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Editorial Board Member of *World Journal of Gastroenterology*, Shozo Osera, MD, PhD, Chief Physician, Department of Gastroenterology, Saku Central Hospital Advanced Care Center, 3400-28, Nakagomi, Saku 385-0051, Nagano, Japan. shou0122@hotmail.com

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REVIEW

Innate and adaptive immune escape mechanisms of hepatitis B virus

Hua-Jun Zhao, Yi-Fei Hu, Qiu-Ju Han, Jian Zhang

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Hua-Jun Zhao, Yi-Fei Hu, Qiu-Ju Han, Jian Zhang, Institute of Immunopharmaceutical Sciences, School of Pharmaceutical Sciences, Shandong University, Jinan 250012, Shandong Province, China

Corresponding author: Jian Zhang, PhD, Professor, Institute of Immunopharmaceutical Sciences, School of Pharmaceutical Sciences, Shandong University, No. 44 Wenhua West Road, Jinan 250012, Shandong Province, China. zhangj65@sdu.edu.cn

Abstract

Chronic hepatitis B virus (HBV) infection is an international health problem with extremely high mortality and morbidity rates. Although current clinical chronic hepatitis B (CHB) treatment strategies can partly inhibit and eliminate HBV, viral breakthrough may result due to non-adherence to treatment, the emergence of viral resistance, and a long treatment cycle. Persistent CHB infection arises as a consequence of complex interactions between the virus and the host innate and adaptive immune systems. Therefore, understanding the immune escape mechanisms involved in persistent HBV infection is important for designing novel CHB treatment strategies to clear HBV and achieve long-lasting immune control. This review details the immunological and biological characteristics and escape mechanisms of HBV and the novel immune-based therapies that are currently used for treating HBV.

Key Words: Hepatitis B virus; Innate immunity; Adaptive immunity; Immune tolerance; Therapeutic strategy

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Core Tip: Chronic hepatitis B (CHB) infection is an international health problem. Current clinical CHB treatment strategies can partly inhibit and eliminate hepatitis B virus (HBV), but cannot achieve long-lasting immune control of the virus. Persistent CHB infection arises as a consequence of the complex interactions between HBV and the host innate and adaptive immune systems. Therefore, it is important to understand the immunological mechanisms involved in CHB infection. In this review, we detail the immune biological characteristics and escape mechanisms of HBV and discuss novel immune-based therapies.

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INTRODUCTION

Hepatitis B virus (HBV) is a small hepatotropic, enveloped DNA virus. Hepatitis B is an international health problem caused by HBV infection. Currently, approximately 257 million people worldwide are chronic HBV carriers with a high risk of developing chronic liver diseases such as liver cirrhosis and hepatocellular cell carcinoma (HCC). Each year, approximately 1 million patients die of HBV-related liver diseases[1,2]. Current clinical treatment strategies for chronic hepatitis B (CHB) mainly include pegylated interferon-α (PEG-IFN-α) and nucleos(t)ide analogues (NAs). Unfortunately, current treatments have limitations and often fail to achieve long-term virologic control.

Generally, the host innate and adaptive immune systems play critical roles in eliminating HBV upon infection. However, HBV has evolved and developed efficient strategies for escaping host immune surveillance, which results in persistent infections. The majority of HBV infections occur in newborn infants with the presence of immunological defects, characterized by a lower quality and quantity of HBV-specific T cells and B cells. In addition, maternal hepatitis B e antigen (HBeAg) can induce the Kupffer cells (KCs) of the offspring by upregulating programmed death-ligand 1 (PD-L1) to suppress the HBV-specific $CD8^+$ T cells response to support HBV persistence after birth[3]. In addition, HBV circumvents endogenous type I interferon (IFN-I) responses[4] and inhibits the function of innate and adaptive immune cells[5]. Prolonged exposure of T cells to large quantities of viral antigens, such as hepatitis B surface antigen (HBsAg) and HBeAg, induces a defective T-cell response with the loss of effector functions and increased inhibitory receptor expression, facilitating viral persistence. Moreover, HBV infection affects the expression of human leukocyte antigen (HLA)-II alleles, including HLA-DP, HLA-DQ, and HLA-DR, on antigen-presenting cells (APCs)[6], which in turn impairs antigen presentation capacity with induction of an inefficient T-cell response, leading to persistent HBV infection.

The clinical outcomes of patients with CHB are highly based on the complex interactions between HBV and the host innate and adaptive immune systems. In this review, we detail the interaction between HBV and the host immune system to understand the immunological and biological characteristics of and escape mechanisms involved in CHB infection, and present the current immune-based therapies for CHB treatment.

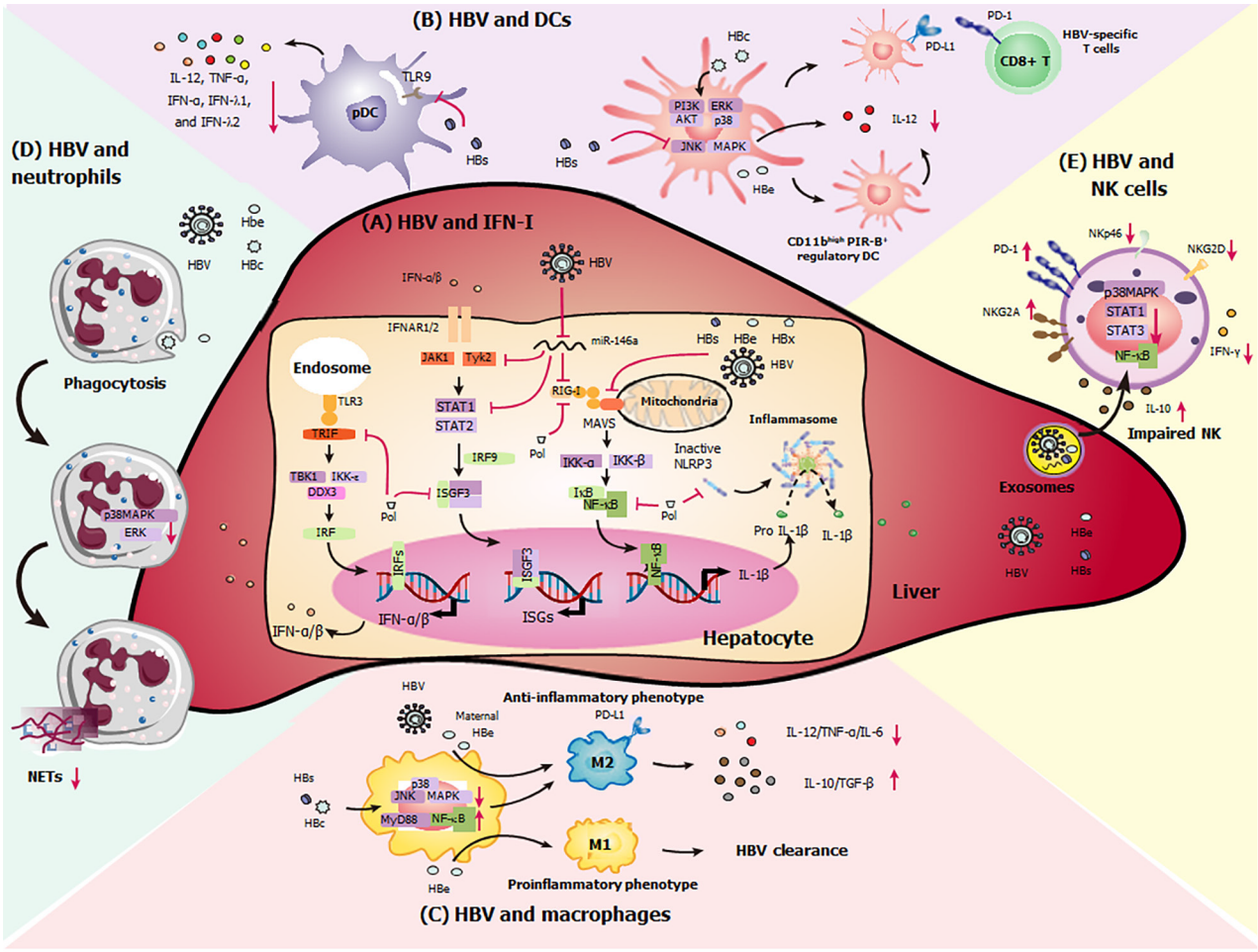
HBV ESCAPES INNATE IMMUNE SURVEILLANCE

Innate immune responses act as the first line of immune defense against viruses, bacteria, and tumors. Complement components, chemokines, and cytokines are soluble factors that form parts of the innate system. Granulocytes, dendritic cells (DCs), macrophages, mast cells, and natural killer (NK) cells are important effector cells[7,8]. Commonly, an effective innate immune response is initiated when pathogen-associated molecular pattern (PAMP) molecules bind pattern recognition receptors (PRRs), which stimulates chemokine and proinflammatory cytokine production, and innate immune cell activation, resulting in the elimination of viruses[9]. Here, we described the interaction between HBV and innate immunity (Figure 1).

HBV infection and IFN-I

PRRs, which are widely expressed by KCs, hepatic DCs, liver sinusoidal endothelial cells (LSECs), and hepatocytes, can recognize PAMPs from HBV and induce antiviral immune responses, resulting in the secretion and IFN-I and other inflammatory cytokines. IFN-I, as a major component of the innate immune response, is critical for HBV clearance. However, HBV circumvents endogenous IFN-I responses through multiple pathways to sustain persistent HBV infection.

Chronic HBV infection downregulates the expression of Toll-like receptor 3 (TLR3), retinoic acid-inducible gene I (RIG-I), and melanoma differentiation-associated protein 5 (MDA-5) in DCs and hepatocytes, leading to the reduction of responsiveness to PAMPs and impairment of IFN-I synthesis [10]. A previous study found that HBV infection upregulates microRNA-146a (miR-146a) expression in hepatocytes, inhibiting the expression of RIG-I-like receptors and in turn suppressing IFN-I transcription[11]. Additionally, HBsAg, HBeAg, HBx, and HBV virions themselves can inhibit IFN-β synthesis by downregulating mitochondrial antiviral signaling (MAVS) and interfering with the interaction between MAVS and RIG-I[12].



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Figure 1 The interaction between hepatitis B virus and innate immunity. A: Hepatitis B virus (HBV) suppression of the type I interferon (IFN-I) response. HBV infection inhibits IFN-I transcription and signal transduction and IFN-β synthesis; B: HBV affects the function of dendritic cells (DCs). HBV upregulates programmed death-ligand 1 and induces regulatory DCs that exhibit extremely low T cell-stimulatory capacity and interleukin-12 production; C: HBV affects the function of macrophages. HBV-related antigens affect macrophage polarization (M1/M2), contributing HBV clearance or HBV persistence; D: HBV affects the function of neutrophils. HBV-related antigens decrease neutrophil extracellular trap release, which facilitates HBV immune escape, replication, and persistence; E: HBV affects the function of natural killer (NK) cells. HBV-related antigens and HBV-derived exosomes dampen the Retinoic acid-inducible gene I, nuclear factor kappa B, and p38 mitogen-activated protein kinase signaling pathways, resulting in the functional suppression of NK cells during chronic hepatitis B infection. DCs: Dendritic cells; DDX3: DEAD-box RNA helicase 3; ERK: Extracellular-regulated kinase; HBc: Hepatitis B core antigen; HBe: Hepatitis B envelope antigen; HBs: Hepatitis B surface antigen; HBx: HBV X protein; HBV: Hepatitis B virus; IFN-α: Interferon-α; IFN-β: Interferon-β; IFN-γ: Interferon-γ; IFN-I: Type I interferon; IFNAR: Interferon-α receptor; IKK-ε: IB kinase ε; IL-1β: Interleukin-1β; IL-6: Interleukin-6; IL-10: Interleukin-10; IL-12: Interleukin-12; IRF3: Interferon regulatory factor 3; ISGs: Interferon-stimulated genes; ISGF3: Interferon-stimulated gene factor 3; JAK: Janus kinase; JNK: c-Jun N-terminal kinase; M1: M1-like macrophages; M2: M2-like macrophages; MAPK: Mitogen-activated protein kinase; MAVS: Mitochondrial antiviral-signaling protein; miR-146a: microRNA-146a; MyD88: Myeloid differentiation primary response gene 88; NET: Neutrophil extracellular trap; NK: Natural killer; NKG2D: Natural killer group 2 member D; NKG2A: Natural killer group 2 member A; NF-B: Nuclear factor kappa B; PD-1: Programmed cell death protein 1; pDCs: Plasmacytoid DCs; PD-L1: Programmed death-ligand 1; PI3K: Phosphatidylinositol 3-kinase; Pol: Hepatitis B virus polymerase; Pro-IL-1β: IL-1β precursor; RIG-I: Retinoic acid-inducible gene I; STAT1: Signal transducer and activator of transcription 1; STAT2: Signal transducer and activator of transcription 2; TBK1: TANK-binding kinase 1; TGF-β: Transforming growth factor-β; TLR2: Toll-like receptor 2; TLR3: Toll-like receptor 3; TLR9: Toll-like receptor 9; TNF-α: Tumor necrosis factor-α; TRIF: Toll/IL-1 receptor domain-containing adaptor inducing IFN-β; Tyk2: Tyrosine kinase 2.

Binding of IFN-I to the IFN receptor can induce the activation of IFN-stimulated genes (ISGs), thereby directly inhibiting HBV infection. However, HBV can extensively impair IFN-I-induced signal transduction and dampen IFN-I-mediated immune responses[4]. HBx is able to reduce transcription of the IFN-α receptor (IFNAR1) and downregulate tyrosine kinase 2, which is essential for cell surface IFNAR1 expression[13]. Additionally, matrix metalloproteinase 9, which is increased in the peripheral blood mononuclear cells of patients with CHB, binds to IFNAR1 and facilitates its phosphorylation, ubiquitination, subcellular distribution, and degradation[14]. HBV can inhibit the activities of IFN-stimulated response elements with lower ISG expression by disrupting the intracellular Janus kinase–signal transducer and activator of transcription 1 (STAT1) signaling pathway. HBV-induced miR-146a downregulates cellular STAT1 levels and blocks STAT1-Tyr701 phosphorylation in hepatocytes[11]. HBV polymerase interferes with the binding of DEAD-box helicase 3 X-linked (DDX3) to the TANK-binding kinase 1/IκB kinase epsilon complex and the induction of IFN-stimulated gene

factor 3 (ISGF3) to inhibit IFN-β induction[10]. In addition, HBV polymerase suppresses interleukin 1 beta (IL-1β) production by inhibiting nuclear factor kappa B (NF-κB) signaling and the inflammasome-caspase-1 pathway, resulting in IFN-α resistance and persistent HBV infection[15].

Based on these findings, IFN-I is used for the treatment of CHB. IFN-α-mediated HBV suppression is correlated with the levels of apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like (known as APOBEC) and the base excision repair gene Nei endonuclease VIII-like 3[16]. IFN-α can also epigenetically regulate the HBV covalently closed circular DNA (cccDNA) minichromosome by disrupting general control non-depressible 5-mediated histone H3 lysine 79 methylation succinylation, resulting in the clearance of HBV cccDNA[17]. Imam *et al*[18] identified sterile alpha motif domain containing 4A as an important anti-HBV ISG that binds to and triggers degradation of the unidentified Smaug-recognition region sequence in viral RNA. ISG20 can induce the degradation of HBV RNA by selectively recognizing and binding N6-methyladenosine. In addition, tripartite motif containing 5 gamma suppresses HBV replication by interacting with the HBx protein, which promotes HBx ubiquitination at residue K48 and its subsequent degradation[19]. MX dynamin like GTPase 2 reduces HBV cccDNA by indirectly impairing the conversion of relaxed circular DNA to cccDNA[20]. Interestingly, IFN-α can also induce soluble factors that compete with HBV for binding to heparin glycosaminoglycans, thereby inhibiting HBV infection[21]. In addition to its direct antiviral effects on HBV, IFN-I indirectly exerts antiviral functions by activating immune cells. IFN-I can quickly recruit and activate NK cells and DCs, promoting the initiation of adaptive immunity[5,22], which in turn contributes to the elimination of HBV.

HBV infection and DCs

As professional APCs, DCs serve as a bridge between the innate and adaptive immune responses[23]. Recent data have shown that functional impairment of DCs by HBV infection fails to induce efficient anti-HBV immunity, leading to CHB infection and the progression of liver disease[24]. Previous data have demonstrated that patients with CHB have significantly fewer peripheral blood DCs than control subjects, accompanied by a functional decline and directly causing HBV-specific T cell dysfunction[24]. Compared to those of healthy donors, myeloid DCs (mDCs) isolated from patients with CHB display limited antigen-presenting capacity and migration capacity, features that are accompanied by the decreased expression of interleukin 6 cytokine family signal transducer (also known as gp130)[24]. Persistent HBV infection downregulates cluster of differentiation 80 (CD80), CD83, CD86, and CD40 expression in DCs, which suppresses the transduction of costimulatory signals to T cells. In addition, HBsAg reduces IL-12 production by mDCs by disrupting the c-Jun N-terminal kinase (JNK)–mitogen-activated protein kinase (MAPK) pathway, resulting in a markedly tolerogenic phenotype[25]. HBcAg upregulates programmed death-ligand 1 (PD-L1) by activating the phosphoinositide 3-kinase (PI3K)–AKT, extracellular-regulated kinase (ERK), and p38 signal transduction pathways, which suppresses HBV-specific T cell immune function[26]. HBeAg induces the conversion of bone marrow-derived DCs into $CD11b^{hi}$PIR-B^{+} regulatory DCs, which exhibit extremely low T-cell stimulatory capacity and IL-12 production[27]. Furthermore, HBV particles, especially HBsAg, downregulate TLR expression and abrogate TLR9-triggered maturation of plasmacytoid DCs, resulting in the significantly decreased secretion of certain cytokines such as IL-12, tumor necrosis factor alpha (TNF-α), IFN-α, IFN-λ1, and IFN-λ2[28-30]. In addition, chronic HBV infection impairs IFN-α secretion and pDC maturation in response to TLR7 ligands[28].

HBV infection and macrophages

Macrophages are important innate immune cells that fight against pathogen infection and can interact with lymphocytes by activating and inhibitory surface molecules. HBV can affect the functions of monocytes and macrophages, thereby contributing to persistent HBV infection. HBV infection promotes the activation of anti-inflammatory macrophages with increased IL-10 production, which support the functional inactivation of $CD8^{+}$ T cells.

KCs, which are localized in liver sinusoids, are the largest population of innate immune cells in the liver[31]. They are stationary and able to effectively phagocytose cellular debris, foreign material, or pathogens, acting as critical sentinels for liver homeostasis[32]. Chronic HBV infection induces the production of immunomodulatory mediators such as IL-10 and transforming growth factor beta (TGF-β), and the expression of PD-L1 and PD-L2 by KCs, suppressing anti-HBV T cell responses. Furthermore, upon HBV infection, elderly mice have a significantly higher number of TNF-α-producing $Ly6C^{+}$ monocytes and a much lower number of IL-10-secreting KCs than younger mice, facilitating HBV clearance[33]. However, KCs can play different roles in the presence of different HBV antigens[34]. Boltjes *et al*[35] found that KCs could interact with HBsAg, which induced secretion of the proinflammatory cytokines IL-6 and TNF that was substantially increased compared with that seen in healthy controls. *In vivo* experiments have demonstrated that HBcAg interacts with KCs upon TLR2 activation, mediating humoral and cellular tolerance *via* IL-10 production during CHB infection, and TLR2 knockout or KC depletion leads to an accelerated HBV clearance and improved HBV-specific $CD8^{+}$ T cell responses[36]. HBeAg suppresses lipopolysaccharide-induced NOD-, LRR- and pyrin domain-containing protein 3 activation and IL-1β maturation in KCs by inhibiting NF-κB phosphorylation and reactive oxygen species production[37]. Nonetheless, HBeAg can play two distinct roles in macrophage

function. Upon HBV infection, maternal HBeAg enhances PD-L1 expression in KCs with an M2-like anti-inflammatory phenotype, which suppresses the HBV-specific cytotoxic T lymphocyte (CTL) response and leads to HBV persistence; however, in control mice born to HBeAg-negative mothers, HBeAg promotes the M1 proinflammatory phenotype, contributing to HBV clearance[3].

Except for KCs, monocyte-derived macrophages are critical for regulating the anti-HBV immune response and disease pathogenesis during HBV infection. Intrahepatic macrophages that had phagocytosed HBcAg show anti-inflammatory over proinflammatory functions and favor the maintenance of infection. In addition, HBsAg inhibits TLR2-induced phosphorylation of p38 MAPK and JNK MAPK with reduced production of IL-6, TNF-α, and IL-12 in human monocytes[38]. Previous findings have also shown that HBsAg can interact with monocytes and induce the MyD88–NF-κB-signaling pathway with high expression of the inhibitory molecules PD-L1, IL-10, and TGF-β, thereby initiating an immunosuppressive cascade[39]. In contrast to HBeAg and HBsAg, HBcAg from HBV-infected hepatocytes upregulates IL-23 secretion in monocyte-derived macrophages and enhances macrophage-mediated angiogenesis[40]. In addition, HBV-induced M2-like macrophages promote the immunosuppressive activity of regulatory T (Treg) cells by enhancing cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4), inducible T cell costimulator, and CD39 expression levels in an amphiregulin-dependent manner[41], impairing T helper type 1 cell immune responses and accelerating liver fibrosis and pathology.

HBV infection and neutrophils

Neutrophils exhibit protective functions against microbial infections *via* phagocytosis, degranulation, and the formation of neutrophil extracellular traps (NETs). Recent results have shown that HBV might suppress the neutrophil response. For example, neutrophils from patients with liver cirrhosis show a decreased capability for NET release, accompanied by the reduced expression of CD69 and CD80[42]. Additionally, HBV-related antigens, such as HBeAg and HBcAg, decrease NET release by decreasing p38 MAPK and ERK phosphorylation and autophagy, which facilitates HBV immune escape, replication, and persistence[43].

HBV infection and NK cells

As the main effector cells of the innate immune system, NK cells constitute up to 40%–50% of human liver lymphocytes, and serve as the first line of defense against pathogens. Activated cytolytic $CD56^{dim}$ NK cells expressing NKp46, NKp30, and perforin are associated with efficient HBV containment[44,45]. Additionally, antibody-mediated activation of NK cells plays a vital role in resolving HBV infection[45]. Abnormal NK cell receptor expression and hepatic NK cell dysfunction contribute to persistent CHB infection and HCC progression, which are related to the poor prognosis and survival of patients with liver cancer[46]. The levels of activating receptors (*e.g.*, NKp30, NKp46, and natural killer group 2 member D [NKG2D]) and cytokines (*e.g.*, IFN-γ and TNF-α) are significantly decreased in patients with CHB, which is accompanied by the higher secretion of inhibitory NK cell receptors such as T cell immunoglobulin and mucin domain-containing protein 3 (Tim-3), NKG2A, and IL-10[47]. In addition, the decreased expression of CD122, the common β chain of the IL-2 receptor on $CD56^{dim}$ NK cells, is associated with NK cell dysfunction during CHB infection[48].

The roles of circulating HBV-related antigens (*e.g.*, HBsAg and HBeAg) in mediating NK cell inhibition remain unclear. Recently, we found that HBsAg and HBeAg directly interact with NK cells and act as inhibitory mediators by interfering with the activation of STAT1, NF-κB, and p38 MAPK, which in turn impairs NK cell cytotoxicity and cytokine production[49]. HBsAg downregulates STAT3 expression, which is partially correlated with degranulation and cytokine production in patients with HBeAg-negative CHB[50]. Additionally, HBsAg-treated monocytes promote the conversion of NK cells into IL-10-producing regulatory NK cells *via* PD-L1 and major histocompatibility complex, class I, E signals, which contribute to persistent CHB infection[39]. Importantly, exosomes derived from patients with CHB shuttle HBV nucleic acids into NK cells and then dampen the RIG-I, NF-κB, and p38 MAPK signaling pathways, resulting in the functional suppression of NK cells during CHB infection[51]. miR-146a is significantly elevated in patients with CHB and modulate both NK and T-cell responses[52]. Interestingly, we found that miR-146a in NK cells can be induced by exogenous IL-10 and TGF-β, which in turn leads to NK cell dysfunction by directly targeting STAT1, accompanied by weakened IFN-γ and TNF-α secretion in patients with CHB[53].

HBV ESCAPES ADAPTIVE IMMUNE SURVEILLANCE

Adaptive immunity plays a critical role in HBV infection, accompanied by antigen specificity and sustained memory responses. Under the stimulation of APCs, HBV-specific $CD4^+$ T cells and $CD8^+$ T cells are activated and then secrete antiviral cytokines such as IL-12, IFN-γ, and TNF-α, and induce CTL responses to kill HBV-infected hepatocytes[54]. In addition, follicular helper T (Tfh) cells promote B cell differentiation into plasma cells, which are capable of producing HBV-specific antibodies[55]. Recent studies have demonstrated that newborn infants have immunological defects, and the inability to

induce HBV-specific T- and B-cell responses have been found in neonatal animals, which develop chronic HBV infection[3,56]. Furthermore, chronic HBV infection can suppress the adaptive immune system and dampen HBV clearance, leading to persistent HBV infection in patients with CHB.

HBV infection and HLA-II

HLA in APCs plays a critical role in initiating the host antiviral immune response against HBV infection due to its capacity to attract and bind peptides. HLA-I molecules can present HBV peptides to $CD8^+$ cytotoxic T lymphocytes, resulting in the direct cytolysis of HBV-infected hepatocytes. HLA-II alleles, including HLA-DP, HLA-DQ, and HLA-DR, encode MHC-II molecules that present exogenous antigens to $CD4^+$ T cells[57,58]. Different HLA-II alleles with particular amino acid polymorphisms determine which peptides can be presented to T cells. Moreover, these HLA alleles expressed on APCs contribute to presenting a broad range of peptides, thus determining the variability in the host immune response to HBV. Compared to HLA-DP and HLA-DQ, HLA-DR allele containing an extra β-chain gene whose product can pair with the DRα chain on APCs, is more important for the induction of sustained HBV-specific immune response. Upon HBV infection, single nucleotide polymorphisms of HLA-II antigens may also contribute to the induction of immune tolerance, leading to persistent HBV infection[57,59-62]. HBV infection reduces the expression of HLA-DP and HLA-DQ molecules on APCs, which in turn results in impaired antigen presentation capacity and an inefficient T-cell response[12,13]. Thus, polymorphisms of HLA-II genes during HBV infection can alter the antigen-binding properties of HLA-II and affect the HBV-specific immune response, partly promoting the persistent HBV infection.

HBV infection and $CD8^+$ T cells

HBV-specific $CD8^+$ T cells function as key cellular effectors against HBV infection[63]. During CHB infection, $CD8^+$ T cells encounter HBV antigens presented by intrahepatic APCs, such as DCs, KCs, or LSECs with weakened costimulatory signals, resulting in immune tolerance[64]. In addition, HBV infection can cause deficient secretion of inflammatory cytokines such as IL-12 and IFN-α/β in response to PAMP stimulation, further dampening the third signal required for $CD8^+$ T-cell activation. Additionally, sustained exposure to high doses of HBV antigens (*e.g.*, HBsAg and HBeAg) leads to exhausted T cells and impaired effector functions during CHB infection. Microarray analyses have revealed that HBV significantly upregulates the expression of the proapoptotic molecule Bcl-2-like protein 11 among HBV-specific $CD8^+$ T cells, suggesting a key mechanism related to the depletion of $CD8^+$ T cells during CHB infection[65]. Exhausted $CD8^+$ T cells show reduced IL-2, IFN-γ, and TNF-α secretion and lost cytotoxic and proliferative capacities[66,67]. Moreover, exhausted HBV-specific $CD8^+$ T cells display multiple inhibitory receptors such as PD-1, CTLA-4, CD244 (2B4), Tim-3, and lymphocyte activation gene 3[68], closely mimicking the transcriptional profiles of $CD8^+$ T cells[67,69,70].

T-bet is essential for successful $CD8^+$ T cell responses against HBV, whereas eomesodermin (EOMES) is a key driver of T cell exhaustion during chronic HBV infection[67,69,71]. Schurich *et al*[71] observed that reduced PD-1 expression on HBV-specific $CD8^+$ T cells is accompanied by high levels of T-bet, which can increase $CD8^+$ T cell functions[71], whereas EOMES might compensate for the lack of T-bet expression during HBV infection[67]. Data from a recent study showed that exhausted $CD8^+$ T cells express elevated levels of the transcription factors interferon regulatory factor 4, basic leucine zipper ATF-like transcription factor, and nuclear factor of activated T cells 1, which in turn promote the expression of multiple inhibitory receptors (such as PD-1) and is accompanied by impaired antiviral function and cellular metabolism. However, transcription factor T cell factor 1 (TCF1) expression is repressed in exhausted $CD8^+$ T cells, which is important considering that TCF1 is essential for memory T cell differentiation[72]. Moreover, when compared with HBV core-specific $CD8^+$ T cells, HBV polymerase-specific $CD8^+$ T cells show higher expression of CD38 and EOMES, accompanied by lower T-bet expression and a reduced expansion capacity[70]. Recently, transcriptome analysis of exhausted HBV-specific $CD8^+$ T cells in patients with CHB revealed a lower mitochondrial potential and substantial mitochondrial dysfunction[73,74], whereas exposure to antioxidants or IL-12 partially reinvigorated the antiviral activity of HBV-specific $CD8^+$ T cells[73,74].

$CD4^+$ T cells

$CD4^+$ T cells are important in regulating $CD8^+$ T cell activation, proliferation, and memory responses during HBV infection[75]. Generally, a loss help of $CD4^+$ T cells is considered the major factor involved in HBV-specific CTL cell failure[76]. Increasing attention has been given to the exhaustion of $CD4^+$ T cells during CHB infection. Previous results have demonstrated that HBV-related antigens, such as HBcAg and HBsAg, can upregulate the expression of inhibitory molecules on $CD4^+$ T cells. For example, Li *et al*[77] found that HBcAg increased PD-1 expression on $CD4^+$ T cells *via* the JNK, ERK, and PI3K/AKT signaling pathways disrupt the function of $CD4^+$ T cells. HBsAg increased the expression of human protein inhibitor of activated STAT1 expression (which is dependent on activation of the ERK and p38 MAPK signaling pathways), thereby contributing to the ineffectiveness of traditional treatments for CHB patients[78]. Data from a recent study showed that $CD4^+$ T cells in patients with CHB expressed high levels of TRAIL receptors, and these T cells could be targeted by $TRAIL^+$ NK cells, leading to a reduction in the number of $CD4^+$ T cells[79]. Additionally, the decreased secretion of

proinflammatory cytokines (such as IL-2, IFN-γ, and IL-21) by HBV-specific $CD4^+$ T cells contribute to the exhaustion of $CD8^+$ T cell responses during chronic HBV infection[80]. $CD4^+$ T cells also differentiate into $CD4^+$ $CD25^+$ $Foxp3^+$ Treg cells, which secrete the suppressive cytokines IL-10 and TGF-β, resulting in a progressive loss of HBV-specific $CD8^+$ T cells[81]. Thus, $CD4^+$ T cells can directly influence HBV clearance by regulating $CD8^+$ T cells.

Tfh cells express C-X-C chemokine receptor type 5 (CXCR5) and can specifically recognize and bind to follicular B cells expressing CXCL13, which promote the formation of affinity-matured, long-lived plasma cells and antibody secretion[82]. A deficiency of Tfh cells can inhibit the formation of germinal centers (GCs) in the spleen[83]. Thus, Tfh cells play important roles in orchestrating the humoral immune response and HBsAg seroconversion in patients with CHB[84]. Previous results have shown that the frequency of Tfh cells correlates negatively with HBsAg levels in patients with CHB after PEG-IFN-α therapy[85]. The recovery of Tfh cell responses induces the production of anti-HBs antibodies and accelerates HBV clearance[55]. Additionally, Wang *et al*[86] found that HBV infection significantly increases the proportion of $CD4^+$ $CXCR5^+$ $CD25^+$ $Foxp3^+$ follicular regulatory T (Tfr) cells. Compared to $CD25^-$ Tfh cells, $CD25^+$ Tfh cells express high levels of inhibitory receptors, such as PD-1 and CTLA-4, with lower levels of IFN-γ and IL-17 and higher TGF-β secretion. Importantly, Tfr cells can suppress the GC reaction of B cells and the antiviral effect of $CD8^+$ T cells. Therefore, Tfh cell dysfunction might disrupt humoral immune responses during CHB infection.

B cells

Anti-HBs antibodies are protective antibodies that can prevent HBV from entering into host hepatocytes and can clear infectious HBV particles from the body[87,88], and B cells are essential for effective HBV control. Although the total number of B cells is enriched during CHB infection[89], previous data revealed that patients with CHB showed a decreased frequency of HBsAg-specific B cells[90]. Furthermore, the production of anti-HBs was defective in patients with CHB[91]. Recent findings indicate that hyperactivated B cells with increased expression of CD69 and CD71, have deficient proliferative capacity and are unable to achieve HBsAg seroconversion in CHB patients.

Burton *et al*[92] found that HBsAg-specific B cells in patients with CHB exhibited a $CD21^-$ $CD27^-$ atypical memory B cell (atMBC) phenotype, which was accompanied by high levels of inhibitory receptors such as PD-1, BTLA, and CD22. atMBCs exhibit impaired survival, proliferation, and cytokine production and cannot normally differentiate into antibody-producing plasma cells, which dampens humoral immune responses in patients with CHB. Poonia *et al*[93] found that HBcAg binding to B cells can induce high expression of the inhibitory receptors Fc receptor-like 5 (FcRL4) and FcRL5 on B cells, as well as dysfunctional phenotypes, and can also suppress the proliferation and activation of B cells mediated by the B cell receptor and TLR signaling. Additionally, HBcAg drives B cell differentiation into IL-10-producing regulatory B (Breg) cells characterized as $CD19^+$ $CD24^{hi}$ $CD38^{hi}$, which suppresses $CD8^+$ T cell responses[89]. Moreover, Breg cells have also been found to promote the conversion of $CD4^+$ $CD25^-$ effector T cells into $CD4^+$ $CD25^+$ Treg cells, thereby participating in the maintenance of immune tolerance during chronic HBV infection[89].

