001170957	*SMG6*	86.32
chr9:108207543-108263690-	hsa_circ_0124055	chr3:49514281-49548252+	NM_001177634	*DAG1*	85.36
chr2:153756037-153769786+	hsa_circ_0059811	chr20:31436477-31438211+	NM_012325	*MAPRE1*	84.18
chr19:40314443-40373578-	hsa_circ_0094611	chr10:97110965-97114724-	ENST00000371247.2	*SORBS1*	93.52
chr16:94611419-94694141+	hsa_circ_0115989	chr21:38792600-38888974+	ENST00000338785.3	*DYRK1A*	91.34
chr7:63891679-63938495-	hsa_circ_0034321	chr15:31619082-31670102+	NM_015995	*KLF13*	85.89
chr9:107847268-107860459-	hsa_circ_0065768	chr3:50000008-50114685+	NM_005777	*RBM6*	90.84
chr1:150413021-150442180+	hsa_circ_0111511	chr1:186294895-186325581-	NM_003292	*TPR*	87.91
chr1:13298706-13325802-	hsa_circ_0113369	chr1:42166586-42254891-	ENST00000247584.5	*HIVEP3*	91.30
chr6:119951703-120038640-	hsa_circ_0024963	chr12:939168-990955+	NM_001184985	*WNK1*	92.02
chr11:23261835-23271205+	hsa_circ_0120688	chr2:61749745-61764803-	ENST00000404992.2	*XPO1*	95.11
chr4:108486454-108508433+	hsa_circ_0012539	chr1:52927184-53018762-	NM_001009881	*ZCCHC11*	92.38
chr11:32283981-32297161+	hsa_circ_0118668	chr2:202780266-202790202-	None	*None*	91.48
chr9:69408311-69432615+	hsa_circ_0035568	chr15:60720627-60748993-	NM_024611	*NARG2*	87.08

demethyltransferases (ALKBH5 and FTO). FTO was reduced at the level of protein, but ALKBH5 was increased in SAP at both the level of mRNA and protein (Figure 8). These results indicated that ALKBH5 might be related to the dynamic process of m^{6}A in SAP.

DISCUSSION

In the present study, we identified transcriptome-wide map of m^{6}A circRNAs and determined their biological significance and potential mechanisms for the first time in SAP. The main findings are: (1) We identified 57 circRNAs with DE m^{6}A peaks and found these DE m^{6}A circRNAs were involved in the key process of SAP by GO and KEGG analysis, such as protein digestion and regulation of autophagy; (2) In m^{6}A circRNA-miRNA networks, several important miRNAs participated in the initiation and development of SAP were found to bind to these m^{6}A circRNAs potentially, suggesting that m^{6}A may affect the interplays with miRNAs; and (3) The total m^{6}A level was reduced in SAP, and the demethylase ALKBH5 was found to be upregulated in SAP, indicating that ALKBH5 may be related to dynamic process of m^{6}A in SAP. These results suggested that m^{6}A modification on circRNAs may be involved in the pathophysiology of SAP, which may provide novel insights to explore the possible pathophysiological mechanism of SAP and seek new potential therapeutic targets.

To find effective therapeutic targets for SAP, many studies have explored the underlying molecular mechanisms of SAP. Our previous study found that many circRNAs are expressed in mice with SAP[9] and these circRNAs play an important role in the pathogenetic mechanism of SAP[9,23]. In recent years, m^{6}A modification of circRNAs was found to be widespread[14] and gained widespread attention in epigenetics. Several important studies have investigated the roles of m^{6}A modification in circRNA metabolism and found that m^{6}A circRNAs play key roles in some diseases [16,24-28]. In circRNA metabolism, m^{6}A modifications can regulate its translation through recognition by YTHDF3 and eIF4G2, and this progress of translation can be enhanced by METTL3/14 and inhibited by FTO[24,25]. In addition, m^{6}A circRNAs associate with YTHDF2 in an HRSP12-dependent manner and are selectively downregulated by RNase P/MRP[26]. In innate immunity, Chen *et al*[27] found that unmodified circRNA adjuvant induces antigen-specific T and B cell responses, but m^{6}A modification could abrogate circRNA immunity though YTHDF2-mediated suppression. In male germ cells, the back splicing tends to occur mainly at m^{6}A-

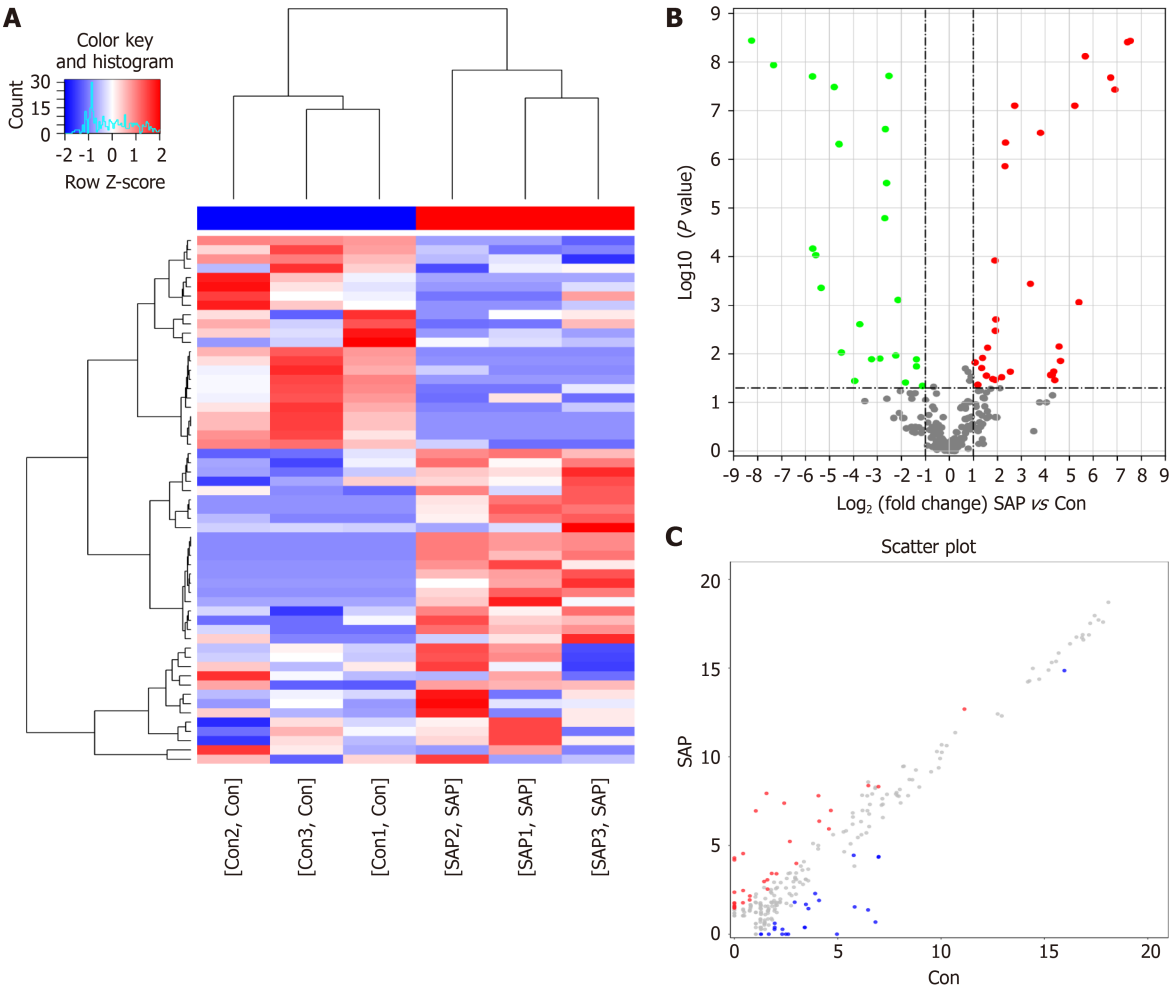

Figure 3 Differential N^6-methyladenosine modification of circRNAs in severe acute pancreatitis. A: Hierarchical clustering graph exhibiting differential N^6-methyladenosine (m^{6}A) modification of circRNAs in control and severe acute pancreatitis (SAP) groups. Higher expression is presented in red and lower expression in blue; B and C: Volcano and scatter plot showing the circRNAs with significant differentially expressed m^{6}A peaks.

enriched sites, which are usually located around the start and stop codons in linear mRNAs, resulting in about half of circRNAs containing large open reading frames. This potential mechanism could ensure long-lasting and stable protein production for specific physiological processes when lacking the corresponding linear mRNAs[28]. These findings showed the important roles of m^{6}A in circRNAs during disease progress. Therefore, it is essential to explore the roles of m^{6}A circRNAs in SAP.

In the present study, the function analysis of DE m^{6}A circRNAs in SAP found that two important pathways were involved in the pathogenesis of SAP, including protein digestion and regulation of autophagy. As an important pathological cellular event, the activation of premature trypsinogen can result in acinar cell necrosis[1]. Many pancreatic injury factors, such as trauma, obstruction of the pancreatic duct and alcohol, can initiate the fusion of lysosomes with zymogen in acinar cells, leading to the activation of trypsinogen through cathepsin B to trypsin. Once trypsin is released, it can cause self-digestion in and outside the acinar cells, and the release of cathepsin B can cause necroptosis. As a cytoprotective mechanism, autophagy can process and recycle various aged, defective or damaged cytoplasmic contents[29]. Selective macroautophagy is a biological process during which specific damaged organelles and misfolded proteins are processed and recycled. Autophagy is accomplished *via* a series of steps, which start with the enucleation of cytoplasmic inclusions in the open double membrane formed by the ER, Golgi apparatus and plasma membrane[30]. Knocking out *ATG7* genes (which are important to form autophagosome) and *LAMP* genes could lead to pancreatitis with extensive inflammation in mice[29,31]. Importantly, impaired autophagy leads to trypsinogen activation, ER stress and mitochondrial dysfunction. These events can together make acinar cells become more susceptible to other insults and cellular death[1]. In addition, RNA transport is enriched in GO terms of note, and Chen *et al*[16] found that m^{6}A modification can modulate the export of circNSUN2 to

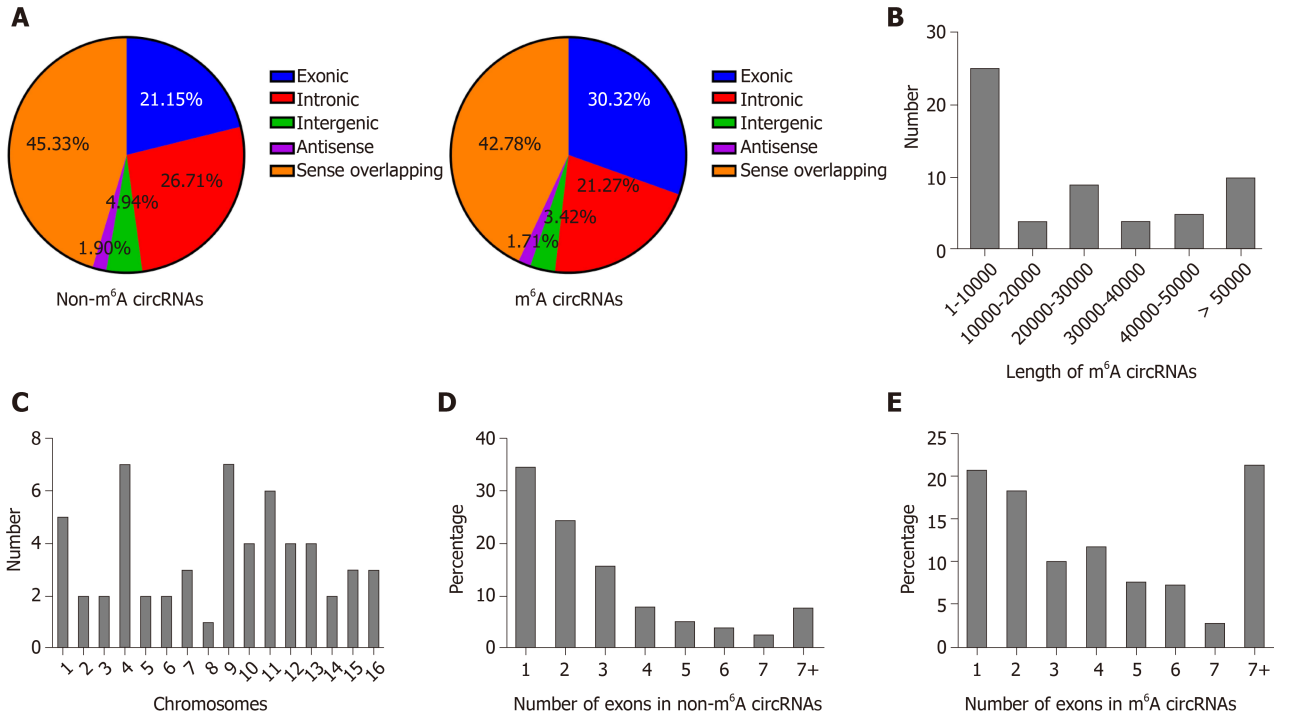

Figure 4 Distribution of N^6-methyladenosine sites in severe acute pancreatitis and control groups. A: Distribution of genomic origins of non-N6-methyladenosine (m^{6}A) circRNAs (left) and m^{6}A circRNAs (right); B: Number of circRNAs with differentially expressed m^{6}A peaks based on the distribution of length; C: Chromosomal distribution of all differential m^{6}A sites within circRNAs; D and E: Distribution of non-m^{6}A and m^{6}A circRNAs based on the number of exons in each circRNA.

the cytoplasm, suggesting that m^{6}A modification regulates transport of circRNAs in SAP. These results were consisted with the hypothesis that m^{6}A modification of circRNAs participated in the progression of SAP.

m^{6}A modification of mRNA can influence its expression by regulating transcription, splicing and degradation[32]. In circRNAs, Zhou *et al*[14] and Su *et al*[33] reported that m^{6}A levels are correlated with expression levels of circRNAs in HeLa cells and a rat model of hypoxia-mediated pulmonary hypertension. However, in SAP, we found m^{6}A modification in circRNAs was not associated with expression of circRNAs, suggesting that m^{6}A circRNAs function in SAP though other mechanisms, such as miRNA sponges. It is worth mentioning that more direct evidence is currently needed to support that m^{6}A can affect circRNA expression.

miRNA sponges is an important function of circRNAs. Cytoplasmic circRNAs can prevent miRNAs from binding to target mRNAs by competitive binding to miRNA response elements, further playing a key role in diseases[8,34]. For instance, in lung squamous cell carcinoma, circTP63 can competitively bind to miR-873-3p and prevent miR-873-3p from decreasing the level of FOXM1. The FOXM1 can upregulate the expression of CENPA and CENPB, ultimately facilitating cell cycle progression[35]. In SAP, circHIPK3 can enhance pyroptosis *via* regulating the miR-193a-5p/GSDMD axis in acinar cells, ultimately aggravating this disease[36]. In our previous study, we found that circZFP644 could sponge miR-21-3p, thereby participating in the pathogenesis of SAP[9]. Recently, Su *et al*[33] found that m^{6}A modification of circRNAs could influence the interactions between circRNAs and miRNAs. Therefore, analysis of m^{6}A circRNA–miRNA networks was performed in this study. Several important miRNAs participated in the pathological process of SAP were found to bind to these m^{6}A circRNAs, such as miR-24-3p, miR-26a, miR-92b, miR-216b, miR-324-5p and miR-762. For example, in caerulein-stimulated AR42J cells, expression of miR-92b-3p was decreased, while overexpression of miR-92b-3p could downregulate the expression of TRAF3 and inhibit the MKK3-p38 pathway, attenuating inflammatory response and autophagy[37]. These results suggest that m^{6}A modification of circRNAs functions by influencing the interactions between circRNAs and miRNAs.

m^{6}A modification is a reversible process that occurs by methyltransferase complex consisting of METTL3, METTL14 and WTAP, and is "erased" by ALKBH5 and FTO [13,15]. In pancreatic cancer, ALKBH5 could regulate the post-transcriptional activation of PER1 through m^{6}A abolishment, thereby inhibiting the cancer[38]. In

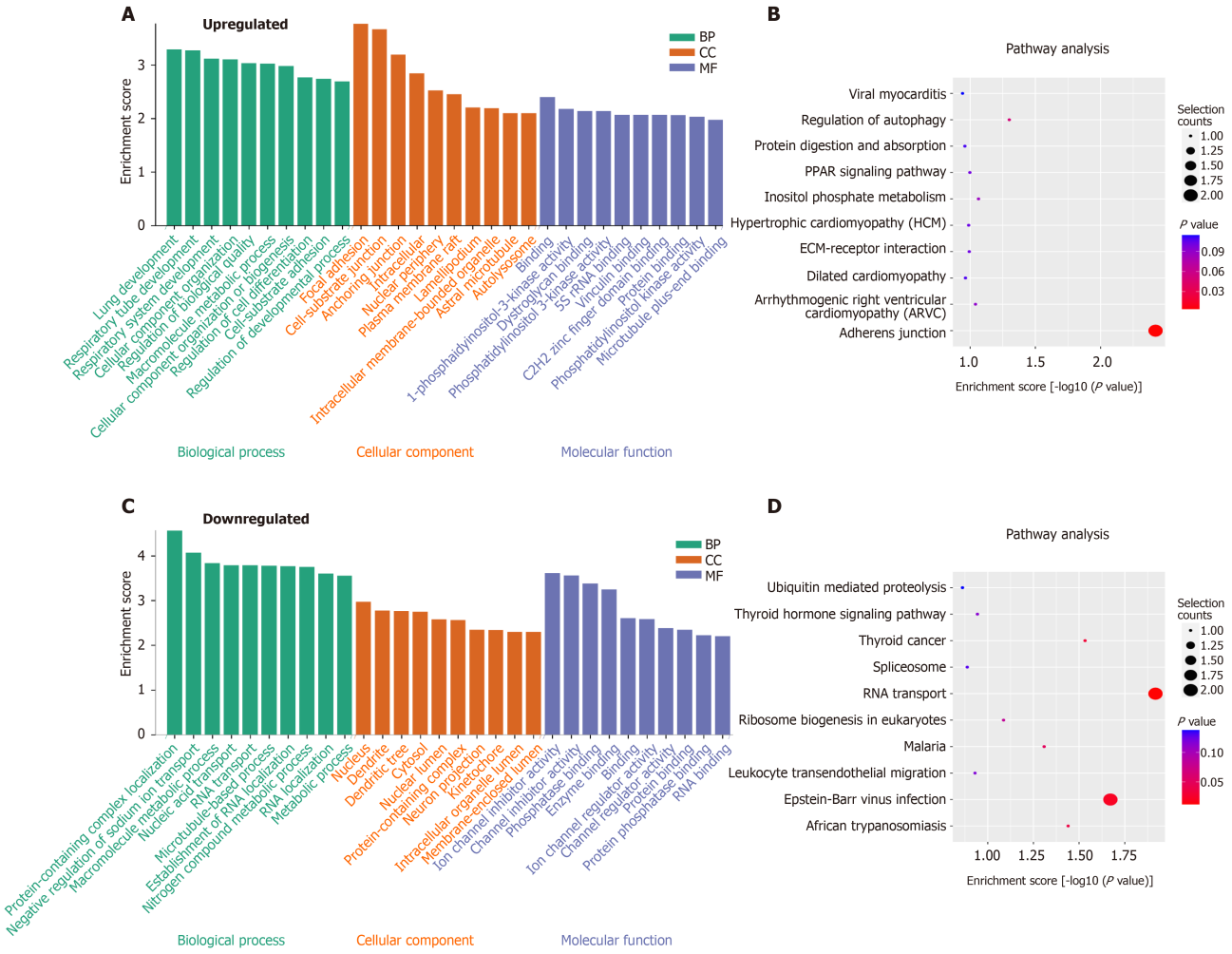

Figure 5 Functional analysis of circRNAs with differentially expressed N^6-methyladenosine peaks though gene ontology and Kyoto Encyclopedia of Genes and Genomes analysis. A and B: Gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis of circRNAs with upregulated N6-methyladenosine (m^{6}A) peaks; C and D: GO and KEGG analysis of circRNAs with downregulated m^{6}A peaks. GO analysis include biological process (BP) analysis, cellular component (CC) analysis, and molecular function (MF) analysis.

hepatocellular carcinoma, ALKBH5 could attenuate expression of LYPD1 by an m^{6}A-dependent manner and act as a tumor suppressor[39]. Overall, this evidence has suggested that ALKBH5 plays an essential role in m^{6}A modification. In this study, we found that expression level of ALKBH5 was upregulated in SAP. Consistent with this result, total m^{6}A level of circRNAs in SAP was reduced, indicating that ALKBH5 may play a role in the dynamic process of m^{6}A in SAP.

However, there are still limitations in our study. Firstly, further *in vivo* and *in vitro* experiments are needed to further explore the m^{6}A circRNA-mediated precise regulatory mechanisms in SAP. Secondly, the conservation analysis of the m^{6}A circRNAs showed that these circRNAs may have similar roles in human SAP. However, their clinical significance and the results should be investigated further in SAP patients. Additionally, the precise mechanism of ALKBH5 in m^{6}A circRNAs during SAP needs to be studied. Actually, these are in our next plans to explore the roles of m^{6}A circRNAs in SAP.

CONCLUSION

In conclusion, our study identified the transcriptome-wide profiling of m^{6}A circRNAs in SAP and predicted their biological significance and possible potential mechanisms, providing new insights to explore the possible pathophysiological mechanism of SAP and seek new potential therapeutic targets.

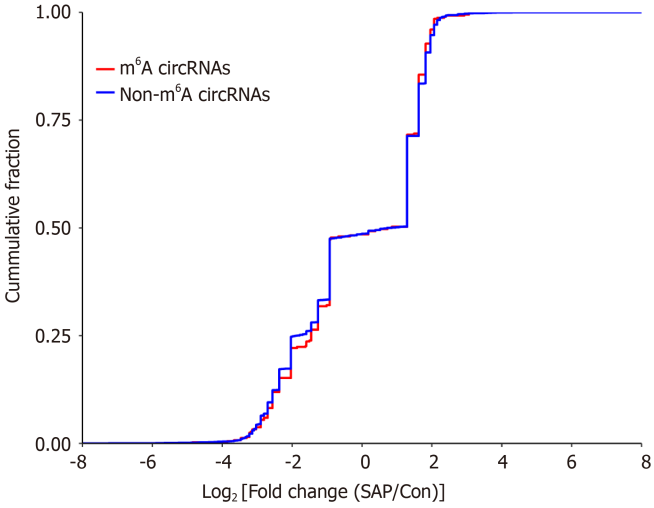

Figure 6 Relationship between N^6-methyladenosine level and expression of circRNAs in severe acute pancreatitis. Cumulative distribution of circRNAs expression between control and severe acute pancreatitis (SAP) groups for N^6-methyladenosine (m^{6}A) circRNAs (red) and non-m^{6}A circRNAs (blue).

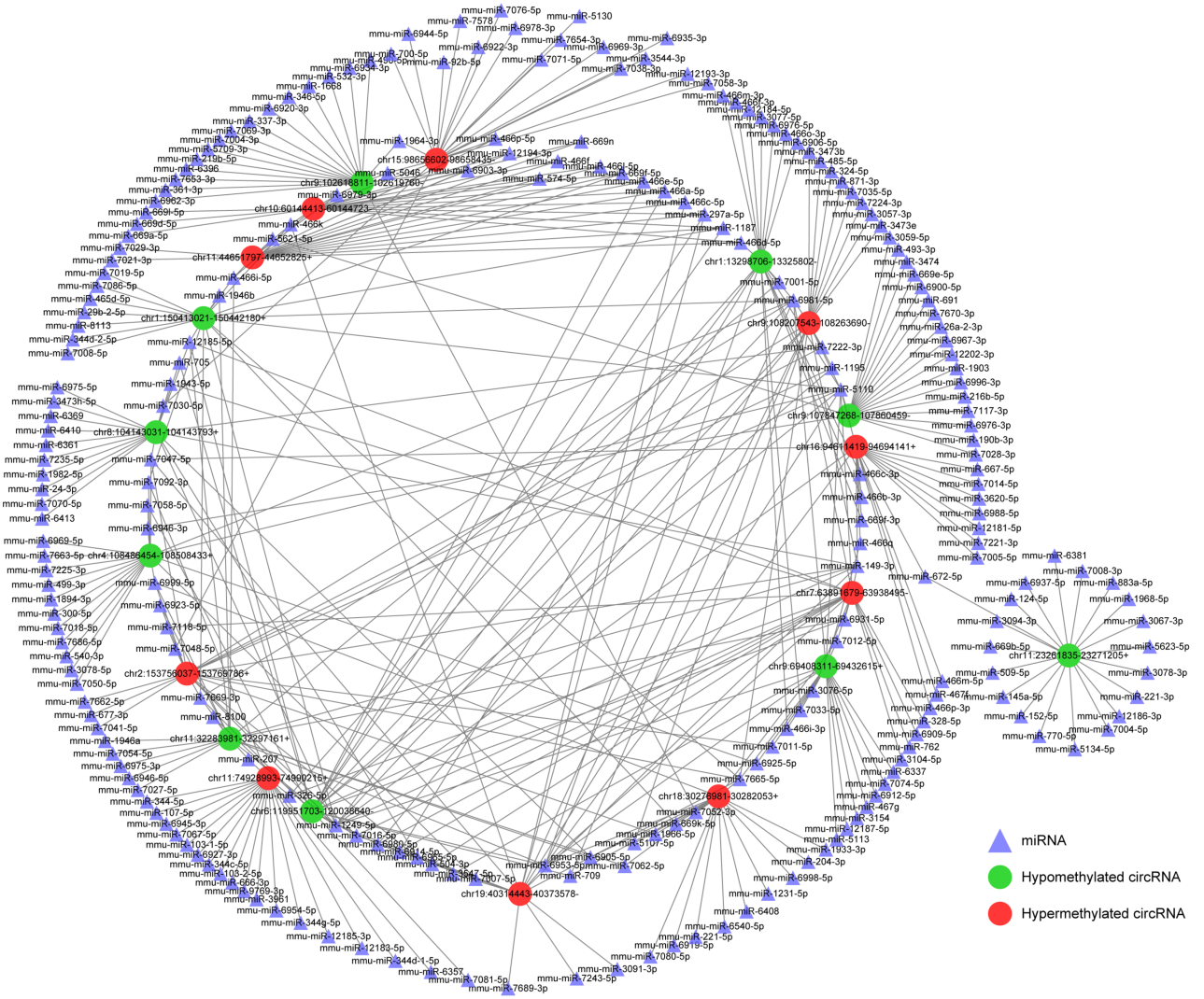

Figure 7 Construction of N^6-methyladenosine circRNA-miRNA networks in severe acute pancreatitis. A map showing the interaction networks of the top 10 upregulated and top 10 downregulated circRNAs according to the level of N6-methyladenosine, and their around 20 target miRNAs with the most stable binding in SAP. Green circles represent hypomethylated circRNAs, red circles represent hypermethylated circRNAs and triangles represent miRNAs, compared with control group.

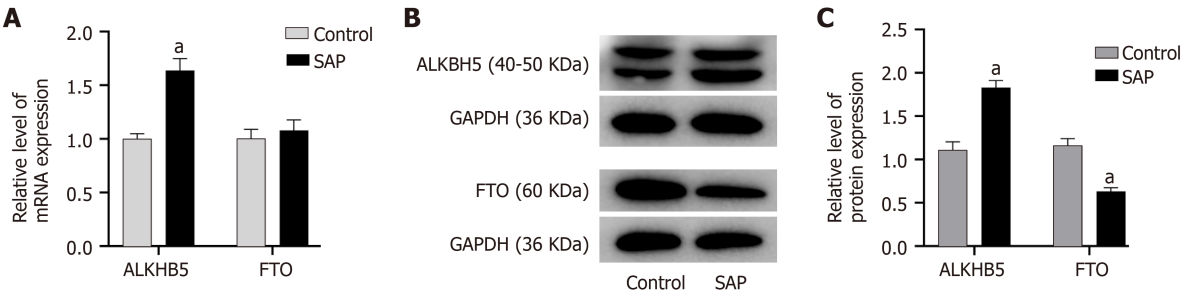

Figure 8 Expression of demethyltransferase in severe acute pancreatitis. A: Relative mRNA levels of alkylation repair homolog 5 (ALKBH5) and fat mass and obesity related protein (FTO) (normalized by the quantity of GAPDH) in each group; B: Representative images of western blot detected with alkylation repair homolog 5 (ALKBH5), FTO, and GAPDH antibodies in control and severe acute pancreatitis (SAP) groups; C: Relative protein levels of ALKBH5 and FTO (measured as the ratio of ALKBH5, FTO to GAPDH by band density) in each group. Data are representative of at least three independent experiments. $^aP < 0.05$ *vs* control group.

ARTICLE HIGHLIGHTS

Research background

Severe acute pancreatitis (SAP) is a lethal inflammatory disease with mortality up to 30%. But the genetic pathological mechanism of SAP remains unclear and SAP is still lack of effective therapeutic options. N^6-methyladenosine (m^{6}A) modification of circular (circ)RNAs plays a key role in many diseases and physiological processes through regulating the metabolism and function of circRNAs. However, the role of m^{6}A circRNA in SAP has been unexplored yet.

Research motivation

The pathophysiology of SAP at the level of gene regulation is complex and remains unclear. circRNAs are found to participate in many physiological processes and play key roles in pathological processes during SAP. m^{6}A modification can affect the "fate" of m^{6}A modified circRNAs, thereby participating in the regulation of diseases. Therefore, we want to explore whether the m^{6}A modification of circRNAs is related to the pathophysiological mechanism of SAP, and determine their biological significance and potential mechanisms.

Research objectives

The present study aims to determine the transcriptome-wide map of m^{6}A circRNAs and explore their biological significance and its possible mechanisms in SAP.

Research methods

The SAP C57BL/6 mice model was induced by retrograde injection of 4% sodium taurocholate salt. m^{6}A-modified RNA immunoprecipitation sequencing was used to determine the transcriptome-wide map of m^{6}A circRNAs. The biological significance of circRNAs with differentially expressed m^{6}A peaks was identified by GO and KEGG analysis. m^{6}A circRNA-microRNA networks was constructed to explore the underlying mechanism of m^{6}A circRNAs in SAP. The expression of demethylases was measured by western blot and qPCR. H&E staining and measurement of serum lipase and amylase were performed to assess the establishment of SAP mice model.

Research results

In the identified transcriptome-wide map of m^{6}A circRNAs, there were 57 circRNAs with differentially expressed m^{6}A peaks; among which, 32 were upregulated and 25 downregulated. Important pathways in the pathogenetic process during SAP were found by functional analysis of these m^{6}A circRNAs, such as protein digestion and regulation of autophagy. m^{6}A circRNA–miRNA networks showed that several important miRNAs in pathogenesis of SAP were bind to these m^{6}A circRNAs, such as miR-24-3p, miR-26a, miR-92b, miR-216b, miR-324-5p and miR-762. To be note, the total m^{6}A level of circRNAs was reduced in SAP, accompanied by the upregulated demethylase ALKBH5.

Research conclusions

The transcriptome-wide profiling of m^{6}A circRNAs in SAP was identified, and the

biological significance and possible potential mechanisms of m^{6}A circRNAs in SAP were predicted, providing new insights into exploring the possible pathophysiological mechanism of SAP and new potential therapeutic targets.

Research perspectives

This present study for the first time identified transcriptome-wide map of m^{6}A circRNAs and determined their biological significance and potential mechanisms. However, the m^{6}A circRNA-mediated precise regulatory mechanisms are need to be explore further *in vivo* and vitro experiments. What's more, further studies are needed to reveal the precise mechanism of ALKBH5 in m^{6}A circRNAs during SAP. In the future, we will explore them and investigate these m^{6}A circRNAs in SAP patients.

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ORIGINAL ARTICLE

Basic Study

Survivin-positive circulating tumor cells as a marker for metastasis of hepatocellular carcinoma

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Institutional review board statement: The study was approved by the ethics committee of Hubei Cancer Hospital (Ethical approval number: LLHBCH2019LW-002).

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Abstract

BACKGROUND

Circulating tumor cells (CTCs) and survivin are indicators for tumor stage and metastasis, as well as epitheliomesenchymal transition, in various cancers, including hepatocellular cancer (HCC).

AIM

To explore the potential of survivin-positive CTCs, specifically, as a marker for tumor progression in HCC patients.

METHODS

We examined the survivin expression pattern in CTCs obtained from 179 HCC patients, and investigated the *in vitro* effects of survivin silencing and overexpression on the proliferation and invasion of HCC cells. CTC count and survivin expression in patient samples were examined using RNA *in situ* hybridization.

RESULTS

All 179 patients were positive for CTC markers, and 94.41% of the CTCs were positive for survivin. The CTC and survivin-positive CTC counts were significantly higher in the HCC patients than in the normal controls, and were significantly associated with tumor stage and degree of differentiation. Further, survivin overexpression was found to induce HepG2 cell proliferation, reduce apoptosis, and improve invasive ability.

CONCLUSION

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Survivin shows upregulated expression (indicative of anti-apoptotic effects) in HCC. Thus, survivin-positive CTCs are promising as a predictor of HCC prognosis and metastasis, and their accurate measurement may be useful for the management of this cancer.

Key Words: Survivin; Circulating tumor cells; Hepatocellular carcinoma; Prognosis; Metastasis

Core Tip: This study first analyzed surviving-positive circulating tumor cells (CTCs) in patients with hepatocellular carcinoma (HCC). The levels of survivin expression in different CTCs were significantly different. The rates of moderate and high expression in the mesenchymal and hybrid CTCs were significantly higher than those in the epithelial CTCs. Survivin overexpression induced HCC cell proliferation, promoted their invasive ability, and reduced apoptosis. The expression of survivin in mesenchymal CTCs (mCTCs) in liver cancer was associated with metastasis and detection of survivin positivity in mCTCs may have potential value in early detection of tumor metastasis and prognostic evaluation.

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INTRODUCTION

According to 2020 statistics, hepatic cancer ranked sixth among malignant cancers in terms of incidence, and it ranked fourth globally as the main reason for cancer-related death[1]. Further, the 5-year overall survival of this cancer is quite abysmal, as it is less than 12%[1]. The prognosis of this disease continues to remain poor, even though there have been advances in the diagnostic and therapeutic strategies[2]. Hence, understanding the mechanisms underlying the metastasis of this cancer and assessing the status of disease progression are necessary.

The number of circulating tumor cells (CTCs) is an effective marker for solid tumors associated with metastasis[2]. CTC analysis is considered as real-time "liquid biopsy" for cancer patients, as it provides real-time monitoring of tumor progression and recurrence[3]. As a non-invasive biomarker, the CTC level can be used for comprehensive surveillance of cancer progression, in the case of both hepatocellular carcinoma (HCC) and cholangiocarcinoma[4]; in particular, it has been shown to be a good prognostic marker for HCC[5-7]. Currently, the most commonly used technique for CTC isolation is the cell search system, which is the only FDA-approved detection method; this method is based on positive immunoselection of epithelial cell adhesion molecule (EpCAM) and negative immunoselection of leukocytes (for which the general target is CD45). By using this technique, CTCs are divided into epithelial CTCs, mesenchymal CTCs (mCTCs), and hybrid CTCs[8]. According to this classification, CTCs have been reported to be useful as a marker for epithelial-mesenchymal transition (EMT) in HCC[9], and EMT is a known marker for the diagnosis and prognosis of cancer progression. Accordingly, previous studies have revealed that CTCs undergoing EMT are useful as indicators for diagnosing HCC and for predicting its prognosis[10]. The prognostic value of CTCs has also been demonstrated after surgery or during chemotherapy and recurrence[11,12].

Survivin is an anti-apoptotic protein (molecular weight, 16.5 kDa)[13] that plays an important role in inhibiting apoptosis in multicellular organisms and is overexpressed in many tumors, including HCC[14]. Studies have also shown that overexpression of survivin is associated with protection against apoptosis and propensity for metastasis in tumor cells, but this effect was not observed in normal cells[15]. Wurmbach *et al*[16] and Roessler *et al*[17] showed gene overexpression of survivin in HCC samples

compared to normal liver samples, and the difference between the two groups was significant, as shown in Figure 1. Additionally, recent research has shown that insulin-like growth factor-1 induced EMT *via* activation of survivin in HCC cells[18]. Accordingly, overexpression of survivin mRNA and protein has been shown to stimulate EMT in HCC cells; this has been shown to increase their ability for invasion and migration and their cell proliferation rate, and decrease their apoptosis rate[15]. Thus, there is evidence for the potential of survivin as a marker for cancer cell proliferation and metastasis.