In addition to antibody production, B cells are also considered professional APCs during CHB infection. However, the levels of costimulatory molecules (CD80 and CD40) are significantly decreased in circulating B cells, which might impair the interactions between B cells and effector T cells, thus inducing T cell exhaustion[94]. B cells can also regulate the immune response by secreting cytokines during CHB infection. HBeAg can stimulate B cell activation by promoting B-cell activating factor production *via* IL-6 and IFN-γ secretion[95,96], where IL-6 can play a non-cytolytic antiviral role against HBV by inducing cccDNA decay, reducing HBV transcription, and downregulating the NTPC receptor [97].

NOVEL IMMUNE THERAPEUTIC STRATEGIES FOR HBV

Current CHB treatments fail to cure HBV and are often accompanied by serious side effects. The long-term use of HBV drugs may even lead to mutations in HBV polymerase and cause drug resistance[98, 99]. Therefore, to overcome the limitations of clinical CHB therapy, researchers are developing new immune strategies to achieve sustained virologic remission (Table 1).

Monoclonal antibodies

Sustained exposure to a high load of viral antigens leads to T cell exhaustion in patients with CHB. Serum HBsAg levels can be as high as 400 μg/mL in patients with CHB; thus, this phenomenon can play a key role in inhibiting HBV-specific immune responses. Neutralizing antibodies against HBsAg or preS1 eliminated HBV and restored HBV-specific immune responses to preventative HBV vaccines in HBV carrier mice. Gao *et al*[100] identified a novel monoclonal antibody (E6F6) that targets the HBsAg-aa119-125 peptide, which can mediate long-lasting HBsAg clearance and facilitates the HBV-specific T cell response *via* Fc-gamma receptor-mediated phagocytosis. Multiple release inhibitors and monoclonal antibodies against HBsAg have been tested in clinical trials, such as GC1102 (a recombinant human

Table 1 Novel immune therapeutic strategies for clinical chronic hepatitis B treatment

Target	Drug name	Sponsor	Phase	Notes	Ref.
HBsAg inhibitor	REP-2139	Replicor	II	Reduce the level of HBsAg	[101,102]
HBsAg inhibitor	REP-2165	Replicor	II	Reduce the level of HBsAg (similar to REP-2139)	[102]
RIG and NOD agonist	SB9200	Spring Bank	IIb/III	Prolonged IFN-α and IFN-β secretion; Reduce hepatitis virus antigen and DNA	[107]
TLR7 agonist	RO7020531	Roche	I	Activate HBV-specific CD8+ T and Tfh cells; Reduce the frequency of Tregs and MDSCs	[108]
TCR-T cells	HBsAg-TCR-T cells	Lion TCR Pte	I	Safely and efficiently reduced HBsAg levels; Reduced level of HBV DNA and HBsAg	[115]
Therapeutic vaccine	TG1050	Transgene	I	Reduced level of HBV DNA and HBsAg; Long-lasting HBV-specific T cell responses	[125]
Therapeutic vaccine	HBsAg-HBIG (YIC)	National Vaccine and Serum Institute	III	Increase the level of IL-2; Long-lasting HBV-specific T-cell responses	[126]
Therapeutic vaccine	Nasvac	CIGB	III	Sustained control of HBV DNA; Clearance of HBeAg	[127]
Therapeutic vaccine	GS-4774	Gilead	III	Strong immune stimulatory effect on T cells	[128]

HBsAg: Hepatitis B surface antigen; HBeAg: Hepatitis B e antigen; HBV: Hepatitis B virus; NOD: nucleotide-binding oligomerization domain; IFN-α: Interferon-α; IFN-β: Interferon-β; IL-2: Interleukin-2; MDSCs: Myeloid-derived suppressor cells; RIG-I: Retinoic acid-inducible gene I; TCR: T-cell receptor; Tfh: Follicular helper T cells; TLR7: Toll-like receptor 7; Tregs: Regulatory T cells.

monoclonal anti-HBs antibody, Green Cross, phase II/III), EYP001 (farnesoid X receptor agonist, Enyo Pharma, phase II), REP-2139 (Replicor, phase II)[101,102], REP-2165 (Replicor, phase II)[102], and RG7834 (Roche, pre-clinical)[103].

Cytokines

Cytokines have been widely used as immunomodulatory agents to regulate immune responses during CHB treatment. For example, IL-12 administration alone can induce IFN-γ secretion and the recovery of exhausted $CD8^+$ T cells in patients with CHB[71]. Moreover, employing IL-12 as an adjuvant combined with the recombinant HBV vaccine (rHBVvac) elicits systemic HBV-specific $CD4^+$ and $CD8^+$ T cell responses and restores HBsAg-specific humoral immunity, thereby overcoming immune tolerance in patients with CHB[104]. Recent findings have shown that co-administering GM-CSF with the rHBVvac induces the secretion of HBsAg-specific IFN-γ and CTL responses to clear HBV *in vivo*[105]. In addition, IL-2, IL-15, IL-21, and IL-33 also efficiently clear persistent HBV infection and produce a long-term immune response against HBV reinfection[106].

TLR and RIG-I agonists

Currently, various TLR ligands are used as drugs for clinical CHB therapy, such as SB9200 (Spring Bank, phase IIb/III)[107], RG-7795 (Roche, phase II), RO7020531 (Roche, phase I)[108], GS-9620 (Gilead, phase II), GS-9688 (Gilead, phase II), RG-7854 (Roche, phase I), and JNJ-4964 (Janssen, preclinical). Data from previous studies have shown that GS9620 (a TLR7 agonist) can upregulate IFN-α production, restore the effector functions of $CD8^+$ T cells and NK cells, and decrease HBsAg and HBeAg titers. Single-stranded RNA40 (a TLR8 agonist) can selectively induce IL-12, IL-18, and IFN-γ secretion by monocytes in patients with CHB, which is beneficial for HBV clearance[109]. SB9200, an agonist of RIG-I and nucleotide binding oligomerization domain containing 2, can stimulate prolonged IFN-α and IFN-β secretion and ISG activation and efficiently reduce hepatic woodchuck hepatitis virus antigen and DNA levels in infected woodchucks[107]. Interestingly, compared with entecavir administration, SB9200 pretreatment better reduces HBV virion production[110]. Additionally, we found that a small interfering RNA targeting HBx (3p-siHBx) induced RIG-I activation, which improved the immune microenvironment and triggered the activation of NK cells and $CD8^+$ T cells in HBV-carrier mice[111].

Immune checkpoint blockade

Prolonged exposure to HBV leads to NK cell dysfunction and hyperexpression of immune checkpoint proteins in T cells. As an alternative approach, blocking the inhibitory receptor NKG2A increases the activity of human NK cells to promote HBsAg clearance[112]. In addition to direct anti-HBV effects,

recent data have shown that NK cells in patients with CHB selectively inhibit HBV-specific T cell responses *via* an IL-10-dependent pathway[39]. Furthermore, NK cells in NA-treated CHB patients expressing high levels of death receptor ligands, such as TNF-related apoptosis inducing ligand (TRAIL) or NKG2D, can mediate the lysis of activated T cells, thereby contributing to the development of chronic HBV infection[46,49]. Depleting inhibitory NK cells and blocking the NKG2D and TRAIL pathways during NA treatment further induces significant improvements in terms of HBV-specific T cell functions to achieve an HBV cure[79]. Additionally, blocking inhibitory receptors, such as PD-1, 2B4, and Tim-3, can restore exhausted HBV-specific $CD8^+$ T cells by promoting the recovery of cytotoxicity, cytokine production, and proliferation, offering an excellent opportunity to achieve sustained virologic control[113].

T cell receptor/chimeric antigen receptor T cells

The adoptive transfer of autologous T cells, such as chimeric antigen receptor (CAR) T cells, is another promising immunotherapeutic option for HBV therapy. These CAR T cells can directly recognize HBV antigens on infected hepatocytes and HBV-related HCC cells independently of HLA, without any need for antigen processing and presentation[114]. Qasim *et al*[115] found that genetically modified T cell receptor (TCR) T cells safely and effectively targeted HBsAg and reduced HBsAg levels in a patient with HBV-related HCC who had undergone liver transplantation. As an alternative strategy, CAR T cells expressing a recombinant HBsAg-specific antibody together with CD28 and CD3 zeta could also recognize and eliminate HBsAg-positive hepatocytes *in vitro*. Moreover, HBsAg-CAR-$CD8^+$ T cells localized to the liver and rapidly reduced HBV replication without significant liver damage after adoptive transfer in a transgenic mouse model of HBV[116]. Additionally, HBsAg-CAR T cells specifically decreased plasma HBsAg, HBV-DNA, and HBV core-positive hepatocytes in persistently HBV-infected chimeric mice with humanized livers after adoptive transfer of HBsAg-CAR-T cells[117], thereby providing a potential therapeutic approach for HBV.

However, specific challenges related to TCR or CAR T-cell therapy remain for HBV treatment, including the risk of developing severe liver damage and the suppressive effects of transferred TCR/CAR T cells due to the immune tolerance of the liver. To prevent severe side effects, such as liver damage and uncontrolled proliferation, Kah *et al*[118] developed a method for transient mRNA electroporation into engineered HBV-TCR T cells. In a separate study, PD-1 knockdown in HBV-TCR-T cells increased their effector functions and ability to kill tumor cells in the PD-$L1^{hi}$ liver microenvironment [119]. In addition, engineered CAR T cells to overexpress c-Jun, an AP-1 transcription factor, showed enhanced expansion and functional capacity with improved antitumor efficiency[120]. These findings have facilitated the development of TCR/CAR T cells for clinical CHB treatment.

Therapeutic vaccines

Therapeutic vaccination presents an attractive strategy for HBV eradication. A novel hepatitis B therapeutic vaccine could overcome immune tolerance; effectively induce powerful $CD4^+$ T cells, $CD8^+$ T cells, and humoral immune responses; and ultimately achieve sustained control of CHB infection. Kosinska *et al*[121] proposed approaches for developing safe and effective therapeutic vaccines, including: Designing potent vaccine components or schemes to prime antigen-specific immune responses; combining checkpoint inhibitors with other strategies to restore exhausted T cells; and Reducing HBV-related antigen levels to prevent T cell attrition and exhaustion. Based on this guidance, we prepared HBsAg nanogels (Ng) with chitosan (CS) and poly γ-glutamic acid (γ-PGA). Interestingly, we found that single-dose HBsAg CS-γ-PGA Ng immunization, especially HBsAg Ng (+), enhanced DC maturation and induced HBV-specific cellular and humoral immunity, and promoted the generation of effector memory T cells for HBV clearance[122]. Recent data also showed that a ferritin NP-preS1 vaccine manifested an efficient antibody response, resulting in efficient viral clearance by delivering preS1 to $SIGNR1^+$ DC[123]. Additionally, we demonstrated that employing poly I:C as an adjuvant combined with rHBVvac also efficiently and safely decreased HBV DNA, HBV RNA, and HBsAg in an HBV carrier mouse model. Importantly, we found that the therapeutic vaccine partially reversed immune tolerance and promoted HBV-specific $CD8^+$ T cell terminal differentiation into $KLRG1^+$ effector T cells, thereby playing a crucial role in HBV clearance[124].

Multiple therapeutic vaccines have been developed and administered for CHB treatment with different clinical outcomes. TG1050, a novel HBV-targeted immunotherapeutic vaccine based on a non-replicative adenovirus vector encoding multiple HBV antigens (S, core, and polymerase), effectively induced polyfunctional and long-lasting HBV-specific T cell responses and reduced HBV DNA and HBsAg levels *in vivo*. The results of a phase Ib trial showed that TG1050 induced the production of IFN-γ-producing HBV-specific T cells and safely achieved effective viral suppression[125]. Currently, TG1050 is being investigated in phase II clinical trials and may be a very promising therapeutic vaccine for HBV, especially in combination with TLR9 agonists. Additional therapeutic HBV vaccines under investigation in clinical trials worldwide including HBsAg-HBIG ("YIC", National Vaccine and Serum Institute, phase III)[126], Nasvac (CIGB, phase II/III)[127], GS-4774 (Gilead, phase II)[128], HepTcell (Altimmune, phase Ib), AIC 649 (AiCuris, phase I), INO-1800 (Inovio, phase I), HB-110 (Ichor, phase I), JNJ-64300535 (Janssen, phase I), TomegaVax HBV (TomegaVax, preclinical), and VR-CHB01 (Vical, preclinical).

CONCLUSION

PEG-IFN-α and NAs are the current major treatment strategies for CHB. Although these antiviral drugs can partly inhibit and eliminate HBV, viral breakthroughs may result from non-adherence due to limitations such as the high cost, the emergence of viral resistance, a long treatment cycle, and adverse side effects. Ideal anti-HBV strategies should meet the following criteria: the strong ability to inhibit virus replication with low drug resistance; stimulation of antiviral immune responses; Long-lasting effects without recurrence; and eventual removal of the virus. Therefore, the design of novel strategies to clear HBV and achieve long-lasting immune control remains a challenging task. Persistent CHB infection arises as a consequence of the complex interactions between HBV and the host innate and adaptive immune systems. Therefore, understanding the immunological mechanisms involved in CHB infection is important for designing potential therapeutic strategies for clinical CHB treatment. In this review, we detailed the immune biological characteristics and escape mechanisms of HBV and discussed novel immune-based therapies. Targeting a combination of viral and host factors provides the best possible chance for achieving a functional cure for CHB.

FOOTNOTES

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Country/Territory of origin: China

ORCID number: Hua-Jun Zhao 0000-0001-6619-8329; Yi-Fei Hu 0000-0001-8619-6558; Qiu-Ju Han 0000-0002-6511-2308; Jian Zhang 0000-0001-5106-1397.

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MINIREVIEWS

Dualistic role of platelets in living donor liver transplantation: Are they harmful?

Chen Liang, Kazuhiro Takahashi, Kinji Furuya, Nobuhiro Ohkohchi, Tatsuya Oda

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Chen Liang, Kazuhiro Takahashi, Kinji Furuya, Nobuhiro Ohkohchi, Tatsuya Oda, Gastrointestinal and Hepatobiliary-Pancreatic Surgery, University of Tsukuba, Tsukuba 3058575, Ibaraki, Japan

Corresponding author: Kazuhiro Takahashi, MD, PhD, Assistant Professor, Surgeon, Gastrointestinal and Hepatobiliary-Pancreatic Surgery, University of Tsukuba, Tennodai 1-1-1, Tsukuba 3058575, Ibaraki, Japan. kazu1123@md.tsukuba.ac.jp

Abstract

Platelets are anucleate fragments mainly involved in hemostasis and thrombosis, and there is emerging evidence that platelets have other nonhemostatic potentials in inflammation, angiogenesis, regeneration and ischemia/reperfusion injury (I/R injury), which are involved in the physiological and pathological processes during living donor liver transplantation (LDLT). LDLT is sometimes associated with impaired regeneration and severe I/R injury, leading to postoperative complications and decreased patient survival. Recent studies have suggested that perioperative thrombocytopenia is associated with poor graft regeneration and postoperative morbidity in the short and long term after LDLT. Although it is not fully understood whether thrombocytopenia is the cause or result, increasing platelet counts are frequently suggested to improve posttransplant outcomes in clinical studies. Based on rodent experiments, previous studies have identified that platelets stimulate liver regeneration after partial hepatectomy. However, the role of platelets in LDLT is controversial, as platelets are supposed to aggravate I/R injury in the liver. Recently, a rat model of partial liver transplantation (LT) was used to demonstrate that thrombopoietin-induced thrombocytosis prior to surgery accelerated graft regeneration and improved the survival rate after transplantation. It was clarified that platelet-derived liver regeneration outweighed the associated risk of I/R injury after partial LT. Clinical strategies to increase perioperative platelet counts, such as thrombopoietin, thrombopoietin receptor agonist and platelet transfusion, may improve graft regeneration and survival after LDLT.

Key Words: Platelet; Liver transplantation; Regeneration; Ischemia/reperfusion injury; Kupffer cell; Oxidative stress

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Core Tip: Perioperative thrombocytopenia is considered to be associated with poor graft regeneration and postoperative morbidity in the short and long term after living donor liver transplantation (LDLT). This review presented recent evidence for the role of platelets in LDLT based on clinical and basic studies. Platelets have both beneficial and detrimental effects on liver grafts, with a generally positive role in liver regeneration and a potentially negative role in ischemia/reperfusion injury. As increasing perioperative platelet counts are suggested to improve graft regeneration and survival, "platelet therapy" may provide prophylactic or therapeutic strategies to enhance the beneficial effects of LDLT.

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INTRODUCTION

Living donor liver transplantation (LDLT) has been developed as an important option for patients with end-stage liver disease, particularly in the virtual absence of deceased donors. During LDLT, changes in the platelet count and platelet function may occur, and these alterations may lead to deterioration of hemostatic function[1]. Transient thrombocytopenia has been considered as a common phenomenon after LDLT[2]. It is characterized by an average reduction of 60% in platelet counts on postoperative day (POD) 3 and recovers to normal levels on POD 10 after LDLT[3]. The reduction in platelet number can be caused by hemodilution, immunologic reactions, decreased platelet production, or sequestration of platelets in the liver graft upon reperfusion[1]. Moreover, platelet function declines during LDLT, as it was demonstrated that a large number of degranulated platelets were detected in the sinusoids of the liver graft after reperfusion[4].

Recent studies have suggested that postoperative thrombocytopenia is not simply an academic observation but is associated with catastrophic events, such as postoperative bleeding, cerebral hemorrhage and infection, which eventually lead to poor graft regeneration, increased postoperative morbidity and decreased patient survival in the short and long term after LDLT[5]. However, the precise mechanism is unknown, and it is unclear whether increasing perioperative platelet counts could improve posttransplant outcomes. The aim of this article is to summarize and discuss the clinical and experimental evidence of the role of platelets in LDLT. We also referred to the potential beneficial and detrimental effects of "platelet therapy" in the form of thrombopoietin (TPO) receptor agonists that augment graft regeneration.

PLATELETS

Platelets are anucleate fragments of cytoplasm derived from megakaryocytes in the bone marrow[6]. The average life span of circulating platelets is approximately 9 d, and they are destroyed by phagocytosis in the spleen and liver[7]. The main function of platelets is to react to hemorrhage by clumping and initiating blood clots[8], which are regulated and kept in balance in hemostasis. However, multiple changes occur in patients with chronic liver disease and post transplantation (LT) conditions, including changes in prohemostatic and antihemostatic pathways, which may consequently lead to either bleeding diatheses or thrombotic disorders[9]. Clinical approaches to increase platelet levels are necessary to compensate for the increased blood loss and requirements for platelets. However, due to the fear of thrombosis and transfusion-related injury[5], the safety and strategies of increasing perioperative platelet counts are still under debate.

Apart from the well-known role of platelets in hemostasis, there is emerging evidence that platelets have other functions in inflammation, angiogenesis, immune response, wound healing, regeneration, and ischemia/reperfusion (I/R) injury[10-12]. Platelets contain three types of secretory granules: alpha granules, dense granules, and lysosomal granules. Each granule contains physiological substances such as platelet-derived growth factor (PDGF), hepatocyte growth factor (HGF), insulin-like growth factor-1 (IGF-1), vascular endothelial growth factor (VEGF), serotonin, epidermal growth factor (EGF), and transforming growth factor-β[13-16]. When platelets are activated in specific situations, these biologically active substances are released and may induce nonhemostatic processes. All these physiological or pathological processes are involved in the alterations that occur in patients undergoing LDLT.

LDLT

LT is one of the most definitive choices for patients with end-stage liver disease and acute liver failure, and LDLT has been recognized as an important option for patients, particularly small pediatric patients and adults who are disadvantaged by the current deceased donor allocation system[17,18]. The feasibility of LDLT is based on the regenerative capacity of the liver, the evolution of surgical techniques in splitting the liver, and the widespread shortage of deceased liver grafts. In the LDLT procedure, a part of the healthy liver is surgically resected from a living person and transplanted to a recipient immediately after the recipient's diseased liver is removed[18]. After LDLT, the liver graft undergoes two different processes, namely, liver regeneration and I/R injury[18]. In liver regeneration, the remnant partial liver graft has to rapidly grow to meet the demands of the recipient's reduced metabolic and synthetic capacities[19]. At the same time, reactive oxygen species (ROS) and inflammatory factors are generated, leading to various responses related to I/R injury[20].

LDLT is sometimes associated with impaired regeneration and severe I/R injury in the liver graft, resulting in small-for-size syndrome (SFSS). SFSS is usually induced by size mismatching between donors and recipients and is characterized by synthetic dysfunction, elevated aminotransferases, and prolonged cholestasis[21]. The increased transaminitis and cholestasis may be attenuated with supportive care and time after LDLT, but sometimes irreversible damage, such as hypoglycemia, cholestasis, encephalopathy, renal failure and acidosis, may occur, which could be critical for recipients [21]. Thus, strategies to improve graft conditions are essential in clinical practice.

LIVER REGENERATION

Liver regeneration is mainly mediated by the proliferation of hepatocytes. In addition, nonparenchymal cells such as Kupffer cells (KCs), liver sinusoidal endothelial cells (LSECs), and hepatic stellate cells contribute to liver growth *via* their own proliferation and proliferation-stimulatory effects on hepatocytes[22]. Proliferation is generated when normally quiescent parenchymal cells and nonparenchymal cells undergo one or two rounds of replication to restore liver mass by a process of compensatory hyperplasia[23]. Liver regeneration is usually induced under two conditions: trauma or surgical resection-induced tissue loss and toxins or virus-induced hepatocellular death[24]. Hepatic progenitor cells are liver stem cells with differentiation capacities that can be activated during hepatic stress or injury. According to the participation of hepatic progenitor cells, the origin of the cells compensating for liver mass could be different. The regenerative process after tissue loss is usually driven by some of the existing cells in the liver without activating the progenitor cell compartments. In contrast, when acute liver failure is induced by some toxins, such as galactosamine, intrahepatic progenitor cells can replicate and differentiate into different cell types, such as cholangiocytes, hepatocytes and epithelial cells, to compensate for impaired liver functions[25].

Due to the central role of the liver in body homeostasis, intensive research was conducted to identify factors that might contribute to hepatic growth and regeneration. The essential circuitry required for liver regeneration encompasses three types of pathways, namely, cytokine, growth factor, and metabolic pathways that link liver function with cell growth and proliferation. Tumor necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6) are important cytokines involved in liver regeneration, as it was reported that both liver mRNA and serum levels of TNF-α and IL-6 stimulated liver regeneration after hepatectomy [26]. The elevations in TNF-α and IL-6 lead to the activation of the transcription factors nuclear factor-kappa B (NF-κB) and signal transducer and activator of transcription 3 (STAT3), which consequently increase the expression levels of cyclin D1 and trigger cellular proliferation[27]. In addition, growth factors, such as HGF, EGF, IGF-1 and PDGF, play essential roles in driving cell cycle progression during liver regeneration[28]. With the release of growth factors, numerous intracellular signaling pathways are activated to regulate liver regeneration.

LIVER I/R INJURY

I/R injury is tissue damage induced when the blood supply returns to tissue after a period of ischemia or hypoxia. It is an important cause of liver damage during hepatectomy and LT, which consequently induces graft dysfunction after surgery[29]. During the ischemic period, the absence of oxygen creates a condition in which inflammation and oxidative damage accumulate in the tissue under oxidative stress, which results in deregulation of the phenotype of all liver cellular components[29].

LSECs, which are essential in controlling vascular homeostasis and toxicant clearance, are especially vulnerable to I/R injury[30]. It was described that I/R injury could induce membrane discontinuation, vacuolization, and cell shape rounding in LSECs[31]. Concomitant with the deregulation of LSECs, the lack of oxygen and energy during the ischemic period produces edema in KCs, and biomolecules, such as damage-associated molecular patterns or pathogen-associated molecular patterns, can be released by neighboring hepatic cells to activate KCs[32]. Activated KCs can initiate the inflammatory response by

releasing ROS and proinflammatory cytokines, including TNF-α, interleukin-1, interferon-c and interleukin-12[33].

I/R injury is associated with two forms of cell death, namely, apoptosis and necroptosis. Apoptosis is a form of programmed cell death that is characterized by a series of cellular alterations, such as DNA breaks, plasma membrane blebbing, cell shrinkage and chromatin condensation[34]. Most of the biochemical and morphological changes in cells are mediated by a subset of the caspase family. Necroptosis, which is a programmed form of necrosis, occurs from extracellular signals or intracellular cues and involves the process of cellular swelling, plasma membrane rupture, and the release of proinflammatory molecules[35]. In the process of apoptosis, TNF-α leads to the activation of initiator caspases such as caspase-8 and caspase-10. These caspases cleave and activate downstream effector caspases, including caspase-3 and caspase-7, which promote the release of pro-apoptotic molecules to execute apoptosis[34]. Necroptosis is also typically driven in response to the engagement of TNF-α. Activation of the TNF receptor facilitates receptor-interacting protein kinase (RIPK) 1 to assemble with RIPK3 and concomitantly phosphorylates mixed lineage kinase domain-like (MLKL), which is a crucial downstream effector protein of necroptosis. The phosphorylation of MLKL induces plasma membrane permeabilization and the release of cell damage-associated molecular patterns, which results in cell destruction[36]. Overall, TNF-α serves as a central regulator in the process of apoptosis and necroptosis during hepatic I/R injury.

EVIDENCE FROM CLINICAL STUDIES

Platelets and partial hepatectomy

Hepatectomy is the surgical resection of the liver mainly performed for the treatment of primary or metastatic hepatic malignancies. This technique is conducted based on the regeneration capacity of the liver. Although surgical techniques and perioperative management have been substantially improved in recent years, partial hepatectomy is still associated with a high postoperative mortality rate of 1% to 5% [37]. Perioperative thrombocytopenia has been recognized as a common phenomenon during liver resection. It was reported that platelet counts drop immediately after surgery with a nadir on POD 2–3 and return to normal levels by POD 14[38]. The potential reasons concerning preoperative thrombocytopenia may be decreased platelet production, hemodilution, splenic sequestration, medications, or infections[2], but the precise mechanism remains unclear.

Recently, the association of the perioperative platelet count with posthepatectomy liver failure and mortality has been investigated. By conducting retrospective studies, several researchers stated that a low postoperative platelet count was associated with poor recovery and worse outcomes after liver surgery[37,39]. Takahashi *et al*[40] reported that a greater than 40% decrease in the platelet count was an independent risk factor for delayed liver function recovery after partial hepatectomy. They observed that the platelet count in the delayed recovery group returned to preoperative levels significantly later than that in the adequate recovery group, which indicated that the extra platelets were consumed to compensate for the delayed recovery, resulting in delayed restoration of the platelet counts in the delayed recovery group[40]. In addition, several other parameters regarding perioperative platelet counts, such as the platelet-to-lymphocyte ratio, alkaline phosphatase-to-platelet ratio index, aspartate aminotransferase to platelet count ratio index, and fibrosis-4 index, were reported to be effective criteria for predicting poor surgical outcomes after partial hepatectomy[5]. Although the underlying mechanisms are not fully understood, these reports indicated that increasing the perioperative platelet count may improve the outcomes after partial hepatectomy.

Platelets and deceased donor liver transplantation

The total number of deceased donor liver transplantation (DDLT) has dramatically increased with innovations in both immune suppression and surgical techniques. Posttransplant thrombocytopenia has been recognized as a common phenomenon since the prevalence of DDLT began to increase[1]. In 1968, it was first reported that an acute drop in platelet count to less than 10×10^3/μL was observed on POD 3 in some patients undergoing DDLT[1]. By using ^{111}In-labeled platelets, researchers found that transplant recipients had a delayed recovery of platelet counts after DDLT[41]. Subsequent studies have demonstrated that retransplantation, low preoperative platelet counts, massive intraoperative platelet transfusions, and poor general preoperative conditions were factors associated with posttransplant thrombocytopenia[42]. However, they did not pay attention to the meaning of posttransplant thrombocytopenia in DDLT.

The first report clarifying the relationship between thrombocytopenia and DDLT was presented in 1992, when McCaughan *et al*[43] conducted an analysis of a large cohort of 541 DDLT patients and identified that the decreased counts after DDLT were an independent risk factor for graft survival. Since then, several consecutive studies have been reported to demonstrate perioperative thrombocytopenia as a negative factor for grafts and patient survival in the short and long term after DDLT[42,44,45]. In 2014, Lesurtel *et al*[9] suggested the 60-5 criteria in which a platelet count of $< 60 \times 10^3$/μL on POD 5 was an independent risk factor associated with severe postoperative complications, early graft failure, and

patient mortality in the short term after DDLT.

Although clinical studies have identified that postoperative thrombocytopenia deteriorates graft and patient survival after DDLT[9], thrombocytosis has not been proven to be a positive factor for DDLT. Some studies stated that a higher preoperative platelet count was associated with I/R injury and arterial thrombosis in DDLT[46,47]. As a result, it is difficult to perform prospective trials by increasing perioperative platelet counts.

Platelets and LDLT

LDLT is different from DDLT in that the partial liver graft needs to regenerate under the condition of I/R injury[18]. Transient thrombocytopenia has been regarded as an independent risk factor for LDLT. Several separate authors stated that a low postoperative count had a higher chance of developing early allograft dysfunction and was a strong predictor of postoperative complications in recipients undergoing LDLT[3,48]. It was demonstrated that an immediate posttransplant platelet count of < 68 × 10^3/μL or a platelet count of < 30 × 10^3/μL on POD 3 was an independent risk factor for major postoperative complications and was associated with early graft dysfunctions[3,48]. Takahashi *et al*[19] reported that a platelet count of < 60 × 10^3/μL on POD 5 was independently associated with the incidence of postoperative morbidity in the mid-term after LDLT and was especially related to small-for-size syndrome such as ascites and infection.

Increasing perioperative platelet counts has been considered to be positively associated with LDLT. Kim *et al*[49] performed a retrospective study in a population of 87 recipients with LDLT and reported that the number of platelets transfused was significantly associated with graft regeneration. Moreover, some consecutive studies were conducted to provide further evidence regarding the benefits and risks of platelet transfusion. They described that platelet transfusion enhanced graft regeneration in recipients after LDLT without increasing morbidity and mortality rates[50,51].

Living donor hepatectomy is sometimes associated with postoperative complications, leading to posthepatecomy liver failure. Previous studies reported that the morbidity rates in liver donors ranged from 8.3% to 78.3%[52,53]. The remnant liver volume ratio, which was recommended to exceed the minimum of 30% to 35% for donor safety[54], is closely related to postoperative morbidity such as liver failure, and platelets have been highlighted as playing an important role in this condition. Yoshino *et al*[55] retrospectively collected data from 254 donors undergoing LDLT and showed that a lower preoperative platelet count was an independent risk factor for postoperative complications, such as bile leakage, subphrenic effusion, infectious ascites, postoperative anemia, and liver failure, after living donor hepatectomy. Emond *et al*[56] demonstrated that even in healthy donors, the fluctuation of platelet count within the normal range was negatively associated with potential portal hypertension and subclinical liver dysfunction, indicating that platelet count might serve as a surrogate marker to predict potential liver failure in healthy donors.

Although postoperative thrombocytopenia after LDLT was associated with low graft regeneration, it is unclear whether postoperative thrombocytopenia is the "cause" of low graft regeneration or just a "result" that appears as an unfavored postoperative condition of the patients. As posttransplant thrombocytopenia was reported to be associated with LDLT, clinical studies concerning this field are necessary. However, due to the fear of thrombosis and other complications, strategies to increase platelet counts are difficult to implement in clinical practice. Thus, basic studies explaining the precise mechanism of platelets in liver regeneration, I/R injury and LT are warranted.

EVIDENCE FROM BASIC STUDIES

The role of platelets in liver regeneration

Platelets are considered to stimulate liver regeneration, as they can secrete physiological substances such as IGF-1 and HGF[57], which play important roles during liver regeneration[22]. In addition, platelet-derived serotonin was demonstrated to be an inducer of liver regeneration, as it was reported that the liver failed to regenerate after partial hepatectomy in mice lacking intraplatelet serotonin[11]. Previous studies revealed that platelets accumulated in the liver after hepatectomy with a 2-fold increase compared with prehepatectomy levels[58], and electron microscopy showed that platelets translocated from the sinusoidal space into the space of Disse and directly contacted hepatocytes[59]. It was shown that marked changes in proliferation-related signaling pathways and mitosis occurred after changing the platelet levels in mice after hepatectomy[59]. These results suggest that platelets accumulate in the liver after hepatectomy and may provide signals for rapid hepatocyte proliferation.

It was suggested that direct contact between platelets and hepatocytes contributed to liver regeneration. When recruited in the liver, platelets translocate from the liver sinusoids to the space of Disse and trigger the release of soluble mediators from platelets such as HGF, IGF-1, serotonin and VEGF, which leads to hepatocyte proliferation[60]. LSECs and KCs were also reported to interact with platelets to stimulate liver regeneration. It was identified that platelets induced the release of IL-6 from LSECs through direct contact with LSECs[61]. On the other hand, platelets could attach to KCs, and the hepatic expression of TNF-α and IL-6, which are predominantly produced by KCs, increased in response

to the interaction between platelets and KCs[62]. Due to the secretion and stimulation capacities of platelets, researchers found that the TNF-α/NF-κB, IL-6/STAT3, and phosphatidylinositol 3-kinase (PI3K)/Akt pathways are the three major cascades in which platelets exert their effects during the process of liver regeneration[62]. The pathways are associated with the transition of quiescent hepatocytes to the cell cycle and progression beyond the restriction point in G1 phase of the cycle[22], which finally stimulates hepatocyte proliferation.