As discussed above, the research so far has indicated that both CTCs and survivin are markers for EMT and, therefore, cancer progression in HCC. However, the serum survivin levels of HCC patients are not different from those of healthy controls and patients with nonmalignant chronic liver diseases[19]. And no study so far has analyzed the prognostic value of survivin-expressing CTCs in HCC. Therefore, in the present study, we explored whether survivin-positive CTC levels are associated with cancer stage and clinicopathological characteristics in HCC, and whether survivin expression in CTCs has potential as a predictor of metastasis in HCC patients.

MATERIALS AND METHODS

Genome data

Cancer genome atlas data were retrieved from GEO database (http://www.ncbi.nlm.nih.gov/geo) and TCGA database (http://www.ualcan.path.uab.edu). The GSE6467 and GSE14520 datasets were downloaded for this study. Data was analyzed using software packages including ClusterProfiler, GSEquery, and pheatmap.

Patient cohort

A study cohort (n = 179) included HCC patients who were enrolled at Hubei Cancer Hospital, China between May 2018 and December 2018. Only patients for whom HCC was confirmed based on pathological evidence, according to the World Health Organization criteria, and patients who had not undergone prior anticancer treatment were included. The tumors were staged as 0-A or B-C according to the Barcelona Clinic Liver Cancer (BCLC) staging system. Further, based on TNM classification, the tumors were staged as I, II, III, or IV. A control group comprised 70 healthy persons and 54 patients who tested positive for hepatitis B/C virus. The study was approved by the ethics committee of Hubei Cancer Hospital. (Ethical approval number: LLHBCH2019LW-002)

Collection of blood samples and isolation of CTCs

Samples of peripheral blood (a volume of 5 mL mixed with EDTA as an anticoagulant) were obtained *via* venipuncture. The CanPatrol CTC enrichment technique (SurExam, Guangzhou, China) was used to isolate CTCs from the blood samples and classify them. The samples were filtered through a membrane (pore diameter, 8 μm; Millipore, Billerica, United States), a vacuum plate that provided various valve settings (SurExam, Guangzhou, China), an E-Z 96 vacuum that also had various settings (Omega, Norcross, United States), and a vacuum pump (Auto Science, Tianjin, China). A cell lysis buffer that comprised 154 mmol/L NH_4Cl, 10 mmol/L $KHCO_3$, and 0.1 mmol/L EDTA (all the reagents were from Sigma, St. Louis, United States) was used to induce lysis of erythrocytes. The cell suspension was then resuspended for 5 min in phosphate-buffered saline (Sigma, St. Louis, United States) that contained 4% formaldehyde (Sigma, St. Louis, United States), and transferred to the specialized filtration system described earlier. The vacuum pump was turned on and set at a pressure of 0.08 MPa. The CTCs were separated using the membrane as mentioned above.

Classification of CTCs by RNA in situ hybridization

CTC phenotypes were detected by RNA *in situ* hybridization (ISH) with CD45 (a leukocyte marker), EpCAM and CK8/18/19 (epithelial cell markers), and vimentin and Twist (mesenchymal cell markers) as markers (Figure 1). A protease (Qiagen, Hilden, Germany) was used to pretreat the blood cells on the membrane. Following this, the cells were hybridized with a capture probe that targeted the genes for the markers mentioned above. Next, the cells were stained by 5-min incubation with 4,6-diamidino-2-phenylindole (Sigma, St. Louis, United States), and viewed under an automated imaging fluorescence microscope (Keyence, United States). Red fluorescence signals were considered to indicate epithelial cell markers, while green

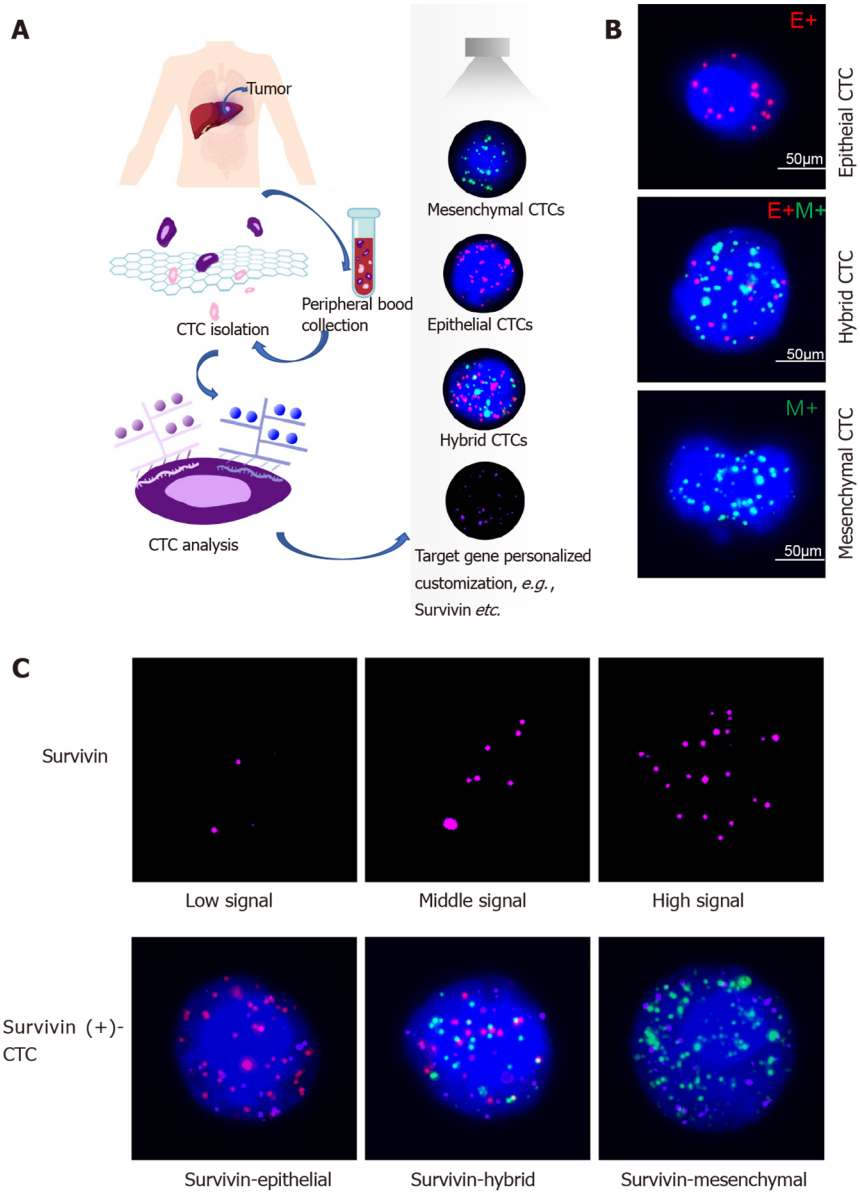


Figure 1 Circulating tumor cell classification and survivin expression in circulating tumor cells by RNA-in situ hybridization. A: Circulating tumor cell (CTC) isolation and RNA *in situ* hybridization analysis of blood samples from patients and healthy controls; B: Detection and classification of CTCs using epithelial-mesenchymal transition markers. CD45 was used as a leukocyte marker, and it is indicated by white fluorescence. Epithelial biomarkers (EpCAM and CK8/18/19) were used for epithelial CTCs, which are represented by red fluorescence, and mesenchymal biomarkers (vimentin and Twist) were used for mCTCs, which are represented by green fluorescence; C: Survivin expression in CTCs is indicated by purple fluorescence (Alexa Fluor 647), with the intensity scored as low, moderate, and high (left to right images). CTC: Circulating tumor cell. CTC: Circulating tumor cell.

fluorescence signals were considered to indicate mesenchymal cell markers. Further, bright white fluorescence signals were considered to indicate CD45 expression.

Survivin expression in CTCs

Survivin expression in CTCs was assessed using the RNA-ISH method (Figure 1C). Survivin expression is indicated by purple fluorescence emitted by the CTCs (Alexa Fluor 647). According to the signal intensity score, survivin expression was classified as absent (0), low (1-2), moderate (3-9), and high (signal > 9), as shown in Figure 1C.

In vitro silencing and overexpression of survivin

The HCC cell line HepG2 was obtained from the cell bank of the Chinese Academy of Science and cultured in Dulbecco's modified Eagle medium with 10% fetal bovine serum and 1% penicillin/streptomycin at 37°C in a 5% CO_2 incubator.

Short hairpin RNAs against the survivin sequence (5'-CTTACCAGGTGAGAA-GTGAGGT-3') were designed to knockdown survivin in HepG2 cells, by transfec-tion with the vector pGPU6 harboring siRNAs against survivin. The lentivirus vector

pCDH-CMV-MCS-EF1-CopGFP-T2A-Puro was used to induce overexpression of survivin in HepG2 cells. The vector and siRNA were obtained from Hualian Biotechnology Company (Wuhan, China). HepG2 cells were stably transfected with the survivin siRNA and lentivirus-siRNA plasmid using Lipofectamine2000 (Invitrogen). The transfected cells were incubated in selection medium containing 2 mg/mL puromycin for 2 wk (Bioswamp), and Western blot analysis was used to assess survivin expression.

Cell proliferation and apoptosis analysis

The CCK-8 kit (Bioswamp Company, China) was used to analyze cell proliferation according to the manufacturer's protocol. The staining reagent used was CCK-8 (at a volume of 10 mL); the cells were incubated with the reagent for 2 h at 37 °C for 12 h, 24 h, 36 h, 48 h, 60 h, and 72 h after transfection (with the survivin siRNA or lentivirus). Following this, the absorbance of the cells was detected at 450 nm. Flow cytometry (Beckman, United States) was used to analyze cell apoptosis with the Annexin V-PE and 7AAD assay kit (Becton, Dickinson Company, United States). Apoptotic cells were defined as PE-positive and 7AAD-negative, while necrotic or dead cells were defined as 7AAD-positive and PE-negative.

Cell invasion analysis

For the invasion experiment, cells were seeded at a density of 5×10^4 cells/well in Transwell inserts (pore diameter, 8 μm) coated with Matrigel and containing cold serum-free medium. After an incubation period of 48 h, non-invasive cells that were present in the upper compartment were removed with a cotton swab, and the cells that had successfully adhered to the lower compartment were subjected to fixation with 10% paraformaldehyde and staining with 0.1% hexamethylpararosaniline for 30 min. The number of cells was counted under a microscope (Olympus BX53) in five selected fields. The average number of adherent cells was calculated.

Western blot and immunohistochemistry analysis of survivin protein expression in HCC tissue

Formalin-fixed paraffin-embedded specimens of HCC tissues from Hubei Cancer Hospital were prepared for immunohistochemical staining. The tissue slides were washed in phosphate-buffered saline (Bioswamp, China) after dewaxing and dehydration. The slides were placed in plastic tubes and incubated with boiling citric acid for antigen retrieval. In the next step, endogenous peroxidase activity was inhibited *via* 10-min incubation in methanol containing 3% H_2O_2. Next, the slides were blocked by 15-min incubation in 1% goat serum at 37 °C. Following this, they underwent incubation with anti-survivin rabbit polyclonal antibody (1:100 dilution; Abcam, United Kingdom) at 4 °C overnight in a moist chamber. After treatment with the secondary antibodies for 30 min, the slides were colored with diaminobenzidine and counterstained with hematoxylin. All the images were captured using an Olympus BX53 microscope. The protocol for Western blot assay of survivin protein expression in the tissue specimens was the same as that described for Western blot analysis of the HepG2 cells.

Statistical analysis

All analyses were conducted using SPSS version 20.0 (IBM Corp., Armonk, NY, United States). The Kolmogorov-Smirnov method was used to determine the normality of the CTC and survivin-positive CTC counts, which were found to be non-normally distributed. The phenotypic CTC counts and survivin expression in different groups were examined by the Fisher exact probability test. Correlations between variables were evaluated with the Spearman rank correlation test. $P < 0.05$ was considered to indicate statistical significance.

RESULTS

Analysis of genome data

Based on the GSE6467 and GSE14520 datasets, gene expression was found to differ between tumor tissue and normal tissue, as evident from the survivin gene expression data shown in Figure 2A and 2C. Next, we found that DNA replication and cell cycle pathways were downregulated in HCC samples, based on the Ingenuity pathway analysis (Z-score < 0; Figure 2B).

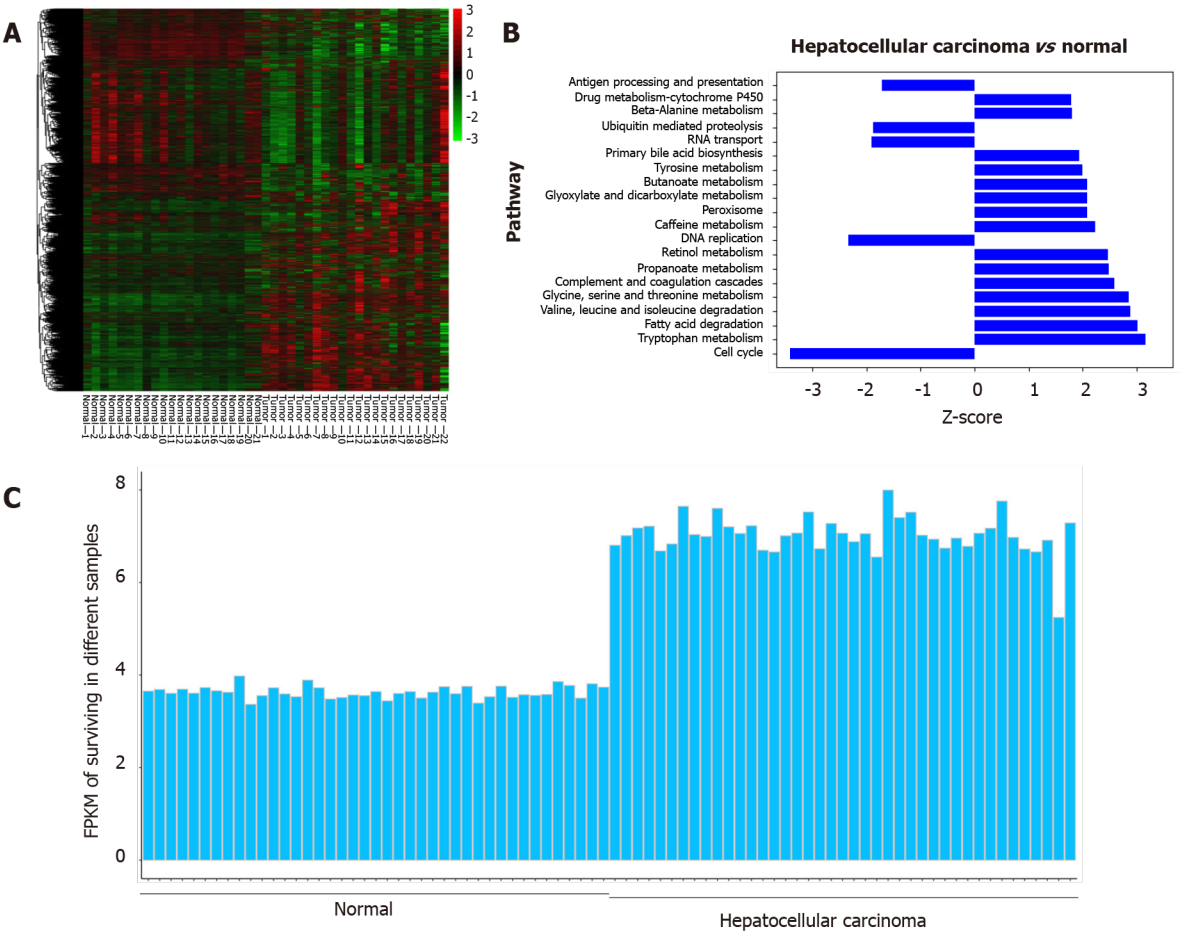


Figure 2 Analysis of survivin gene expression in normal liver tissue and hepatocellular cancer tissue from previously reported genome data. A: The expression patterns in 21 normal tissue samples and 22 hepatocellular carcinoma samples were analyzed by hierarchical clustering; B: Ingenuity pathway analysis was used to identify the upregulated pathways (Z-score > 0) and the downregulated pathways (Z-score < 0) in the normal tissue samples and hepatocellular cancer (HCC) samples; C: The FPKM (fragments per kilobase of transcript per million fragments mapped) of survivin was determined for different samples. Compared with normal liver tissue samples, the expression of the survivin gene was upregulated in HCC samples.

Based on the TCGA data analysis, the expression of survivin in HCC tissue was significantly associated with stage and tumor grade. Survivin expression (fragments per kilobase of transcript per million fragments mapped) in patients with stages III and IV HCC was significantly higher than that in patients with stages I and II HCC ($P < 0.05$). And survivin expression (fragments per kilobase of transcript per million fragments mapped) in patients with grades 3 and 4 HCC was significantly higher than that in patients with grades 1 and 2 HCC ($P < 0.05$) (Supplementary Figure 1).

Survivin expression in HCC specimens and normal hepatic tissues

The survivin expression level in tissue samples from HCC patients ($n = 8$) was significantly higher than that in normal hepatic tissues ($n = 4$), as indicated in the Western blot in Figure 3A and the immunohistochemical staining images in Figure 3B.