In addition, platelet-derived messenger RNA was considered to have an impact on liver regeneration. By coculturing platelets with hepatocytes, it was found that platelets accumulated in the perinuclear region of hepatocytes, and messenger RNA from platelets was transferred throughout the hepatocyte cytoskeleton[63]. This result suggested that platelets were internalized into hepatocytes and transferred proliferation-related messenger RNA and stimulated hepatocyte proliferation[63].

Overall, basic studies have identified that platelet-derived liver regeneration occurs through four different mechanisms: (1) direct effects on hepatocytes; (2) cooperative effects with LSECs; (3) collaborative effects with KCs; and (4) the transfer of messenger RNA to hepatocytes (Figure 1).

The role of platelets in I/R injury

There is emerging evidence that platelets have pathological functions in hepatic I/R injury. Cywes *et al* [12] used a reperfusion model to study the contribution of platelets to I/R injury. They isolated the rat liver and perfused the liver *ex vivo* with Krebs-Henseleit solution containing platelets. They speculated that the degree of platelet adherence to LSECs was related to hepatic injury in perfused rat livers[12], and the number of apoptotic LSECs increased by 6-fold in isolated liver perfused with platelets. These reports indicated that platelets are directly responsible for hepatic injury and contribute to the development of apoptosis in LSECs after reperfusion. Adhesion molecules such as selectins and integrins, which are expressed on platelets and LSECs, are thought to mediate the interaction between platelets and LSECs and result in liver damage[10].

KCs are considered to act in synergy with platelets in the mechanism of I/R injury, as activated KCs release a large amount of both proinflammatory and anti-inflammatory mediators, such as TNF-α, IL-6, interleukin-10 and interleukin-13, which aggravate liver injury[64]. Electron microscopy showed platelets attached to KCs in the early period after hepatic ischemia[65]. It was demonstrated that platelet-related I/R injury after hepatic reperfusion was mainly characterized by the activation of KCs, which potentially release proinflammatory cytokines and generate ROS[66].

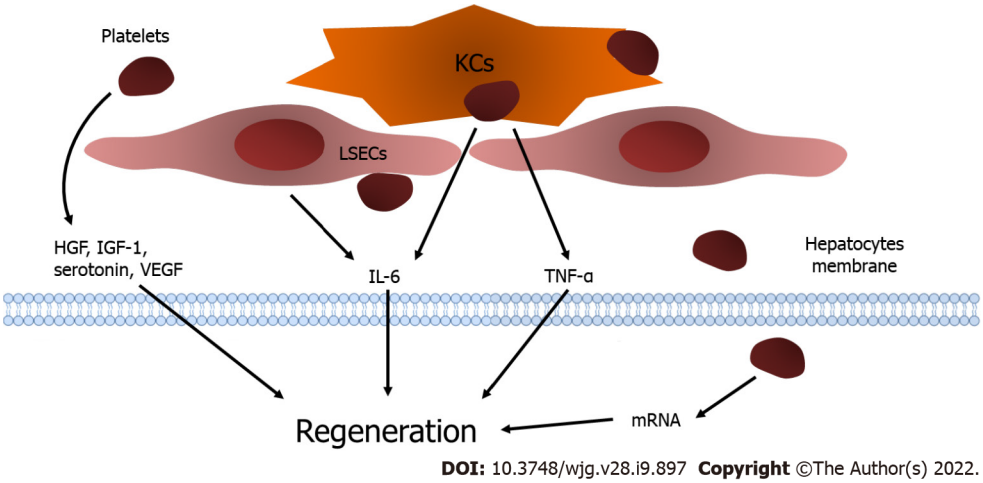
ROS, which contribute to inflammatory responses in I/R injury[67], are pivotally related to platelets. First, oxidases or proinflammatory molecules located in platelets are able to produce ROS[68]. Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase is considered to be the most relevant source of ROS in platelets. Patients with a hereditary deficiency of NADPH oxidase had an almost complete loss of platelet-related ROS products[69]. Xanthine oxidase is another potential source of ROS[70], but its precise relationship with platelet physiology is still unclear. In addition, platelet proinflammatory molecules, such as P-selectin and CD40 ligand, are demonstrated to be associated with intraplatelet ROS generation[71]. Second, ROS formation is functionally associated with platelet activation. It has been reported that catalase, which can reduce the cytosolic concentration of hydrogen peroxide, inhibits platelet aggregation[72]. Moreover, the inhibition of NADPH oxidase by chemical inhibitors, such as diphenyleneiodonium and apocynin, was observed to be related to the suppression of platelet activation [73].

In contrast, platelets have been demonstrated to indirectly inhibit I/R injury. Oberkofler *et al*[74] reported that the platelet-serotonin-VEGF-interleukin 10/matrix metalloproteinase 8 axis mediated the protective effects of preconditioning on I/R injury in mice. Additionally, it was reported that inducible nitric oxide synthase, an aggravating enzyme for I/R injury[75], was inhibited in macrophages after coculture with platelets under lipopolysaccharide-induced inflammatory conditions[76].

Although the role of platelets in hepatic I/R injury is controversial, it is supposed that platelets could directly aggravate hepatic I/R injury in three ways: (1) adhesion to LSECs; (2) cooperative effects with KCs; and (3) platelet-derived ROS formation (Figure 2).

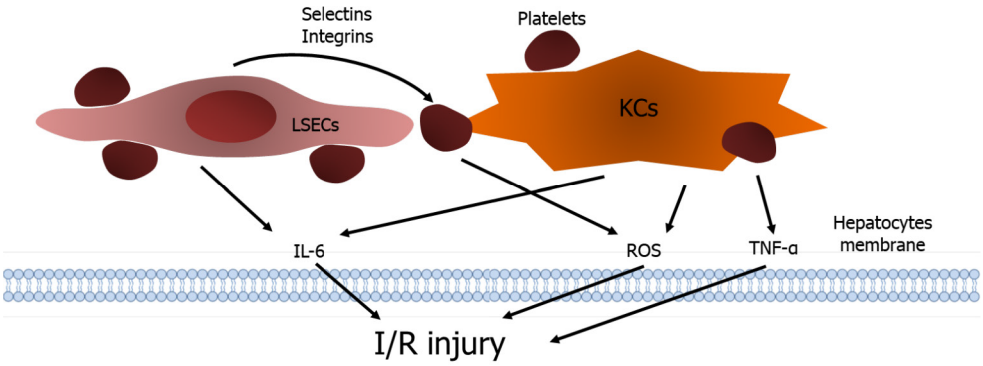
The role of platelets in partial LT

Platelets are suggested to be positively associated with LDLT in that partial liver grafts require postoperative liver regeneration under I/R injury[77]. This is compatible with previous studies that proved that platelets stimulate liver regeneration after hepatectomy in animal models[59]. Although the positive role of higher perioperative platelet counts has been suggested, the precise mechanisms clarifying how platelets interact with other cells under I/R conditions were reported recently. Liang *et al* [61] reported that TPO-induced preoperative thrombocytosis contributed to a better outcome in a rat model of partial LT. In this study, platelets stimulated liver regeneration after partial LT *via* several proliferation-related cytokines and pathways. I/R injury was not aggravated, as shown by unchanged levels of aggravating parameters such as ROS, apoptosis or necrosis. They further used a critical model of 20% partial LT and identified that thrombocytosis could prolong the survival rate in rats. This research explained that thrombocytopenia is not a "result" but a "cause" of postoperative complications.



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Figure 1 Platelets and liver regeneration. Platelets translocate into the space of Disse and release insulin-like growth factor-1, hepatocyte growth factor, and vascular endothelial growth factor. The direct contact of platelets with liver sinusoidal endothelial cells (LSECs) results in the excretion of interleukin-6 (IL-6) from LSECs. In addition, the attachment of platelets activates Kupffer cells (KCs) and enhances the release of tumor necrosis factor-alpha and IL-6 from KCs to promote liver regeneration. Moreover, platelets are internalized into hepatocytes and trigger the functional transfer of messenger RNA stored in platelets, which stimulates hepatocyte proliferation. KCs: Kupffer cells; LSECs: Liver sinusoidal endothelial cells; IGF-1: Insulin-like growth factor-1; HGF: Hepatocyte growth factor; VEGF: Vascular endothelial growth factor; LSECs: Liver sinusoidal endothelial cells; IL-6: Interleukin-6; KCs: Kupffer cells; TNF-α: Tumor necrosis factor-alpha.



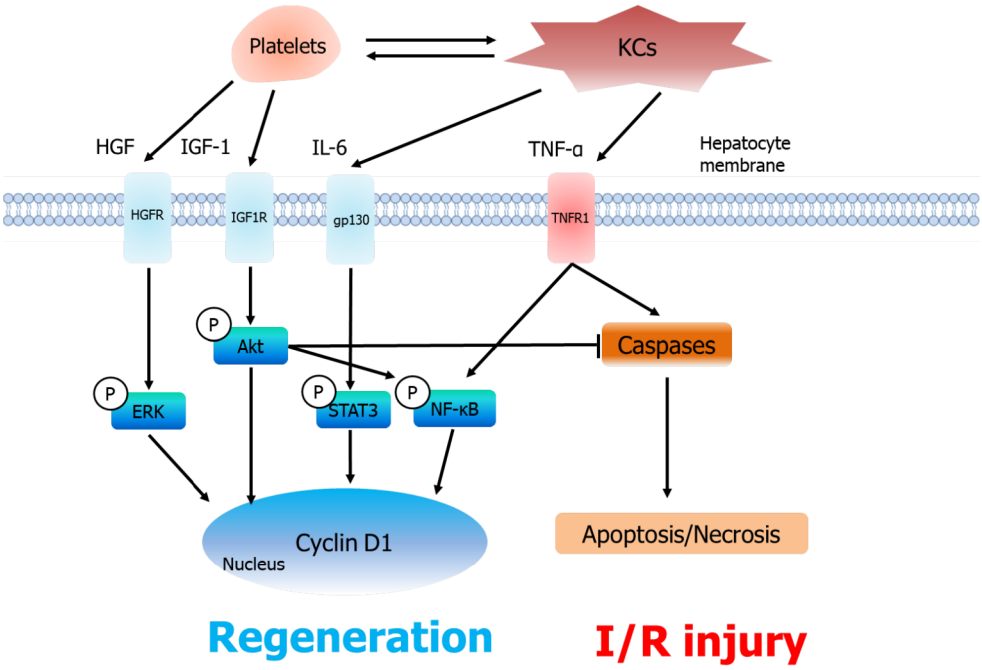
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Figure 2 Platelets and ischemia/reperfusion injury. Liver sinusoidal endothelial cells (LSECs) express selectins and integrins to stimulate the interaction between platelets and LSECs, and platelets result in the excretion of interleukin-6 (IL-6) from LSECs. The generation of tumor necrosis factor-alpha, IL-6 and reactive oxygen species (ROS) from KCs is elevated after the cooperative effect between platelets and KCs. Furthermore, platelets can produce ROS independently and consequently aggravate ischemia/reperfusion injury. KCs: Kupffer cells; LSECs: Liver sinusoidal endothelial cells; IL-6: Interleukin-6; TNF-α: Tumor necrosis factor-alpha; ROS: Reactive oxygen species; I/R injury: Ischemia/reperfusion injury.

The most ambiguous factor concerning platelets and partial LT is TNF-α, which is a pleiotropic cytokine possessing two opposite effects on hepatocytes, namely, promoting proliferation and inducing apoptosis. TNF-α binds to its receptor and activates signaling pathways such as the NF-κB pathway and cyclin protein families to stimulate cellular proliferation[78]. On the other hand, TNF-α can induce apoptosis through caspase cascades[78]. The Akt signaling pathway, which could be activated by IGF-1 [59], was reported to suppress TNF-α-mediated apoptosis through NF-κB activation[78,79]. It was supposed that the elevated secretion of IGF-1 under thrombocytosis enhanced the phosphorylation of Akt and NF-κB and consequently prevented liver grafts from undergoing apoptosis[61]. However, direct evidence proving the interaction between the Akt pathway and IGF-1 or TNF-α was not provided in previous studies. Partial transplantation models using Akt agonists or inhibitors are necessary to clarify the precise mechanisms (Figure 3).

PERSPECTIVES FOR PLATELET THERAPY

Platelet transfusion and TPO receptor agonists are some alternatives to increase perioperative platelet levels in the clinical setting. Platelet transfusion in LT has been controversial, as prophylactic platelet transfusion was reported to have a prothrombotic effect in patients with liver disease[80]. On the other



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Figure 3 Platelets, liver regeneration and ischemia/reperfusion injury after partial liver transplantation. After accumulating in the liver graft, platelets excrete hepatocyte growth factor (HGF) and insulin-like growth factor-1 (IGF-1) and collaborate with KCs to increase the release of interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF-α). As a result, the serum levels of HGF, IGF-1, IL-6 and TNF-α increase under thrombocytosis, which consequently induces the phosphorylation of the ERK, Akt, STAT3 and nuclear factor-kappa B signaling pathways to promote liver regeneration (Cyclin D1). On the other hand, platelets do not aggravate in ischemia/reperfusion injury. The phosphorylated Akt pathway inhibits TNF-α-induced apoptosis and necrosis in the liver graft. KCs: Kupffer cells; HGF: Hepatocyte growth factor; IL-6: Interleukin-6; IGF-1: Insulin-like growth factor-1; TNF-α: Tumor necrosis factor-alpha; HGFR: HGF receptor; IGF1R: IGF-1 receptor; gp130: Glycoprotein 130; TNFR1: Tumor necrosis factor receptor; ERK: Extracellular signal-regulated kinase; STAT3: Signal transducer and activator of transcription 3; NF-κB: Nuclear factor-kappa B; Caspase: Cysteinyl aspartate specific proteinase; I/R injury: Ischemia/reperfusion injury.

hand, platelet transfusion has been considered to have positive effects on LDLT due to the regeneration capacity of the liver graft, according to previous studies[49-51]. However, several potential problems, such as anaphylaxis reaction, platelet transfusion refractoriness, and transfusion-related lung injury, could be critically harmful to patients[81,82]. Clinical studies have indicated that TPO receptor agonists are more effective than platelet transfusion for chronic liver disease with thrombocytopenia, as shown by the success ratio, effect duration, and nonincidence rate of adverse events[83].

TPO receptor agonists are currently the main focus of pharmaceutical treatment options for thrombocytopenia[84]. There are currently four types of TPO receptor agonists on the market: eltrombopag, avatrombopag, lusutrombopag, and romiplostim. Eltrombopag, avatrombopag, and lusutrombopag are oral TPO receptor agonists approved for patients with thrombocytopenia, and romiplostim is a subcutaneous TPO receptor agonist[85]. Eltrombopag was revealed to increase platelet counts significantly in patients with chronic liver disease, along with an antitumor effect on hepatocellular carcinoma[86]. However, eltrombopag was reported to induce hepatic decompression and thromboembolic events[5]. In addition, romiplostim was shown to have serious side effects leading to bone marrow reticulin fibrosis[87]. Avatrombopag was proven to be generally well tolerated without the occurrence of hepatotoxicity[88]. Although a few thrombotic events were reported[85], there were no serious adverse effects or critical events reported in previous studies. Lusutrombopag was recently released for patients with thrombocytopenia in Japan and the USA[83], with additional effects being reported such as an increase in hematocytes in a patient with compensated liver cirrhosis[89]. There is still no report regarding the side effects of lusutrombopag in clinical practice. For these reasons, avatrombopag and lusutrombopag are promising and may serve as a suitable "platelet therapy" to increase perioperative platelet counts.

CONCLUSION

This review presented accumulated evidence for the role of platelets in LT, especially LDLT, based on clinical and basic studies. Platelets have both beneficial and detrimental effects on liver grafts, with generally positive roles in liver regeneration and potentially negative roles in I/R injury. Clinical and basic studies have broadened our horizons about altering platelet counts in patients undergoing LDLT, and "platelet therapy" may provide prophylactic or therapeutic strategies to enhance the beneficial

effects on LDLT.

FOOTNOTES

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Country/Territory of origin: Japan

ORCID number: Chen Liang 0000-0002-4528-7303; Kazuhiro Takahashi 0000-0003-1089-0644; Kinji Furuya 0000-0002-2630-3072; Nobuhiro Ohkohchi 0000-0003-2779-1247; Tatsuya Oda 0000-0001-6115-0158.

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MINIREVIEWS

Applications of endoscopic ultrasound elastography in pancreatic diseases: From literature to real life

Clara Benedetta Conti, Giacomo Mulinacci, Raffaele Salerno, Marco Emilio Dinelli, Roberto Grassia

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Clara Benedetta Conti, Giacomo Mulinacci, Marco Emilio Dinelli, Interventional Endoscopy Unit, ASST Monza, Ospedale San Gerardo, Monza 20900, Italy

Raffaele Salerno, Gastroenterology and Endoscopy Unit, ASST Fatebenefratelli-Sacco, Milan 20121, Italy

Roberto Grassia, Gastroenterology and Endoscopy Unit, ASST Cremona, Cremona 26100, Italy

Corresponding author: Clara Benedetta Conti, MD, Consultant Physician-Scientist, Interventional Endoscopy Unit, ASST Monza, Ospedale San Gerardo, Via G.B. Pergolesi 30, Monza 20900, Italy. benedetta.conti1@gmail.com

Abstract

Elastography is a non-invasive method widely used to measure the stiffness of the tissues, and it is available in most endoscopic ultrasound machines, using either qualitative or quantitative techniques. Endoscopic ultrasound elastography is a tool that should be applied to obtain a complementary evaluation of pancreatic diseases, together with other imaging tests and clinical data. Elastography can be informative, especially when studying pancreatic masses and help the clinician in the differential diagnosis between benign or malignant lesions. However, further studies are necessary to standardize the method, increase the reproducibility and establish definitive cut-offs to distinguish between benign and malignant pancreatic masses. Moreover, even if promising, elastography still provides little information in the evaluation of benign conditions.

Key Words: Elastography; Pancreas; Pancreatic stiffness; Pancreatitis; Pancreatic cancer; Endosonography; Endoscopic ultrasound; Quantitative elastography; Strain elastography; Pancreatic diseases

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Core Tip: Elastography is largely available in most endoscopic ultrasound machines, using either qualitative or quantitative methods. The application of elastography in the study of pancreatic diseases should be considered as a complementary test, together with other imaging and clinical data. Elastography can help the clinician in the diagnosis of pancreatic masses, whereas there is still little information in the case of pancreatitis. Further studies are necessary to standardize the method and above all to establish definitive cut-offs to distinguish between benign and malignant pancreatic masses.

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INTRODUCTION

Elastography is a non-invasive method used to measure the stiffness of different tissues[1,2].

Nowadays, it is widely used in various medical fields, and it is available in most endoscopic ultrasound (EUS) machines as an integrated software. EUS stiffness measurement can be assessed using either a qualitative or a quantitative method.

Qualitative elastography

The qualitative technique, known as strain elastography, is a method based on the use of a colored scale: red-green areas indicate softer tissues, whereas blue areas indicate stiffer tissues. This type of measurement is useful but does not allow a quantitative measurement. Its clinical application is simple and intuitive, and this explains its large use. However, since the interpretation of different colors and images is largely operator dependent, it lacks reproducibility. Thus, it provides little information when comparing different patients, lesions, or diffuse diseases[3,4].

Semi-quantitative and quantitative elastography

Semi-quantitative analysis allows a numeric measurement of the tissue stiffness by using both the strain ratio (SR) and the strain histogram technique.

When using the SR, the operator sets a bigger region of interest (ROI), usually a circle or a square, on the target tissue (*i.e.* a lesion) and a smaller ROI above a reference tissue, usually a homogeneous soft area. The SR is calculated by dividing the stiffness data of the two regions, expressed either by the speed wave through the tissues (m/s) or converted to the Young's modulus (kPa)[5]. This technique is largely used in the evaluation of pancreatic lesions, where the reference tissue is the normal pancreas. However, despite its quantitative nature, this analysis only allows partial information of the tissue, without giving the mean and median stiffness values of the lesion.

Conversely, the strain histogram method generates an average hue histogram that graphically represents the colors and therefore the stiffness distribution within the tissue. The mean value of the histogram can thus be considered a good esteem of the real elasticity of the target[6,7]. This method can be more informative when facing a large tissue, such as a diffuse pancreatic disease, as it collects the stiffness of different areas and gives the operator a unique average value.

EUS shear wave measurement (SWM) allows a quantitative measurement of the stiffness, and it can be expressed in either m/s or in kPa[5]. This method provides a numeric value, usually a mean and a median value of the tissue stiffness. This technique is theoretically more informative than the semi-quantitative one, as it simplifies a comparison between individuals or diseases. The final value can be obtained by a single measurement or by calculating the mean and median value of repeated measurements. The operator sets the ROI within the target tissue or lesion and obtains several values through the repetition of the measurement, usually five or ten. Successively, the mean and median measurement value can be calculated. Hence, a more accurate stiffness assessment is provided[3,8,9]. Overall, the best image quality can be achieved when the lesion of interest covers up to 50% of the ROI [10]. This method, largely used in United States machines, is rarely available in EUS machines.

The large application of EUS elastography in the diagnosis of pancreatic diseases is relatively new. Moreover, its use in routine practice lacks a high diagnostic accuracy, especially due to the large use of the qualitative method. Accounting for the poor reproducibility and the absence of definitive diagnostic cut-offs, the chance to standardize the method is low. Therefore, the use and the interpretation of elastography in pancreatic diseases are still challenging for clinicians.

This review aims to describe the main applications of EUS elastography in pancreatic diseases, its practical value, and its main limitations.

We performed an accurate literature search using the following MESH terms: "Pancreas;" "pancreatitis;" "chronic pancreatitis;" "acute pancreatitis;" "pancreatic cancer;" "pancreatic lesion;" "elastography;" "elastography AND pancreas;" "pancreatic stiffness;" "endoscopic ultrasound;" "EUS AND elastography;" "ultrasonography AND elastography;" "quantitative elastography AND pancreatitis;" "elastography AND pancreatic cancer;" "autoimmune pancreatitis;" "elastography AND acute pancreatitis;" "focal autoimmune pancreatitis AND diagnosis;" "inflammatory pancreatic masses;" "elastography AND cancer;" and "NET AND elastography." We identified all the pertinent articles (reviews, original articles, and meta-analyses) published between 2000 and 2021 with an available abstract and/or full text. The reference lists from the selected studies were examined to identify further relevant reports. Only English language papers were included. The level of evidence and strength of recommendations were graded according to the Oxford Centre of Evidence-Based Medicine system as of the March 2009 update (http://www.cebm. net/oxford-centre-evidence-based-medicine-levels-evidence-march-2009).

CLINICAL APPLICATION OF EUS ELASTOGRAPHY IN PANCREATIC DISEASES

Application of elastography in benign pancreatic diseases

Chronic pancreatitis: The application of qualitative elastography in cases of chronic pancreatitis (CP) conventionally shows a predominantly green pattern with heterogeneous small red or blue areas[11] (Figure 1). Even if the colored scale adds more information to the B-mode imaging, it is still not pathognomonic for any specific disorder. Semi-quantitative elastography, using histogram or shear waves, aids the interpretation of the elastography data. This technique was used to retrospectively examine 96 patients with either diagnosed or suspected CP. Each patient was classified using Rosemont criteria (Table 1)[12], and elastography values were significantly different for each stage ($P < 0.001$)[13].

Similarly, another study measured pancreatic stiffness (PS) in 84 patients and revealed a significantly positive correlation with the Rosemont classification stage (rs = 0.54) and the number of EUS features (rs = 0.47). Area under the receiver operating characteristic curve (AUROC) for the accuracy of SWM-elastography (consistent with CP and suggestive for CP *vs* normal and indeterminate for CP) was 0.77 (sensitivity 77.1%, specificity 64.9%)[14]. In a multivariate linear regression, hyperechoic foci with shadowing and lobularity with honeycombing were independent features related to PS[10,11].

This data was confirmed by a prospective study with 191 patients, of which 92 were diagnosed with CP. A significant linear correlation was confirmed between the number of EUS criteria of CP and the SR ($r = 0.813$; $P < 0.0001$), with an AUROC of 0.949 [95% confidence interval (CI): 0.916-0.982]. The accuracy of EUS-elastography for diagnosing CP was 91.1% (cut-off SR of 2.25), and the SR varied significantly among different Rosemont classification groups ($P < 0.001$)[15]. Another study measured PS in both pancreatic tumors and healthy surrounding parenchyma of 58 consecutive patients before pancreatectomy. Histological fibrosis was divided into four stages: normal, mild, marked, and severe fibrosis. Using the mean PS value, the AUROC for the diagnosis of each fibrosis stage was 0.90[16]. These findings support the theory of PS measurement as a possible surrogate marker for pancreatic fibrosis.

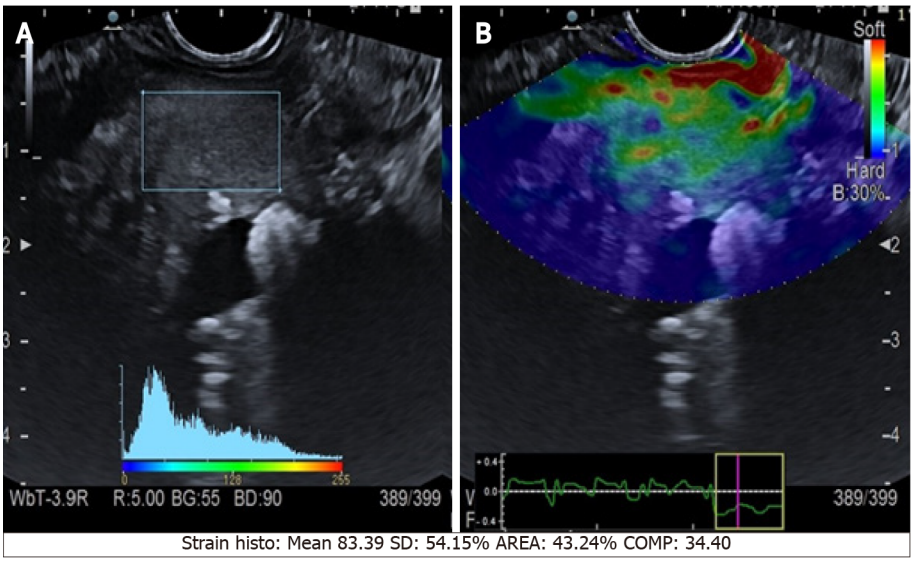
Moreover, a study evaluated 40 patients who underwent pancreatic EUS-SWM. They were classified following the Japan Pancreatic Society criteria for CP. They compared EUS-SWM values of the healthy pancreas with early, probable, and definite CP groups. Notably, the relationship between EUS-SWM value and exocrine/endocrine dysfunction was also assessed. A positive correlation resulted between PS values measured through EUS-SWM and the Japan Pancreatic Society criteria stages, with higher values registered in the CP group. AUROCs for the diagnostic accuracy of EUS-SW measurement for CP, exocrine dysfunction, and endocrine dysfunction were 0.92, 0.78, and 0.63, respectively. The cut-off values of 1.96, 1.96, and 2.34 for diagnosing CP, exocrine dysfunction, and endocrine dysfunctions had a sensitivity of 83%, 90%, and 75% and a specificity of 100%, 65%, and 64%, respectively[17]. Similar results were found by Domínguez-Muñoz *et al*[18]. In the final analysis, elastography seems a promising approach in the evaluation of pancreatitis, even if diagnostic cut-offs are still lacking. Further, large scale studies are awaited to improve the diagnostic accuracy.

Autoimmune pancreatitis: Autoimmune pancreatitis (AIP) is a rare condition, even if it is likely underdiagnosed. There is a wide spectrum of radiological features of AIP, from a normal pancreas to diffuse parenchymal enlargement or focal mass-like lesion. The latter radiological appearance is recognized as focal AIP, and it can be easily confused with pancreatic cancer (PC). Thus, it is important to avoid unnecessary surgeries. A correct diagnosis can be reached through the assessment of clinical, laboratory, and histocytological data combined with the EUS evaluation and the assessment of Rosemont Criteria (Table 1). Of note, elastography may help the clinician to differentiate AIP from PC. Indeed, the former usually shows a homogeneous increase of stiffness in the whole organ, while in the latter the increased stiffness is limited within the tumoral area[19]. When using qualitative elastography in AIP, the inflamed area should have a predominantly green pattern with slight red or yellow lines, while a homogeneous green pattern should appear in the normal parenchyma[11]. However, since this does not always occur, the clinician should not completely rely on this pattern presentation to reach the

Table 1 Rosemont criteria for chronic pancreatitis[12]

Diagnostic criteria		Major A	Major B	Minor
Pancreas	Parenchyma	Hyperechoic foci with acoustic shadows; body/tail	Honeycomb-like lobulation; body/tail	Lobulation without honeycombing; body/tail
				Hyperechoic foci without acoustic shadows; body/tail
				Cysts
				Echo-dense septa; body/tail
	Duct	Stones in the duct	None	Irregular duct; body/tail
				Dilated side ducts; body/tail
				Dilated main duct; body/tail
				Hyperechoic contours on the main duct; body/tail

According to them, it is defined as: Definitive: 1 major A + ≥ 3 minor or 1 major A + 1 major B or 2 major A; Suspected: 1 major A + < 3 minor or 1 major B + ≥ 3 minor or ≥ 5 minor; Possible: 3 or 4 minor, no major or major B alone or with < 3 minor; Normal: < 3 minor, no major.



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Figure 1 Quantitative endoscopic ultrasound elastography of chronic pancreatitis. A: Endoscopic ultrasound B-mode with region of interest square; B: Elastographic image.

diagnosis.

The quantitative elastography could theoretically be more informative to diagnose AIP. As such, a study measured the stiffness of 123 lesions (78 PC cases and 45 focal AIP cases), and the SR between the lesion and the surrounding parenchyma correlated significantly with the actual presence of a malignancy[20]. Similarly, in a prospective study of 325 patients assessing the SR lesion/parenchyma, a cut-off value of 4.2 *vs* 10.9 correlated to sensitivity, specificity, positive predictive value, negative predictive value, and accuracy of 95%, 63%, 89%, 81%, and 87%, respectively *vs* 75%, 88%, 95%, 54%, and 79%, respectively[21].

Moreover, in a meta-analysis that included 17 studies (1544 lesions), the pooled sensitivity and specificity for qualitative elastography in the differential diagnosis between PC and AIP were 0.97 (95%CI: 0.95–0.99) and 0.67 (95%CI: 0.59–0.74), respectively; the pooled sensitivity and specificity for SR were 0.98 (95%CI: 0.96–0.99) and 0.62 (95%CI: 0.56–0.68), respectively. Interestingly, quantitative elastography has been investigated to assess AIP activity in a small series of patients under steroid therapy[22]. A significant decrease of the mean share wave elastography of the pancreas (P = 0.023) was found. Nevertheless, no definitive cut-offs are available either to diagnose AIP and to distinguish between focal AIP and PC.

Therefore, elastography does not represent the main tool to diagnose AIP, and even though helpful, it is not a crucial test in the differential diagnosis between PC and focal AIP. However, its simple application coupled with serial imaging examinations and fine needle biopsies could help clinicians reach a correct diagnosis[19].

Acute pancreatitis: The application of PS in the evaluation of acute pancreatitis is scarce. Unfortunately, up to now no significant data are available in the literature to help the clinician either in the diagnosis or in the stratification of the severity of the condition.

Application of elastography in the evaluation of malignant diseases

Evaluation of PC: Qualitative elastography applied on the pancreatic focal lesions usually shows a geographic appearance, characterized by a heterogeneous, predominantly blue pattern with small green and red areas (Figure 2). A more homogeneous blue pattern is more typical in pancreatic neuroendocrine malignant tumors[11] (Figure 3). Even if not pathognomonic, these findings can be helpful, in particular when there is a clear demarcation from the healthy pancreatic parenchyma, which is usually of a homogenous green color.

The quantitative assessment is apparently much more informative, as it allows the clinician to compare the SR of a mass over the normal surrounding pancreatic parenchyma (Figure 4). Malignant pancreatic masses show a higher SR than the normal parenchyma[23]. However, it seems that no significant difference exists between the accuracy of quantitative and qualitative methods. In a meta-analysis including 10 studies and 893 pancreatic masses (646 malignant, 72.3%), the positive and negative likelihood ratios were 3.15 and 0.03 for qualitative EUS elastography, and 3.94 and 0.05 for quantitative EUS elastography, respectively[24]. Both qualitative and quantitative methods were useful for excluding the presence of PC, but they had a poor diagnostic specificity[24].