Association between survivin expression and cell proliferation, apoptosis, and invasion abilities in HepG2 cells

Silencing of survivin expression and overexpression of survivin were successfully induced by siRNA and lentivirus transfection, respectively (Figure 4A). At 12 h, 24 h, 48 h, 60 h, and 72 h post-transfection, the cell proliferation rate was significantly higher in the survivin-overexpression cells than in the control cells and survivin-silenced cells, while it was significantly lower in the survivin-silenced cells than in the control cells and survivin-overexpressing cells (Figure 5A). Accordingly, the apoptosis rate of survivin-silenced HepG2 cells was significantly higher than that of survivin-overexpressing and normal control cells (Figure 4B). Finally, the results for cell invasion rates obtained from the Transwell migration assays indicated that the survivin-overexpressing HepG2 cells had significantly higher invasive ability than the survivin-

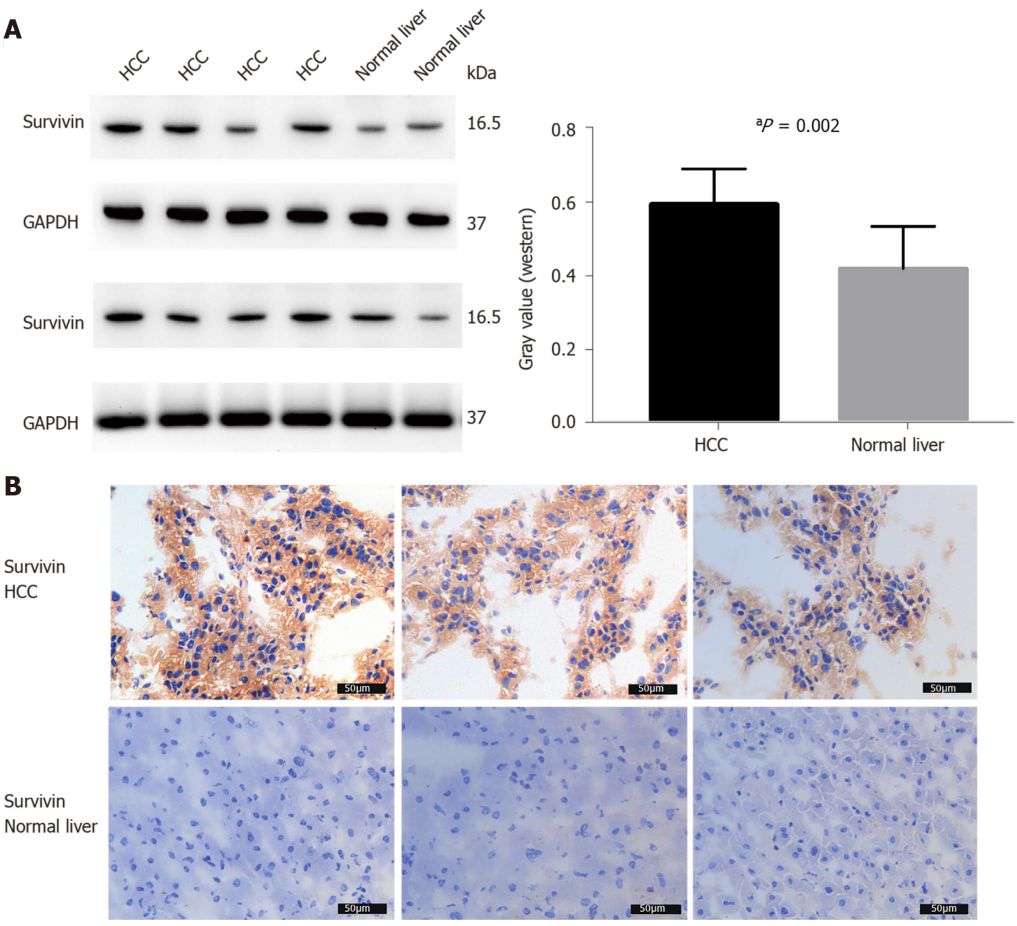


Figure 3 Survivin expression in hepatocellular cancer and normal adjacent tissue samples. A: Western blot analysis showing the protein expression of survivin in hepatocellular cancer (HCC) (n = 8) and normal liver tissue (n = 4); B: Immunohistochemical staining for survivin in HCC (n = 8) and normal liver tissue (n = 4). Survivin expression was higher in HCC than in normal liver tissue. $^aP < 0.05$. HCC: Hepatocellular cancer.

silenced and normal control cells (Figure 5B).

Isolation and classification of CTCs

The observations of RNA-ISH analysis of CTCs based on EMT markers are shown in Figure 1B. The expression of survivin in these subtypes of CTCs is also indicated. The proportion of mCTCs is shown in Figure 6C. The number of mCTCs seemed to be higher in advanced-stage HCC (stage B-C) than in metastatic HCC.

CTC count and its association with clinicopathological characteristics of HCC patients

Table 1 shows the clinicopathological characteristics of the study cohort. A CTC count of > 5/5 mL was detected in 168 out of 179 blood samples (93.85%), and the median CTC count was 16/5 mL (range, 1-193 CTCs/5 mL). Further, survivin-positive CTCs were detected in 94.41% (169 of 179) of the patients. As shown in Figure 6, the median CTC count (Figure 6A) and survivin-positive CTC count (Figure 6B) in HCC patients were significantly higher than those in the HBV/HCV patients and healthy controls (P < 0.05). Patients with stage B/C HCC (or advanced HCC) had a significantly greater median CTC count than those with stage 0/A HCC (or early-stage HCC) (P = 0.024) (Table 1), and the median CTC count in patients with poorly differentiated tumors was significantly higher than that in patients with well-differentiated tumors (P = 0.002) (Table 1).

Correlation between mCTC counts and clinicopathological features

Out of the 179 patients, 138 had an mCTC count of > 2/5 mL. The mCTC count was not significantly associated with AFP level, tumor stage, or degree of differentiation (Table 1). However, the proportion and count of mCTC were associated with BCLC stage and metastasis (Figure 6C).

Table 1 Clinical characteristics of 179 hepatocellular cancer patients

Clinical factor	*n*	[1]CTC count/5 mL Median, SE	[2]mCTC count/5 mL Median, SE	[3]Survivin (+)CTC count/5 mL Median, SE	*P* value
Sex					
Male	119	17, 3.57	6, 1.11	9, 3.05	0.251[1], 0.819[2], 0.909[3]
Female	60	12, 9.61	6, 3.19	9.5, 7.88	
Age					
< 55 yeara	89	19, 4.28	9, 1.50	11, 3.54	0.700[1], 0.141[2], 0.467[3]
≥ 55 years	90	14, 5.75	4, 1.71	8, 4.88	
HBV/HCV					
Positive	130	17, 3.57	7, 1.16	10, 3.03	0.402[1], 0.539[2], 0.436[3]
Negative	49	13, 9.90	4, 3.12	6.5, 8.21	
AFP levels					
< 7.0	80	14, 5.85	5, 1.85	7, 4.66	0.285[1], 0.81[2], 0.254[3]
≥ 7.0	99	22.5, 4.42	7.5, 1.45	11, 3.92	
Cirrhosis					
Yes	119	16, 3.43	6, 1.19	9.0, 2.78	0.498[1], 0.671[2], 0.829[3]
No	60	19.5, 10.03	7.5, 2.94	10, 8.67	
TNM stage					
I-II	79	14, 2.93	6, 1.04	6, 1.49	0.111[1], 0.433[2], 0.002[3]
III-IV	100	24, 6.29	8.50, 2.00	18, 5.95	
BCLC stage					
0-A	58	12.5, 3.04	4, 0.75	4, 1.25	0.024[1], 0.000[2], 0.000[3]
B-C	121	25, 4.94	9, 1.56	20, 4.64	
Differentiation					
Well	29	13, 2.04	5, 2.46	5, 1.70	0.002[1] (well *vs* poor)
Moderate	92	14.5, 5.35	6, 1.77	8.5, 5.15	0.970[2] (well *vs* poor)
Poor	58	37, 6.18	7.5, 1.55	24, 5.44	0.005[3] (well *vs* poor)

[1]Presents circulating tumor cell counts analysis.
[2]Presents mesenchymal circulating tumor cell counts analysis.
[3]Presents survivin positive circulating tumor cell counts analysis.
CTC: Circulating tumor cell; mCTC: Mesenchymal circulating tumor cell; AFP: α-fetoprotein.

ROC curve analysis of CTCs, survivin-positive CTCs, and mCTC proportion

The area under the ROC curve was 0.84 [95% confidence interval (CI) = 0.77-0.91] for CTCs, 0.82 (95%CI = 0.75-0.89) for survivin-positive CTCs, and 0.82 (95%CI = 0.75-0.90) for the proportion of mCTCs (Figure 6D). The rational cut-off value for diagnosis was 12 for the CTC count (sensitivity = 60.89%, specificity = 91.13%), 4 for the survivin-positive CTC count (sensitivity = 81.01%, specificity = 78.23%), and 1.85% for the proportion of mCTCs (sensitivity = 84.92%, specificity = 72.58%).

Correlation between survivin expression in CTCs and clinicopathological factors of HCC patients

The survivin-positive CTC counts were not significantly associated with AFP levels. However, the median survivin-positive CTC count in patients with stages III and IV HCC was significantly higher than that in patients with stages I and II HCC (Table 1). Further, patients with stage B/C HCC (or advanced HCC) had a significantly greater median survivin-positive CTC count than those with stage 0/A HCC (or early-stage HCC) ($P < 0.001$) (Table 1). Finally, patients with poorly differentiated HCC had a

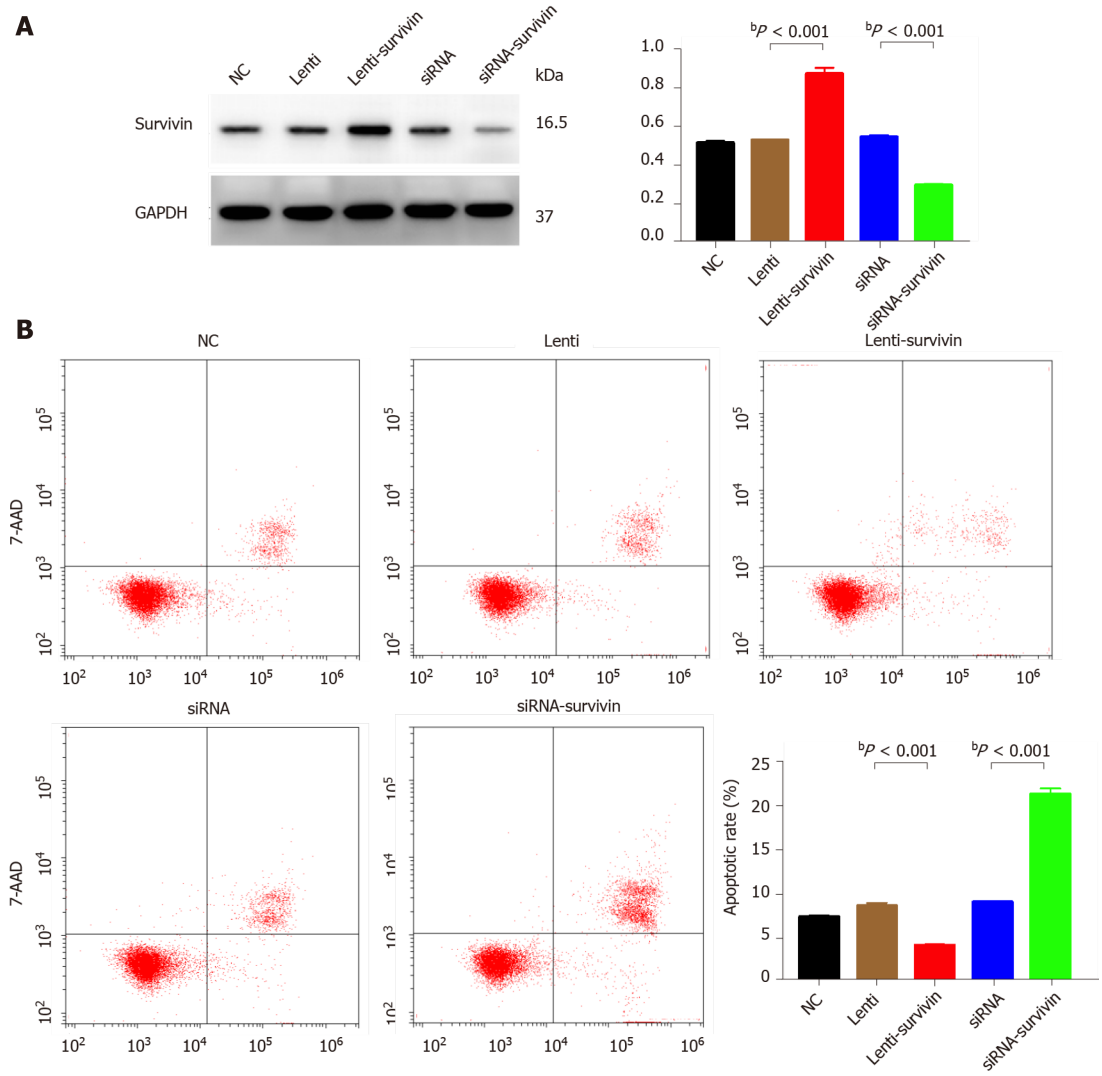


Figure 4 Effect of survivin silencing and overexpression on apoptosis ability of HepG2 cells. A: Western blot analysis confirming survivin protein knockdown and upregulation in the selected clones (siRNA, siRNA-survivin, Lenti, and Lenti-survivin) and controls; B: Cell apoptosis was detected by flow cytometric analysis (n = 3). The apoptosis rate was significantly lower in the survivin-overexpressing cells ($^bP < 0.01$).

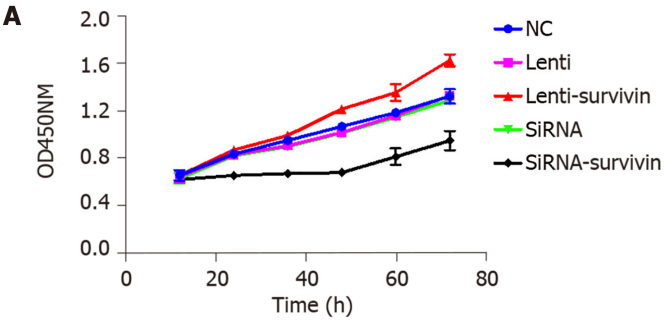
significantly greater median survivin-positive CTC count than those with well-differentiated tumors (P = 0.005) (Table 1).

Survivin expression in CTC subtypes

Survivin expression was detected in all CTC-positive patients (Figure 7), but the level of expression (based on the intensity of the purple fluorescent signal) differed between epithelial, mesenchymal, and hybrid CTCs, although not significantly: Survivin expression was observed in 62.7% of mCTCs, 74.61% of hybrid CTCs, and 73.53% of epithelial CTCs. However, the signal intensities were significantly different: Moderate- and high-intensity signals were observed in 48.53% and 31.91% of mCTCs, respectively, and in 47.65% and 16.5% of hybrid CTCs, respectively. The proportion of mesenchymal and hybrid CTCs with moderate- and high-intensity signals was significantly higher than that of epithelial CTCs (P < 0.001) (Figure 7A and B). In contrast, a significantly greater proportion of epithelial CTCs tended to have low signal intensity for survivin expression: 72.47% of epithelial CTCs *vs* 33.85% and 35.83% of hybrid and mCTCs, respectively (Figure 7A and B).

Dynamic changes in survivin-positive CTC count following surgery and its prognostic significance

The survivin-positive CTC counts were detected in 22 patients at 1 wk following surgical resection. The total CTC count exhibited a considerable decrease after resection, as did the survivin-positive CTC and mCTC counts (Figure 8A-C). During the postoperative period, in 10 out of 22 HCC patients, the total CTC, mCTC, and



P-value	Lenti *vs* lenti-sur	SiRNA *vs* siRNA-sur
60 h	$P < 0.001$	$P < 0.001$
72 h	$P < 0.001$	$P < 0.001$

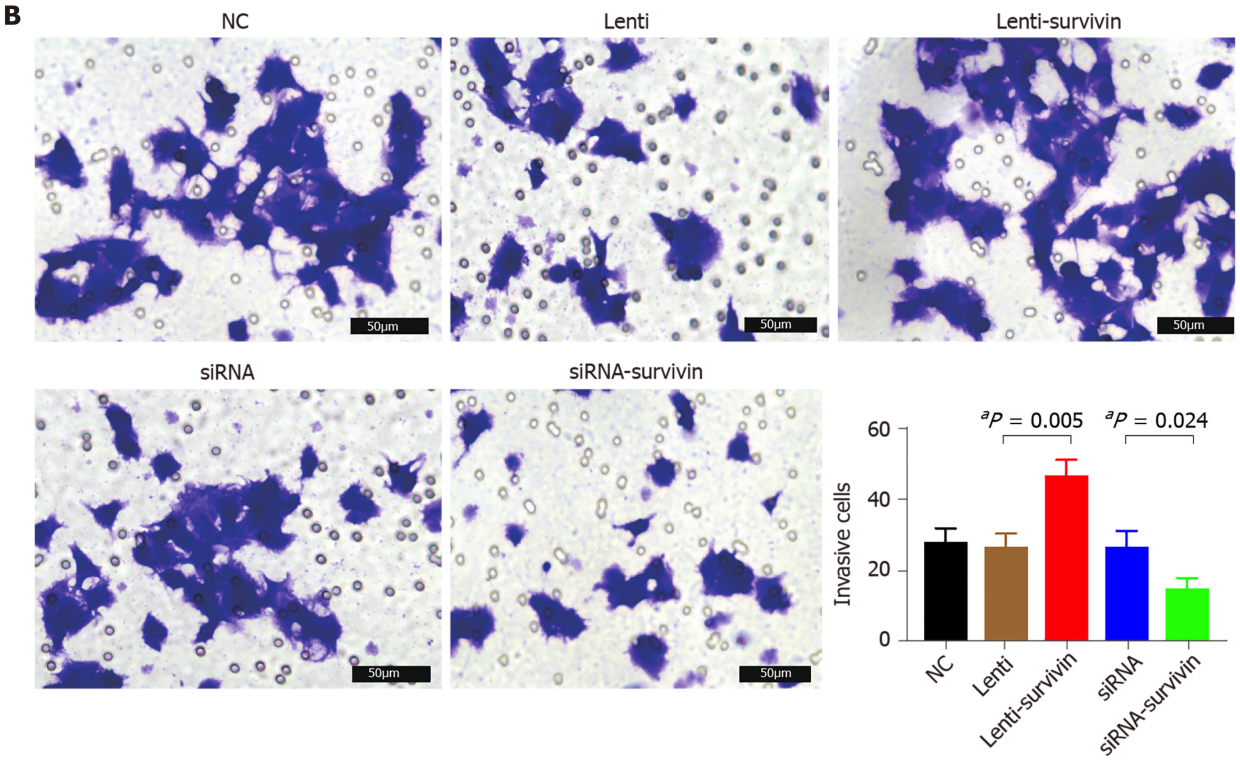


Figure 5 Effect of survivin silencing and overexpression on proliferation and invasion abilities of HepG2 cells. A: Cell proliferation was evaluated at 12 h, 24 h, 48 h, 60 h, and 72 h after transfection with the siRNA or lentivirus using the CCK8 assay ($n = 5$), and it was significantly higher in the survivin-overexpressing cells ($^aP < 0.05$); B: Invasion ability of cells was examined by transwell assay ($n = 3$). The invasive cell count was significantly higher in the survivin-overexpressing cells ($^aP < 0.05$).

survivin-positive CTC counts had increased over the thresholds (12 for CTCs, 4 for mCTCs and sur-CTCs) at 86 d before imaging recurrence or metastatic lesions were detected, while no increase was noted in the other 12 patients for more than 1 year (Figure 8D). Additionally, log-rank test revealed that the recurrence free survival rate was significantly associated with survivin-positive CTC count (Figure 9A). In all 10 cases, over 4 survivin-positive CTCs were detected before recurrence (Figure 9B).

In two HCC patients, initial overexpression of survivin was observed in CTCs (counts/5 mL), while recurrence or metastasis (Figure 10A and B) was observed 4-5 mo after therapy in patients who were in the same BCLC stage before treatment. And increased surviving-positive CTC count is showed in Figure 10C.

DISCUSSION

In the present study, we analyzed the correlation between survivin-positive CTC expression patterns and the prognosis of HCC, in order to explore the potential of survivin-positive CTCs as markers for HCC stage and predictors of HCC metastasis. The prognostic value of survivin-positive CTCs in patients after surgical resection was also demonstrated.

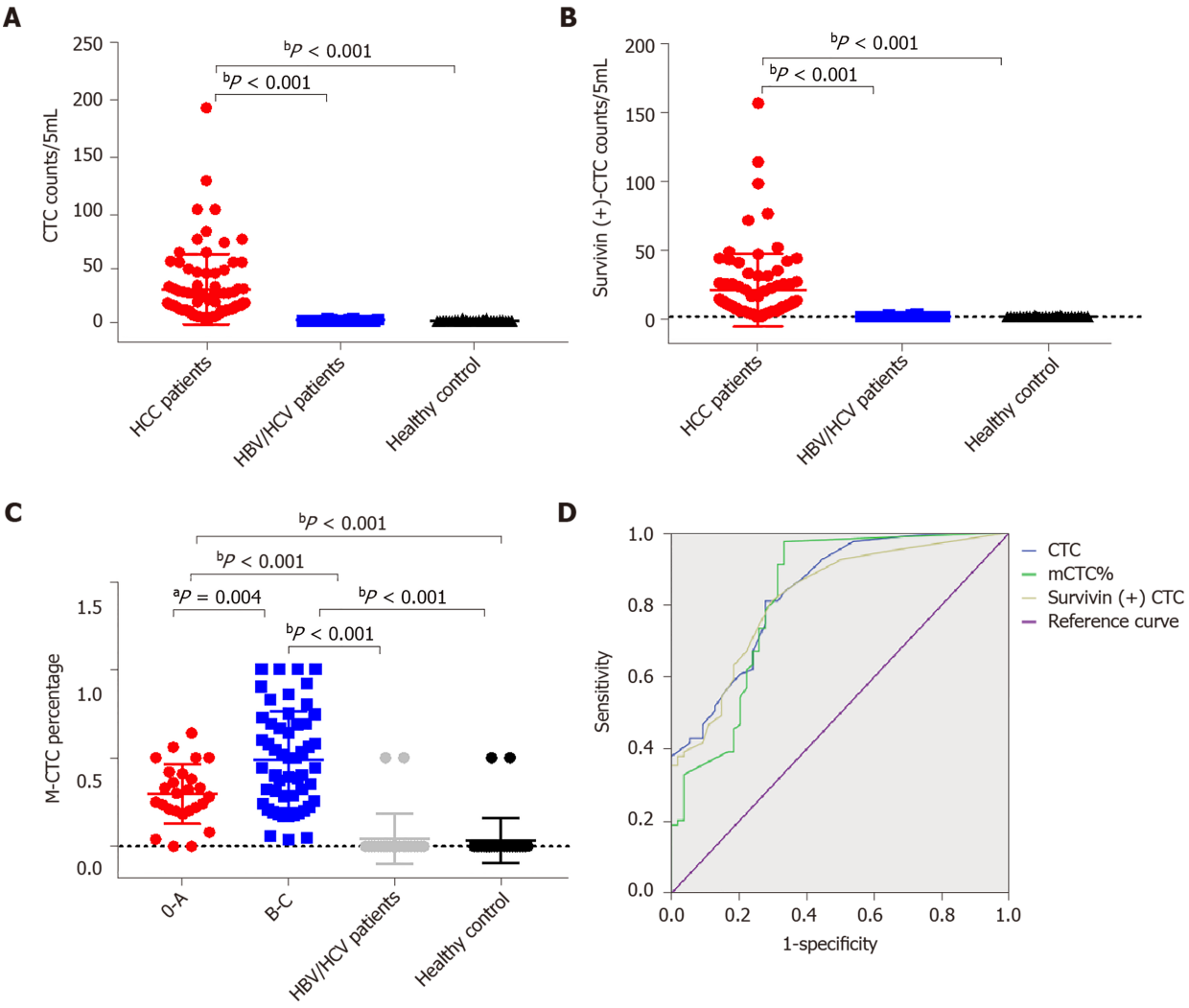


Figure 6 Total circulating tumor cell count and survivin-positive circulating tumor cell count in the study cohort. A and B: Both the median circulating tumor cell (CTC) count (A) and survivin-positive CTC count (B) in hepatocellular cancer (HCC) patients were significantly higher than those in the HBV/HCV patients and healthy controls; C: The proportion of mesenchymal CTCs (mCTCs) in HCC at the advanced BCLC stage (B-C) was significantly higher than that in HCC at the early stage (0-A); D: ROC curves for survivin-positive CTC count, total CTC count, and mCTC proportion. The rational cut-off for diagnosis was 12 for the CTC count, 4 for the survivin-positive CTC count, and 1.85% for the mCTC proportion. $^{a}P < 0.05$; $^{b}P < 0.01$. CTC: Circulating tumor cell.

Our *in vitro* experiments with HepG2 cells showed that siRNA-induced silencing of survivin expression resulted in a significant decrease in the proliferation and invasive ability of these cells, and a significant increase in their apoptosis rate; opposite results were obtained with overexpression of survivin. In agreement with this finding, previous studies have confirmed that survivin deficiency induces apoptosis in HepG2 cells[20,21]. These findings together indicate that survivin promotes the metastasis of HCC, and they are in agreement with a previous study which showed that knockdown of survivin expression suppressed HCC metastasis[22].

In our study, 94.41% of CTCs obtained from the HCC patients were positive for survivin expression. Further, we examined the survivin expression pattern in different phenotypes of CTCs, including mCTCs, hybrid CTCs, and epithelial CTCs. The findings showed that survivin expression was much higher in mesenchymal and hybrid CTCs than in epithelial CTCs, and a major proportion (62.7%) of mCTCs were positive for survivin expression. These findings indicate that survivin plays an important role in promoting EMT in HCC cells. During the process of carcinogenesis, tumor cells gain invasive ability by undergoing EMT, which occurs *via* downregulation of the epithelial cell marker E-cadherin (among others) and upregulation of the mesenchymal cell markers vimentin and N-cadherin (among others)[23]. Accordingly, it has also been reported that survivin mediates EMT in HCC cells *via* modulation of EMT marker expression[24]. Thus, based on these findings, it appears that survivin promotes metastasis of HCC through mediation of EMT events.

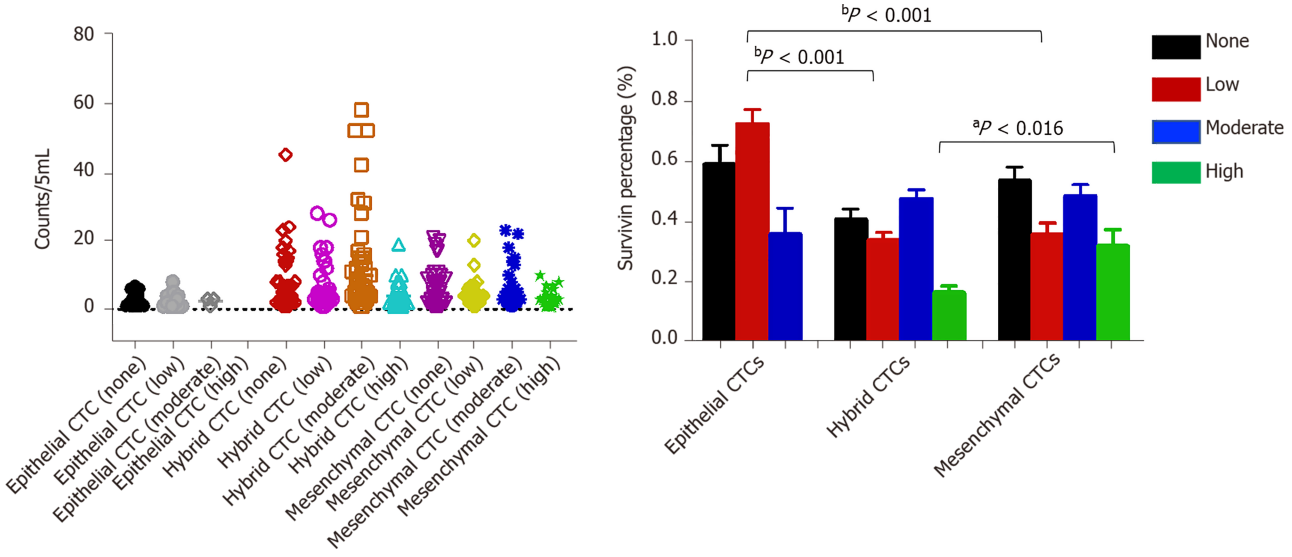


Figure 7 Analysis of survivin gene expression patterns in epithelial, mixed, and mesenchymal circulating tumor cells. A significantly lower percentage of epithelial circulating tumor cells (CTCs) showed moderate staining intensity than mixed and mesenchymal CTCs (mCTCs), while a significantly higher percentage of epithelial CTCs showed low staining intensity than hybrid and mCTCs. $^{a}P < 0.05$; $^{b}P < 0.01$. CTC: Circulating tumor cell.

Our findings showed that survivin was expressed at significantly greater levels in HCC tissue than in normal liver tissue, and the expression levels were significantly associated with the degree of tumor differentiation, that is, poorly differentiated tumors had significantly higher survivin expression. We also found that survivin expression level was significantly greater in HCC tissue than in normal tissue. The high frequency of cytoplasmic survivin expression in HCC observed in our study is in agreement with that reported in previous studies[24,25]. Many studies have demonstrated that survivin plays a crucial role in the early and late stage of carcinogenesis[26]. In agreement with our findings, other *in vitro* studies have reported increased expression of survivin in tumor cells[27,28]. Based on these findings, we assumed that survivin plays an anti-apoptotic role and aids in the progression of HCC. Hence, HCC patients who are positive for survivin should be monitored carefully, and enhanced adjuvant therapy should be considered to help improve their prognosis[29,30]. It is not easy to obtain HCC tissue samples at every follow-up appointment; therefore, detection of survivin in CTCs may be a useful supplementary method.

CONCLUSION

Previous studies have mostly investigated the expression of survivin in cancer cells or tissue specimens from patients[25,31,32], but the strength of our study lies in its investigation of survivin expression in different subtypes of CTCs. This is the first study to have pursued this line of investigation. We found that the survivin-positive CTC counts are significantly associated with the TNM tumor stage, BCLC stage, and degree of differentiation. Thus, the survivin-positive CTC count may particularly be important as an indicator of tumor stage and cancer progression. Additionally, the proportion of mCTCs also increases with tumor progression, but the mCTC counts are not significantly different between the different tumor grades and AFP. This variable needs to be explored further in future studies for its potential as a tumor stage marker.

One of the limitations of this study is the small sample size. Future investigations on survivin-positive CTCs should use a larger sample. Additionally, because of the limited follow-up time, the relationship between survivin-positive CTC counts and overall survival could not be explored. Therefore, an analysis with a longer follow-up duration would be highly useful.

Thus, the present findings indicate that the survivin-positive CTC count might have potential as an indicator for disease stage and a predictor of recurrence in HCC patients. Thus, an efficient and reliable technique to measure survivin expression in CTCs from liver cancer patients could prove useful for predicting the prognosis and recurrence of this cancer, and this could help clinicians select an optimal and customized management strategy for the treatment of this malignancy.

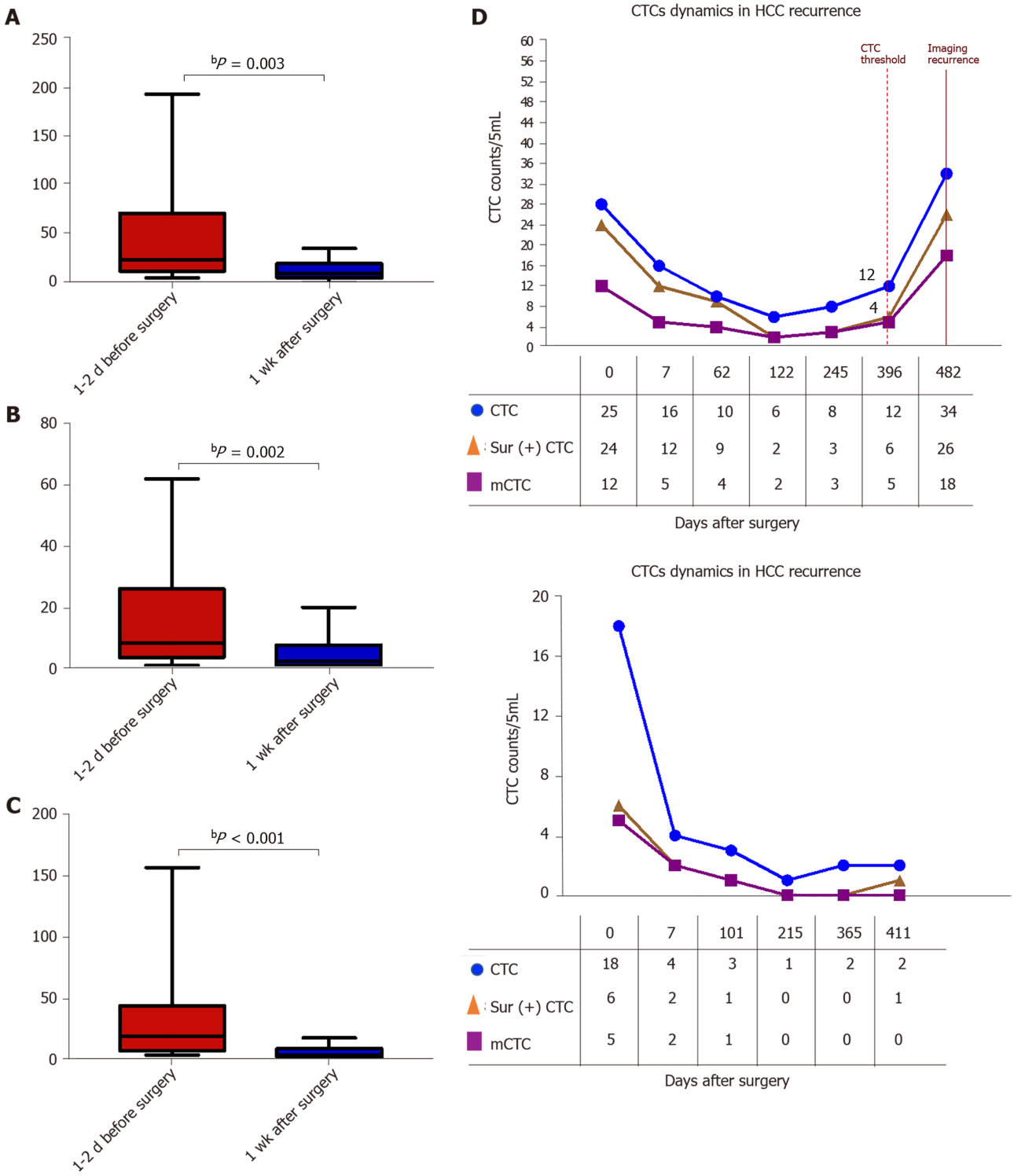


Figure 8 Dynamic changes in circulating tumor cell, mesenchymal circulating tumor cell, and survivin-positive circulating tumor cell counts following surgery. A-C: The total circulating tumor cell (CTC) count, mesenchymal CTC proportion, and survivin-positive CTC count decreased dramatically 1 wk after resection. D: Increased survivin-positive CTC count in postoperative hepatocellular cancer patients (n = 10) at 1-12 mo before detectable recurrence or appearance of metastatic lesions, and decreased surviving-positive CTC count in the last 12 hepatocellular cancer patients with no recurrence. $^{b}P <$ 0.01. CTC: Circulating tumor cell.

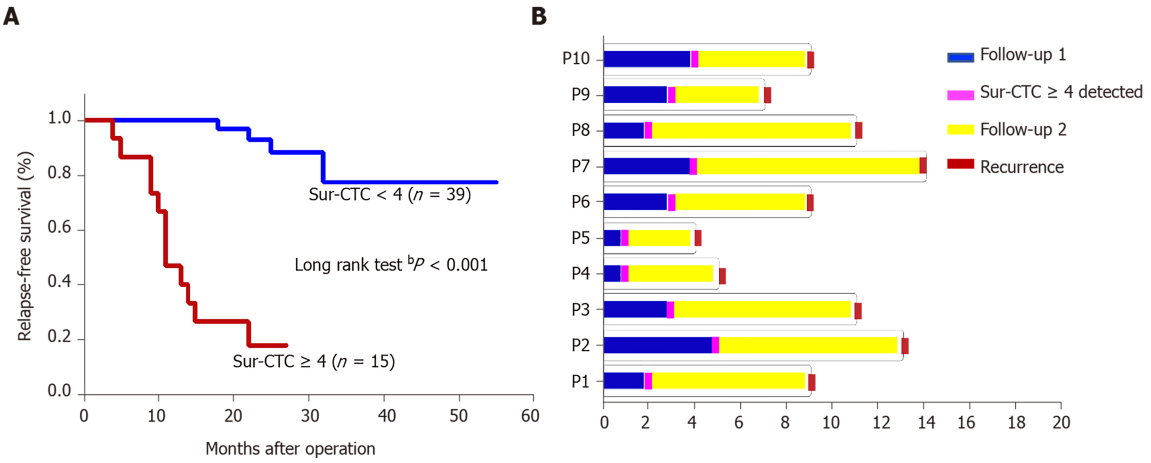


Figure 9 Prognosis of hepatocellular cancer patients with different survivin-positive circulating tumor cell counts. A: Recurrence free survival time after surgery by change in survivin-positive circulating tumor cell (CTC) count; B: The survivin-positive CTCs detected during follow-up of 10 hepatocellular cancer patients after operation predict tumor recurrence. $^{b}P < 0.01$. CTC: Circulating tumor cell.