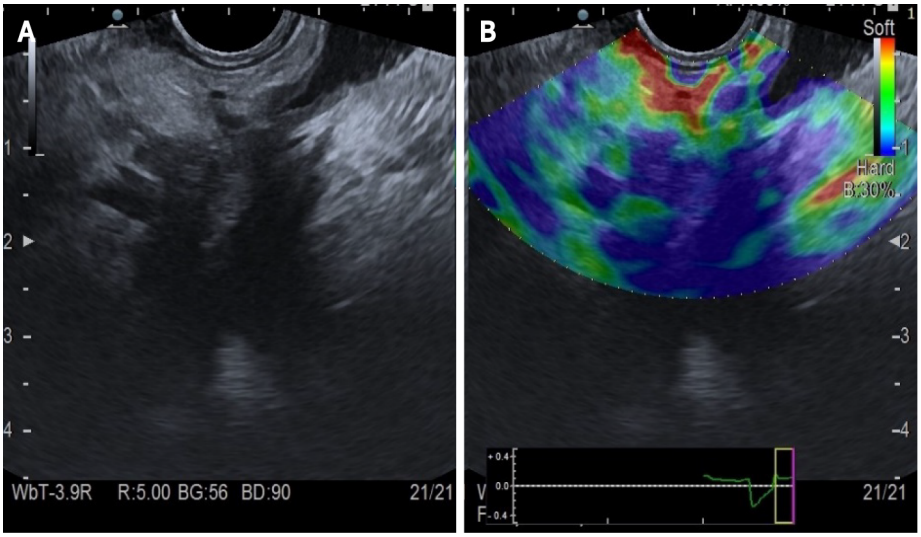
Similar results were found in another meta-analysis[25] involving 1044 patients, in which the pooled sensitivity, specificity, and diagnostic odds ratio of EUS elastography to distinguish benign from malignant solid pancreatic masses were 0.95 (95%CI: 0.94-0.97), 0.67 (95%CI: 0.61-0.73), and 42.28 (95%CI: 26.90-66.46), respectively. The AUROC was 0.9046[26]. Another meta-analysis involving 17 studies (1544 lesions) showed a pooled sensitivity and specificity for qualitative methods of 0.97 (95%CI: 0.95-0.99) and 0.67 (95%CI: 0.59-0.74), respectively[27]. The pooled sensitivity and specificity for strain histograms were 0.97 (95%CI: 0.95-0.98) and 0.67 (95%CI: 0.61-0.73), and the pooled sensitivity and specificity for SR were 0.98 (95%CI: 0.96-0.99) and 0.62 (95%CI: 0.56-0.68). Furthermore, they investigated the pooled sensitivity and specificity for contrast enhancement EUS, which were 0.90 (95%CI: 0.83-0.95) and 0.76 (95%CI: 0.67-0.84), respectively. To complete, they analyzed the pooled sensitivity and specificity for EUS-fine needle aspiration (FNA), which were 0.84 (95%CI: 0.77-0.90) and 0.96 (95%CI: 0.88-1.00), respectively[27].

Overall, data available in the scientific literature show that the sensitivity of elastography for the diagnosis of PC ranges between 92% to 98%. Despite this observation, the specificity is low, as it varies between 67% and 76%[25-27]. Nevertheless, elastography combined with contrast EUS and FNA is likely to increase the diagnostic accuracy for PC detection[28].

Considering the epidemiological relevance of the pancreatic ductal adenocarcinoma (PDAC) among all the pancreatic lesions, the biggest diagnostic effort when facing a pancreatic mass is directed to exclude it (Figure 4). The definition of elastography cut-offs to distinguish PDAC from other types of pancreatic tumors would be of extreme importance. Nevertheless, no definitive cut-offs are available, even if some studies tried to compare different types of pancreatic lesions.

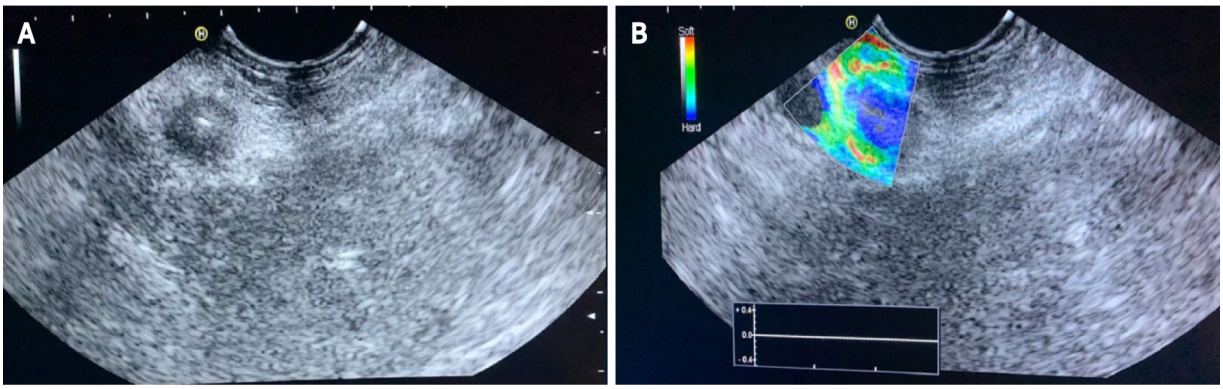
A study included different types of pancreatic solid lesions: 49 PDAC, 27 inflammatory masses, 6 pancreatic neuroendocrine neoplasms, two metastatic cell lung cancers, one pancreatic lymphoma, and one pancreatic solid pseudopapillary tumor. The authors compared these lesions with 20 controls. The mean SRs were: 1.68 (95%CI: 1.59–1.78) for normal pancreatic tissue, 3.28 for inflammatory masses, and 18.12 for pancreatic adenocarcinoma. Interestingly, the highest mean SR was found among neuroendocrine malignant tumors (52.34)[29]. Conversely, in a group of 218 patients with small lesions (23% PDAC, 52% neuroendocrine malignant tumors, 8% metastases, 17% other entities; 66% benign lesions), the high stiffness of the lesion for the diagnosis of malignancy had a sensitivity of 84% (95%CI: 73%-91%), a specificity of 67% (58%-74%), positive predictive value of 56% (50%-62%), and negative predictive value of 89% (83%-93%). For the diagnosis of PDAC, the sensitivity, specificity, positive predictive value, and negative predictive value were 96% (87%-100%), 64% (56%-71%), 45% (40%-50%), and 98% (93%-100%), respectively. In a group of neuroendocrine neoplasms, 64% had a soft pattern on elastography[30].

Targeted biopsy: Another role of EUS elastography can be the detection of pancreatic areas of increased stiffness within a focal lesion to better target the needle during EUS-FNA or fine needle biopsy. To achieve this aim, the application of qualitative elastography can be considered. Indeed, it allows the clinician to examine the lesion, giving the possibility to the operator to perform the needle insertion in the most suitable region, which usually is the hardest part (the softer is often necrotic tissue)[31]. Overall, the diagnostic accuracy of EUS-elastography guided FNA is good, with diagnostic accuracy, sensitivity, and specificity for the diagnosis of malignancy of 94%, 93%, and 100%, respectively[32]. No



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Figure 2 Qualitative endoscopic ultrasound elastography of pancreatic adenocarcinoma of the head. A: Endoscopic ultrasound B-mode; B: Elastographic image.



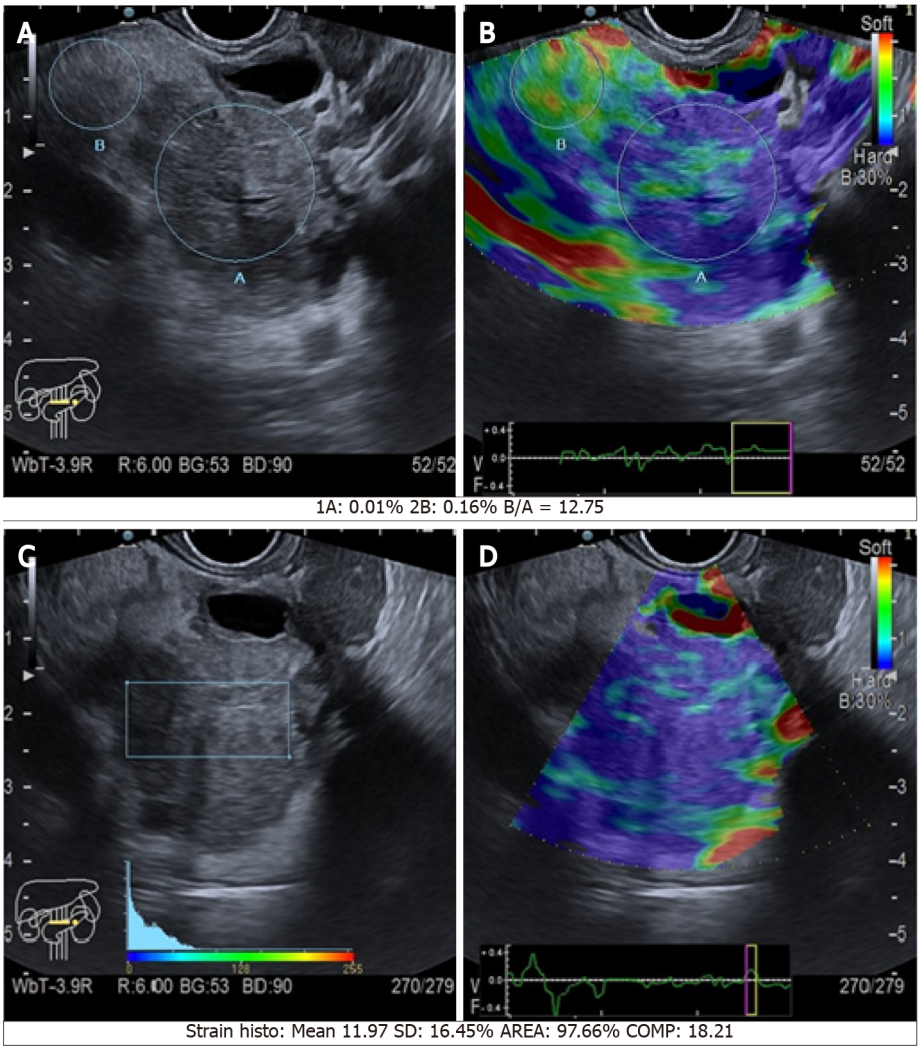
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Figure 3 Qualitative endoscopic ultrasound elastography of a neuroendocrine malignant tumor of the pancreatic tail. A: Endoscopic ultrasound B-mode; B: Elastographic image.

large studies that compare EUS-FNA/fine needle biopsy with and without elastography guide are available. Thus, this use of elastography remains possible but not mandatory.

CONCLUSION

EUS elastography is a technically simple, widely available, risk-free, and cheap exam. Thus, its use should be implemented and encouraged. Among its possible roles, EUS elastography helps the clinicians to add some information to the EUS study of pancreatic diseases, either benign or malignant. Elastography should therefore be considered a complementary tool to B-mode imaging. Notwithstanding its potential, clinicians must be aware that the current application of elastography still has little information[13-16], in particular for concerns of pancreatic diffuse benign diseases[11,15-18]. A potential application of this technique would be the ability to non-invasively stratify patients according to different risk categories for developing PC, similarly to the application of elastography in liver diseases [31]. However, so far, no definitive cut-offs have been recognized for the recognition of benign diseases through elastography. One of the main limitations to standardize the PS application is the lack of corresponding histologic samples as a reference standard, both from the healthy pancreas and pancreatic diseases. This happens because performing a pancreatic biopsy in cases of benign conditions would be unethical and risky.



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Figure 4 Quantitative endoscopic ultrasound elastography of pancreatic cancers of the body. A and C: B-mode with region of interest circles and square; B and D: Elastographic images.

Conversely, the use of elastography in the study of solid pancreatic lesions seems to be more informative[25-29], thanks to its high negative predictive value for the diagnosis of PC[22-25]. In particular, soft focal solid pancreatic lesions are rarely PDACs, whereas stiff lesions might be malignant or benign[30].

This makes elastography a fair test to exclude the presence of cancer. Moreover, the combination of elastography with FNA and contrast enhanced EUS could improve the accuracy of the diagnosis, even if no definitive data are available[32].

Nevertheless, it should also be highlighted that elastography cannot be considered mandatory during EUS examination of the pancreas, and there are no data supporting a higher diagnostic accuracy for the endosonographers that routinely use elastography *vs* who do not use this method. This latter consideration is mainly supported by the low reproducibility of pancreatic elastography, especially for the qualitative methods[25-28]. One of the major limitations in the use of elastography is the lack of a standardization of the technique. Moreover, the large use of EUS-FNA/fine needle biopsy has increased the accuracy of the diagnosis of PC, and in the case of inadequate sampling or negative cytology/histology, elastography does not have any key role[33]. Summarizing, EUS-related elastography is a tool that should be considered to obtain a complementary evaluation of pancreatic diseases, together with the other imaging tests and clinical data. Elastography can be informative, especially when studying pancreatic masses and help the clinician in the diagnosis. However, further studies are necessary to first standardize the method, increase the reproducibility and establish definitive cut-offs to distinguish between benign and malignant pancreatic lesions.

FOOTNOTES

Author contributions: Conti CB and Grassia R conceived the idea of the manuscript and supervised the findings of the work; Conti CB and Mulinacci G performed the literature search and wrote the draft of the paper with input from all authors; Salerno R and Dinelli ME verified the methods and the contents; Salerno R and Grassia R provided the illustrations by performing the examinations; All authors discussed the results and contributed to the final manuscript.

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Country/Territory of origin: Italy

ORCID number: Clara Benedetta Conti 0000-0001-9774-2374; Giacomo Mulinacci 0000-0002-9398-893X; Raffaele Salerno 0000-0001-9201-7755; Marco Emilio Dinelli 0000-0003-0214-0333; Roberto Grassia 0000-0003-4491-4050.

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ORIGINAL ARTICLE

Basic Study

Cystic fibrosis transmembrane conductance regulator prevents ischemia/reperfusion induced intestinal apoptosis *via* inhibiting PI3K/AKT/NF-κB pathway

Zhi-Wei Dong, Hui Liu, Fei-Fei Su, Xiao-Zhou Fan, Yong Zhang, Peng Liu

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Zhi-Wei Dong, Department of General Surgery, Air Force Medical Center, Beijing 100000, China

Hui Liu, Department of Gastroenterology, Second Affiliated Hospital of Dalian Medical University, Dalian 116023, Liaoning Province, China

Fei-Fei Su, Department of Cardiology, Air Force Medical Center, Beijing 100000, China

Xiao-Zhou Fan, Department of Ultrasound, Air Force Medical Center, Beijing 100000, China

Yong Zhang, School of Chemistry and Biological Engineering, University of Science and Technology, Beijing 100000, China

Peng Liu, Research Laboratory of Aero-Medical Support, Air Force Medical Center, Beijing 100000, China

Corresponding author: Peng Liu, PhD, Research Scientist, Research Laboratory of Aero-Medical Support, Air Force Medical Center, No. 30 Fucheng Road, Haidian District, Beijing 100000, China. liupeng-81@foxmail.com

Abstract

BACKGROUND

Intestinal ischemia/reperfusion (I/R) injury is a fatal syndrome that occurs under many clinical scenarios. The apoptosis of intestinal cells caused by ischemia can cause cell damage and provoke systemic dysfunction during reperfusion. However, the mechanism of I/R-induced apoptosis remains unclear. Cystic fibrosis transmembrane conductance regulator (CFTR) is a cAMP-activated chloride channel. Few researchers have paid attention to its role in intestinal I/R injury, or the relationship between CFTR and intestinal apoptosis induced by hypoxia/reoxygenation (H/R).

AIM

To investigate the effects of CFTR on I/R-induced intestinal apoptosis and its underlying molecular mechanisms.

METHODS

An intestinal I/R injury model was established in mice with superior mesenteric

artery occlusion, and Caco2 cells were subjected to H/R for the simulation of I/R *in vivo*.

RESULTS

The results suggested that CFTR overexpression significantly increased the Caco2 cell viability and decreased cell apoptosis induced by the H/R. Interestingly, we found that the translocation of p65, an NF-κB member, from the cytoplasm to the nucleus after H/R treatment can be reversed by the overexpression of CFTR, the NF-κB P65 would return from the nucleus to the cytoplasm as determined by immunostaining. We also discovered that CFTR inhibited cell apoptosis in the H/R-treated cells, and this effect was significantly curbed by the NF-κB activator BA, AKT inhibitor GSK690693 and the PI3K inhibitor LY294002. Moreover, we demonstrated that CFTR overexpression could reverse the decreased PI3K/AKT expression induced by the I/R treatment *in vivo* or H/R treatment *in vitro*.

CONCLUSION

The results of the present study indicate that the overexpression of CFTR protects Caco2 cells from H/R-induced apoptosis; furthermore, it also inhibits H/R-induced apoptosis through the PI3K/AKT/NF-κB signaling pathway in H/R-treated Caco2 cells and intestinal tissues.

Key Words: Apoptosis; Cystic fibrosis transmembrane conductance regulator; Intestinal ischemia-reperfusion injury; PI3K/AKT/NF-κB; Hypoxia/reoxygenation; Caco2 cells

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Core Tip: Intestinal ischemia/reperfusion (I/R) injury is a fatal syndrome that occurs under many clinical scenarios. The apoptosis of intestinal cells caused by ischemia can cause cell damage and provoke systemic dysfunction during reperfusion. However, the mechanism of I/R-induced apoptosis remains unclear. In our paper, our data demonstrate that cystic fibrosis transmembrane conductance regulator (CFTR) is downregulated in hypoxia/reoxygenation (H/R)-treated Caco2 cells, and overexpression of CFTR protects Caco2 cells from H/R-induced apoptosis. Additionally, CFTR is involved in inhibiting H/R-induced apoptosis, and it inhibits apoptosis through PI3K/AKT/NF-κB signaling pathway. In all, this work suggests that overexpression of CFTR attenuates H/R-induced apoptosis through PI3K/AKT/NF-κB signaling pathway in H/R-treated Caco2 cells.

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INTRODUCTION

Intestinal ischemia/reperfusion (I/R) injury is a fatal syndrome that occurs in different critical clinical scenarios, including acute mesenteric ischemia, burn injury, sepsis, and hemorrhagic shock, and is characterized by high incidence and mortality rates[1]. Intestinal I/R causes local injuries in the intestine and multiple organ dysfunction syndromes or even multiple organ failure (MOF) in distant organs with a mortality rate ranging from 30%–90%[2]. The underlying mechanisms are very complex, including damage by oxygen radicals, the release of large numbers of inflammatory factors, bacterial translocation, and cell apoptosis. Protecting intestinal apoptosis is one of the critical targets in treating patients with intestinal I/R injury.

The innate and adaptive apoptosis resulting from intracellular damage and translocation of gut pathogens play a critical role in the pathological process of intestinal I/R[3]. The apoptosis of intestinal cells induced by ischemia can cause cell damage and activate epithelial barrier dysfunction during reperfusion, leading to system dysfunction[4]. However, the mechanism of apoptosis induced by H/R remains unclear.

Cystic fibrosis transmembrane conductance regulator (CFTR), a cAMP-activated chloride channel, mutations of which cause the most common lethal genetic disease[5]. Mutation in CFTR causes cystic fibrosis (CF) and induces severe damage to body organs, especially the lungs and digestive system. Extensive research on the role of CFTR has focused on epithelial cell function, while its role in other types of cells, such as endothelial cells, is largely unclear[6]. Many studies have indicated that patients

with CF had endothelial perturbation and microvascular dysfunction, suggesting that CFTR deficiency contributes to endothelial dysfunction[7]. Furthermore, CFTR protects against endothelial apoptosis from oxidative stress and inflammation[8]. However, the relationship between CFTR and I/R-induced apoptosis in enterocytes has not been reported.

It was reported that the activation of the NF-κB signaling pathway is involved in apoptosis, autophagy, and inflammatory gene transcription[1,9]. Moreover, NF-κB can be the target for disease treatment. Inhibition of NF-κB activation could attenuate LPS-induced apoptosis[1]. H/R stress was reported to trigger apoptosis by impairing NF-κB survival signaling in malignant B cells[10]. Additionally, the PI3K/AKT pathway is considered an essential regulatory factor in NF-κB activation. Conditional CFTR knockout mice showed increased inflammatory cell infiltration and activation of the NF-κB signaling pathway[4]. In endothelial cells, CFTR modulates inflammation by regulating the NF-κB signaling[11]. But how CFTR modulates PI3K/AKT/NF-κB signaling in apoptosis induced by H/R has not been fully elucidated.

It was hypothesized that CFTR could affect I/R-induced intestinal apoptosis through the NF-κB signaling pathway. In this study, a CFTR activator and CFTR overexpression vector were used to investigate the effects of CFTR on I/R-induced intestinal apoptosis and its underlying molecular mechanisms.

MATERIALS AND METHODS

Cell culture

Caco2 cells purchased from American Type Culture Collection were maintained in a high-glucose DMEM medium (Gibco, Rockville, MD, United States) supplemented with 10% fetal bovine serum (Gibco, Rockville, MD, United States), 100-U/mL penicillin, 100-mg/mL streptomycin (Sigma, St. Louis, MO, United States), and 5% CO_2 at 37 °C in a humidified atmosphere for 48 h. To mimic hypoxic conditions, the cells were incubated in a microaerophilic system (Thermo Fisher Scientific, Waltham, MA, United States) with 5% CO_2 and 1% O_2 balanced with 94% N_2 for 12 h. Then, the cells were cultured under normal conditions for 0, 12, 24, and 48 h to achieve reoxygenation.

For inhibitor treatment, the cells were incubated with NF-κB inhibitor BAY11 (40 μmol/L) (Beyotime, Nantong, China), AKT activator IGF-1 (20 μmol/L) (Beyotime, Nantong, China), PI3K activator (740 Y-P, 10 μmol/L) (Beyotime, Nantong, China), NF-κB activator Betulinic acid (BA) (20 μmol/L) (Beyotime, Nantong, China), AKT inhibitor GSK690693 (20 μmol/L) (Beyotime, Nantong, China), and PI3K inhibitor LY294002 (10 μmol/L) (Beyotime, Nantong, China) for 1–8 h and then subjected to H/R. The cells were used for various experiments at different times after the H/R treatment.

Lentivirus transfection

To overexpress CFTR, Caco2 cells were transfected with the expression vector Human CFTR lentivirus (LV-CFTR), which was bought from Genechem Company (Shanghai Genechem Co., Ltd., Shanghai, China). The lentivirus was transfected into the Caco2 cells (80% confluence) at the multiplicity of infection (MOI) 20 before the experiment. After 6 h, the cell culture medium was replaced with a fresh medium, and the cells were used for the experiments 2 d later.

Cell viability assay

Cell counting kit-8 (CCK8; Dojindo Laboratories, Kumamoto, Japan) assay was employed to investigate cell survival following the manufacturer's instructions. In brief, Caco2 cells after various treatments were plated in 96-well plates at a density of 2.5 × 10^3 cells/well. On the other day, 15-μL CCK-8 was added to each well and incubated at 37 °C for 4 h. Absorbance was measured at 450 nm using a microplate spectrophotometer (Molecualr Devices LLC, Sunnyvale, CA, United States).

TUNEL

According to instruction, one-step TUNEL apoptosis assay kit (Beyotime, Nantong, China) was used to conduct TUNEL staining. The images of the FITC-labeled TUNEL-positive cells were captured using a fluorescence microscope (Nikon Corporation, Tokyo, Japan). The nick-ends labeled in red indicated apoptotic cells. The cell nucleus was labeled in blue using DAPI (Invitrogen, Carlsbad, CA, United States).

Western blotting

Western blotting was conducted as previously described[11]. Briefly, total proteins were obtained from cultured Caco2 cells using RIPA (Beyotime, Nantong, China), and the concentrations were tested using the bicinchoninic acid (BCA) Protein Assay Kit (Pierce, Rockford, IL, United States). The intestinal tissues were taken and washed thrice with PBS, then stored at -80 °C. A 100-μg intestinal tissue was removed for the Western blotting using the same protocol used for the isolation of the cell protein; further, 50-μg protein was separated in 10% SDS-PAGE and transferred to PVDF membranes. The

membranes were blocked in 5% nonfat dry milk and then probed with mouse monoclonal anti-p-NF-κB p65 and anti-NF-κB p65 (1:2000 dilution, Cell Signaling Technology, Danvers, MA, United States), and rabbit anti-GAPDH (1:1000 dilution; Santa Cruz Biotechnology, Dallas, TX, United States) overnight at 4 °C. On the other day, membranes were then incubated with the HRP-conjugated anti-rabbit or goat anti-mouse secondary antibody (1:3000; Santa Cruz Biotechnology, Dallas, TX, United States) for 1 h at room temperature. The protein bands were visualized using Amersham Hyperfilm™ ECL (Thermo Fisher Scientific; former, MA, United States). ImageJ 1.41o software (National Institutes of Health (Bethesda, MD, United States) was used to quantify protein expression.

Intestinal I/R model and treatment

C57BL/6J mice aged 8–12 wk old were supplied by the Experimental Animal Center at the southwest hospital. All experimental procedures conformed to the institutional guidelines and protocols approved by the Animal Care and Use Committee of Air Force Medical Center. All animal studies complied with the principle for replacement, refinement, or reduction. The mice were randomly separated into five groups (n = 8): Sham and I/R groups (45 min of ischemia and reperfusion for 30, 90, 720, and 1440 min). All animals were group-housed with 2–3 mice *per* cage on a 12 h light/dark cycle in a temperature-controlled (25 °C ± 2 °C) room with free access to water and food. Before the experiment, all mice were fasted for 24 h, but the water was supplied. The animals were anesthetized with intraperitoneal injection, 1.0–1.25 g/kg 10% urethane in 0.9% saline (w/v) (Sigma-Aldrich, St. Louis, MO, United States), and all mice were kept under anesthesia during the surgery period[12]. In the Sham group, the wound was closed after finding the superior mesenteric artery using laparotomy. In I/R groups, the abdominal cavity was opened, the superior mesenteric artery and its adjacent tissues were cautiously isolated and clamped using a microvascular clip for 45 min to induce ischemia, and then the clip was gently removed to allow reperfusion for 30, 90, 720, and 1440 min. After the procedure, the surgical incision was sutured with sutures. The mice were placed on a warming plate and sacrificed after the injury during the process. Then, the whole intestines were obtained and stored at -80 °C, which was used for the test[13]. FSK, an adenylate cyclase activator, was administered to activate the CFTR chloride channel for Con + FSK and I/R + FSK groups through intraperitoneal injection (2 mg/kg, Sigma-Aldrich, United States)[14].

Statistical analysis

The GraphPad Prism 7.0 statistical software (GraphPad Software, Inc., La Jolla, San Diego, CA, United States) was used for statistical analysis. Data are presented as mean ± SD. Multiple comparisons between more than two groups were analysed by one-way ANOVA test or Kruskal–Wallis test (non-parametric). $P < 0.05$ was considered statistically significant.

RESULTS

Apoptosis was induced in Caco2 cells after H/R treatment

To investigate the effect of H/R injury on cell injuries, cell viability was examined in vitro. As shown in Figure 1A, after a reoxygenation period of 12 h and 24 h, cell viability decreased significantly compared with the control group, and returned to normal 48 h after reoxygenation. Meanwhile, as shown in Figure 1B, after the reoxygenation period, the percentage of apoptosis was elevated compared with the control group, and it also returned to normal 48 h after reoxygenation. The western blotting was used to test the cell apoptosis after H/R treatment. The results showed that the ratio of Bcl-2/Bax was significantly decreased at 12 h and 24 h, indicating that apoptosis was remarkably elevated by the H/R treatment (Figure 1C).

CFTR can protect the Caco2 cells from apoptosis induced by H/R

To explore the effect of CFTR on H/R-induced apoptosis, the expression of the CFTR protein before and after H/R was assessed using western blotting analysis of protein lysate from the H/R treatment Caco2 cells. The results indicated that CFTR proteins were detectable at high levels in the control group, while its level was remarkably downregulated 12 h and 24 h after reoxygenation (Figure 2A). To assess whether CFTR was responsible for altering the levels of apoptosis markers, the endogenous CFTR in Caco2 cells was increased by approximately 80% using the LV-CFTR. Infection of Caco2 cells with LV-CFTR 2 d before the experiment modestly increased CFTR levels compared with Caco2 cells infected with LV-NC (lentivirus expressed with empty vector). In addition, LV-CFTR also increased CFTR levels in Caco2 cells treated with H/R treatment (Figure 2B). Considering that the apoptosis reached its maximum at 12 h (Figure 1B and C), this time point was used to test the effect of CFTR on cell apoptosis. It was discovered that the CFTR overexpression significantly increased the viability of Caco2 cells induced by H/R treatments (Figure 2C), and Caco2 cells with high LV-CFTR expression had increased levels of Bcl-2/Bax ($P < 0.001$) compared to the control infected with LV-NC (Figure 2D). These results indicate that CFTR can protect the Caco2 cells from apoptosis induced by the H/R treatment.

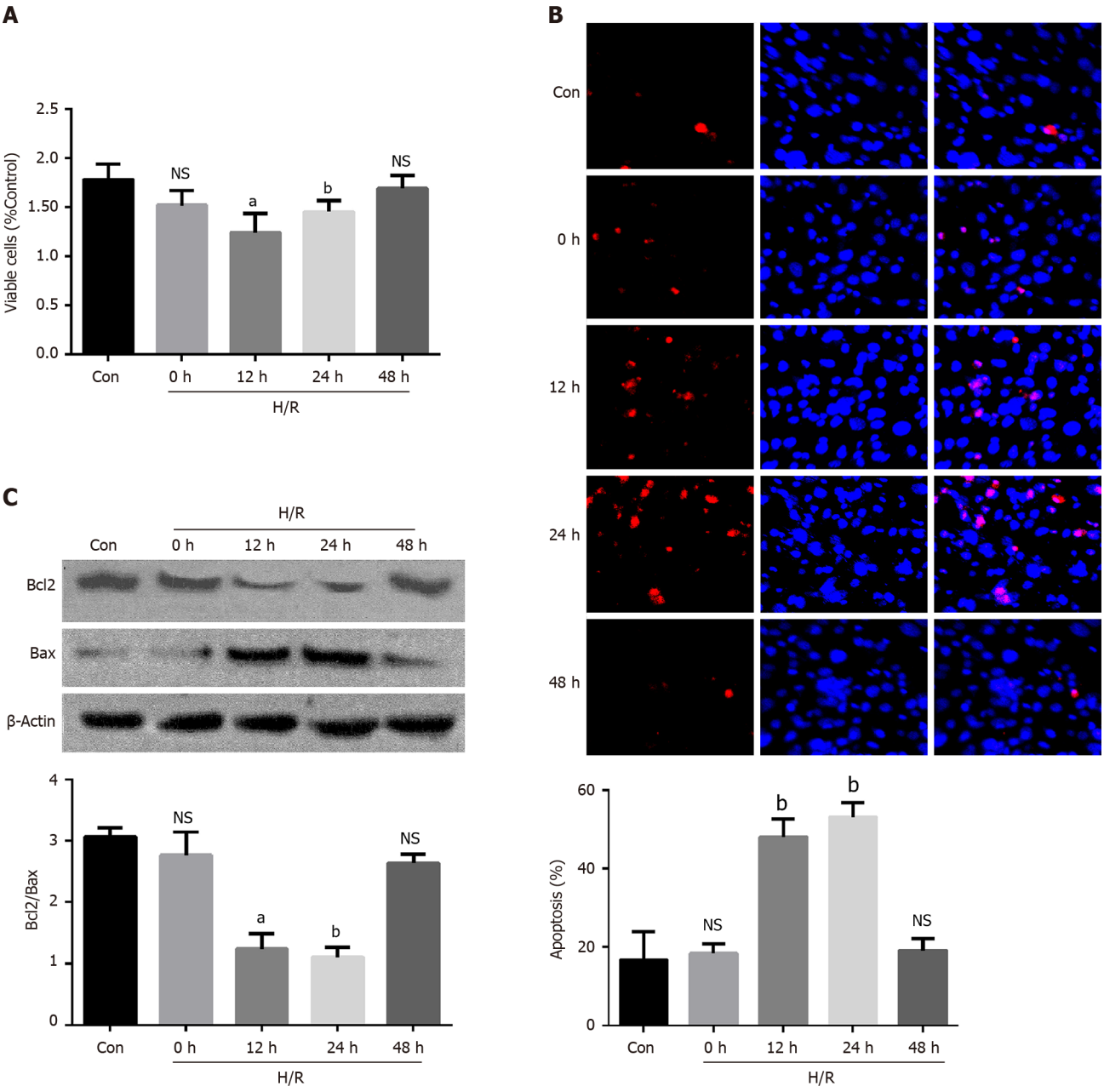


Figure 1 Apoptosis was induced in Caco2 cells after hypoxia/reoxygenation treatment. A: Cell viability was tested using the CCK8 kit on different times after the hypoxia/reoxygenation (H/R) treatment, $^{a}P < 0.01$ and $^{b}P < 0.001$ *vs* control ($n = 3$); B: The quantification data of apoptosis tested by the TUNEL at different times after the H/R treatment, $^{b}P < 0.001$ *vs* control ($n = 3$); C: Representative Western blotting and quantification data for cell apoptotic proteins Bcl-2 and Bax at different times after the H/R treatment. Data are presented as mean ± SD, $^{a}P < 0.01$ and $^{b}P < 0.001$ *vs* control ($n = 3$). NS: No significant difference; H/R: Hypoxia/reoxygenation.