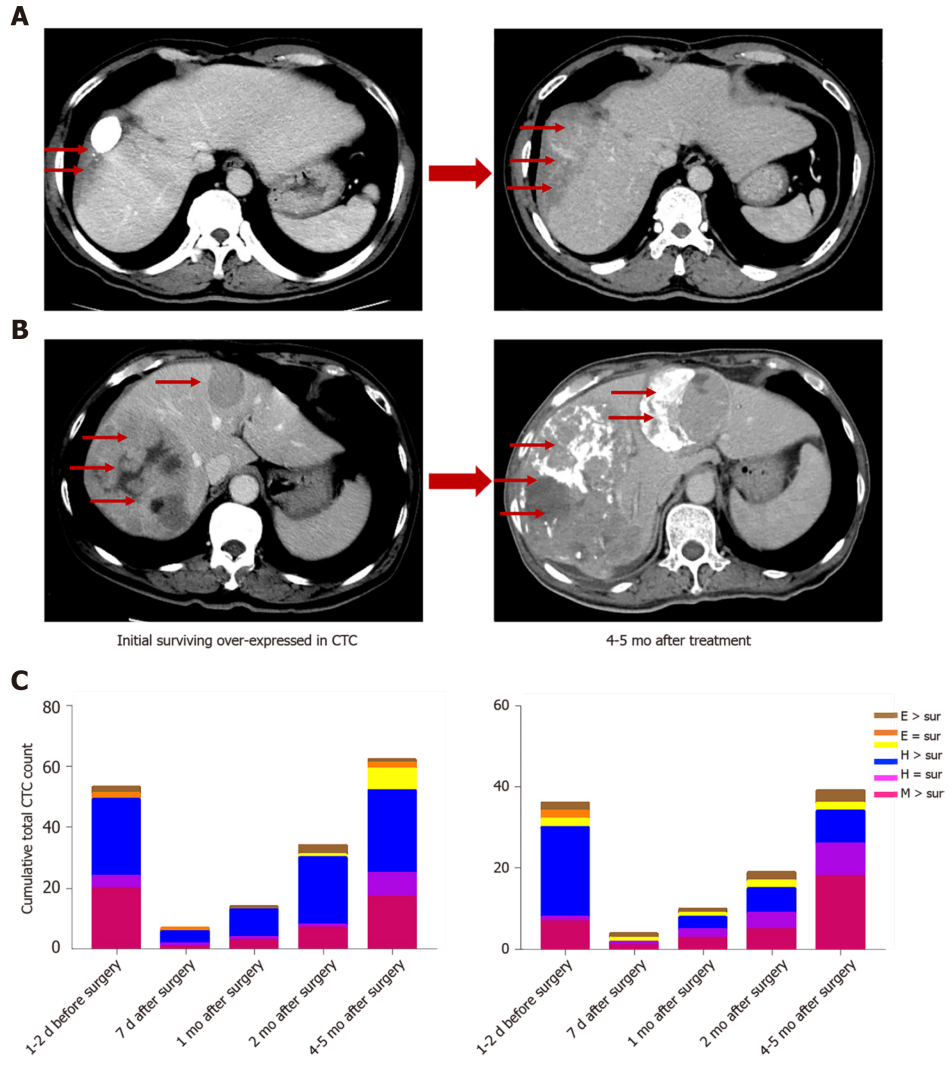


Figure 10 Survivin-positive circulating tumor cells detected during follow-up predict recurrence (m). A and B: Images of two hepatocellular cancer (HCC) patients at the same BCLC stage who expressed survivin in circulating tumor cells (CTCs). At 4 and 5 mo after treatment, the HCC tumors showed more rapid progression in patients with higher survivin expression in CTCs at baseline; C: Cumulative total CTC count by three cell types and survivin-positive CTC count in the two patients before and after operation. The patients' CTC and survivin-positive CTC count increased at the 2-mo follow-up. Letter E represents epithelial CTCs; Letter H represents hybrid CTCs; Letter M represents mesenchymal CTCs. CTC: Circulating tumor cell.

ARTICLE HIGHLIGHTS

Research background

Circulating tumor cells (CTCs), which are also known as liquid biopsy, are mainly used as a predictor to monitor the recurrence and metastasis of tumors including hepatocellular carcinoma (HCC). Survivin is an anti-apoptotic protein that plays an important role in inhibiting apoptosis and is overexpressed in many tumors, including HCC. No study has analyzed the value of survivin-expressing CTCs in HCC.

Research motivation

We aimed to study the expression of survivin in CTCs of HCC and indicate the role of survivin-expressing CTCs as a marker for EMT and cancer progression in HCC.

Research objectives

To explore the prognostic value of survivin-expressing CTCs in HCC, and estimate survivin-positive CTCs as a potential predictor of metastasis in HCC patients.

Research methods

We examined the survivin expression patterns in CTCs of HCC patients, and investigated the *in vitro* effects of survivin silencing and overexpression on the proliferation and invasion of HCC cells. We also analysed the survivin protein expression in HCC tissue. And we observed the dynamic changes in survivin-positive CTC count following surgery and its prognostic significance.

Research results

The CTC and survivin-positive CTC counts were significantly higher in the HCC patients than in the normal controls. Further, survivin overexpression was found to induce HepG2 cell proliferation, reduce apoptosis, and improve invasive ability. Additionally, log-rank test revealed that the recurrence free survival rate was significantly associated with survivin-positive CTC count.

Research conclusions

Survivin-positive CTCs are promising as a predictor of HCC prognosis and metastasis.

Research perspectives

The dynamic monitoring of survivin-positive CTC count would help clinical therapy and improve the prognosis of HCC patients.

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ORIGINAL ARTICLE

Prospective Study

Immunoglobulin G in non-alcoholic steatohepatitis predicts clinical outcome: A prospective multi-centre cohort study

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Author contributions: De Roza MA contributed to study design, data collection and analysis, manuscript writing, critical Review and submission of manuscript; Lamda M contributed to study design, data collection and analysis, manuscript writing and review of manuscript; Goh GBB contributed to study design and critical review of manuscript; Cheah MCC and Lum JHM contributed to data collection; Ngu JHJ contributed to study design, writing and critical review of manuscript; and all authors read and approved the final manuscript.

Institutional review board statement: This study conforms to ethical guidelines and was approved by our institutional review board with waiver of patient consent.

Clinical trial registration statement: This study does not include any intervention and is not a

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Abstract

BACKGROUND

Autoimmune markers including plasma cells (PC), anti-smooth-muscle antibody (ASMA), anti-nuclear antibody (ANA), and raised immunoglobulin G (IgG) are commonly observed in non-alcoholic steatohepatitis (NASH), however their clinical significance is unknown.

AIM

To determine if autoimmune markers in NASH patients are independently associated with poorer clinical outcomes.

METHODS

Consecutive patients with biopsy proven NASH from Christchurch Hospital, New Zealand and Singapore General Hospital (SGH) were included between 2005 to 2016 in a prospective multi-centre cohort study. Patients with other causes of chronic liver disease were excluded. IgG > 14 g/L or globulin fraction > 50%, ANA ≥ 1:40, SMA ≥ 1:40 were considered positive. Multivariate analysis was performed to assess which markers were independently associated with mortality and hepatic decompensation.

RESULTS

randomized controlled trial.

Informed consent statement: Consent was not obtained as data was anonymized and protected with little to no risk of identification.

Conflict-of-interest statement: All authors declare there are no conflicts of interest. None of the authors received financial support or grants for this study.

Data sharing statement: Statistical code, and dataset is available from the corresponding author at jeffrey.ngu@cdhb.health.nz.

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Grade A (Excellent): A
Grade B (Very good): 0
Grade C (Good): 0
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Total 261 patients were included of which 201 were from SGH. The median age was 53 and 51.9% were male. Advanced fibrosis was present in 31.4% at diagnosis. PC, ASMA, ANA and raised IgG were observed in 13.1%, 4.9%, 27.8% and 30.1% of patients respectively. After multivariate analysis, elevated IgG [Hazard Ratio (HR) 6.79, 95%CI: 2.93-17.15] and fibrosis stage (HR 1.37, 95%CI: 1.03-1.87) were found to be independently associated with increased risk of liver decompensation. Age (HR 1.06, 95%CI: 1.02-1.10) and elevated IgG (HR 3.79, 95%CI: 1.90-7.68) were independent factors associated with higher mortality risk.

CONCLUSION

Elevated IgG, rather than ANA, ASMA or plasma cells, is independently associated with increased risk of hepatic decompensation and mortality in NASH. It could hence be important for prognostication.

Key Words: Non-alcoholic fatty liver disease; Non-alcoholic steatohepatitis; Immunoglobulin G; Autoantibodies; Mortality; Hepatic decompensation

Core Tip: Autoantibodies such as anti-nuclear antibody (ANA) and anti-smooth-muscle antibody (ASMA) can be present in up to 20%-30% of patients with non-alcoholic steatohepatitis (NASH). However, clinical significance is not well studied and there is no published data on the impact of immunoglobulin G (IgG) and plasma cells on hepatic decompensation and mortality outcomes. Our study found that elevated IgG but not ANA, ASMA or plasma cells is associated with higher risk of mortality, including liver related death, as well as increased risk of hepatic decompensation events. Patients with IgG positive NASH should hence be identified early and monitored closely as they are at higher risk of poorer clinical outcomes.

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INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is a growing phenomenon with an estimated global prevalence of 25%. Non-alcoholic steatohepatitis (NASH) in particular is a progressive form of NAFLD and is associated with poorer clinical outcomes and higher liver related mortality[1]. Independent predictors for poor outcomes include fibrosis[2], obesity and metabolic syndrome, diabetes mellitus (DM)[3], as well as genetic polymorphisms such as PNPLA3[4]. NASH is characterized histologically by hepatic steatosis, inflammation, hepatocellular injury and varying degrees of fibrosis[5]. The inflammatory process in NASH is a complex and heterogeneous "multi-hit" pathway in which the innate immune system plays a critical role, driving the progression of liver fibrosis and leading to cirrhosis, liver failure, the need for liver transplantation and death[6-8]. Less is known, however, about the role of the adaptive immune system and autoantibodies. Autoantibodies are produced by humoral immune responses against self-cellular proteins and nucleic acids and can be physiological or pathological[9]. When used in tandem with clinical findings, they are serological hallmarks for inflammatory autoimmune liver diseases. However, their significance in NAFLD is not well studied despite autoantibodies being present in 25%-35% of patients with NAFLD[10,11].

Adams *et al*[10] reported a higher grade of inflammation in NAFLD patients with positive antinuclear antibodies (ANA) and/or anti smooth muscle antibodies (ASMA) but the difference was slight (1.0 *vs* 1.2, P = 0.02) and there was no correlation to clinical significance or outcomes. More recent data from McPherson *et al*[12] looking specifically at serum immunoglobulins in NAFLD, showed that elevated serum

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immunoglobulin A was significantly associated with advanced fibrosis. Similarly, there was no correlation to immunoglobulins being a predictor for mortality or hepatic events independently from fibrosis and other factors. Despite evidence showing association of autoantibodies and immunoglobulins with higher histological grades of inflammation or fibrosis[10,12,13], other studies dispute these findings[14,15], and correlation to clinical outcomes is still not established in NASH. Ravi *et al*[16] reported no significant difference on liver disease outcomes in steatohepatitis patients with positive ANA or ASMA , however, the major limitation of the study was that overall follow-up was short (median 3 years) and alcoholic and non-alcoholic hepatitis were grouped together.

Overall, autoimmune markers such as ANA, ASMA, plasma cells and immunoglobulins with immunoglobulin G (IgG) in particular, are not well studied in NASH and their clinical significance is unknown in this population. Hence, the aim of this study is to determine if autoimmune markers in patients with biopsy proven NASH are independently associated with poorer outcomes. The outcomes measured being all cause mortality and liver decompensation events.

MATERIALS AND METHODS

Patient selection

Consecutive patients who underwent liver biopsy at Christchurch Hospital, New Zealand (CH) and Singapore General Hospital, Singapore (SGH) were assessed for inclusion in the study. CH cohort consisted of patients with liver biopsy performed from 2008 to 2016 and the SGH cohort from 2005 to 2015. Patients with chronic liver diseases of other aetiologies such as viral hepatitis, alcohol, toxins or drugs, autoimmune including IgG4 related disease, vascular, metabolic and hereditary causes were excluded. All patients, as per local hospital protocol underwent non-invasive liver testing including serology analysis and imaging to exclude other causes of chronic liver disease. Patients were included in the study if the final histological diagnosis was NASH with NAFLD activity score (NAS) ≥ 3 on biopsy with scores for steatosis, lobular inflammation and hepatocyte ballooning[17].

ANA and ASMA were considered positive if titres were observed to be ≥ 1:40. IgG was considered elevated if > 14g/L. Additionally, if quantitative values were not available, globulin fraction (GF) was calculated by the following equation: total protein/(total protein - albumin). Since IgG is the commonest globulin type[18], individuals with GF > 50% were defined as elevated IgG. To assess presence of plasma cells, histology reports were reviewed. Plasma cells were scored as positive if any plasma cells were identified on histology specimens by the pathologist. This study conforms to ethical guidelines and was approved by our respective institutional review boards.

Follow-up

Patients from CH and SGH were followed up for clinical events of liver decompensation and all-cause mortality. Liver decompensation event was defined as the development of any of the following: ascites, gastrointestinal haemorrhage secondary to portal hypertension, hepatic encephalopathy, hepatorenal syndrome. Patients were followed up till 31st December 2017. Follow-up was censored at development of first liver decompensation event, death, liver transplantation, or last clinical contact in case of patients lost to follow-up.

Statistical analysis

Continuous variables were expressed as mean ± SD or median (interquartile range, IQR) and were compared using unpaired *t*-test. Categorical variables were compared using χ^2 test. The associations of putative risk factors and outcomes were analyzed using Cox proportional hazards regression and are summarized as hazard ratios (HR) with 95%CI. The time to event outcomes were also summarized using Kaplan-Meier curves. A two-tailed *P* value of < 0.05 was taken to indicate a statistical significance. All analyses were undertaken using statistical software SPSS version 20.

RESULTS

A total of 261 patients met the study criteria. Of these, 201 patients were recruited from

SGH and 60 patients from CH. Baseline characteristics of patients are listed in Table 1. Majority of patients from CH were of European origin (91.7%) while 97.5% patients from SGH were of Asian origin, reflecting the local population demographic. Median follow-up per patient was 5.1 years (IQR 3.5-7.5). Median age at inclusion in the study was 53 years (± 12.9) and 51.9% were male. The median NAS score at diagnosis was 4 (IQR 3-5) and the mean Metavir fibrosis score was 1.7 (± 1.4). 77% of patients had data available for body mass index (BMI), and comorbidities including presence of DM, of which the mean BMI was 30.61 and DM was present in 45.02% of patients. There were no significant differences in baseline characteristics between patients from SGH and CH (Table 1).

Prevalence of autoimmune markers

ANA was the most common positive autoimmune marker present in 27.8% patients followed by elevated IgG observed in 23.4%. Plasma cells were found on histological examination in 13% of patients with NASH. ASMA was the least common autoimmune marker and was positive in only 4.9% of NASH patients.

Clinical end-points: Liver decompensation and all-cause mortality

During a cumulative follow-up of 1464 person years, 25 patients developed liver decompensation. There was no significant difference between risk of liver decompensation or all-cause mortality between patients from CH and SGH. The 5-year risk of developing liver decompensation after diagnosis of NASH was 8.1% (95%CI: 6.1-11.8) and the 5-year mortality risk was 11.7% (95%CI: 4.4-22.9) (Figure 1). Ten patients developed hepatocellular carcinoma of which 7 were male. Median age at diagnosis of HCC was 65.7 years. Advanced fibrosis or cirrhosis was present in 6 of 10 patients at diagnosis of HCC. During the follow-up period, 36 patients died. Data on cause of death were available for 30 (83.3%) patients. Liver related causes of death were observed in 12 cases (40%), followed by malignancy (30%), septicaemia (17%) and cardiovascular causes of death (13%). Overall, 5-year risk of all-cause mortality was 11.7% (95%CI: 4.4-22.9) (Figure 1).

Predictors of Liver decompensation and all-cause mortality

Liver decompensation: In univariate analysis (Table 2), factors associated with increased risk of liver decompensation were increasing age (HR 1.04, 95%CI: 1.00-1.08), stage of fibrosis (HR 1.67, 95%CI: 1.25-2.26) and elevated IgG (HR 8.20, 95%CI: 3.61-20.30). Other autoimmune markers (ANA, ASMA or Plasma cells) were not found to be associated with risk of liver decompensation (Table 2; Figure 1). Multivariate model was constructed with variables found to be significant in univariate analysis. In multivariate analysis, elevated IgG (HR 6.79, 95%CI: 2.93-17.15) and stage of fibrosis (HR 1.37, 95%CI: 1.03-1.87) were found to be independently associated with increased risk of liver decompensation during follow-up (Table 2).

In a sub-group analysis where only patients with quantitative IgG values (> 14 g/L) were included (n = 43), a trend of association was observed between elevated IgG and increased risk of liver-decompensation during follow-up. (HR 3.1, 95%CI: 0.92-10.8, P = 0.054) (Figure 2).

Mortality: In univariate analysis, predictors of all-cause mortality included: increasing age (HR 1.06, 95%CI: 1.03-1.10), stage of fibrosis (HR 1.27, 95%CI: 1.00-1.61) and elevated IgG (HR 4.5, 95%CI: 2.29-9.00) (Table 2). In multivariate analysis, age (HR 1.06, 95%CI: 1.02-1.10) and elevated IgG (HR 3.79, 95%CI: 1.90-7.68) were found to be independent factors associated with increased risk of mortality (Table 2; Figure 2). Median survival in patients with elevated IgG at baseline was 9.4 years.

DISCUSSION

In this multicentre cohort study, we examined the association between the presence of autoimmune markers such as ANA, ASMA, elevated IgG and plasma cells on histology with clinical outcomes in patients with NASH. The most pertinent finding of our study is that elevated IgG at diagnosis of NASH was associated with increased risk of liver decompensation and reduced overall survival.

Autoimmune markers are commonly encountered in patients with NASH, however their clinical significance is not well defined. In a study of 225 patients with histologically confirmed NAFLD, 20% and 3% respectively were found to have the presence of ANA and ASMA[10]. Similarly, in another cohort study of NASH patients, the

Table 1 Baseline characteristics, *n* (%)

	Overall	CH	SGH	*P* value
Patients	261	60	201	-
Regions				0.07
European	60 (23.0)	55 (91.7)	5 (2.5)	
Asian	197 (75.5)	2 (3.3)	195 (97.5)	
Others	4 (1.5)	3 (5.5)	1 (0.5)	
Age, mean ± SD	53 ± 12.9	52.9 ± 16.9	53 ± 11.7	0.30
Male	51.9%	53.3%	51.7%	0.83
ALT, mean ± SD	112.3 ± 373.9	80.2 ± 66.6	121.9 ± 425.5	0.45
AST, mean ± SD	80.3 ± 216.7	60.8 ± 42.3	86.1 ± 246.1	0.43
NAS, Median (IQR)	4 (3-5)	4 (3-5)	4 (3-5)	0.54
Metavir Fibrosis score, mean ± SD	1.7 ± 1.4	1.6 ± 1.4	1.7 ± 1.3	0.58
F0	53 (20.3)	13 (21.7)	40 (19.9)	
F1	95 (36.3)	25 (41.7)	70 (34.8)	
F2	31 (11.9)	5 (8.3)	26 (12.9)	
F3	48 (18.4)	7 (11.7)	41 (20.4)	
F4	34 (13.1)	10 (16.6)	24 (11.9)	

CH: Christchurch Hospital, New Zealand; SGH: Singapore General Hospital, Singapore; IQR: interquartile range; NAS: Non-alcoholic fatty liver disease activity score.

Table 2 Univariate and multivariate analysis of predictors of clinical outcomes

Predictors	Liver decompensation				All-cause mortality			
	Univariate analysis		Multivariate analysis		Univariate analysis		Multivariate analysis	
	HR (95%CI)	*P* value	HR (95%CI)	*P* value	HR (95%CI)	*P* value	HR (95%CI)	*P* value
Age[1] (per 1 yr)	1.04 (1.00-1.08)	0.04	1.01 (0.98-1.05)	0.46	1.06 (1.03-1.10)	< 0.0001	1.06 (1.02-1.10)	0.001
Sex	0.99 (0.44-2.19)	0.98	-	-	1.16 (0.60-2.27)	0.65	-	-
Fibrosis[2]	1.67 (1.25-2.26)	< 0.001	1.37 (1.03-1.87)	0.03	1.27 (1.00-1.61)	0.05	1.01 (0.80-1.29)	0.91
NAS[3]	0.96 (0.67-1.32)	0.79	-	-	0.88 (0.66-1.16)	0.37	-	-
IgG	8.20 (3.61-20.30)	< 0.0001	6.79 (2.93-17.15)	< 0.0001	4.50 (2.29-9.00)	< 0.0001	3.79 (1.90-7.68)	0.0001
ANA	1.15 (0.41-2.86)	0.78	-	-	1.14 (0.47-2.51)	0.76	-	-
ASMA	0		-	-	0.94 (0.05-4.53)	0.95	-	-
Plasma cells	0.97 (0.23-2.81)	0.96	-	-	1.20 (0.41-2.83)	0.71	-	-

[1]Age as continuous variable for every one year increase in age.
[2]Increase in Metavir fibrosis stage.
[3]Increase in non-alcoholic fatty liver disease activity score by 1.
IgG: Immunoglobulin G; ANA: Anti-nuclear antibody; ASMA: Anti smooth muscle antibody; HR: Hazard ratio.

presence of ANA and ASMA was observed in 34% and 6% of all patients respectively [15]. The findings of our study are consistent with the reported prevalence estimates. While inflammation involving plasma cells is typically observed in AIH, the prevalence of plasma cell infiltration in NASH is not known. In the present study, plasma cell infiltration was observed in 13% of patients with histological diagnosis of NASH.

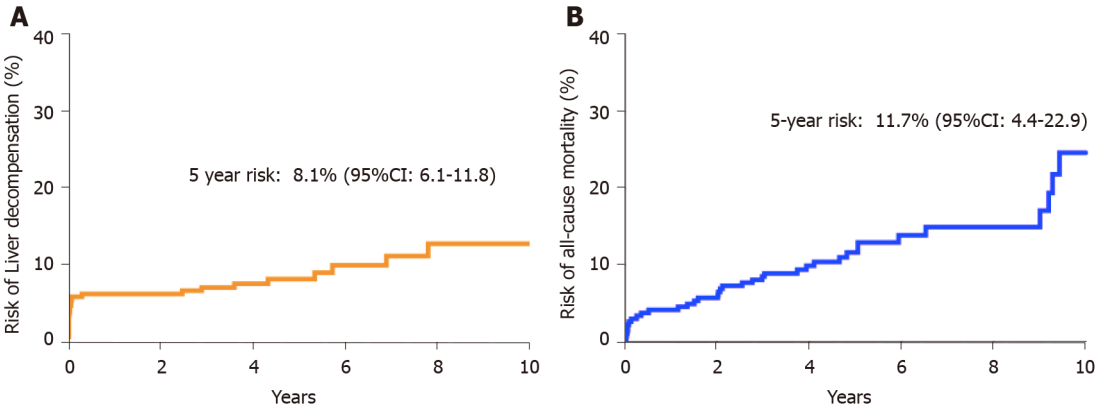

Figure 1 Overall risk of liver decompensation and all cause mortality. A: Overall risk of liver decompensation; B: Overall risk of all cause mortality.

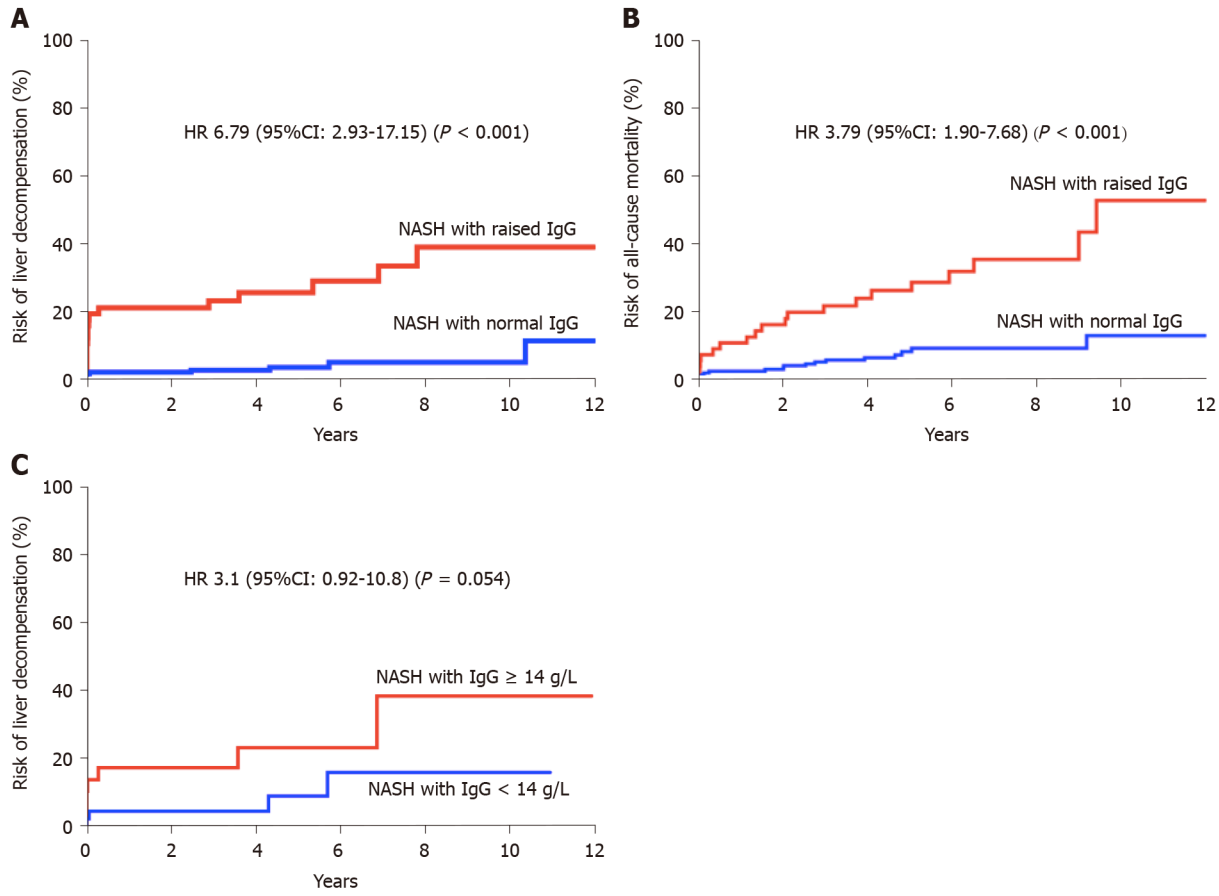

Figure 2 Raised immunoglobulin G and risk of liver decompensation and mortality on multivariate analysis. A: Raised immunoglobulin G (IgG) and risk of liver decompensation; B: Raised IgG and risk of all-cause mortality; C: Raised absolute IgG > 14 and risk of liver decompensation. IgG: Immunoglobulin G.

Association of ANA and/or ASMA with histological severity of NASH has been examined previously in multiple cohort studies and yielded conflicting results[10,12-15,19]. None of the studies, to our knowledge, have assessed association of autoimmune markers with long-term clinical outcomes. We found that the risk of liver decompensation or all-cause mortality were not associated with the presence of either ANA, ASMA or plasma cells, thereby suggesting that these are non-specific markers of inflammation and unlikely to be pathogenically relevant.

On the contrary, elevated IgG at diagnosis of NASH was independently associated with increased risk of liver decompensation (HR 6.79, 95%CI: 2.93-17.15, $P < 0.0001$) and all-cause mortality (HR 3.79, 95%CI: 1.9-7.68, $P = 0.0001$) during follow-up. Majority of the excess mortality in the elevated IgG cohort were liver-related. It needs to be highlighted that diagnosis of probable or definite AIH was conclusively ruled out

in all patients based on the international standardised criteria and no patients were treated with immunosuppression except in cases of organ transplantation.

We do not have a concrete biological explanation for the observed pathogenic role of IgG in NASH, however several mechanisms are plausible. Firstly, oxidative stress in NASH may induce production of IgG by deployment of adaptive humoral response[5,20]. Animal and human based studies have shown that IgG directed against products of lipid peroxidation such as Malondialdehyde and 4-hydroxynonenal appears to be elevated in NASH and correlates with disease severity[20]. Secondly, anti-endotoxins IgG levels were observed to be higher in patients with NASH compared to controls (48 vs 10 GMU/mL), and IgG levels corelated with severity of NASH[20,21]. Endotoxins are generally derived from the gut microbiota[21] and are potential triggers for inflammation and insulin resistance, driving oxidative stress in NASH. Therefore, elevated IgG may be representative of high endotoxemic burden leading to rapid progression of NASH. Lastly, it is possible that elevated IgG in patients with NASH may represent an overlap with a mild degree of autoimmune hepatitis. However, currently no diagnostic criteria exist to define NASH-AIH overlap syndrome.

Our study has several limitations which ought to be acknowledged. Quantitative immunoglobulin values were not available for all patients and globulin fraction was used as a surrogate marker of elevated IgG. While globulin fraction has previously been utilised as an effective surrogate marker of hyper/hypogammaglobulinemia[22] it is possible that we may have under or overestimated the IgG effect on clinical outcomes. However, upon restricting analysis to those patients with quantitative IgG values, a similar effect was observed (although statistically non-significant), suggesting that the observed association between IgG and poor clinical outcomes is true rather than a type-1 error. Data for pre-existing medical comorbidities and current medications were only available for 77% of patients, which may have confounded overall results. However, all patients were on follow up with a specialist and would have received standard of care for hepatic decompensation, regardless of compliance to treatment. Lastly, the diagnosis of NASH was based on unblinded histology interpretation by the local pathology team, consequently an element of inter-observer bias cannot be ruled out. Our study design involved two different population cohorts from two large tertiary centres. Despite having different ethnic compositions, there were no significant differences in baseline-characteristics at inclusion in the study between the two centres suggesting that our results are generalizable. Importantly, both centres have national electronic records available making complete data ascertainment of clinical events possible.

CONCLUSION

In conclusion, we report that ANA, ASMA and plasma cells are commonly present in patients with NASH but carry no prognostic significance. On the contrary, elevated IgG is an independent predictor of increased risk of liver decompensation and reduced overall survival in patients with NASH. Presence of elevated IgG therefore represents a more aggressive NASH phenotype. Identification and close monitoring of these patients is prudent to improve overall clinical outcomes.

ARTICLE HIGHLIGHTS

Research background

Autoimmune markers such as immunoglobulin G (IgG), anti-nuclear antibody (ANA), anti-smooth-muscle antibody (ASMA) can be present in patients with Non-alcoholic steatohepatitis (NASH) but their clinical significance is not well studied.

Research motivation

Knowing the clinical significance of autoimmune markers in patients with biopsy proven NASH can pave the way for future research to better understand why certain sub-groups of patients with NASH deteriorate more rapidly.

Research objectives

This study aimed to determine if any of the autoimmune markers were independently associated with worse outcomes such as mortality and hepatic decompensation. This is important as such patients can be identified for closer monitoring.

Research methods

This is a prospective, multi-center study. Patients with biopsy proven NASH were included and multivariate analysis was performed to determine if any of the autoimmune markers (IgG, ANA, ASMA) were independent risk factors for mortality and hepatic decompensation

Research results

Elevated IgG was an independent risk factor for both mortality and liver decompensation after multivariate analysis with adjustment for age and fibrosis stage. The exact pathophysiology is unknown but IgG levels could possibly correlate to disease severity due to anti-endotoxins IgG and oxidative stress.

Research conclusions

Elevated IgG is an independent predictor of increased risk of liver decompensation and reduced survival in patients with NASH. It could represent a more aggressive NASH phenotype.

Research perspectives

Further research is needed to validate and reproduce this finding and to also establish the pathophysiology and underlying biochemical mechanisms for this observation.

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SYSTEMATIC REVIEWS

Minimum sample size estimates for trials in inflammatory bowel disease: A systematic review of a support resource

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Grade B (Very good): B, B

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Abstract

BACKGROUND

Of 25% of randomised controlled trials (RCTs) on interventions for inflammatory bowel disease (IBD) have no power calculation.

AIM

To systematically review RCTs reporting interventions for the management of IBD and to produce data for minimum sample sizes that would achieve appropriate power using the actual clinical data.

METHODS

We included RCTs retrieved from Cochrane IBD specialised Trial register and CENTRAL investigating any form of therapy for either induction or maintenance of remission against control, placebo, or no intervention of IBD in patients of any age. The relevant data was extracted, and the studies were grouped according to the intervention used. We recalculated sample size and the achieved difference, as well as minimum sample sizes needed in the future.

RESULTS

A total of 105 trials were included. There was a large discrepancy between the estimated figure for the minimal clinically important difference used for the calculations (15% group differences observed *vs* 30% used for calculation) explaining substantial actual sample size deficits. The minimum sample sizes indicated for future trials based on the 25 years of trial data were calculated and grouped by the intervention.

CONCLUSION

A third of intervention studies in IBD within the last 25 years are underpowered, with large variations in the calculation of sample sizes. The authors present a sample size estimate resource constructed on the published evidence base for

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future researchers and key stakeholders within the IBD trial field.

Key Words: Inflammatory bowel disease; Crohn's disease; Ulcerative colitis; Gastroenterology; Statistics; Sample size

Core Tip: This work has identified a large variation in the estimated minimal clinically important difference (MCID) between study groups in inflammatory bowel disease trials in the literature, with no standard to support study designers or reviewers. We have provided a resource to support sample size estimation based on observed MICD in the literature over the last 25 years.

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INTRODUCTION

Sample size estimation (SSE) is an extremely important calculation for designing a clinical trial. Failure to produce an appropriate calculation may lead to imprecise results[1]. If a sample size is too large, statistically significant outcomes may be theoretically detected that may not be clinically relevant (type 1 error). This, however, is rarely a concern as studies are rarely overpowered to balance the study power with the cost. On the other hand, if a sample size is too small then a clinically significant outcome may not be detected statistically (type 2 error)[2,3]. The reporting of SSE in randomised controlled trials (RCTs) is a standard requirement according to the consolidated standards of reporting trials (CONSORT) statement which was introduced as a guide to conducting RCTs in 1996[4].

In a previous systematic review[5], we showed that 25% of RCTs on interventions for inflammatory bowel disease (IBD) have no power calculation (PC). A third of those who report PC do not achieve their target sample size. Based on those results, we decided to conduct a further systematic review.

We set out to systematically review RCTs on interventions for the IBD management, extract the vital parameters needed for sample size calculations, and synthesise the data to demonstrate whether trials across the field are adequately powered. We also set out to use the actual clinical data across these comparisons to synthesise data for minimum sample sizes that would achieve appropriate power to support future researchers designing trials and performing SSEs.

MATERIALS AND METHODS

This review was performed in alignment with Cochrane guidelines[6] in April 2020 and reported in line with the Preferred Reporting Items for Systematic Reviews and Meta-analyses statement[7].

Eligibility criteria

We followed the sampling methodology described within our systematic review protocol (uploaded within our institutional repository)[8] used for our previous review of the reporting of sample size calculations[5].

In brief, we included RCTs investigating either induction or maintenance therapy with biologics, immunomodulators, and microbiome against control, placebo, or no intervention. We conducted a comprehensive search of the Cochrane IBD Specialized Trials Register, CENTRAL, Cochrane library of IBD reviews for primary RCTs. The search terms are presented in Supplementary material.

We included RCTs published since 1996 (after the publication of the CONSORT statement). We excluded reports lacking clear information on the number of participants; cluster RCTs; pilot or feasibility studies; studies with mixed population of people with and without IBD; studies on secondary analyses of follow-up data collection after discontinuation of treatment. We excluded abstracts as these rarely allow space for such information to be presented. As we wanted to assess the established evidence for a PC of treatment for the IBD, we excluded RCTs describing all interventions where work may be at phase 3 (pharmacological: *e.g.* ustekinumab, golimumab, tofacitinib) or not under the three core headings (biologic, immunomodulators or anti-inflammatories).