PI3K/AKT/NF-κB pathway was involved in H/R-induced apoptosis

It has been reported that PI3K/AKT/NF-κB mediates the H/R injury in many physiological and pathological means, including apoptosis, inflammation, autophagy, *etc*. The effect of H/R on the PI3K/AKT/NF-κB pathway was quantified in the Caco2 cells. As shown in Figure 3A, the protein expressions of PI3K, phosphorylated AKT^{ser473} (p-AKT^{ser473}), phosphorylated AKT^{Thr308} (p-AKT^{Thr308}) were decreased 12 h and 24 h after the H/R treatment, and returned to normal at 48 h; on the contrary, the activation of the NF-κB P65 was upregulated 12 h and 24 h after the H/R treatment. More importantly, H/R treatment provoked the translocation of p65, an NF-κB member, from the cytoplasm to the nucleus at 12 h and 24 h, as observed through immunofluorescence staining, and then returned to the cytoplasm at 48 h (Figure 3B). Furthermore, the cells were treated with NF-κB inhibitor BAY11 (40 μmol/L), AKT activator IGF-1(20 μmol/L), and PI3K activator (740 Y-P, 10 μmol/L) 6 h before the H/R experiment, apoptotic activity was checked 12 h after the H/R treatment. Interestingly, we discovered that NF-κB inhibitor, AKT activator, and PI3K activator could reverse the H/R-induced cell apoptosis (Figure 3C). Furthermore, the PI3K and AKT activator significantly decreased the NF-κB elevations induced by the

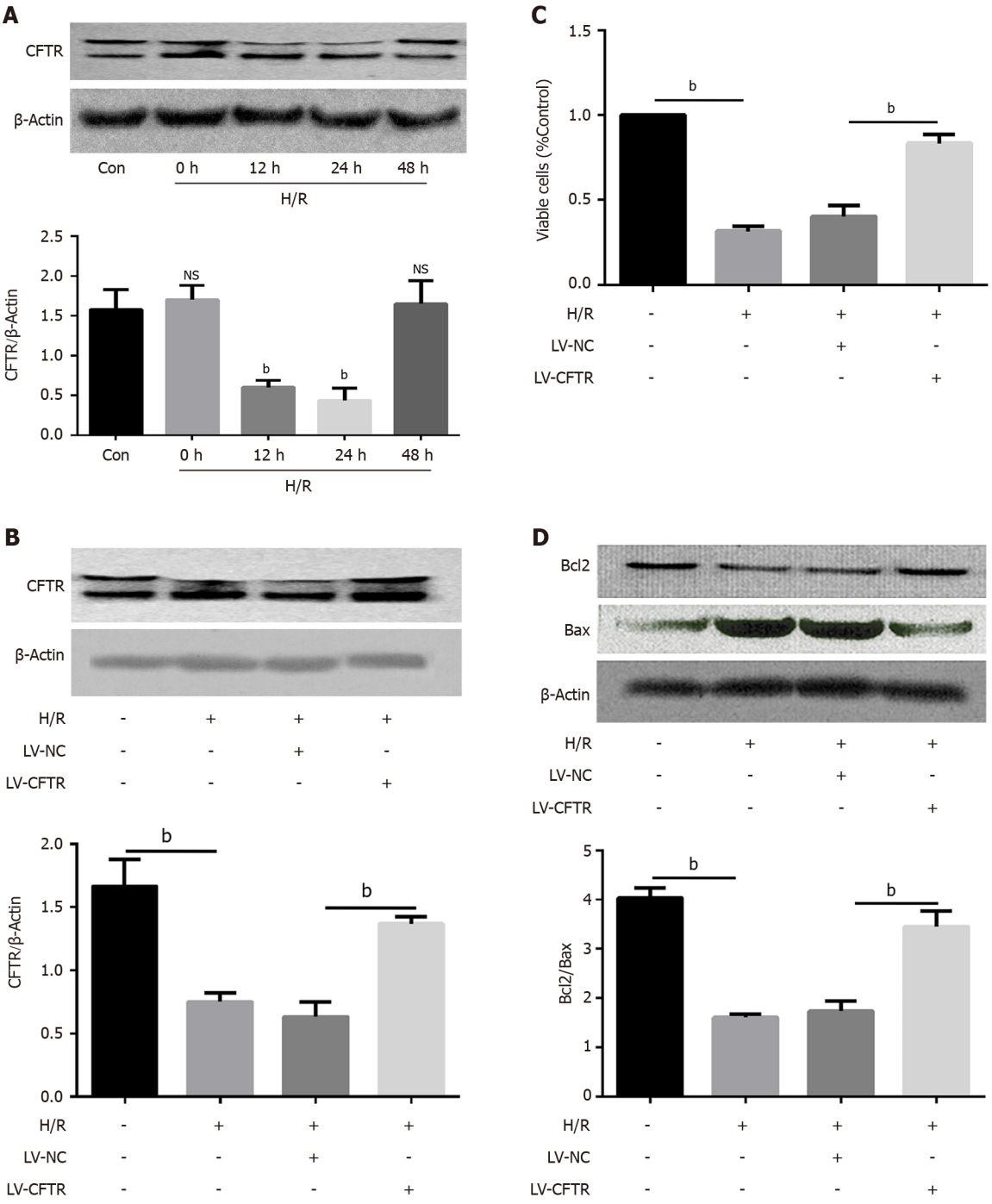
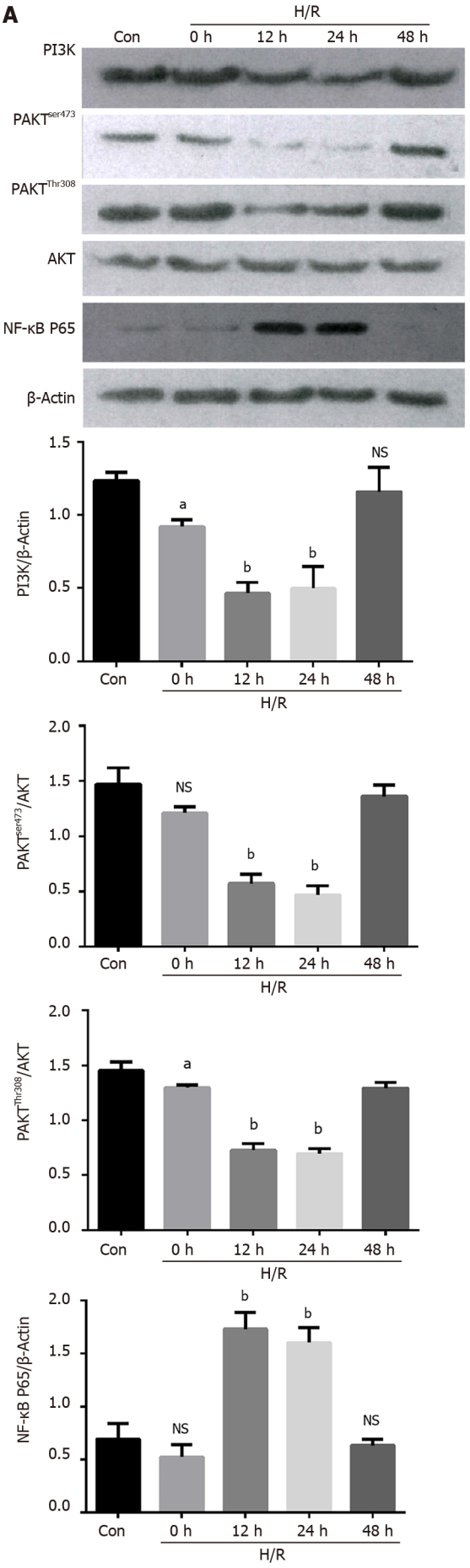


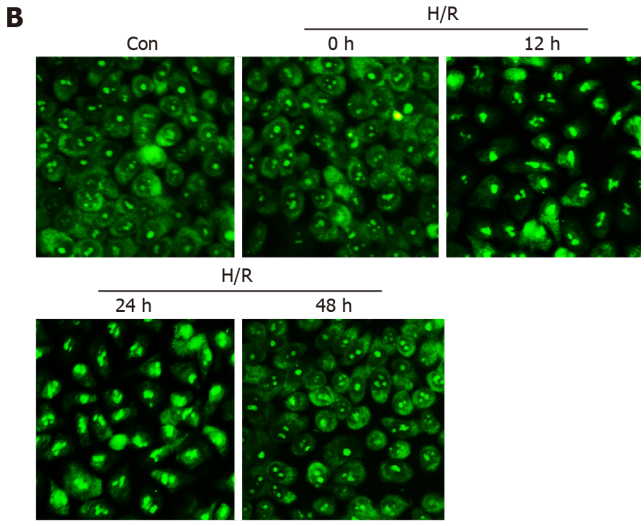
Figure 2 Cystic fibrosis transmembrane conductance regulator can protect the Caco2 cells from apoptosis induced by the hypoxia/reoxygenation. A: Representative Western blotting and quantification data for cystic fibrosis transmembrane conductance regulator (CFTR) after the hypoxia/reoxygenation (H/R) treatment, $^{b}P < 0.001$ *vs* control ($n = 3$); B: Representative Western blotting and quantification data for CFTR after the LV-CFTR transfection, $^{b}P < 0.001$ *vs* control ($n = 3$); C: Cell viability was tested using the CCK8 kit after the LV-CFTR transfection, $^{b}P < 0.001$ *vs* control ($n = 3$); D: Representative Western blotting and quantification data for cell apoptotic proteins Bcl-2 and Bax after the LV-CFTR transfection. Data are presented as mean ± SD. $^{b}P < 0.001$ *vs* control ($n = 3$). CFTR: Cystic fibrosis transmembrane conductance regulator; H/R: Hypoxia/reoxygenation.

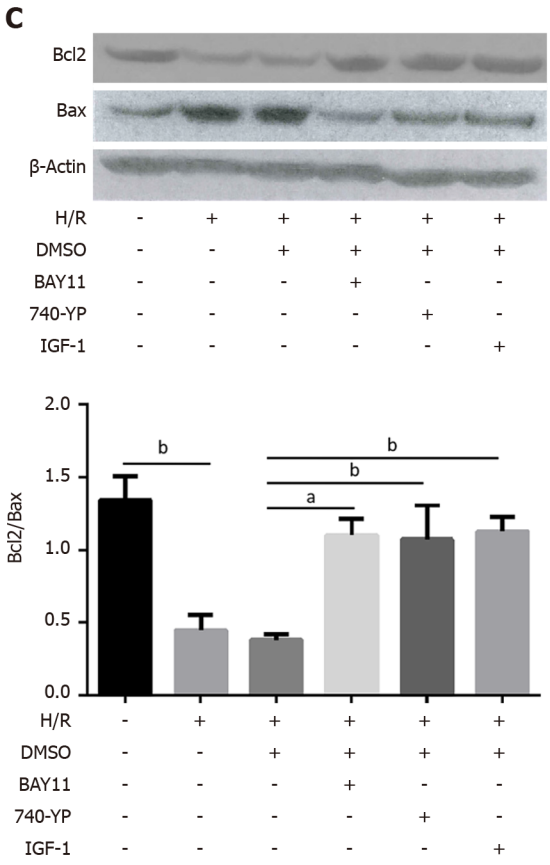
H/R (Figure 3D), whereas the NF-κB inhibitor could not impend the decline of PI3K and AKT in H/R-treated cells (Figure 3E), indicating that NF-κB was downstream of PI3K and AKT in the H/R-treated cells. All of these results suggested that PI3K/AKT/NF-κB pathway is involved in H/R-induced apoptosis.

CFTR protected the Caco2 cells from H/R-induced apoptosis through the PI3K/AKT/NF-κB pathway

Previous research has established that CFTR can regulate PI3K/AKT/NF-κB pathway in tumor cell migration and cell permeability. To assess the relationship between CFTR and PI3K/AKT/NF-κB in H/R-induced apoptosis, CFTR was overexpressed and the H/R condition stimulated. Interestingly,


A
H/R
Con
0 h
12 h
24 h
48 h
PI3K
PAKTser473
PAKTThr308
AKT
NF-κB P65
β-Actin
PI3K/β-Actin
PAKTser473/AKT
PAKTThr308/AKT
NF-κB P65/β-Actin
NS
a
b


B
H/R
Con
0 h
12 h
24 h
48 h


C
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IGF-1
Bcl2/Bax
a
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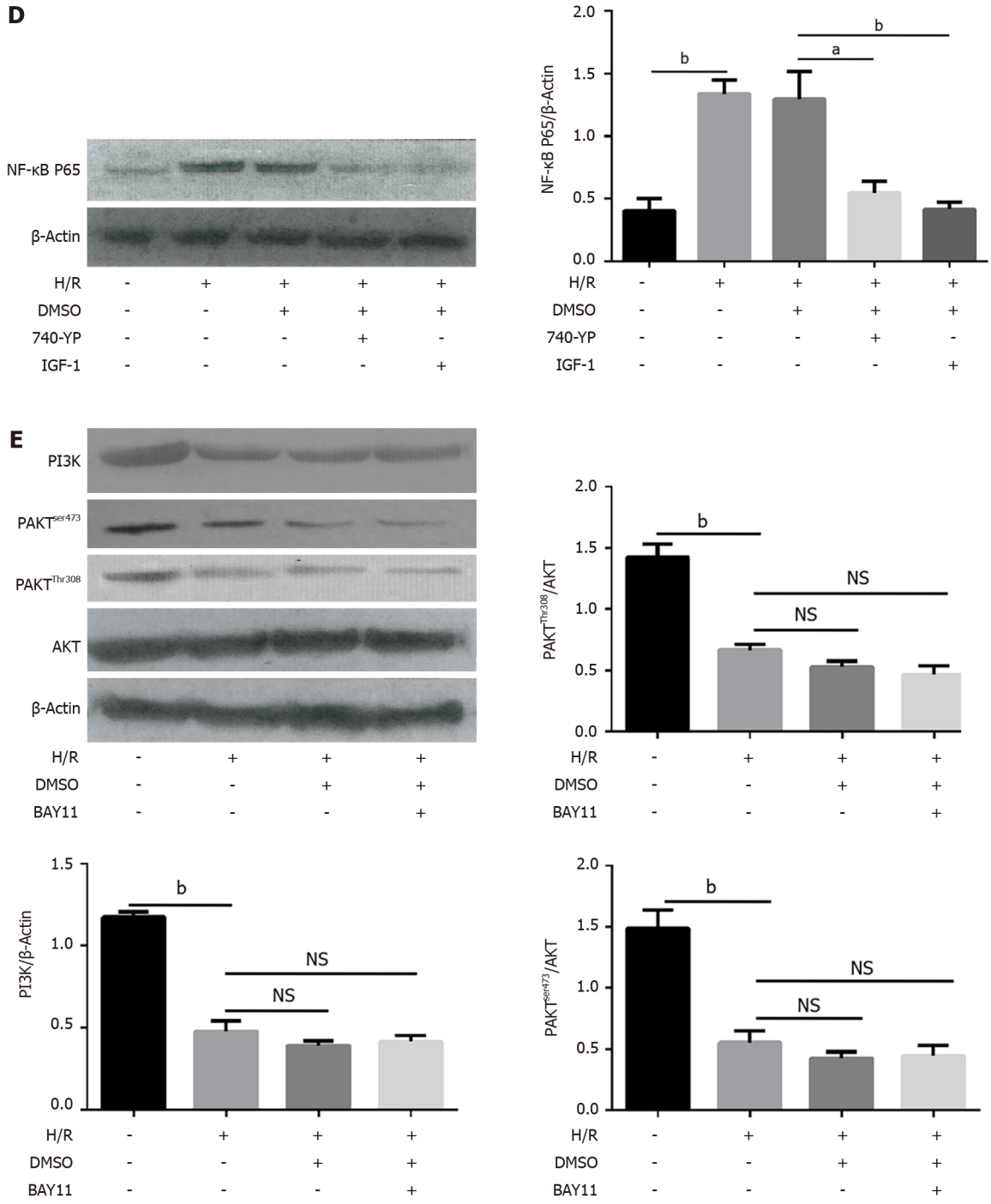


Figure 3 PI3K/AKT/NF-κB pathway was involved in hypoxia/reoxygenation-induced apoptosis. A: Representative western blotting and quantification data for PI3K, p-AKT^{ser473}, p-AKT^{Thr308}, NF-κB P65, and β-actin at different time points after hypoxia/reoxygenation (H/R) treatment. ns, no significant difference, $^{a}P < 0.01$ and $^{b}P < 0.001$ *vs* control ($n = 3$); B: Representative images of immunofluorescence staining for NF-κB P65 in Caco2 cells before (Con) and after the H/R treatment at different time, Bars = 100-μm ($n = 3$); C: Representative Western blotting and quantification data for cell apoptotic proteins Bcl-2, and Bax after NF-κB inhibitor BAY11, AKT activator IGF-1 and PI3K activator 740 Y-P treatment, $^{a}P < 0.01$ and $^{b}P < 0.001$ *vs* control ($n = 3$); D: Representative Western blotting and quantification data for NF-κB P65 after AKT activator IGF-1 and PI3K activator 740 Y-P treatment, $^{a}P < 0.01$ and $^{b}P < 0.001$ *vs* control ($n = 3$); E: Representative Western blotting and quantification data for PI3K, p-AKT^{ser473}, and p-AKT^{Thr308}, after NF-κB inhibitor BAY11 treatment. Data are presented as mean ± SD. $^{b}P < 0.001$ *vs* control ($n = 3$). NS: No significant difference.

compared with the H/R treatment groups, the overexpression of CFTR can inhibit the NF-κB P65 activation and instigate the return of NF-κB P65 from nuclei to the cytoplasm (Figure 4A). The results showed that overexpression of CFTR significantly limited the activation of the NF-κB p65 induced by the H/R treatment, and can also increase the PI3K/AKT expression (Figure 4B). Furthermore, cells were treated with NF-κB activator betulinic acid (BA) (20 μmol/L), AKT inhibitor GSK690693 (20 μmol/L), and PI3K inhibitor LY294002 (10 μmol/L) 6 h before the H/R treatment experiment. Our results implied that CFTR increased cell viability and inhibited cell apoptosis in the H/R-treated cells, and this effect was significantly inhibited by BA, GSK690693, and LY294002 (Figure 4C). Furthermore, results from

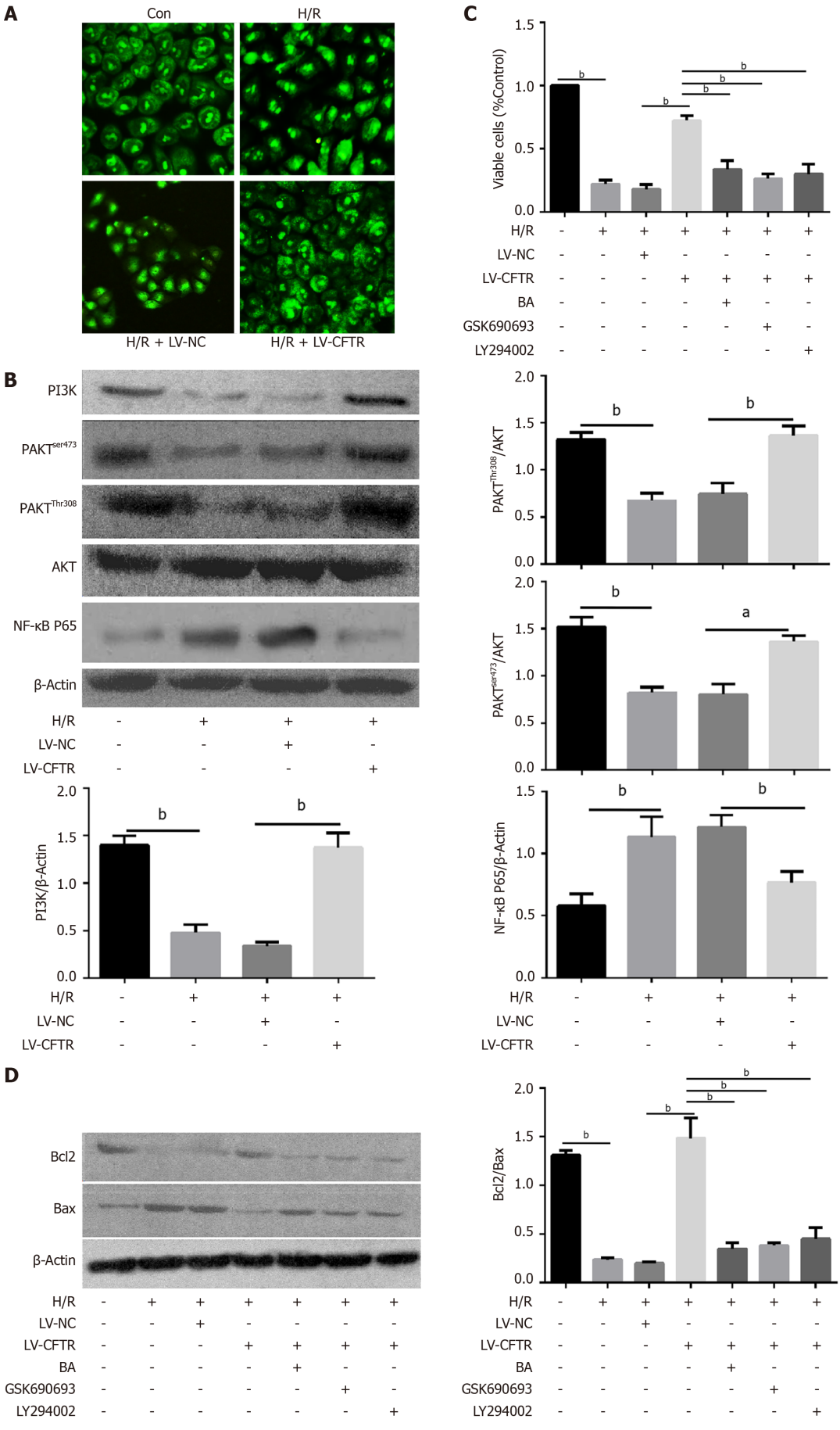


Figure 4 Cystic fibrosis transmembrane conductance regulator protected the Caco2 cells from hypoxia/reoxygenation-induced apoptosis

by PI3K/AKT/NF-κB pathway. A: Representative images of immunofluorescence staining for NF-κB P65 in Caco2 cells after the LV-cystic fibrosis transmembrane conductance regulator (CFTR) transfection (n = 3); B: Representative western blotting and quantification data for PI3K, p-AKT^{ser473}, p-AKT^{Thr308}, and NF-κB P65 after LV-CFTR transfection, $^{a}P < 0.01$ and $^{b}P < 0.001$ *vs* control (n = 3); C: Cell viability was tested using the CCK8 kit after LV-CFTR transfection and treatment with NF-κB activator Betulinic acid (BA), AKT inhibitor GSK690693 and PI3K inhibitor LY294002, $^{b}P < 0.001$ *vs* control (n = 3); D: Representative Western blotting and quantification data for cell apoptotic proteins Bcl-2 and Bax after LV-CFTR transfection and treatment with NF-κB activator BA, AKT inhibitor GSK690693 and PI3K inhibitor LY294002. Data are presented as mean ± SD. $^{b}P < 0.001$ *vs* control (n = 3).

western blotting showed that CFTR could not increase Bcl-2/Bax ration after BA, GSK690693, and LY294002 treatment (Figure 4D). Together, these results indicate that the inhibition of PI3K/AKT or the activation of NF-κB both impeded the protective effect of CFTR on H/R-induced apoptosis, and also demonstrate that CFTR protects the Caco2 cells from H/R-induced apoptosis by inhibiting the PI3K/AKT/NF-κB pathway.

CFTR overexpression protects the intestinal cell from apoptosis induced by I/R via inhibiting the PI3K/AKT/NF-κB pathway in vivo

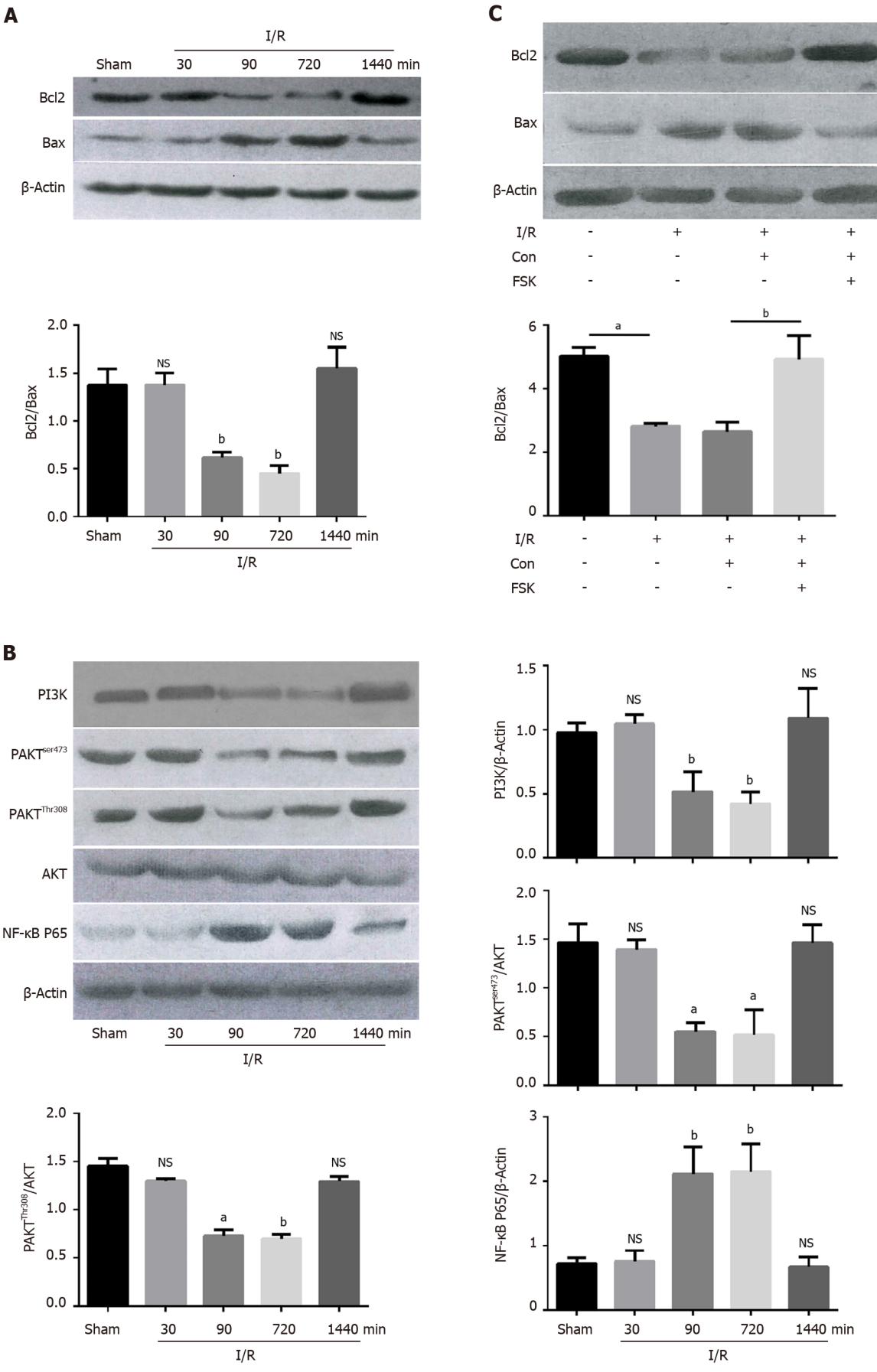
To assess whether the findings of CFTR in Caco2 cell lines were relevant to animal models *in vivo*, we performed the I/R-induced intestinal injury in animal models. Results from the western blotting demonstrated that the ratio of Bcl-2/Bax was significantly decreased at 90 min and 720 min, indicating that I/R treatment could significantly instigate apoptosis *in vivo* (Figure 5A), and the expressions of PI3K, p-AKT^{ser473} and p-AKT^{Thr308} were all decreased at 90 and 720 min, but they all returned to the normal level at 1440 min (Figure 5B). Similarly, the activation of the NF-κB P65 was increased at 90 and 720 min after the I/R treatment (Figure 5B). Furthermore, we treated the animals with the FSK, an activator of adenylate cyclase, and checked the apoptosis after I/R treatment. As shown in Figure 5C, the apoptosis of the intestines after FSK administration was significantly lower than those who received control treatment. The NF-κB was also activated in the I/R treatment and downregulated by the FSK administration. FSK administration could also increase the expression of PI3K and AKT, which was downregulated by I/R treatment (Figure 5D). To sum up, results indicate that CFTR can attenuate intestinal apoptosis in the I/R treatment model relating to the PI3K/AKT/NF-κB pathway.

DISCUSSION

To the best of our knowledge, this study is the first to investigate the possible involvement of CFTR, as an essential therapeutic target, in I/R injury. Additionally, it has been demonstrated that CFTR/PI3K/AKT/NF-κB pathway is the underlying signaling mechanism in the I/R-induced intestinal cell apoptosis.

I/R can cause intestinal mucosal injury, ultimately leading to bacterial translocation, sepsis, MOF, and eventually death[15,16]. I/R injury is caused by complex pathological damage with different factors involved. Early tissue ischemia could induce intestinal mucosal barrier dysfunction, while subsequent reperfusion could cause oxidative stress, cell apoptosis, and immune activation[1,5]. Many studies have shown that H/R can induce apoptosis and necrosis in various cell lines, including lymphocytes, blood cells, and cancer cells[12]. However, the sensitivity of different cell lines differ widely according to the H/R exposure[17]. In our study, the focus was on the effect of H/R on intestinal cells. After hypoxia, cell viability significantly reduced compared with the control group in the time point of 12 h and 24 h after reo-xygenation, and it returned to normal 48 h after reoxygenation. Meanwhile, after 12 h and 24 h of reoxygenation, the percentage of apoptosis increased compared with the control group, and it also returned to normal 48 h after reoxygenation, showing that apoptosis was remarkably elevated by the H/R treatment and the percentage of apoptosis relied on the time point after reoxygenation.

The effect of H/R stimulation on cells is multifaceted and holistic, including all intracellular biochemical reactions and intracellular structures. In the process of H/R, several physiological changes were induced after H/R treatment in the cell, such as the cytoskeleton, intracellular calcium concentration, HSP and mitochondrial, *etc.*[18]. Thus, it is necessary to investigate the primary mechanism of H/R-induced apoptosis in Caco2 cells, and it is vital to identify new targets for the therapy of intestinal injury. Activation of NF-κB controls multiple cellular processes and several molecules involved in apoptosis[19]. CFTR maintains NF-κB activation by inhibiting the degradation of kappa B kinase γ (IKKγ) inhibitor[20]. It was also reported that the phosphorylation of NF-κB p65 was reduced in CFTR knockdown mice[21]. A recent study on intestinal Caco2 cells also found that knockdown of CFTR significantly increased IκB phosphorylation compared to control cells under IL-1β stimulation[7]. Despite the well-demonstrated inverse relationship between CFTR and NF-κB activation in various experimental settings[22,23], the solid evidence linking CFTR mutation to aberrant NF-κB activation remains mysteriously missing. Moreover, most studies focus on the relationship between CFTR/NF-κB and apoptosis in lung epithelial injury[7,24]. And the role of CFTR and NF-κB in intestinal apoptosis caused by H/R remains unclear. In our study, the activation of NF-κB P65 was upregulated at 12 h and


A
I/R
Sham
30
90
720
1440 min
Bcl2
Bax
β-Actin
Bcl2/Bax
NS
b
I/R
C
Bcl2
Bax
β-Actin
I/R
Con
FSK
a
b
B
PI3K
PAKT ser473
PAKT Thr308
AKT
NF-κB P65
PI3K/β-Actin
PAKT ser473/AKT
PAKT Thr308/AKT
NF-κB P65/β-Actin

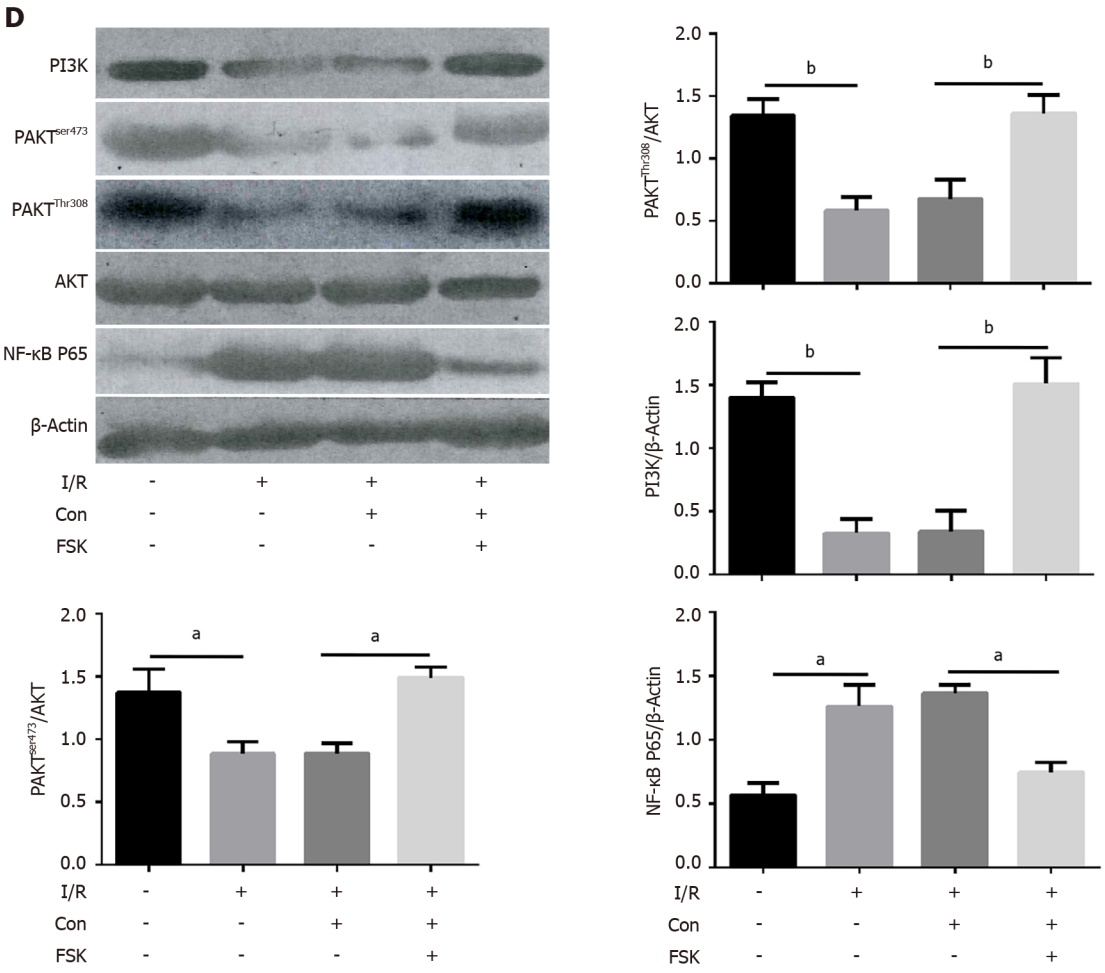


Figure 5 Cystic fibrosis transmembrane conductance regulator protects the intestinal cell from apoptosis induced by ischemia/reperfusion by inhibiting PI3K/AKT/NF-κB pathway *in vivo*. A: Representative Western blotting and quantification data for cell apoptotic proteins Bcl-2 and Bax at different time after the ischemia/reperfusion (I/R) treatment, $^{b}P < 0.001$ *vs* control ($n = 3$); B: Representative Western blotting and quantification data for PI3K, p-AKT^{ser473}, p-AKT^{Thr308}, and NF-κB P65 at different time after I/R treatment, $^{a}P < 0.01$ and $^{b}P < 0.001$ *vs* control ($n = 3$); C: Representative Western blotting and quantification data for cell apoptotic proteins Bcl-2 and Bax after the FSK treatment in I/R treatment animal model, $^{a}P < 0.01$ and $^{b}P < 0.001$ *vs* control ($n = 3$); D: Representative Western blotting and quantification data for PI3K, p-AKT^{ser473}, p-AKT^{Thr308}, and NF-κB P65 after FSK treatment in the I/R treatment animal model. Data are presented as mean ± SD. $^{a}P < 0.01$ and $^{b}P < 0.001$ *vs* control ($n = 3$).