Complying to the above search strategy, two authors (SL and MG) identified RCTs titles that appeared to be applicable. These were independently screened and in cases of disagreement, a third review author (VS) was involved to reach consensus. Two review authors independently extracted and recorded data on a predefined checklist. When disagreements occurred, a third review author was involved, and the consensus was reached.

We created an excel document to extract data regarding the trials. Firstly, we separated the studies into 8 categories [Crohn's disease (CD)–clinical relapse, clinical remission, endoscopic relapse, endoscopic remission; ulcerative colitis (UC)–clinical relapse, clinical remission, endoscopic relapse, endoscopic remission]. Secondly, we grouped the studies according to the intervention used. One author extracted the data, and in case of any problems, the data was checked by the second author.

The extracted data although is not available publicly can be obtained *via* direct contact with authors. The references of the included stuidies can be found in Supplementary material.

Extracted data included

(1) Number of events and participants originally assigned to each group; (2) Characteristics of participants; (3) The proportion that we calculated according to the number of events and participants (x = n/N), in which n is a number of events and N is a number of participants); (4) The difference achieved that we calculated according to the proportions of two groups (proportion 1–proportion 2); (5) Intervention and control details; (6) Presence of SSE and calculation details [minimal clinically important difference (MCID) used for PC, power, significance level, target sample size]; and (7) Outcomes (the number of patients recruited and completing study; the number of treatment success/failures; and the difference achieved).

We used the studies in which intervention was compared to the control or placebo. We grouped those studies according to the interventions, type of treatment (induction, maintenance), and outcomes (relapse, remission) and calculated mean difference and mean MCID where it was possible.

After resolving all the inconsistencies with data extraction regarding the use of sample size calculations for the studies with achieved difference of less than 10%, we produced two tables (Tables 1 and 2). We recalculated sample size for those groups using the power of 80%, probability of type 1 error 0.05, and the achieved difference. We used those parameters as they were the most commonly used amongst the studies. The parameters we used were two independent groups, dichotomous outcomes. In group 1 we have put the rate reported by the study of the intervention drug, and in group 2 we have put the rate of the placebo.

The small lest MCID that was reported by the studies was 10%, thereby, we decided to not reproduce PC for those studies with the achieved difference of less than 10%. We also calculated the mean sample deficit in percentage based on the target sample size and achieved sample size reported by the studies.

After receiving the sample size of participants, we made a decision whether the study is underpowered, and if yes, then by how many people.

Data synthesis

We produced descriptive statistics regarding the sample sizes for the studies grouped according to the interventions (Tables 1 and 2).

Ethical statement

As all data included already existed within the published scholarly output, no ethical approval was sought.

Table 1 Overall summary of power calculations and sample size deficits

	Total studies	Studies with power calculation	Studies with difference of 10% and less[1]	How many studies didn't achieve target sample size	Mean sample size underpowered (range)	Mean sample size needed	How many studies are underpowered[2]
CD induction	39	26	12	6	28 (2-70)	231	11
CD maintenance	25	19	9	3	52 (7-79)	300	10
UC induction	27	19	8	3	22 (1-55)	219	4
UC maintenance	16	10	0	1 + 1 didn't report	21	196	7

[1]Those studies were not included in analysis.
[2]Either didn't achieve their target sample size, or their achieved sample size is less than mean sample size needed.
UC: Ulcerative colitis; CD: Crohn's disease.

RESULTS

A total of 7451 potential citations were screened and 308 full texts assessed for eligibility. There were 209 texts excluded, 106 because they were published prior to the release of the CONSORT statement and 103 because they did not match our inclusion outcome. This left a total of 99 trials included, with 60 pertaining to CD and 39 to UC. The full details are shown in Figure 1.

The mean proportion of patients achieving clinical remission reported within the placebo groups of induction studies was 34.34% in CD trials and 26.79% for UC. For endoscopic remission, 0% in CD and 29.6% for UC. The mean proportion of patients achieving clinical relapse for maintenance studies were 55% for CD and 46.79% for UC. For endoscopic relapse, 78.85% in CD, and 28.7% in UC.

Within CD induction studies, 26 out of 41 (63.4%) reported a PC and 19 of 26 (73.1%) in maintenance studies. Within UC induction studies, 22 out of 31 (71%) reported a PC and 10 of 17 (58.8%) in maintenance studies.

When considering the MCID that those studies reporting a PC employed for this calculation, within CD induction studies the mean difference was 33% (range 20%-50%) and 27% difference for maintenance studies (15%-40%). Within UC induction studies the mean was 26% (range 19%-40%) and 27% for maintenance studies (18%-40%). The MCIDs these studies reported rarely matched the actual differences achieved by these studies. In fact, the discrepancy between this estimated figure for the MCID used for the PC and the actual differences seen were a mean of 22.8% higher in CD induction studies, 13.8% higher in maintenance studies, 15.7% higher in UC induction studies, and 10.2% higher in maintenance studies.

These discrepancies are proportionally large and in the context of PCs are clearly substantial and led to large numbers of studies being underpowered. These are summarised in Table 1. Study specific data with further details is available upon request.

Table 2 gives the results of our sample size calculations at the intervention specific level that employed the actual achieved clinical differences from previous studies, using the power of 80% and the probability of type 1 error 0.05. This shows the minimum sample sizes that would be indicated for RCTs compared with placebo to use. Within comparisons where the mean difference was less than 10%, no calculation has been given as this would be a very high indicative figure.

DISCUSSION

Within this review, it has been demonstrated that there is no clear basis or accepted standard for current practice for MCID estimation when producing a PC for a primary RCT within IBD. This has led to huge variations in suggested figures for recruitment. These trials present practical and logistical challenges to organisers, with potential inconvenience to patients, as well as the cost to those funding such research. Having an accurate figure for calculations is important to ensure this investment of resource is used most efficiently and effectively. It is key to note that we are not commenting at

Table 2 Proposals for minimum clinically important difference and associated power calculations for future studies

Ulcerative colitis-comparison	Ulcerative colitis-difference achieved (Group 1–Placebo)	Ulcerative colitis-Minimum sample size needed based on data	Crohn's disease-comparison	Crohn's disease-difference achieved (Group 1–Placebo)	Crohn's disease-Minimum sample size needed based on data
Induction studies					
Outcome–clinical remission			Outcome–clinical remission		
Vedolizumab *vs* Placebo	14.8%	190	Glutamine-enriched diet *vs* Placebo	-11.1	634
			Azathioprine *vs* Placebo	-3.6%	NA
			6-MP *vs* Placebo	5%	NA
Fecal Transplant *vs* Control	20.3%	150	6-MP *vs* Placebo	5%	NA
Budesonide *vs* Placebo	6.5%	NA	Interventional diet *vs* Control diet	20.9%	160
Type 1 IFNs *vs* Placebo	5.9%	NA	Elemental diet *vs* Non elemental diet	1.6%	NA
Etrolizumab *vs* Placebo	13.4%	140	N6/N9 rich feeds *vs* non N6/N9 rich food	-1.1%	NA
			Low dose naltrexone *vs* Placebo	9%	NA
5-ASA *vs* Placebo	11.8%	422	GM-CSF *vs* Placebo	7.8%	NA
Outcome–endoscopic remission			Brakinumab *vs* Placebo	8.5%	NA
Vedolizumab *vs* Placebo	37.7%	182	Ustekinumab *vs* Placebo	8.6%	NA
			Natalizumab *vs* Placebo	14.8%	310
Fecal Transplant *vs* Control	26.4%	160	Methotrexate *vs* Placebo	-14.8%	350
Budesonide *vs* Placebo	13.9%	NA	Antibiotics *vs* Placebo	10%	780
Methotrexate *vs* Placebo	46.7%	NA	Outcome–endoscopic remission		
Etrolizumab *vs* Placebo	7.7%	NA	Low dose naltrexone *vs* Placebo	22.2%	60
5-ASA *vs* Placebo	53.7%	306			
Maintenance studies					
Outcome–clinical relapse			Outcome–clinical relapse		
5-ASA *vs* Placebo	-16.4%	290	5-ASA *vs* Placebo, medically induced	3.1%	NA
Vedolizumab *vs* Placebo	-27.4	84	5-ASA *vs* Placebo, surgically induced	-5.4%	NA
Interventional diet *vs* Control diet	-3.6%	NA	Anti-TB *vs* Placebo	-23%	130
Probiotics *vs* Control	-16.7	154	Azathioprine *vs* Placebo, medically induced	-9.9%	NA
Azathioprine *vs* Placebo	-22.4	154	Azathioprine *vs* Placebo, surgically induced	-17.3%	254
Methotrexate *vs* Placebo	19.9%	194	6-MP *vs* Placebo, surgically induced	-10.9%	646
Rectal 5-ASA *vs* Placebo	-29%	90	Omega -3 fatty acids diet *vs* Control diet	-8.5%	NA
Curcumin *vs* Placebo	-9.6%	NA	Elemental diet *vs* No supplemets	-29.4%	88

Outcome–endoscopic relapse			Interventional diet *vs* Control diet	-2.5%	NA
Vedolizumab *vs* Placebo	-34	60	Antibiotics *vs* Placebo	-14.6%	360
			Methotrexate *vs* Placebo	-24.2%	128
5-ASA *vs* Placebo	-16.4%	290	Methotrexate *vs* Placebo	-24.2%	128
			Outcome–endoscopic relapse		
			5-ASA *vs* Placebo	2.7%	NA
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			6-MP *vs* Placebo	-3.8%	NA
			Antibiotics *vs* Placebo	6.6%	NA

NA is put when the difference achieved is less than 10% (which is the least Minimal Clinically Important Difference used by the studies).

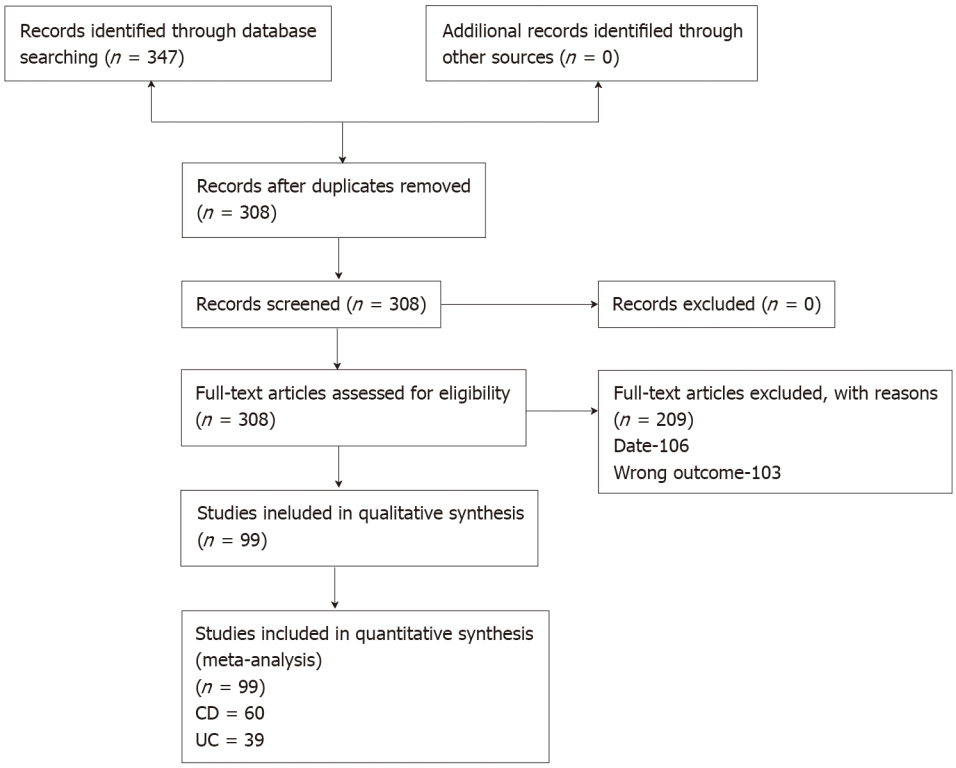

Figure 1 Study flow diagram. UC: Ulcerative colitis; CD: Crohn's disease.

the individual study level. It is inappropriate to look at the projected MCID and PC for a project, if calculated on a reasonable basis, to then retrospectively suggest that the findings of a lesser MCID mean it is underpowered. This not just statistically inappropriate, but methodologically flawed. However, these findings propose that the basis for such MCID estimations is at worst unclear and often can be seen as flawed.

There are further ethical issues these problems raise, such as being forced to give treatments to people without having a statistically proved effect or a high certainty result within the Grading of Recommendations Assessment, Development and

Evaluation analysis (due to reasons of imprecision from statistical sampling issues). The power of a study, therefore, has huge implications on the precision of estimates in the future analysis of data and in turn clinical practice guidelines. Within this review, 30% of studies appeared to be underpowered based on actual achieved clinical differences within the wider comparable evidence base, with mean sample size deficits up to 79 patients per trial. This does impact the overall certainty of the global evidence base within IBD, with precision a key limitation downgrading many outcomes within key guidelines across dozens of interventions.

Within this review, we present a resource for SSE not just for future study authors, but for study peer reviewers and most importantly professionals and the patients. This table gives an estimated PC result for a minimum sample size based on all existing studies within this period. Rather than being based on just single studies or clinical judgement, these represent estimates based on actual achieved clinical data and to our knowledge are the first time such a resource has ever been provided for researchers in the field or indeed for readers of future research. Additionally, for those wishing to calculate key statistics and measures of outcome from their primary studies, this paper provides a systematic and objective resource for baseline risk. This could be used for calculating numbers needed to treat or harm, for example.

This resource can be used by study designers to prevent PCs based on studies that offer a high MCID and as such a lower minimum sample size than is actually warranted. Conversely, it prevents unnecessary over recruitment. Funders can use this to appropriately budget and ensure viability of studies. Ethics boards and other governance groups will be able to consult this resource to support their consideration of research proposals.

There were a number of comparisons where the difference in practice was below 10% and it was deemed inappropriate to make a calculation in such cases, as no previous study has ever indicated an MCID below 10% as clinically significant to patients or practice. In these cases, consideration should be given to the overall figures presented in Table 2 or minimum sample size and MCID in practice in a similar context.

We would also recommend that in practice, patients and key stakeholders should be involved in deciding on an MCID for a given intervention prior to a new study. They may indicate that in spite of any existing MCID evidence that such a difference is not significant enough to matter to those who are most impacted by the findings and such views must be reflected in the process of SSE. It is also worth noting that there will always be settings and contexts when deviation may be warranted, thereby, a resource is not prescriptive but rather presented as evidence-based guidance. We would, however, propose that such deviations can and should be justified to support transparency for the readings these trials report.

There are weaknesses and exceptions to these approaches. The search methods used limited the parameters of the search for pragmatic reasons. However, this does not represent any systematic bias, hence we do not believe it invalidates the findings, and in the future this resource can be updated prospectively. When the achieved difference was less than 10%, rather than reporting extremely large sample size calculations, no such calculation was made. Additionally, in studies comparing active agents, accurate estimates are needed based on the contexts as the hypothesis may not be of the inferiority or superiority but of no difference, which requires a different approach to calculations.

There were some limitations to this review. There are obvious issues of heterogeneity limiting the appropriateness of pooling the data, however, the only way to obtain the previously used MCID was through looking at the past studies. These are mainly related to missing or unclear information in primary studies regarding SSE and as authors were not contacted, assumptions were made for the basis of these calculations which could confer some inaccuracy in our estimations. We also limited our studies to those from after the CONSORT statement release as we felt this was a fair time from which to expect SSE to occur, but earlier studies could potentially have offered more insight. Finally, we have focussed on studies comparing treatment with placebo or no intervention. This was a pragmatic decision as many studies of agents choose to make this comparison, although often these do not reflect current standard clinical practice. In the cases of such comparisons, SSE may not have to be based on a MCID but instead assume clinical equivalency and therefore be informed differently. In essence, this guidance may not be relevant for these scenarios, although may inform statistical considerations within similar contexts. Finally, such a resource of course is likely to become inaccurate rapidly, with the need for updates, but as often no such resource is employed, we believe this is still an improvement on current practices.

Future researcher is needed to potentially validate the calculations with clinical and patient input to ensure the SSE and MCID that the data informs has clinical, as well as statistical relevance. This could lead to a more triangulated resource that is statistically and evidentially sound, but also clinically sound and patient informed. This could conceivably lead to increases or decreases in minimally important differences to reflect complexity in specific clinical scenarios and interventional contexts.

CONCLUSION

In conclusion, a third of intervention IBD studies within the last 25 years are underpowered, with large variations in the approaches to calculating sample sizes and the minimum clinically important differences. The authors present a sample size estimate resource based on the published evidence base for future researchers and other key stakeholders within the IBD trial field.

ARTICLE HIGHLIGHTS

Research background

A third of randomised controlled trials (RCTs) on interventions for inflammatory bowel disease (IBD) have no adequate power calculation (PC).

Research motivation

A key element of PCs is an estimation of a minimally important clinical difference. The basis of these is capricious within the literature, with many not based on any existing or prior studies and as such can lead to massive shifts in PCs for similar studies, with concerns as to the underlying power.

Research objectives

We systematically reviewed RCTs reporting interventions for the management of IBD and to producted a resource for minimum clinically important difference using clinical data for the future researchers to use as a starting point.

Research methods

We included RCTs retrieved from Cochrane IBD trial register and CENTRAL investigating anti-inflammatory, immunomodulator and biologic therapies for either induction or maintenance of remission against control, placebo, or no intervention of IBD in patients of any age. The data was extracted and synthesized. We recalculated sample size and the achieved difference, as well as minimum sample sizes and presented in a tabular format.

Research results

Of 105 trials were included. A large discrepancy between the estimated figure for the minimal clinically important difference used for the calculations (15% differences observed *vs* 30% used for calculation) was observed explaining substantial actual sample size deficits. The minimum sample sizes indicated for future trials based on the 25 years of trial data were calculated and grouped by the intervention.

Research conclusions

There are large variations in the sample size calculatins in the studies of interventions for IBD with a third of all studies being underpowered. The authors present a sample size estimate resource constructed on the published evidence base for future researchers and key stakeholders within the IBD trial field.

Research perspectives

The use of this resource will support research staff, ethics committees and journal editors in ensuring adequate sample sizing and powering of studies across the field.

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