24 h after the H/R treatment. And H/R treatment provoked the translocation of p65 from the cytoplasm to the nucleus at 12 h and 24 h, as observed through immunofluorescence staining, and then returned to the cytoplasm at 48 h. Additionally, NF-κB inhibitor BAY11 could reduce the apoptotic activity induced by the H/R treatment. And CFTR overexpression could inhibit the NF-κB P65 activation and make the NF-κB p65 come back from the nucleus to the cytoplasm. These results indicated that overexpression of CFTR significantly limited the activation of the NF-κB p65, which was caused by the H/R treatment.

PI3K/AKT plays a role in inducing inflammation, oxidative stress, and apoptosis in tissue injuries[15, 25]. The key signaling molecule of PI3K is Akt[26]. Previous studies have observed the correlation of CFTR with PI3K/AKT pathway in both physiological and pathological conditions. Aberrant activity of AKT has been observed in CF cells and mice. For instance, the macrophages obtained from CF mice shed a significant decrease in AKT phosphorylation at serine 473 compared with those obtained from wild-type mice after LPS challenge, which might contribute to the extravagant inflammatory response manifested by CF mice. Accordingly, insulin-induced activation of Akt1 and Akt2 signaling was diminished in human airway epithelia expressing F508del-CFTR compared with wild-type CFTR cells. In this study, the protein expressions of PI3K, p-AKT^{ser473}, and p-AKT^{Thr308} were reduced at 12 h and 24 h after the H/R treatment and returned to normal at 48 h. Interestingly, it was discovered that the AKT activator and PI3K activator could reverse the H/R-induced cell apoptosis. The results indicated that overexpression of CFTR can significantly enhance the PI3K/AKT expression in the H/R-induced apoptosis. Our results implied that CFTR increased cell viability and inhibited cell apoptosis in the H/R-treated cells, and BA, GSK690693, and LY294002 significantly inhibited this effect. Altogether, based on our findings and previous studies, these results indicate that the inhibition of PI3K/AKT or the activation of NF-κB both impedes the protective effect of CFTR on H/R-induced apoptosis and showed that CFTR protects the Caco2 cells from H/R-induced apoptosis through the PI3K/AKT and

NF-κB pathway.

Since the above experiments seemed to indicate that PI3K/AKT and NF-κB are involved in the same signaling pathway, we proceeded to test the relationship between PI3K/AKT and NF-κB activation. Recent studies have confirmed that the PI3K/AKT pathway reduces nuclear translocation of p65[27,28]. But some studies have shown that AKT overexpression increases NF-κB-dependent gene expression[2, 29]. In our study, it was found that PI3K/AKT is upstream of NF-κB in H/R-induced response since PI3K inhibitor can abrogate the elevation of NF-κB P65 induced by H/R, whereas NF-κB inhibitor does not affect the activation of PI3K/AKT. And it was also found that activation of PI3K/AKT is sooner than that of NF-κB after H/R treatment was detected using Western blotting, supporting the notion that PI3K/AKT is the upstream of NF-κB. Additionally, the H/R-induced apoptosis can be reversed through PI3K/AKT or NF-κB inhibitor alone, with no additive effect if treated in combination, suggesting that PI3K/AKT and NF-κB are on the same pathway.

Although this study has achieved certain results, there are some limitations. First, we use an immortal human intestinal epithelial cell instead of primary cells, which limits the research's authenticity and reliability to a certain extent. Second, the simulated hypoxic environment *in vitro* may be different from the real intestinal ischemia-reperfusion. However, the mechanism of ischemia-reperfusion affecting intestinal barrier permeability is complex, including the activity of inflammatory cytokines, endotoxemia of mitochondria or endoplasmic reticulum, and congenital immune dysfunction, which may become the mutual network of cellular pathways. Thus, further research is needed to clarify this mechanism.

CONCLUSION

In conclusion, our data demonstrate that CFTR is downregulated in H/R-treated Caco2 cells, and overexpression of CFTR protects Caco2 cells from H/R-induced apoptosis. Additionally, CFTR is involved in inhibiting H/R-induced apoptosis, and it inhibits apoptosis through PI3K/AKT/NF-κB signaling pathway. In all, this work suggests that overexpression of CFTR attenuates H/R-induced apoptosis through PI3K/AKT/NF-κB signaling pathway in H/R-treated Caco2 cells.

ARTICLE HIGHLIGHTS

Research background

Intestinal ischemia/reperfusion (I/R) causes local injuries in the intestine and multiple organ dysfunction syndromes or even multiple organ failure in distant organs with a mortality rate ranging from 30%–90%. The underlying mechanisms are very complex. preventing intestinal apoptosis is one of the critical targets in treating patients with intestinal I/R injury.

Research motivation

Exploring the mechanism of apoptosis in intestinal I/R injury and preventing intestinal apoptosis is one of the critical targets in treating patients with intestinal I/R injury.

Research objectives

To apoptosis and its mechanism.

Research methods

An intestinal I/R injury model was established in mice with superior mesenteric artery occlusion, and Caco2 cells were subjected to hypoxia/reoxygenation (H/R) for the simulation of I/R *in vivo*.

Research results

Cystic fibrosis transmembrane conductance regulator (CFTR) overexpression significantly increased the Caco2 cell viability and decreased cell apoptosis induced by the H/R. And CFTR overexpression could reverse the decreased PI3K/AKT expression induced by the I/R treatment *in vivo* or H/R treatment *in vitro*.

Research conclusions

Overexpression of CFTR attenuates H/R-induced apoptosis through PI3K/AKT/NF-κB signaling pathway in H/R-treated Caco2 cells.

Research perspectives

CFTR/PI3K/AKT/NF-κB signaling pathway is potential mechanism to protect intestinal cell from apoptosis in intestinal I/R injury and critical targets in treating patients with intestinal I/R injury.

FOOTNOTES

Author contributions: Dong ZW and Liu H contributed equally to this study; Dong ZW, Liu H and Liu P designed the research; Su FF, Fan XZ, and Zhang Y conducted experiments and analyzed the data; Dong ZW and Liu H wrote the manuscript; Liu P revised the manuscript; all authors approved the final version of the article.

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Institutional animal care and use committee statement: All animal experiments conformed to the internationally accepted principles for the care and use of laboratory animals, No. 2020-43-YJ01.

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Data sharing statement: No additional data are available.

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

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Country/Territory of origin: China

ORCID number: Zhi-Wei Dong 0000-0001-7009-9331; Hui Liu 0000-0003-4721-560X; Fei-Fei Su 0000-0001-9816-1491; Xiao-Zhou Fan 0000-0002-8280-4146; Yong Zhang 0000-0001-9650-4294; Peng Liu 0000-0002-8063-6214.

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ORIGINAL ARTICLE

Retrospective Cohort Study

Sex-based differences in histology, staging, and prognosis among 2983 gastric cancer surgery patients

Yonghoon Choi, Nayoung Kim, Ki Wook Kim, Hyeong Ho Jo, Jaehyung Park, Hyuk Yoon, Cheol Min Shin, Young Soo Park, Dong Ho Lee, Hyeon Jeong Oh, Hye Seung Lee, Young Suk Park, Sang-Hoon Ahn, Yun-Suhk Suh, Do Joong Park, Hyung-Ho Kim, Ji-Won Kim, Jin Won Kim, Keun-Wook Lee, Won Chang, Ji Hoon Park, Yoon Jin Lee, Kyoung Ho Lee, Young Hoon Kim

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Yonghoon Choi, Nayoung Kim, Ki Wook Kim, Hyeong Ho Jo, Jaehyung Park, Hyuk Yoon, Cheol Min Shin, Young Soo Park, Dong Ho Lee, Ji-Won Kim, Jin Won Kim, Keun-Wook Lee, Department ofInternal Medicine, Seoul National University Bundang Hospital, Seongnam 13620, South Korea

Nayoung Kim, Dong Ho Lee, Keun-Wook Lee, Department of Internal Medicine, Seoul National University College of Medicine, Seoul 03080, South Korea

Hyeon Jeong Oh, Department of Pathology, Seoul National University Bundang Hospital, Seongnam 13620, South Korea

Hye Seung Lee, Department of Pathology, Seoul National University College of Medicine, Seoul 03080, South Korea

Young Suk Park, Sang-Hoon Ahn, Yun-Suhk Suh, Hyung-Ho Kim, Department of Surgery, Seoul National University Bundang Hospital, Seongnam 13620, South Korea

Yun-Suhk Suh, Do Joong Park, Hyung-Ho Kim, Department of Surgery, Seoul National University College of Medicine, Seoul 03080, South Korea

Won Chang, Ji Hoon Park, Yoon Jin Lee, Kyoung Ho Lee, Young Hoon Kim, Department of Radiology, Seoul National University Bundang Hospital, Seongnam 13620, South Korea

Kyoung Ho Lee, Young Hoon Kim, Department of Radiology, Seoul National University College of Medicine, Seoul 03080, South Korea

Corresponding author: Nayoung Kim, MD, PhD, Professor, Department of Internal Medicine, Seoul National University Bundang Hospital, 82, Gumi-ro 173 Beon-gil, Bundang-gu, Seongnam 13620, South Korea. nakim49@snu.ac.kr

Abstract

BACKGROUND

Few studies have been conducted on sex differences in the incidence, pathophysiology, and prognosis of gastric cancer (GC).

AIM
To analyze the differences in GC characteristics according to sex in patients who underwent surgical treatment for GC.

METHODS
A total of 2983 patients diagnosed with gastric adenocarcinoma who received surgical treatment at the Seoul National University Bundang Hospital between 2003 and 2017 were included. Baseline clinicopathological characteristics, histologic type of GC, overall and GC-specific survival rates, and associated risk factors were analyzed.

RESULTS
Among the 2983 patients, 2005 (67.2%) and 978 (32.8%) were males and females, respectively. The average age of the female group (59.36 years) was significantly younger than that of the male group (61.66 years; $P < 0.001$). Cancer of the gastric body ($P < 0.001$) and diffuse-type histology ($P < 0.001$) were more common in females than in males. This trend was more prominent in females younger than 60 years of age, with a significantly higher proportion of diffuse-type cancer than in the male group. Regardless of sex, diffuse-type GC was more common in younger patients, and the proportion of intestinal-type GC increased with age. The overall survival rate was significantly higher in females ($P < 0.001$). However, this difference disappeared for GC-specific survival ($P = 0.168$), except for the poor GC-specific survival rate in advanced-stage cancer (stage III or above) in females ($P = 0.045$). The risk factors for GC-related mortality were older age, upper location of GC, and diffuse- or mixed-type histology. In terms of comorbidities, more males died from diseases other than GC, including other malignancies such as lung cancer, hepatocellular carcinoma, and pancreatic cancer, and respiratory diseases such as interstitial lung disease and chronic obstructive pulmonary disease, while there were relatively more cardiovascular or cerebrovascular deaths in females.

CONCLUSION
Sex-based differences in GC were observed in clinicopathological features, including age at diagnosis, tumor location, histologic type, survival rate, and comorbidities.

Key Words: Gastric cancer; Histology; Prognosis; Sex difference; Survival

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Core Tip: In the analyses of sex differences in gastric cancer (GC), the sex ratio between males and females was 2:1, but the incidence of diffuse-type cancer was higher in females until the age of 60 years. The average age of the female group was significantly younger, and cancer of the gastric body and diffuse-type histology were more common than those in the males. In addition, there was poor GC-specific survival rate in advanced-stage cancer in females, while comorbidities including cancers of other organs and respiratory diseases were more common in males.

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INTRODUCTION

Gastric cancer (GC) is the fifth most common cancer and the third most common cause of cancer-related deaths worldwide[1]. Age-standardized incidence rates are approximately twice as high in males than in females[2]. Major risk factors for developing GC include *Helicobacter pylori* (*H. pylori*) infection, family history of GC, dietary habits, ionizing radiation, smoking, alcohol, and pernicious anemia, and the difference in incidence between males and females is likely due to the difference in exposure to these risk factors[3-5]. However, these factors alone do not fully explain the different characteristics of GC between the sexes. Recent research has revealed the role of sex hormones in various diseases and the resulting sex differences. It is well known that sex differences exist in the location and prognosis of

various cancers, including colorectal cancer[6,7], renal cell carcinoma[8], and bladder cancer[9]. In addition, sex differences are also known in central nervous system diseases such as cognitive disorders, Alzheimer's disease[10], Parkinson's disease[11], and autoimmune diseases[12]. However, the role of sex hormones such as testosterone and estrogen in the etiology, response to therapy, and survival of patients with GC, and the involved mechanisms and pathways remain unclear. Also, few studies to date have detailed the epidemiological and prognostic differences in GC between males and females.

Lauren classification is an independent prognostic factor in patients with GC[13]. That is, intestinal-type GC shows better clinicopathological characteristics and prognosis than diffuse-type GC. Diffuse-type cancer exhibits a higher recurrence rate than intestinal-type cancer, and the clinical appearance and survival of mixed-type cancers are known to be similar to those of diffuse-type GC[14]. Environmental factors reportedly play an important role in the development of intestinal-type versus diffuse-type GC [15]. There is also a sex-based difference in the histologic type of GC. That is, there is a marked predominance of older age and male sex in intestinal-type GC and a younger female predominance in diffuse-type GC. Younger female patients seem to exhibit a higher percentage of diffuse-type GC, resulting in more aggressive tumor behavior[14]; therefore, treatment methods may vary according to the Lauren type[16]. From this point of view, we hypothesized that an accurate analysis of sex-based differences in GC is possible in a well-designed surgical cohort with regular follow-up observations, clear histologic results, accurate information on family history, and social history such as smoking and alcohol consumption. Based on this background, the aim of this study was to analyze the sex-based differences in clinicopathological features and staging in GC, and to investigate prognostic factors including survival and death.

MATERIALS AND METHODS

Study population

Initially, 3074 patients aged > 18 years were selected from a prospective surgical cohort of patients who were diagnosed with gastric adenocarcinoma and underwent surgical treatment at Seoul National University Bundang Hospital (SNUBH) between 2003 and 2017 (Figure 1). Analyses of the effects of *H. pylori* eradication treatment, P53 overexpression and the incidence of metachronous GC in this cohort were previously published by our team[17-19]. The following patients were excluded: those with incomplete medical records or unclassified histology, who were lost to follow-up, had a prior history of other cancers at the time of diagnosis, or those who had other diseases with inoperable severity were excluded from the study. Finally, 2983 patients were included in the analysis (Figure 1). The medical records of these patients, including sex, age, death (including cause), histologic type of cancer, and social history such as alcohol consumption, smoking, and family history of GC were collected from surgical and medical cohorts, and reviewed using the Clinical Data Warehouse. The dates and causes of death of the enrolled patients were cross-reviewed with data from the National Statistical Office for verification.

Statistical analysis

The outcomes were overall survival and GC-specific survival. Univariate and multivariate Cox proportional hazards analyses were used to identify risk factors, and variables with a P value < 0.2 in the univariate analyses, were used as covariates for the multivariate analysis. The Kaplan–Meier estimator method and log-rank tests were used to compare survival. Analyses were performed using IBM SPSS Statistics software (version 25.0; IBM Corp., Armonk, NY, United States). Statistical significance was set at $P < 0.05$. All data are available upon request from the corresponding author.

Ethical considerations

The study was reviewed and approved by the Institutional Review Board of SNUBH (IRB No. B-1902–523-107) and registered at clinicaltrials.gov (NCT03978481). All authors have access to the study data and have approved the final manuscript.

RESULTS

Baseline clinicopathological characteristics

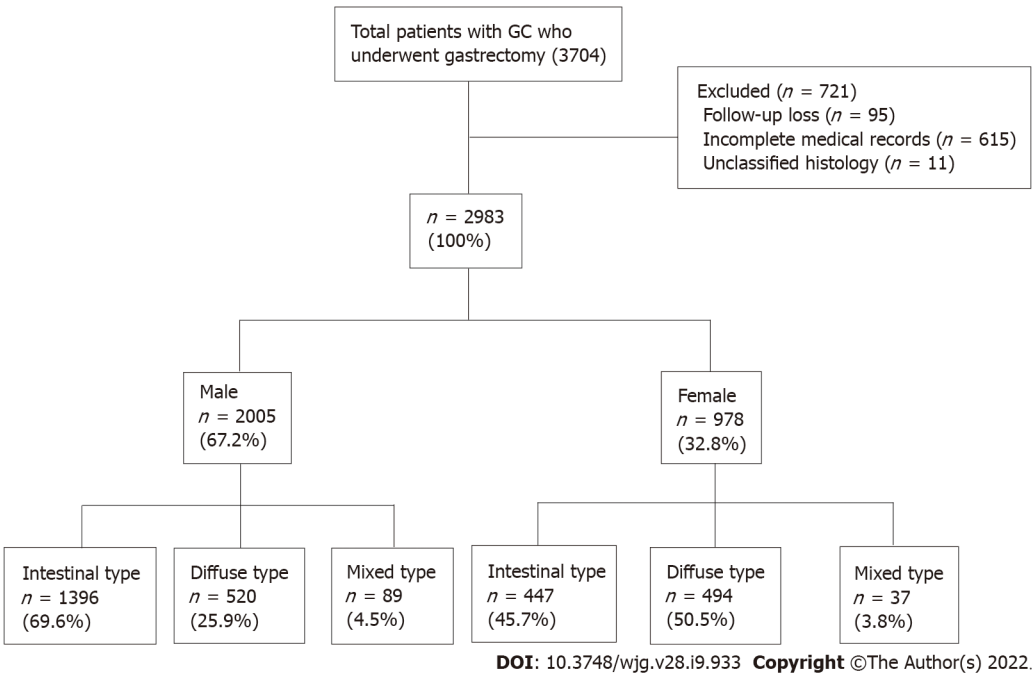
The baseline clinicopathological features of the subjects are shown in Table 1. Of the 2983 patients, 2005 were males and 978 were females, indicating a 2:1 sex ratio, with an average age of 61.66 for males and 59.36 for females, indicating a significantly younger onset age in females ($P < 0.001$). A higher proportion of males had a history of alcohol consumption and smoking (drinking history, $P < 0.001$; smoking history, $P < 0.001$). Cancer of the gastric body and diffuse-type cancer were more common in females (tumor location, $P < 0.001$; histologic type, $P < 0.001$, respectively). Overexpression of P53 was more common in males than in females ($P < 0.001$). There were no differences in family history, cancer

Table 1 Clinicopathological features of patients with gastric cancer

Characteristics	Total (*N* = 2983)	Female (*n* = 978)	Male (*n* = 2005)	*P* value
Age (yr, mean ± SD)	60.91 ± 12.31	59.36 ± 13.47	61.66 ± 11.63	< 0.001[a]
Drinking history, *n* (%)				
No	1631 (54.7)	790 (80.8)	841 (41.9)	< 0.001[a]
Yes	1352 (45.3)	188 (19.2)	1164 (58.1)	
Smoking history, *n* (%)				
No	1645 (55.1)	902 (92.2)	743 (37.1)	< 0.001[a]
Yes	1338 (44.9)	76 (7.8)	1262 (62.9)	
Family history, *n* (%)				
No	2467 (82.7)	803 (82.1)	1664 (83.0)	0.548
Yes	516 (17.3)	175 (17.9)	341 (17.0)	
Tumor location, *n* (%)				
Upper	77 (2.6)	19 (2.0)	58 (2.9)	< 0.001[a]
Middle	1332 (44.6)	497 (50.8)	835 (41.6)	
Lower	1574 (52.8)	462 (47.2)	1112 (55.5)	
Atrophic gastritis, *n* (%)				
No	2162 (72.5)	751 (76.8)	1411 (70.4)	< 0.001[a]
Yes	821 (27.5)	227 (23.2)	594 (29.6)	
Intestinal metaplasia, *n* (%)				
No	1680 (56.3)	560 (57.3)	1120 (55.9)	0.469
Yes	1303 (43.7)	418 (42.7)	885 (44.1)	
T stage, *n* (%)				
T1	2134 (71.5)	696 (71.2)	1438 (71.7)	0.669
T2	330 (11.1)	102 (10.4)	228 (11.4)	
T3	420 (14.1)	144 (14.7)	276 (13.8)	
T4	99 (3.3)	36 (3.7)	63 (3.1)	
N stage, *n* (%)				
N0	2215 (74.3)	696 (71.2)	1519 (75.7)	0.014[a]
N1	423 (14.2)	163 (16.7)	260 (13.0)	
N2	174 (5.8)	66 (6.7)	108 (5.4)	
N3	171 (5.7)	53 (5.4)	118 (5.9)	
Stage, *n* (%)				
I	2312 (77.5)	743 (76.0)	1569 (78.3)	0.189
II	405 (13.6)	151 (15.4)	254 (12.7)	
III	212 (7.1)	69 (7.1)	143 (7.1)	
IV	54 (1.8)	15 (1.5)	39 (1.9)	
Cancer type, *n* (%)				
EGC	2133 (71.5)	696 (71.2)	1437 (71.7)	0.774
AGC	850 (28.5)	282 (28.8)	568 (28.3)	
Histologic type, *n* (%) (Lauren classification)				
Intestinal	1843 (61.8)	447 (45.7)	1396 (69.6)	< 0.001[a]
Diffuse	1014 (34.0)	494 (50.5)	520 (25.9)	

Mixed	126 (4.2)	37 (3.8)	89 (4.5)	
H. pylori status, *n* (%)				
Negative	1267 (42.5)	379 (38.8)	888 (44.3)	0.004[a]
Positive	1716 (57.5)	599 (61.2)	1117 (55.7)	
P53, *n* (%)				
Negative	1917 (64.3)	706 (72.2)	1211 (60.4)	< 0.001[a]
Positive	1066 (35.7)	272 (27.8)	794 (39.6)	

[a]*P* < 0.05 indicates statistical significance. SD: Standard deviation; EGC: Early gastric cancer; AGC: Advanced gastric cancer; *H. pylori*: *Helicobacter pylori*.



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Figure 1 Study flow chart of patient enrollment and exclusion process. GC: Gastric cancer.

staging, or *H. pylori* infection at the time of diagnosis between males and females (family history, P = 0.548; cancer stage, P = 0.189; and *H. pylori* status, P = 0.062, respectively).

Differences in cancer histology by sex and age

To identify the histological changes in GC by age, the entire group of patients was divided into groups of under 40, 40-49, 50-59, 60-69, and 70+ years, and the trend of an increasing percentage of intestinal-type cancers with age, in both males and females, was noted (Supplementary Table 1). Considering the number of patients and histological ratios, there were more female patients under the age of 40 years and older male patients (Supplementary Figure 1). A higher number of female GC patients were under 40 years of age, while diffuse-type cancer was more common in both males and females than the other histological types (Figure 2). Among the male patients, the proportion of intestinal-type cancer increased steeply from age 50 years, whereas in female patients, the proportion of diffuse-type cancer remained high at 50-59 years of age (Figure 2). The ratio of intestinal- and diffuse-type GC in females approximately 20 years after menopause was similar to that of male patients aged ≥ 70 years (Figure 2).

Meanwhile, a significant correlation was observed between histological type and GC location, with a high ratio of diffuse-type cancer and stomach body cancer in females and a high ratio of intestinal-type cancer and stomach antral cancer in males (Pearson correlation analysis, P < 0.001).

Overall and cancer-specific survival

A statistically significant female predominance was identified in overall survival (P < 0.001), while a non-significant male predominance was identified in GC-specific survival (Figure 3). Increasing age, proximal tumor location, and diffuse- or mixed-type histology were identified as risk factors for GC-related morbidity (Table 2). In terms of cancer stage, there were no significant differences in patients

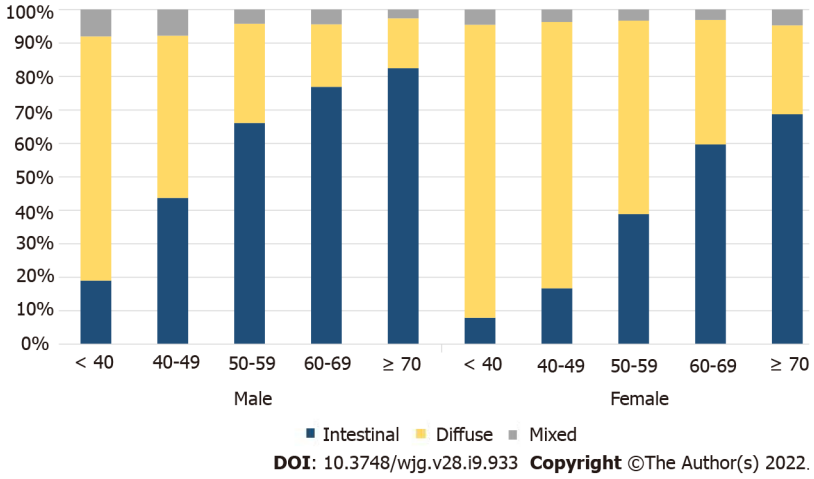
Table 2 Univariate and multivariate analyses for gastric cancer related morbidity

Variable	Univariate analysis	*P* value	Multivariate analysis	*P* value
Sex				
Male	Ref	0.169	Ref	0.672
Female	1.22 (0.92-1.61)		1.06 (0.80-1.42)	
Age				
< 60	Ref	0.001[a]	Ref	< 0.001[a]
≥ 60	1.64 (1.23-2.18)		2.02 (1.50-2.73)	
Drinking history				
No	Ref	0.996		
Yes	1.00 (0.76-1.32)			
Smoking history				
No	Ref	0.283		
Yes	1.16 (0.88-1.53)			
Family history				
No	Ref	0.189	Ref	0.165
Yes	0.77 (0.51-1.14)		0.75 (0.51-1.12)	
Tumor location				
Upper	Ref	0.003[a]	Ref	< 0.001[a]
Middle	1.65 (0.41-6.71)		1.40 (0.34-5.71)	
Lower	2.61 (0.65-10.54)		2.63 (0.65-10.64)	
Atrophic gastritis				
No	Ref	0.871		
Yes	0.97 (0.71-1.34)			
Intestinal metaplasia				
No	Ref	0.412		
Yes	0.89 (0.67-1.18)			
Histologic type (Lauren classification)				
Intestinal	Ref	< 0.001[a]	Ref	< 0.001[a]
Diffuse	2.16 (1.62-2.89)		3.07 (2.25-4.19)	
Mixed	2.25 (1.29-3.92)		2.50 (1.43-4.35)	
P53				
Negative	Ref	0.651		
Positive	1.07 (0.80-1.42)			

[a]$P < 0.05$ indicates statistical significance.
$P < 0.2$ were used for multivariable analyses.

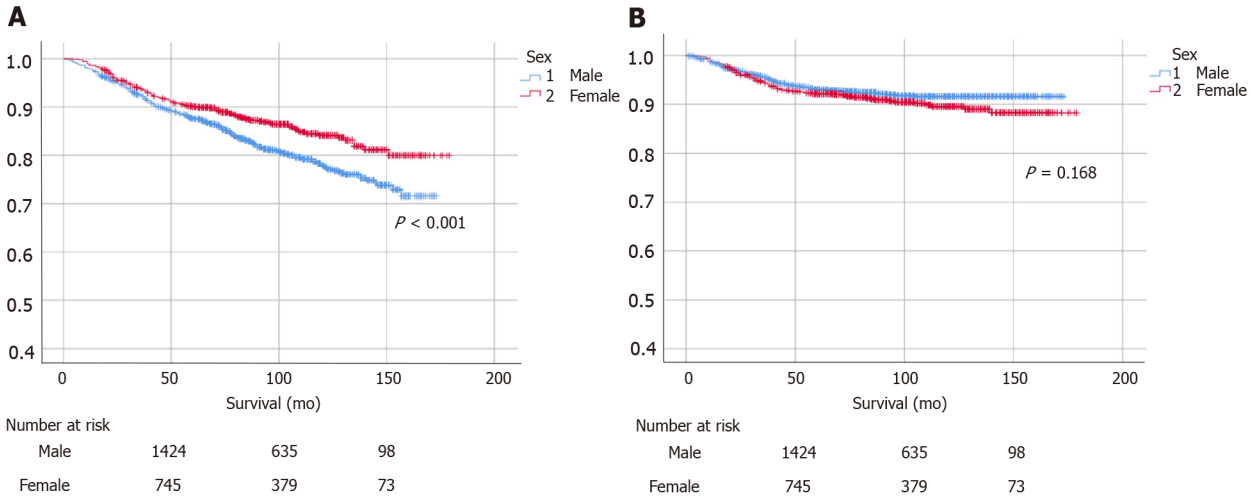
with stage I or II GC, whereas a statistically significant male predominance was observed in patients with advanced-stage cancer (stage III or above, $P = 0.045$; Figure 4). Histologically, patients with intestinal-type GC had a significantly higher survival rate than those with diffuse-type GC, and there were no statistically significant differences between males and females in intestinal- or diffuse-type GC (Supplementary Figure 2).

In the assessment of comorbidities, we investigated sex-based causes of death. Patients with a prior history of other cancers at the time of diagnosis or having severe diseases with inoperable conditions were excluded from the study, as mentioned above. Among the patients, 453 died including 135 males (6.7%) and 86 females (8.8%) died of GC. Significantly more males died from diseases other than GC (193 males and 39 females). In males, there were more deaths from malignancies such as lung cancer,



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Figure 2 Proportion of histological types of gastric cancer according to sex and age. The trend of an increasing proportion of intestinal-type cancers with increasing age was observed in both males and females. In males, the proportion of intestinal-type cancer increased steeply from an age of 50 years. In females, the proportion of diffuse-type cancer remained high until 60 years of age. The ratio of intestinal- and diffuse-type gastric cancer in females became similar to that of male patients aged 70 years or older, about 20 years after menopause.



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Figure 3 Survival according to sex and initial cancer stage. (A) Overall and (B) gastric cancer–specific survival. A statistically significant female predominance in overall survival was identified ($P < 0.001$), while a non-significant male predominance was identified in gastric cancer-specific survival. *P* values were calculated using the log-rank test.

hepatocellular carcinoma, pancreatic cancer, and respiratory diseases such as interstitial lung disease and chronic obstructive pulmonary disease, while there were relatively more cardiovascular or cerebrovascular deaths in females. Details regarding these are given in Supplementary Tables 2-4.

Subgroup analyses by sex and histology

The results of the subgroup analyses based on sex and histology are presented in Table 3 and Supplementary Table 3. In females, intestinal-type GC was associated with older age and a family history of GC, while diffuse-type GC was associated with younger age and P53 negativity. In males, intestinal-type GC was associated with older age, while diffuse-type GC tended to be associated with younger age and smoking history.

DISCUSSION

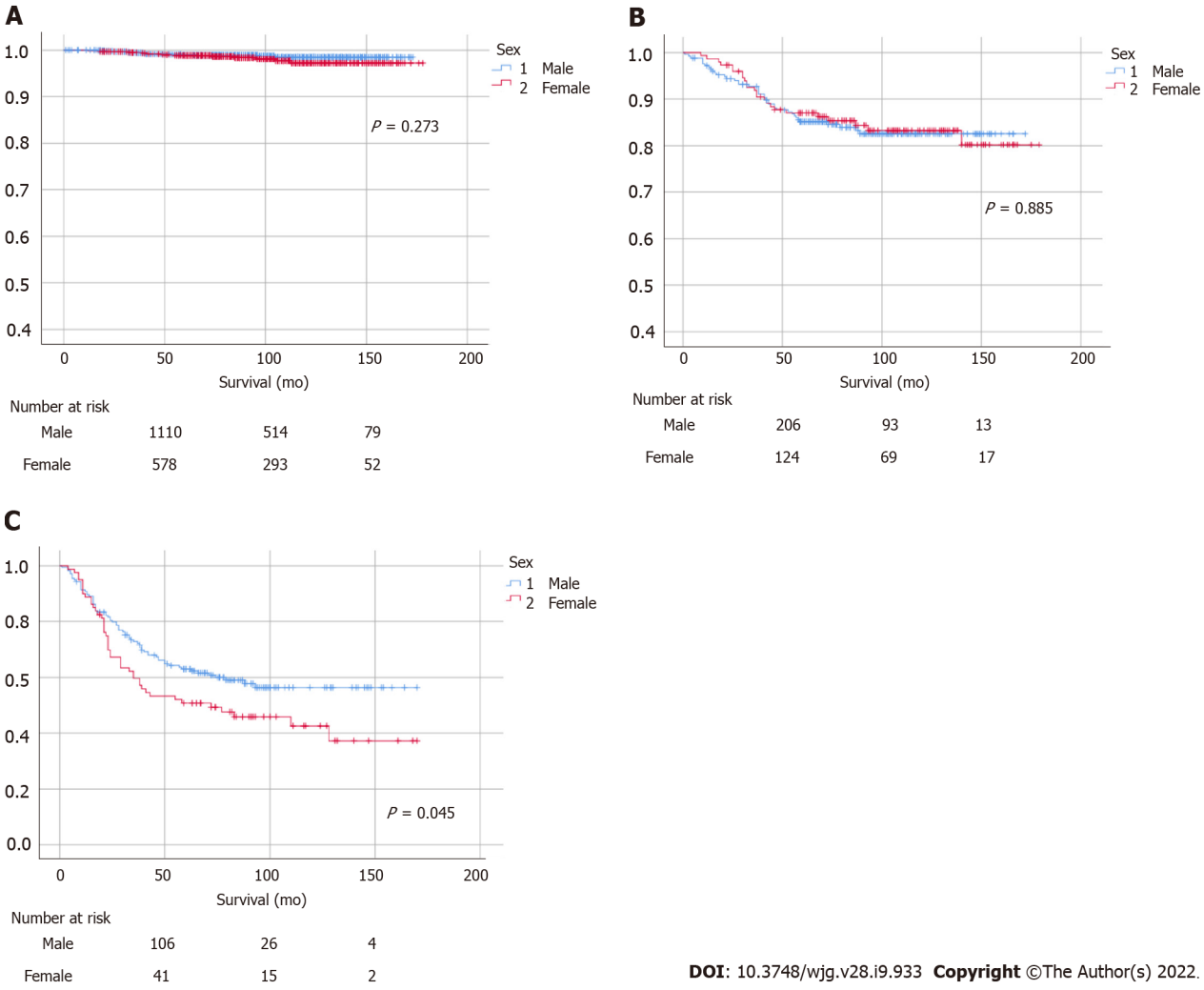
To the best of our knowledge, this study is the first to provide evidence of sex differences in GC with exact histologic diagnosis and long-term follow-up in nearly 3000 patients. In our data, a statistically significant overall survival benefit in females and a non-significant GC-specific survival in males were

Table 3 Clinicopathological features by sex and histologic type

Characteristics	Intestinal type			Diffuse type		
	Female (*n* = 447)	Male (*n* = 1396)	*P* value	Female (*n* = 494)	Male (*n* = 520)	*P* value
Age (yr, mean ± SD)	65.72 ± 10.71	64.06 ± 10.34	0.792	53.60 ± 13.06	55.82 ± 12.48	0.229
Drinking history, *n* (%)						
No	385 (86.1)	619 (44.3)	< 0.001[a]	374 (75.7)	183 (35.2)	< 0.001[a]
Yes	62 (13.9)	777 (55.7)		120 (24.3)	337 (64.8)	
Smoking history, *n* (%)						
No	420 (94.0)	530 (38.0)	< 0.001[a]	447 (90.5)	175 (33.7)	< 0.001[a]
Yes	27 (6.0)	866 (62.0)		47 (9.5)	345 (66.3)	
Family history, *n* (%)						
No	348 (77.9)	1138 (81.5)	0.088	419 (84.8)	447 (86.0)	0.606
Yes	99 (22.1)	258 (18.5)		75 (15.2)	73 (14.0)	
Tumor location, *n* (%)						
Upper	12 (2.7)	44 (3.2)	0.053	7 (1.4)	10 (1.9)	< 0.001[a]
Middle	139 (31.1)	517 (37.0)		334 (67.6)	284 (54.6)	
Lower	296 (66.2)	835 (59.8)		153 (31.0)	226 (43.5)	
Atrophic gastritis, *n* (%)						
No	320 (71.6)	951 (68.1)	0.168	400 (81.0)	389 (74.8)	0.018[a]
Yes	127 (28.4)	445 (31.9)		94 (19.0)	131 (25.2)	
Intestinal metaplasia, *n* (%)						
No	229 (51.2)	751 (53.8)	0.344	303 (61.3)	306 (58.8)	0.418
Yes	218 (48.8)	645 (46.2)		191 (38.7)	214 (41.2)	
T stage, *n* (%)						
T1	355 (79.4)	1087 (77.9)	0.529	314 (63.6)	305 (58.6)	0.445
T2	40 (9.0)	138 (9.9)		57 (11.5)	66 (12.7)	
T3	40 (9.0)	145 (10.4)		99 (20.0)	118 (22.7)	
T4	12 (2.6)	26 (1.8)		24 (4.9)	31 (6.0)	
N stage, *n* (%)						
N0	356 (79.6)	1140 (81.7)	0.745	319 (64.6)	335 (64.4)	0.055
N1	56 (12.5)	149 (10.7)		97 (19.6)	85 (16.4)	
N2	20 (4.5)	62 (4.4)		42 (8.5)	38 (7.3)	
N3	15 (3.4)	45 (3.2)		36 (7.3)	62 (11.9)	
Stage, *n* (%)						
I	375 (83.9)	1171 (83.9)	0.49	342 (69.2)	347 (66.7)	0.152
II	47 (10.5)	142 (10.2)		96 (19.5)	89 (17.1)	
III	23 (5.1)	65 (4.6)		43 (8.7)	66 (12.7)	
IV	2 (0.5)	18 (1.3)		13 (2.6)	18 (3.5)	
Cancer type, *n* (%)						
EGC	355 (79.4)	1086 (77.8)	0.469	314 (63.6)	305 (58.7)	0.109
AGC	92 (20.6)	310 (22.2)		180 (36.4)	215 (41.3)	
P53, *n* (%)						
Negative	293 (65.5)	770 (55.2)	< 0.001[a]	385 (77.9)	393 (75.6)	0.374

Positive	154 (34.5)	626 (44.8)	109 (22.1)	127 (24.4)

[a]*P* < 0.05 indicates statistical significance. SD: Standard deviation; EGC: Early gastric cancer; AGC: Advanced gastric cancer.



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Figure 4 Gastric cancer-specific survival in (A) stage I, (B) stage II, and (C) stage above III. There were no significant differences between males and females in stages I and II, but a statistically significant male predominance was observed in advanced-stage cancer (stage III or above, *P* = 0.045). *P* values were calculated using the log-rank test.

observed. In both males and females, a high proportion of diffuse-type cancers was observed among younger patients, while intestinal-type cancer became more prominent with increasing age. However, more females of all ages had diffuse-type cancer, while the ratio of diffuse-type to intestinal-type cancer was higher in females until the age of 60 years. In addition, the incidence of higher proportion of diffuse-type and gastric body cancers in females, compared to intestinal-type and antral cancers in males could be the reasons for higher N stage and poor GC-specific survival in females. Furthermore, there were also differences in comorbidities, including causes of death other than GC, between males and females.

There are few studies on the prognosis of GC by sex, and sex-based differences in GC are not clear, as in other cancers such as colorectal cancer[6,7]. A recent study based on the Surveillance, Epidemiology, and End Results (SEER) database in the United States reported survival advantages in females[20]. A female advantage was observed in both overall survival and GC-specific survival, and the prognosis of GC and the risk of developing GC were significantly worse in males than in females in that study, so the authors insisted on the necessity for early intervention in high-risk male patients due to their relatively poor prognosis[20]. However, this difference from our results could be due to a difference in the histologic type of GC, with a higher proportion of adenocarcinoma and a lower proportion of signet ring cell carcinoma (SRC), especially in females. In a large meta-analysis of data obtained from the Korea Central Cancer Registry and National Statistical Office reported by Song *et al*[21], the prognosis of female GC patients was also better than that of male GC patients, with differential incidence and

mortality patterns among age groups. However, females tend to have a worse prognosis when they are diagnosed later than 40 years of age. In that study, the histologic type or anatomic subsites of GC could not be identified. Since the 2000s, many early GC (EGC) patients have been identified and treated through a national endoscopic surveillance project in Korea, possibly showing different results from data prior to the 2000s[22]. Contrary to earlier results, a recent study in Korea reported poor prognosis in females[23], similar to the present study. The authors concluded that female GC patients were significantly younger, had more poorly differentiated adenocarcinomas, and were more likely to have SRC than male GC patients. In addition, females with advanced GC (AGC) and SRC had significantly poorer overall survival rates. In our data, among patients with advanced-stage disease (stage III or above), females had significantly lower GC-specific survival rates than males. In our data, the ratio of diffuse-type (undifferentiated) GC was relatively high, especially among younger females. Diffuse-type GC is known to be related to genetic factors such as E-cadherin mutations, feature a poorer prognosis due to rapid growth and poor treatment response, and is generally more common in younger patients [24,25]. Comparing the characteristics of GC in Korea and the United States, more upper-third and differentiated cancers were observed in the United States, while Korean patients showed fewer upper-third cancers with poorer cancer differentiation, deeper invasion, and poorer prognosis; hence, a difference in GC characteristics between Korean and United States populations is suspected[26].

The same results have been shown in previous studies in relation to histologic type and GC location, as more diffuse-type, gastric body location cases were noted in females versus more intestinal-type, stomach antrum location cases in males[27,28]. Based on previous reports on GC location, the distribution was reported as cardia 4%-8%, body 15%-30%, and antrum 60%-80% in a study of EGC in Korea [29]. In another Korean study of patients who underwent endoscopic resection for EGC, the most common location for EGC was the antrum (57.5%) and lesser curvature (37.8%), and body cancers were associated with younger patient age, larger tumor size, and more frequent poorly differentiated or SRC histology than cancers at other sites[30]. Our data are consistent with those of the aforementioned studies, and this relation to histologic type and GC location is believed to be due to differences in the composition of gastric mucous cells, such as the gastric body with a large distribution of parietal cells and the antrum with a large distribution of G cells[31].

The effects of sex hormones may cause this sex-based difference in GC. Epidemiological studies have reported that exogenous sex hormone exposure reduces the risk of esophageal adenocarcinoma[32,33] and esophageal squamous cell carcinoma[34], and a decrease in the risk of GC[35] and colorectal cancer [33,36] have been reported in females taking oral contraceptives or hormone replacement therapy. A large cohort study in Japan also reported that females in early menarche had a decreased risk of GC, especially differentiated-type GC, in subgroup analyses of histologic subtypes[37]. In addition, in a Chinese study of approximately 2000 surgically treated GC patients, the proportion of female GC patients showed a decreasing tendency, and the proportion of male GC patients showed an increasing tendency with age, but this trend stopped after 60 years of age[38]. Furthermore, a recent study in Korea reported that no premenopausal females had intestinal-type GC and that the ratio of intestinal-type GC increased in females after menopause and became similar to that of males about 10 years after menopause; this parity was associated with an increased risk of intestinal-type GC in females after menopause[27].

These results suggest that estrogen plays a role in curbing the development of GC in females, especially in intestinal-type GC. However, the specific mechanisms of estrogen in different histologic subtypes have not yet been established. Several studies have attempted to explain this by investigating the role of estrogen receptors (ER) in GC. First, Yi *et al*[39] showed that ERα expression was associated with diffuse-type GC and shorter disease-free survival. Wang *et al*[40] reported that well-differentiated gastric adenocarcinoma has a higher expression rate of ERβ and that poorly differentiated gastric adenocarcinoma is associated with a reduction or loss of ERβ. According to previous studies, diffuse-type GC may be initiated by the downregulation of E-cadherin by 17β-estradiol (E2), the most potent isoform of estrogen, through ERα[41-43].

In addition to the action of estrogen, Gan *et al*[44] reported that the four sex hormone receptors, ERα, ERβ, progesterone receptor, and androgen receptor (AR), were expressed independently and showed a decreased expression pattern in gastric tumors compared to adjacent normal tissues, suggesting that sex hormone receptors may be partly involved in gastric carcinogenesis. Jukic *et al*[2] reported a significantly higher frequency of cases with AR-positive cells in the stroma of intestinal-type GC in males than in females, which may be the reason for the greater invasiveness of this cancer type in males and presented the possibility of AR-targeted agents in GC treatment. Another study by Hsu *et al*[45] showed that males were more likely to develop tumor recurrence and liver metastasis than females, especially in cases of stage III GC. The authors suggested that the cause was higher programmed death ligand 1 expression in males and GC patients aged 65 years or older, and supporting data suggest that sex hormones are the basis of these differences[46,47].

The changes in the proportion of intestinal-type and diffuse-type cancers in the present study suggest that estrogen might have a protective effect on intestinal-type GC[27,28]. Thus, intestinal-type GC is much less common in young females than in males, and the prevalence of intestinal-type GC increases in females after menopause, which is likely to be similar to males about 20 years after menopause according to our data (approximately 70 years of age). Additional in-depth studies are needed to

confirm the role of sex hormones, including estrogen, in the pathogenesis and progression of GC, depending on the tissue type.

The pattern of P53 overexpression also differed by sex; P53 overexpression was more frequent in males and intestinal-type GC patients. In our previous report on P53 overexpression, the clinical and prognostic significance differed by histological type of GC; P53 overexpression was more common in intestinal-type GC, but was associated with a poor prognosis for diffuse-type GC[18]. Therefore, it is also likely to act as a factor that affects GC prognosis differently in males and females.

Our study has several limitations. First, the enrolled subjects were patients who underwent surgical treatment after receiving a diagnosis of GC; therefore, early cases treated with endoscopic resection and advanced inoperable cases were not included. Hence, in terms of GC-related survival and mortality, the data from our study are likely to be slightly different from those of all patients with GC. To compensate for this limitation, we are conducting a follow-up study of patients diagnosed with and treated for over 14000 GC in SNUBH. The results of our data analyses to this point showed no significant differences between males and females according to the treatment method. Second, there are no data on estrogen exposure such as menopause, childbirth, and breastfeeding in this study, making it difficult to provide additional evidence that estrogen has protective effects against intestinal-type cancer. Further research, including a history of sex hormone use, is required. Third, the eradication of *H. pylori* was not confirmed in all patients, although postoperative *H. pylori* eradication treatment may affect prognosis or survival [17]. In the future, additional research is needed that considers both *H. pylori* infection and sex. In contrast, our research has several strengths over existing studies. Studies involving subjects before the year 2000 reported that the prognosis of GC was relatively good in females; however, these studies did not reflect the situation in East Asia, where the prevalence of GC is high[20], or the exact histologic type of GC was not analyzed[21]. A relatively recent large-scale Korean study reflecting histological types reported that the prognosis of GC was poorer in females than in males, similar to our results[23]. In this study, the changes in the histology of GC according to age was examined, and the change in the ratio according to aging and menopause was confirmed, suggesting that female hormones would affect the development and progression of GC. However, we further analyzed the comorbidities of GC patients with respect to survival.

CONCLUSION

In conclusion, differences in the epidemiology of GC incidence, including a higher proportion of diffuse-type histology and mortality, and poorer survival in AGC in females, were observed. The proportion of diffuse-type cancer was found to be higher in younger patients, the frequency of intestinal-type histology increased with age, and the ratio of diffuse-type cancer was higher until the age of 60 years in females. Differences in Lauren histologic type and tumor location by sex were also observed, with a high proportion of diffuse-type and gastric body location in females. Comorbidities, including other malignancies and respiratory diseases, are more common in males. These differences may originate from hormonal factors and should be considered in the diagnosis, treatment, and prediction of prognosis of GC in individuals.

ARTICLE HIGHLIGHTS

Research background

Despite the nationwide large-scale screening campaign, the incidence of gastric cancer (GC) in Korea is still high. The incidence is approximately twice as high in males than in females.

Research motivation

However, studies so far have not fully explained the different characteristics of GC between the sexes. These differences might be due to the difference in exposure to the known risk factors for GC, such as frequent *Helicobacter pylori* infection, smoking, and alcohol consumption in males, but we thought that there is a possibility that sex hormones were based on this difference.

Research objectives

This study aimed to analyze sex-based differences in clinicopathological features, staging, survival, and comorbidities in GC.

Research methods

A total of 2983 patients diagnosed with gastric adenocarcinoma who received surgical treatment at the Seoul National University Bundang Hospital between 2003 and 2017 were included, and clinicopathological characteristics, histologic type of GC, overall and GC-specific survival rates, and associated risk

factors were analyzed.

Research results

The male to female ratio was 2:1, and the average age of the female group was lower than that of the male group. Diffuse-type GC was more common in younger patients, especially in females younger than 60 years of age, and the proportion of intestinal-type GC increased with age. The overall survival rate was significantly higher in females, whereas GC-specific survival tended to be higher in males. Comorbidities, including other malignancies and respiratory diseases, are more common in males.

Research conclusions

Differences in the epidemiology of GC incidence, including a higher proportion of diffuse-type histology, mortality, including poorer survival in the advanced stage in females, and comorbidities were observed. These differences may be due to hormonal factors.

Research perspectives

We believe that a larger study including patients who received non-surgical treatment is needed. Individual sex hormone data, including menopause, childbirth, and breastfeeding, would be analyzed to prove the protective effect of estrogen against intestinal-type GC.

FOOTNOTES

Author contributions: Choi Y analyzed the data, provided statistical support, and drafted the article; Kim N designed this study, collected the data, and edited the manuscript; Kim KW, Jo HH, Park J, Yoon H, Shin CM, Park YS, and Lee DH performed endoscopies for the diagnosis of gastric cancer and edited the text; Park YS, Ahn SH, Suh YS, and Park DJ performed surgeries for gastric cancer; Kim HH kindly provided surgical cohort information, advised on the design of this study and supervised the manuscript preparation; HJO and HSL performed the histologic diagnosis of gastric cancer; Kim JW, Kim JW and Lee KW administered chemotherapy to patients with advanced gastric cancer; and Chang W, Park JH, Lee YJ, Lee KH, and Kim YH performed the radiologic studies; all authors reviewed the final manuscript and provided comments.

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

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

Data sharing statement: To gain access to data, data requestors will need to sign a data access agreement. Proposals should be directed to the Ethics Committee of Seoul National University Bundang Hospital.

STROBE statement: The authors have read the STROBE Statement—checklist of items, and the manuscript was prepared and revised according to the STROBE Statement—checklist of items.

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Country/Territory of origin: South Korea

ORCID number: Yonghoon Choi 0000-0002-1331-969X; Nayoung Kim 0000-0002-9397-0406; Ki Wook Kim 0000-0003-3936-2490; Hyeong Ho Jo 0000-0002-4950-5435; Jaehyung Park 0000-0003-1142-264X; Hyuk Yoon 0000-0002-2657-0349; Cheol Min Shin 0000-0003-2265-9845; Young Soo Park 0000-0001-8945-0507; Dong Ho Lee 0000-0002-6376-410X; Hyeon Jeong Oh 0000-0002-9998-3988; Hye Seung Lee 0000-0002-1667-7986; Young Suk Park 0000-0002-6352-9759; Sang-Hoon Ahn 0000-0001-8827-3625; Yun-Suhk Suh 0000-0003-3319-8482; Do Joong Park 0000-0001-9644-6127; Hyung-Ho Kim 0000-0002-8916-0048; Ji-Won Kim 0000-0001-6426-9074; Jin Won Kim 0000-0002-1357-7015; Keun-Wook Lee 0000-0002-8491-703X; Won Chang 0000-0001-7367-9841; Ji Hoon Park 0000-0002-6794-4909; Yoon Jin Lee 0000-0002-3572-029X; Kyoung Ho Lee 0000-0001-6045-765X; Young Hoon Kim 0000-0001-5554-3828.

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ORIGINAL ARTICLE

Retrospective Study

Postoperative morbidity adversely impacts oncological prognosis after curative resection for hilar cholangiocarcinoma

Zhi-Peng Liu, Wei-Yue Chen, Yan-Qi Zhang, Yan Jiang, Jie Bai, Yu Pan, Shi-Yun Zhong, Yun-Ping Zhong, Zhi-Yu Chen, Hai-Su Dai

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Zhi-Peng Liu, Yan Jiang, Jie Bai, Yu Pan, Shi-Yun Zhong, Yun-Ping Zhong, Zhi-Yu Chen, Hai-Su Dai, Department of Hepatobiliary Surgery, Southwest Hospital, Third Military Medical University (Army Medical University), Chongqing 400038, China

Wei-Yue Chen, Department of Clinical Center of Oncology, Lishui Hospital of Zhejiang University, Lishui 323000, Zhejiang Province, China

Yan-Qi Zhang, Department of Health Statistics, Third Military Medical University (Army Medical University), Chongqing 400038, China

Corresponding author: Hai-Su Dai, MD, Professor, Department of Hepatobiliary Surgery, Southwest Hospital, Third Military Medical University (Army Medical University), No. 30 Gaotanyan Road, Chongqing 400038, China. daihaisu@163.com

Abstract

BACKGROUND

Postoperative morbidity after curative resection for hilar cholangiocarcinoma (HCCA) is common; however, whether it has an impact on oncological prognosis is unknown.

AIM

To evaluate the influence of postoperative morbidity on tumor recurrence and mortality after curative resection for HCCA.

METHODS

Patients with recently diagnosed HCCA who had undergone curative resection between January 2010 and December 2017 at The First Affiliated Hospital of Army Medical University in China were enrolled. The independent risk factors for morbidity in the 30 d after surgery were investigated, and links between postoperative morbidity and patient characteristics and outcomes were assessed. Postoperative morbidities were divided into five grades based on the Clavien-Dindo classification, and major morbidities were defined as Clavien-Dindo ≥ 3. Univariate and multivariate Cox regression analyses were used to evaluate the risk factors for recurrence-free survival (RFS) and overall survival (OS).

RESULTS

Postoperative morbidity occurred in 146 out of 239 patients (61.1%). Multivariate

logistic regression revealed that cirrhosis, intraoperative blood loss > 500 mL, diabetes mellitus, and obesity were independent risk factors. Postoperative morbidity was associated with decreased OS and RFS (OS: 18.0 mo *vs* 31.0 mo, respectively, $P = 0.003$; RFS: 16.0 mo *vs* 26.0 mo, respectively, $P = 0.002$). Multivariate Cox regression analysis indicated that postoperative morbidity was independently associated with decreased OS [hazard ratios (HR): 1.557, 95% confidence interval (CI): 1.119-2.167, $P = 0.009$] and RFS (HR: 1.535, 95%CI: 1.117-2.108, $P = 0.008$). Moreover, major morbidity was independently associated with decreased OS (HR: 2.175; 95%CI: 1.470-3.216, $P < 0.001$) and RFS (HR: 2.054; 95%CI: 1.400-3.014, $P < 0.001$) after curative resection for HCCA.

CONCLUSION

Postoperative morbidity (especially major morbidity) may be an independent risk factor for unfavorable prognosis in HCCA patients following curative resection.

Key Words: Hilar cholangiocarcinoma; Morbidity; Surgery; Oncology; Survival; Recurrence

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Core Tip: In this study, postoperative morbidity was found to be an independent risk factor for poor overall survival and recurrence-free survival following curative resection for hilar cholangiocarcinoma. In addition, this study revealed the independent risk factors associated with increased postoperative morbidity, which could help to reduce the incidence of postoperative morbidity and improve oncological prognosis.

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INTRODUCTION

Cholangiocarcinoma is a common malignancy of the liver, second only to hepatocellular carcinoma (HCC) in incidence and accounting for approximately 10% of primary liver tumors[1,2]. Hilar cholangiocarcinoma (HCCA), also referred to as a Klatskin tumor, represents 60% of cholangiocarcinomas[3]. The HCCA incidence is increasing, and tumors have a poorer prognosis than any other hepatobiliary tumor, such as HCC, with five-year survival rates of 20% to 40%[4,5]. Radical surgery offers a possible cure for eligible HCCA patients. However, the oncological prognosis after liver resection for HCCA is often uncertain, as the tumor recurs within five years in over 60% of patients[6,7]. Consequently, identifying the risk factors that influence HCCA recurrence is important to improve outcomes.

Previous studies have demonstrated that postoperative morbidity is linked to greater recurrence and lower survival rates than many other gastrointestinal tumors, such as HCC[8], pancreatic[9], gastric[10,11], and colorectal carcinomas[12,13], as well as intrahepatic cholangiocarcinoma[14]. Systemic inflammation may result from postoperative morbidity, which could, in turn, reduce the effectiveness of the immune response against the tumor[15]. This may explain the relationship between poorer prognosis and postoperative morbidity. Regrettably, because HCCA surgery is one of the most complicated operations in hepatobiliary surgery, there is a high incidence of postoperative morbidity, ranging from 30% to 70%[16]. Postoperative morbidity is linked to both surgical factors and patients' underlying diseases[17-19]. From our point of view, surgery should be both safe and effective, avoiding postoperative morbidity to improve oncological prognosis. However, few studies have investigated the effects of postoperative morbidity on oncological prognosis in patients with HCCA after curative resection.

Therefore, this study aimed to determine if there is a link between the presence of postoperative morbidity and oncological prognosis following curative resection for HCCA. Additionally, the study assessed the independent risk factors for the occurrence of postoperative morbidity.

MATERIALS AND METHODS

Patients

The data of patients with HCCA who had undergone curative resection for newly diagnosed HCCA between January 2010 and December 2017 at The First Affiliated Hospital of Army Medical University (Southwest Hospital) in China were collected and analyzed. The HCCA diagnosis was confirmed by postoperative pathological evaluation. Extrahepatic bile duct resection and partial hepatectomy were performed on all patients. Regardless of preoperative computed tomography (CT), magnetic resonance imaging (MRI) or suspicion of lymph node metastasis, all patients underwent locoregional lymphadenectomy. To achieve curative resection, combined pancreaticoduodenectomy and/or vascular resection was conducted, with curative resection classified as complete tumor (both macroscopic and microscopic) removal, with clear resection margins visible on microscopy (R0 resection). Patient exclusion criteria were: (1) Having received adjuvant chemotherapy and radiotherapy; (2) Unresectable tumor at exploration; (3) Having undergone R1 or R2 resection; (4) Recurrent HCCA; (5) Age < 18 years; (6) Postoperative death within 30 d; and (7) Incomplete data. The study was conducted in accordance with the Declaration of Helsinki. The study was approved by the Institutional Review Board of the South-West Hospital of Chongqing, China (No. KY2021129). Patients were not required to give informed consent for the study because the analysis used anonymous clinical data that were obtained after each patient agreed to treatment by written consent.

Variables

Variables related to patients and liver pathology included age, sex, American Society of Anesthesiologists (ASA) score, obesity (BMI > 30), diabetes mellitus, cirrhosis, and preoperative drainage. Cirrhosis was verified by postoperative histopathology. Tumor-related variables included preoperative carbohydrate antigen 19-9 (CA19-9) levels, maximum tumor size, macrovascular and microvascular invasion, peripheral nerve invasion, metastasis to lymph nodes, Bismuth type, and poorly differentiated tumors. A preoperative CA19-9 level of 150 U/L was used as a threshold to separate patients into two groups[20]. The operative variables were intraoperative blood loss, blood transfusion, and the extent of hepatectomy (minor or major). Resection of three or more Couinaud liver segments was called major hepatectomy, whereas resection of fewer segments was labeled minor hepatectomy.

Perioperative outcomes

Postoperative morbidity was classified according to the Clavien-Dindo classification[21], with minor morbidity defined as Clavien-Dindo grades I-II and major morbidity defined as grades III-V. The occurrence of postoperative morbidity within 30 d was recorded, as was the postoperative morbidity hospital stay. Morbidity included posthepatectomy liver failure (PHLF); blood, lung, abdominal, and biliary infection; pleural effusion; bile leakage; ascites; intestinal leakage cholangitis; abdominal hemorrhage; delayed gastric emptying; and wound dehiscence, among others. PHLF was recognized by the "50-50 criteria" five days or more after surgery[22]. A severe drop of > 3 g/dL in the postoperative hemoglobin level compared with the preoperative level was indicative of abdominal hemorrhage, with or without the need for transfusion and/or reoperation. Bile leakage was defined as a drain bilirubin concentration of more than three times higher than that of serum. Ascites or pleural effusions requiring diuretic administration or paracentesis were also recognized. Surgical site infection was diagnosed based on the Prevention of the National Nosocomial Infections Surveillance and Centers for Disease Control[23].

Follow-up procedures

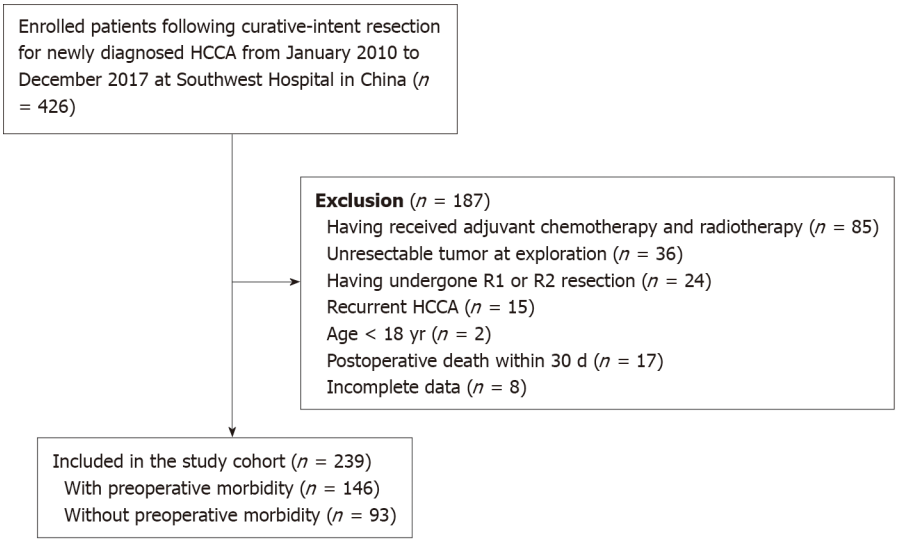
The patients were followed up at regular intervals (approximately 1-2 mo) after discharge. A standard protocol was used to evaluate the presence of HCCA recurrence. This included clinical symptoms, physical examinations, laboratory tests (liver function and tumor biomarkers), and abdominal ultrasonography. CT, MRI, or ultrasonic contrast was performed every two months after surgery or when tumor recurrence was suspected. The presence of new lesions seen on MRI or CT was defined as recurrence that was treated by surgery, drugs, or supportive therapy.

Endpoints

The primary endpoint was overall survival (OS), and the secondary endpoint was recurrence-free survival (RFS). OS was considered to be the interval from curative resection to death or last follow-up. For patients with recurrence, RFS was considered to be the interval from curative resection to the diagnosis of tumor recurrence. For patients without recurrence, RFS was taken as the interval from curative resection to death or last follow-up. Until the study's termination on July 15, 2020, all patients were followed up on until death or loss to follow-up.

Statistical analysis

Continuous variables are expressed as the means ± SD or medians (range), and categorical variables are expressed as the frequencies and percentages. Student's t test or the Mann–Whitney U test was used for



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Figure 1 Selection of the study population. HCCA: Hilar cholangiocarcinoma.

continuous variables, and Pearson's chi-square test was used for categorical variables. The Kaplan–Meier method and log-rank test were used to calculate and compare the OS and RFS rates. Variables showing significance levels of $P < 0.1$ on univariate analyses were used for multivariate analysis by the Cox proportional hazard model. In univariate and multivariate Cox regression studies, hazard ratios (HRs) and their 95 percent confidence intervals (CIs) were calculated. SPSS® version 26.0 (IBM, Armonk, New York, United States) was used for statistical analysis. *P* values were two-sided, and statistical significance was defined as a *P* value of less than 0.05.

RESULTS

Perioperative outcomes

In our study, 239 patients were included based on established inclusion criteria (Figure 1). All patients performed open surgery. Table 1 presents the perioperative outcomes for the 239 patients. Of these patients, 146 (61.1%) experienced morbidity within 30 d of surgery, with minor morbidity occurring in 78 (32.6%) and major morbidity in 68 (28.5%) patients. The top three causes of morbidity were surgical site infection (36/239, 15.1%), bile leak (32/239, 13.4%), and pleural effusion (24/239, 10.0%).

Patient characteristics

Table 2 shows the comparisons of patients' clinicopathologic and operative variables between those with and without postoperative morbidity. Notably, obesity, diabetes mellitus, cirrhosis, and intraoperative blood loss > 500 mL were more common in patients with morbidity ($P < 0.05$).

Risk factors for postoperative morbidity

In the multiple logistic regression model using significant ($P < 0.1$) factors shown in Table 2, cirrhosis [odds ratio (OR): 2.867; 95%CI: 1.207-6.810; $P = 0.017$], intraoperative blood loss > 500 mL (OR: 2.240; 95%CI: 1.162-4.318; $P = 0.016$), diabetes mellitus (OR: 3.395; 95%CI: 1.082-10.651; $P = 0.036$), and obesity (OR: 3.694; 95%CI: 1.197-11.394; $P = 0.023$) were identified as independent risk factors for postoperative morbidity (Table 3).

Survival outcomes

Table 4 shows the relationships between the patient survival outcomes and perioperative morbidity. Over the median 19.0-mo follow-up period, tumor recurrence and death were apparent in 76.7% (112/146) and 71.9% (105/146), respectively, of patients with postoperative morbidity and in 64.5% (60/93) and 59.1% (55/93), respectively, of patients who did not experience morbidity (recurrence, $P = 0.041$; death, $P = 0.041$). The median OS and RFS were significantly lower in the patients with postoperative morbidity, as shown in Figure 2 (OS: 18.0 mo *vs* 31.0 mo, $P = 0.003$; RFS: 16.0 mo *vs* 26.0 mo, $P = 0.002$).

Table 1 Postoperative outcomes of 239 patients who underwent curative resection for hilar cholangiocarcinoma

Postoperative outcomes (*n* = 239)	Patients (%)
Postoperative 30-d morbidity	146 (61.1)
Minor morbidity (Clavien-Dindo grade I-II)	78 (32.6)
Major morbidity (Clavien-Dindo grade III-IV)	68 (28.5)
Types of postoperative 30-d morbidity	
PHLF	15 (6.3)
Blood infection	14 (5.9)
Lung infection	12 (5.0)
Bile leakage	32 (13.4)
Pleural effusion	24 (10.0)
Ascites	4 (1.7)
Intestines leak	9 (3.8)
Abdominal hemorrhage	10 (6.8)
Delayed gastric emptying	17 (7.1)
Surgical site infection	36 (15.1)
Others	11 (4.6)
Postoperative hospital stay, days[1]	19 (15, 26)

[1]Values are median (interquartile range).
PHLF: Post-hepatectomy liver failure.

Prognostic factors for survival

Tables 5 and 6 present the results of the univariate and multivariate Cox regression analyses, respectively, for survival prediction. The multivariate analysis identified postoperative morbidity was independently associated with decreased OS (HR: 1.557, 95%CI: 1.119-2.167, P = 0.009) and RFS (HR: 1.535, 95%CI: 1.117-2.108, P = 0.008). Furthermore, preoperative CA19-9 > 150 U/L, maximum tumor size > 3 cm, lymph node metastasis, macrovascular invasion, and poor tumor differentiation were also observed to be risk factors for both OS and RFS.

Furthermore, based on the severity of postoperative morbidity, major morbidity was associated with both lower OS and RFS, as shown in Figure 3 (OS: HR: 2.175; 95%CI: 1.470-3.216, P < 0.001; RFS: HR: 2.054; 95%CI: 1.400-3.014, P < 0.001).

DISCUSSION

It is difficult to verify and define the quality of surgery, whether assessed on the level of outcome, process, or system[24]. As a tumor-related surgical quality measure, postoperative morbidity has been an increasingly interesting topic. In assessing a potential link between postoperative complications and outcomes in cancer patients, it is necessary to determine the factors leading to postoperative morbidity and the level of morbidity that may result in an unfavorable outcome[25]. Postoperative morbidity, therefore, is an indication of the quality of the surgery and may also act as a reliable prognostication of outcomes with the potential for therapeutic application. Thus, to reduce the incidence of perioperative morbidity, it is important to identify its contributory factors.

Here, we examined the prognostic impacts of 30-d morbidity in 239 HCCA patients after curative resection. The findings showed that perioperative morbidity negatively impacted both OS and RFS, indicating the value of reducing postoperative morbidity to improve patient outcomes. In this study, postoperative morbidity occurred in 146 (61.1%) patients, of which 68 (28.5%) experienced major morbidity. These findings support those of other studies. Hasegawa *et al*[19] observed a major postoperative complication (grade 3 or more) rate of 46.8%[26]. Gerhards *et al*[27] described a postoperative morbidity rate of 65.0% in patients following hemihepatectomy[27], while Dar *et al*[28] observed complications in approximately 66.7% of patients within 90 d[28]. In addition, a study from Japan observed that 21 patients (35.0%) had remarkable postoperative complications, while the presence of complications predicted worse outcomes in intrahepatic cholangiocarcinoma patients[14]. A large-

Table 2 Comparisons of patients' clinicopathologic and operative variables between those with and without postoperative morbidity

Variables	Total (*n* = 239)	Without postoperative morbidity (*n* = 93)	With postoperative morbidity (*n* = 146)	*P* value
Age > 60 yr	54 (22.6)	22 (23.7)	32 (21.9)	0.754
Male sex	144 (60.3)	54 (58.1)	90 (61.6)	0.581
ASA score > 2	23 (9.6)	6 (6.5)	17 (11.6)	0.185
Obesity	28 (11.7)	4 (4.3)	24 (16.4)	0.004
Diabetes mellitus	24 (10.0)	4 (4.3)	20 (13.7)	0.018
Cirrhosis	39 (16.3)	8 (8.6)	31 (21.2)	0.010
Preoperative CA19-9 > 150 U/L	129 (54.0)	44 (47.3)	85 (58.2)	0.099
Maximum tumor size > 3 cm	68 (25.8)	21 (22.6)	47 (32.2)	0.108
Macrovascular invasion	144 (60.3)	55 (59.1)	89 (61.0)	0.779
Microvascular invasion	85 (35.6)	28 (30.1)	57 (39.0)	0.160
Peripheral nerve invasion	80 (33.5)	30 (32.3)	50 (34.2)	0.751
Poor tumor differentiation	77 (32.2)	29 (31.2)	48 (32.9)	0.785
Intraoperative blood transfusion	159 (66.5)	57 (61.3)	102 (69.9)	0.171
Intraoperative blood loss > 500 mL	185 (77.4)	65 (69.9)	120 (82.2)	0.027
Major hepatectomy	171 (71.5)	65 (69.9)	106 (72.6)	0.651
Hepatic artery reconstruction	12 (5.0)	5 (5.4)	7 (4.8)	0.841
Portal vein reconstruction	45 (19.6)	22 (23.7)	23 (15.8)	0.128
Pringle maneuver	175 (73.2)	73 (78.5)	102 (69.9)	0.142
Number of examined LNs > 4	125 (52.3)	52 (55.9)	73 (50.0)	0.372
LN metastasis	54 (22.6)	18 (19.4)	36 (24.7)	0.339
Bismuth type, III-IV	135 (56.5)	52 (55.9)	83 (56.8)	0.887
Preoperative drainage	71 (29.7)	28 (30.1)	43 (29.5)	0.914

ASA: American Society of Anesthesiologists; CA19-9: Carbohydrate antigen 19-9; LN: Lymph node.

Table 3 Univariable and multivariable logistic regression analyses of risk factors associated with postoperative morbidity following curative resection for hilar cholangiocarcinoma

Variables	Multivariable analyses[1]	
	P value	OR (95%CI)
Obesity	0.023	3.694 (1.197-11.394)
Diabetes mellitus	0.036	3.395 (1.082-10.651)
Cirrhosis	0.017	2.867 (1.207-6.810)
Preoperative CA19-9 > 150 U/L	0.155	1.493 (0.859-2.593)
Intraoperative blood loss > 500 mL	0.016	2.240 (1.162-4.318)

[1]Factors with $P < 0.1$ in Table 2 were applied to multiple logistic regression model.
CA19-9: Carbohydrate antigen 19-9; CI: Confidence interval; OR: Odds ratio.

sample multicenter study from China indicated that 758 (35.1%) out of 2161 HCC patients experienced morbidity within 30 d, and the median OS and time-to-recurrence in these patients were poorer (48.1 mo *vs* 91.6 mo and 19.8 mo *vs* 46.1 mo, respectively)[8]. However, no studies have investigated the link between postoperative morbidity and prognosis in HCCA patients.

Table 4 Comparisons of survival outcomes between patients with and without postoperative morbidity

Survival outcomes	Total (*n* = 239)	Without postoperative morbidity (*n* = 93)	With postoperative morbidity (*n* = 146)	*P* value
Period of follow-up, months[1]	19.0 (11.0, 34.0)	15.0 (23.0, 41.0)	16.0 (9.8, 30.0)	0.001
Death during the follow-up	160 (66.9)	55 (59.1)	105 (71.9)	0.041
Recurrence during the follow-up	172 (72.0)	60 (64.5)	112 (76.7)	0.041
OS, months[2]	23.0 (20.0-26.0)	31.0 (22.4-39.6)	18.0 (13.0-23.0)	0.003
1-yr OS rate, %	73.4	83.8	66.7	
3-yr OS rate, %	34.0	45.5	26.7	
5-yr OS rate, %	22.9	31.7	17.0	
RFS, month[2]	19.0 (16.1-21.9)	26.0 (14.1-37.9)	16.0 (12.2-19.8)	0.002
1-yr RFS rate, %	64.6	75.1	54.3	
3-yr RFS rate, %	28.7	40.8	22.4	
5-yr RFS rate, %	18.2	30.4	13.3	

[1]Values are median (interquartile range).
[2]Values are median and 95% confidence interval.
OS: Overall survival; RFS: Recurrence-free survival.

Both clinicopathological and operative variables were found to differ significantly in relation to postoperative morbidity, including obesity, diabetes mellitus, cirrhosis, and intraoperative blood loss > 500 mL. Many previous studies have used propensity score matching to balance the intergroup baseline features in evaluating the effect of postoperative complications on outcomes[29,30]. However, as postoperative 30-d morbidity is itself a short-term outcome, it is not appropriate to adopt this statistical approach, which may increase, rather than decrease, selection bias between the groups. In contrast, classical statistical approaches are appropriate to determine the link between postoperative morbidity and outcomes with adjustment for confounding factors.

It is important to identify the risk factors for postoperative morbidity to reduce its incidence. Here, we specifically investigated the independent risk factors for morbidity and identified obesity, diabetes mellitus, cirrhosis, and intraoperative blood loss > 500 mL. These findings are significant for guiding clinical practice. Similar conclusions have been reported; for example, a major morbidity rate of 40% was observed after liver resection in obese or overweight patients[31,32]. There is evidence to explain this phenomenon, namely, hepatic steatosis associated with obesity may adversely affect the regeneration of liver remnants and thus influence morbidity[33]. During the perioperative period, obese patients should be instructed by dieticians to adjust their dietary habits and properly match their nutritional structure. It is known that obesity is closely related to chronic liver diseases, such as steatosis, nonalcoholic steatohepatitis, and other comorbidities, including diabetes[34]. Moreover, the presence of diabetes mellitus is known to be linked to postoperative complications after HCCA surgery [35]. For severe diabetes, the clinician needs to effectively control blood glucose levels before surgery with the assistance of endocrinologists. In addition to the above two risk groups related to metabolism, for patients with cirrhosis, due to their worse liver function, surgeons should evaluate the remaining liver volume and reserve function more carefully before surgery and pay more attention to the prevention of complications, including PHLF, pleural effusion, abdominal hemorrhage, and biliary infection. Notably, cirrhosis may cause poor blood coagulation, making it more difficult to control the amount of bleeding during surgery[36]. For patients with poor liver function and coagulation dysfunction, intraoperative infusion of plasma or cryoprecipitate may help to reduce intraoperative bleeding. For patients with severe liver cirrhosis, the surgeon should use Pringle's maneuver to obstruct the temporary hilar of the liver for hepatectomy. In addition, the anesthetist should ensure low central venous pressure to reduce the amount of bleeding during the surgery. Moreover, the vast majority of intraoperative bleeding occurs during liver resection. With new medical advances, many kinds of instruments can be used for liver resection: Ultrasonic knife, electrocautery (bipolar, monopolar, or water sealed bipolar), and radiofrequency-assisted liver resection. However, which can better prevent intraoperative bleeding may be related to the patient's liver condition and the operator's habits, and it is worthy of further study. As this study showed that postoperative morbidity (especially major morbidity) can affect the oncological prognosis of HCCA after curative resection, adjusting the above risk factors can reduce complications and also improve the prognosis of patients. In our opinion, only through multidisciplinary treatment can we reduce the postoperative morbidity of patients who undergo curative HCCA resection.

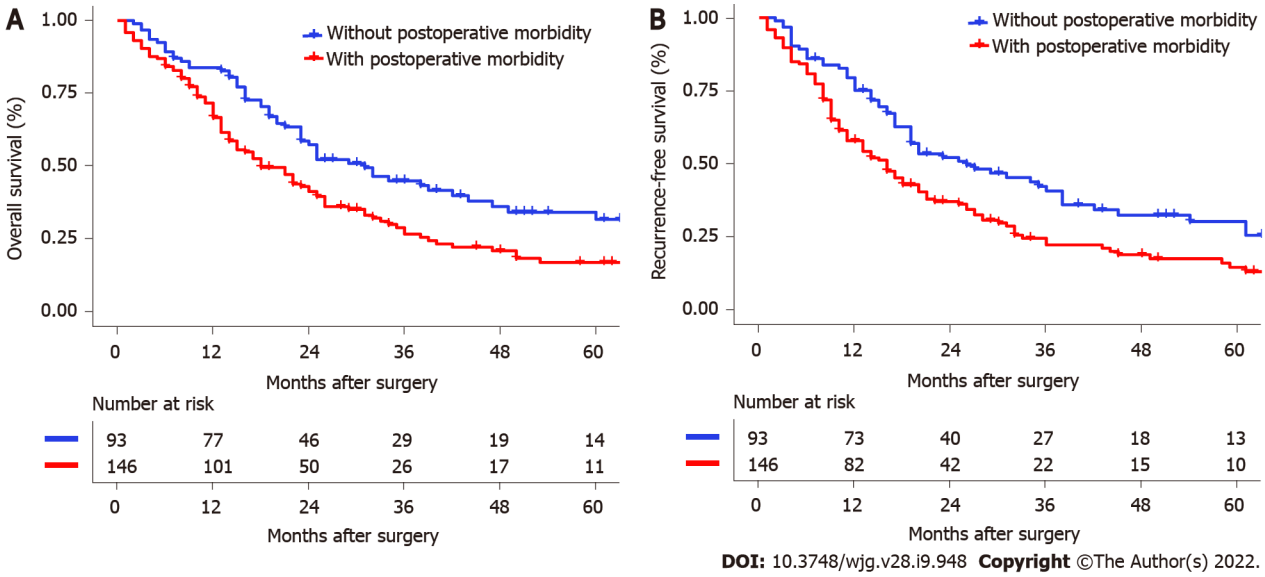
Table 5 Univariable and multivariable Cox regression analyses of risk factors associated with overall survival following curative resection for hilar cholangiocarcinoma

Variables	Univariable analyses		Multivariable analyses[1]	
	P value	HR (95%CI)	*P* value	HR (95%CI)
Age > 60 yr	0.341	1.190 (0.832-1.730)		
Male sex	0.754	1.052 (0.766-1.445)		
ASA score > 2	0.333	1.282 (0.775-2.120)		
Obesity	0.772	0.928 (0.561-1.536)		
Diabetes mellitus	0.063	1.595 (0.975-2.609)	0.288	1.324 (0.789-2.224)
Cirrhosis	0.222	1.283 (0.861-1.912)		
Preoperative CA19-9 > 150 U/L	0.009	1.522 (1.112-2.083)	0.015	1.485 (1.079-2.044)
Maximum tumor size > 3 cm	< 0.001	1.809 (1.296-2.525)	0.001	1.805 (1.290-2.526)
Macrovascular invasion	0.014	1.507 (1.088-2.087)	0.012	1.527 (1.099-2.122)
Microvascular invasion	0.005	1.588 (1.151-2.192)	0.102	1.324 (0.946-1.853)
Peripheral nerve invasion	0.663	1.075 (0.776-1.488)		
Poor tumor differentiation	0.005	1.608 (1.158-2.231)	0.003	1.654 (1.188-2.302)
Intraoperative blood transfusion	0.316	1.186 (0.850-1.654)		
Intraoperative blood loss > 500 mL	0.593	1.108 (0.761-1.612)		
Major hepatectomy	0.634	1.087 (0.771-1.531)		
LN metastasis	0.016	1.551 (1.086-2.215)	0.021	1.527 (1.067-2.186)
Bismuth type, III-IV	0.346	1.163 (0.849-1.593)		
Preoperative drainage	0.721	0.939 (0.665-1.326)		
Postoperative morbidity	0.003	1.635 (1.178-2.269)	0.009	1.557 (1.119-2.167)

[1]Those variables found significant at $P < 0.100$ in univariable analyses were entered into multivariable Cox regression analyses.
ASA: American Society of Anesthesiologists; CA19-9: Carbohydrate antigen 19-9; CI: Confidence interval; HR: Hazard ratio; LN: Lymph node.

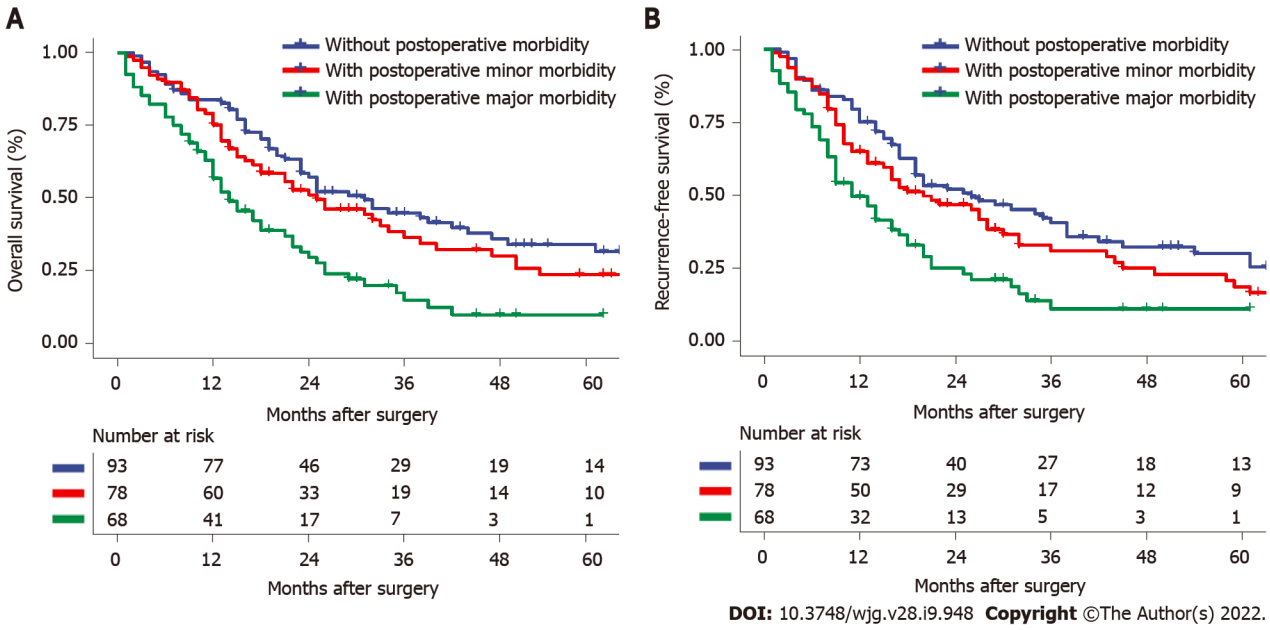
In other cancers, postoperative morbidity may be an independent predictor of poor prognostic outcome, including colorectal liver metastasis[37], HCC[8,38,39], pancreatic cancer[40], and esophageal cancer[41]. Although the precise association between postoperative morbidity and unfavorable prognostic outcomes remains to be elucidated, there are several possible explanations. Previous studies have shown that major surgery can induce systemic inflammation, with increased secretion of inflammatory cytokines, including interleukin-1 and interleukin-6, which contributes to cancer angiogenesis, proliferation, growth, and metastases[42-44]. In addition, severe systematic inflammation caused by postoperative morbidity may lead to an immunosuppressive condition and state, which can regulate the reduction of tumor monitoring and may lead to both metastasis and disease-specific death[15,42]. Notably, the postoperative stress response can inhibit cell-mediated immune function. Consequently, during the period of postoperative morbidity and relative immunosuppression caused by postoperative stress, residual malignant cells may proliferate[45]. Therefore, postoperative 30-d morbidity may negatively impact long-term oncological outcomes.

There are several limitations to this study. Specifically, it was a single-institution study with a retrospective design. Despite this, the database was established by standardized surgical techniques and perioperative management, thus preventing some limitations of multicenter, population-based, or national studies. Nevertheless, the impact of postoperative morbidity on the prognosis of HCCA patients still requires evaluation using a larger prospective study. In addition, in this study, patients who received adjuvant chemotherapy and radiotherapy were excluded. Some previous studies have demonstrated a benefit of prognosis for patients following surgery who received postoperative adjuvant therapy[46,47]. However, adjuvant therapy cannot be administered immediately when morbidities occur after surgery. As a result, we believed it was better to exclude patients who received adjuvant therapy to more accurately reflect the impact of postoperative complications on prognosis.



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Figure 2 Overall survival and recurrence-free survival curve comparisons between patients without and with postoperative morbidity. A: Overall survival, $P = 0.003$; B: Recurrence-free survival, $P = 0.002$.



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Figure 3 Overall survival and recurrence-free survival curve comparisons among patients without postoperative morbidity, with minor postoperative morbidity, and with major postoperative morbidity. A: Overall survival, $P = 0.231$ (with minor postoperative morbidity *vs* without postoperative morbidity), $P < 0.001$ (with major postoperative morbidity *vs* without postoperative morbidity); B: Recurrence-free survival, $P = 0.132$ (with minor postoperative morbidity *vs* without postoperative morbidity), $P < 0.001$ (with major postoperative morbidity *vs* without postoperative morbidity).

CONCLUSION

In summary, the results of this study clearly show that postoperative morbidity both lessens long-term survival and raises tumor recurrence in HCCA patients following curative resection. Independent risk factors for postoperative morbidity included diabetes, obesity, liver cirrhosis, and intraoperative blood loss > 500 mL. Clinicians should further optimize preoperative management, surgical procedures, and perioperative care to prevent complications and thus improve both short-term and long-term oncological prognoses.

Table 6 Univariable and multivariable Cox regression analyses of risk factors associated with recurrence-free survival following curative resection for hilar cholangiocarcinoma

Variables	Univariable analyses		Multivariable analyses[1]	
	P value	HR (95%CI)	*P* value	HR (95%CI)
Age > 60 yr	0.330	1.201 (0.850-1.696)		
Male sex	0.998	1.002 (0.793-1.136)		
ASA score > 2	0.457	1.210 (0.732-1.997)		
Obesity	0.911	0.973 (0.604-1.568)		
Diabetes mellitus	0.035	1.654 (1.036-2.264)	0.177	1.403 (0.858-2.295)
Cirrhosis	0.247	1.260 (0.852-1.863)		
Preoperative CA19-9 > 150 U/L	0.002	1.617 (1.193-2.192)	0.012	1.487 (1.092-2.024)
Maximum tumor size > 3 cm	0.002	1.695 (1.223-2.351)	0.002	1.665 (1.198-2.314)
Macrovascular invasion	0.008	1.534 (1.120-2.100)	0.011	1.514 (1.101-2.081)
Microvascular invasion	0.009	1.524 (1.118-2.088)	0.121	1.295 (0.934-1.794)
Peripheral nerve invasion	0.683	1.068 (0.780-1.462)		
Poor tumor differentiation	0.007	1.547 (1.124-2.129)	0.006	1.575 (1.141-2.173)
Intraoperative blood transfusion	0.251	1.208 (0.875-1.668)		
Intraoperative blood loss > 500 mL	0.819	1.043 (0.729-1.490)		
Major hepatectomy	0.978	0.995 (0.718-1.379)		
LN metastasis	0.010	1.573 (1.114-2.220)	0.017	1.528 (1.080-2.157)
Bismuth type, III-IV	0.788	1.042 (0.771-1.410)		
Preoperative drainage	0.517	0.895 (0.640-1.252)		
Postoperative morbidity	0.003	1.169 (1.180-2.220)	0.008	1.535 (1.117-2.108)

[1]Those variables found significant at $P < 0.100$ in univariable analyses were entered into multivariable Cox regression analyses.

ASA: American Society of Anesthesiologists; CA19-9: Carbohydrate antigen 19-9; CI: Confidence interval; HR: Hazard ratio; LN: Lymph node.

ARTICLE HIGHLIGHTS

Research background

Postoperative complications after surgery for hilar cholangiocarcinoma (HCCA) are common; but, whether it has an adverse impact on oncological prognosis is still unknown.

Research motivation

Our study aimed to determine whether there is an association between the presence of postoperative complication and oncological prognosis following surgery for HCCA. Moreover, our study assessed the independent risk factors for the occurrence of postoperative complication.

Research objectives

We aimed to evaluate the influence of postoperative morbidity on tumor recurrence and mortality after curative resection for HCCA.

Research methods

Patients with diagnosed HCCA following curative resection between January 2010 and December 2017 at our hospital were enrolled. The independent risk factors for postoperative comlication within 30 d after surgery were investigated, and links between postoperative morbidity and patient characteristics and survival outcomes were assessed. Postoperative morbidities were divided into five grades according to the Clavien-Dindo classification, and major morbidities were defined as Clavien-Dindo ≥ 3. Univariate and multivariate Cox regression analyses were used to evaluate the risk factors for recurrence-free survival (RFS) and overall survival (OS).

Research results

Postoperative complication occurred in 146 out of 239 patients (61.1%). Multivariate logistic regression revealed that cirrhosis, intraoperative blood loss > 500 mL, diabetes mellitus, and obesity were independently associated with postoperative complication. And, postoperative complication was associated with decreased OS and RFS (OS: 18.0 mo *vs* 31.0 mo, respectively, $P = 0.003$; RFS: 16.0 mo *vs* 26.0 mo, respectively, $P = 0.002$). Multivariate Cox regression analysis indicated that postoperative morbidity was independently associated with decreased OS [hazard ratios (HR): 1.557, 95% confidence interval (CI): 1.119-2.167, $P = 0.009$] and RFS (HR: 1.535, 95%CI: 1.117-2.108, $P = 0.008$). Moreover, major morbidity was independently associated with decreased OS (HR: 2.175; 95%CI: 1.470-3.216, $P < 0.001$) and RFS (HR: 2.054; 95%CI: 1.400-3.014, $P < 0.001$) after curative resection for HCCA.

Research conclusions

Postoperative complication (especially major complication) may be independently associated with poor prognosis in HCCA patients following curative resection.

Research perspectives

Clinicians should further optimize preoperative management, surgical procedures, and perioperative care to prevent complications and thus improve both short-term and long-term oncological prognoses.

FOOTNOTES

Author contributions: Dai HS had full access to all the data in the study and take responsibility for the integrity of the data and accuracy of the data analyses; Liu ZP, Chen ZY, Zhang YQ, Chen WY, Bai J, and Jiang Y contributed to the study concept and design; Liu ZP, Chen ZY, Zhang YQ, Chen WY, Zhong SY, Zhong YP, and Pan Y contributed to the acquisition, analyses, or interpretation of data; Liu ZP, Dai HS, Chen ZY, and Zhang YQ drafted the manuscript; Dai HS, Chen ZY, and Zhang YQ contributed to the critical revision of the manuscript for the important intellectual content; Liu ZP, Dai HS, and Zhang YQ performed the statistical analyses; Dai HS contributed to the study supervision.

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Country/Territory of origin: China

ORCID number: Zhi-Peng Liu 0000-0001-7652-1783; Wei-Yue Chen 0000-0001-6922-8888; Yan-Qi Zhang 0000-0001-9438-0953; Yan Jiang 0000-0003-4628-7150; Jie Bai 0000-0003-3031-8434; Yu Pan 0000-0003-3827-2418; Shi-Yun Zhong 0000-0001-7148-8777; Yun-Ping Zhong 0000-0001-9027-6104; Zhi-Yu Chen 0000-0002-1321-1793; Hai-Su Dai 0000-0001-9896-6249.

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ORIGINAL ARTICLE

Observational Study

Relationship between clinical remission of perianal fistulas in Crohn's disease and serum adalimumab concentrations: A multi-center cross-sectional study

Laura Sirmai, Anne-Laure Pelletier, Nathalie Gault, Camille Zallot, Guillaume Bouguen, Dominique Bouchard, Pascale Roland Nicaise, Marine Peyneau, Sandrine Sironneau, Marcelo De Carvalho Bittencourt, Antoine Petitcollin, Pedro Fernandez, Xavier Roblin, Laurent Siproudhis, Laurent Abramowitz

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Laura Sirmai, Anne-Laure Pelletier, Division of Hepato-Gastroenterology and Digestive Oncology, Bichat Claude Bernard University Hospital, Paris 75018, France

Laura Sirmai, Division of Hepato-Gastroenterology, Hospital Croix Saint Simon, Paris 75020, France

Nathalie Gault, Division of Epidemiology, Biostatistics and Clinical Research, University Hospital Center Bichat, Paris 75018, France

Nathalie Gault, National Institute of Health and Medical Research CIC-EC1425, University Hospital Center Bichat, Paris 75018, France

Camille Zallot, Division of Gastroenterology, Nancy Regional and University Hospital Center, Nancy 54035, France

Guillaume Bouguen, Laurent Siproudhis, Imphy CIC 1414 Group and Division of Gastroenterology and Hepatology, University Hospital of Rennes, Pontchaillou, Rennes 35033, France

Dominique Bouchard, Division of Proctology, Hospital Bagatelle, Talence 33400, France

Pascale Roland Nicaise, Marine Peyneau, Division of Immunology, University Hospital Center Bichat, Paris 75018, France

Marine Peyneau, Inflammation, Microbiome and Immunosurveillance, Faculty of Pharmacy, Université Paris-Saclay, Châtenay-Malabry 92290, France

Sandrine Sironneau, Division of Radiology, Bagatelle Hospital Center, Talence 33400, France

Marcelo De Carvalho Bittencourt, Division of Immunology, Nancy Regional and University Hospital Center, Nancy 54000, France

Marcelo De Carvalho Bittencourt, University of Lorraine, CNRS UMR 7365, IMoPA, Nancy 54000, France

Antoine Petitcollin, Department of Clinical and Biological Pharmacology and Pharmacovigilance, Pharmacoepidemiology and Drug Information Center, Rennes University Hospital, Rennes 35700, France

Pedro Fernandez, Division of Radiology, University Hospital Center Bichat, Paris 75018, France

Pedro Fernandez, Orangerie Center, Le Perreux-sur-Marne 94170, France

Xavier Roblin, Division of Gastroenterology, CHU Saint Etienne, Saint-Priest-en-Jarez 42270, France

Laurent Abramowitz, Division of Gastroenterology and Hepatology and Proctology, University Hospital Center Bichat, Paris 75018, France

Laurent Abramowitz, Ramsay GDS Clinique Blomet, Paris 75018, France

Corresponding author: Laura Sirmai, MD, Doctor, Division of Hepato-Gastroenterology and Digestive Oncology, Bichat Claude Bernard University Hospital, 46 Rue Henri Huchard, Paris 75018, France. laura.sirmai@gmail.com

Abstract

BACKGROUND

Crohn's disease (CD) is complicated by perianal fistulas in approximately 20% of patients. Achieving permanent fistula closure remains a challenge for physicians. An association between serum anti-tumor necrosis factor-α concentrations and clinical outcomes in patients with CD has been demonstrated; however, little information is available on serum adalimumab (ADA) concentrations and remission of perianal fistulas in such patients.

AIM

To study the relationship between serum ADA concentrations and clinical remission of CD-associated perianal fistulas.

METHODS

This cross-sectional study of patients with CD-associated perianal fistulas treated with ADA was performed at four French hospitals between December 2013 and March 2018. At the time of each serum ADA concentration measurement, we collected information about the patients and their fistulas. The primary study endpoint was clinical remission of fistulas defined as the absence of drainage (in accordance with Present's criteria), with a PDAI ≤ 4, absence of a seton and assessment of the overall evaluation as favorable by the proctologist at the relevant center. We also assessed fistula healing [defined as being in clinical and radiological (magnetic resonance imaging, MRI) remission] and adverse events.

RESULTS

The study cohort comprised 34 patients who underwent 56 evaluations (patients had between one and four evaluations). Fifteen patients had clinical remissions (44%), four of whom had healed fistulas on MRI. Serum ADA concentrations were significantly higher at evaluations in which clinical remission was identified than at evaluations in which it was not [14 (10-16) *vs* 10 (2-15) μg/mL, $P = 0.01$]. Serum ADA concentrations were comparable at the times of evaluation of patients with and without healed fistulas [11 (7-14) *vs* 10 (4-16) μg/mL, $P = 0.69$]. The adverse event rate did not differ between different serum ADA concentrations.

CONCLUSION

We found a significant association between high serum ADA concentrations and clinical remission of CD-associated perianal fistulas.

Key Words: Crohn's disease; Clinical pharmacology; Peri-anal disorders; Adalimumab

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Core Tip: Perianal fistulas (PAFs) are a complication of Crohn's disease (CD) in approximately 20% of patients. Adalimumab (ADA) was shown to treat CD-associated PAFs; however, little information is available on serum ADA concentrations and their remission. We performed a cross-sectional study at four hospitals in France to investigate this relationship, including 34 patients with 56 evaluations. Fifteen patients had clinical remission (44%). Serum ADA concentrations were significantly higher in evaluations showing clinical remission compared to those without. Thus, ADA serum concentrations that are required for PAF remission should be higher compared with the previously described concentrations associated with luminal remission.

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INTRODUCTION

Crohn's disease (CD) is complicated by perianal fistulas (PAFs) in approximately 20% of patients and these PAFs can severely and negatively affect patients' quality of life[1]. Treatment of CD-associated PAFs was revolutionized by the advent of anti-tumor necrosis factor (TNF)-α antibodies[2]. A meta-analysis of treatment with adalimumab (ADA), a TNF-α blocker, found that complete PAF closure occurred in 36% of patients with CD-associated fistulas (95%CI: 0.31-0.41) and partial responses in 31% (95%CI: 0.031-0.61)[3]. Its efficacy is better in anti-TNF-α-naive patients but it can also be effective after infliximab failure[4]. Adding surgical drainage with a seton has been shown to be superior to anti-TNF-α alone, with better responses and fewer recurrences[5,6]. Resolution of complex PAFs takes longer and timing of seton withdrawal depends on the effectiveness of the medical treatment and findings on clinico-radiological evaluation[5]. However, achieving permanent fistula closure remains a challenge for physicians.

Optimization of anti-TNF-α therapy (increasing the drug dosage and/or shortening the intervals between injections) is advised in patients with confirmed active disease, good compliance, and subtherapeutic drug serum concentrations in the absence of anti-drug antibodies[7]. Adding an immunosuppressive drug (in combination therapy) should also be considered[8]. Thus, optimization of treatment on the basis of anti-TNF-α (including ADA) and antibody serum concentrations has been evaluated[9-11]. Retrospective trials have suggested that higher target serum infliximab concentrations are required to reach remission in patients with CD-associated PAF than in those with luminal disease only[12-16]. Two recent retrospective trials also suggested that higher target serum ADA concentrations are associated with remission in patients with CD-associated PAF[15,16]. However, in those studies the evaluation criteria were mostly clinical, the studies were not confined to assessment of ADA, and they were small single-center studies. Our primary objective was, therefore, to study the relationship between serum ADA concentrations and clinical remission of CD-associated PAFs.

MATERIALS AND METHODS

Study cohort and setting

This cross-sectional study was conducted in four French centers between December 2013 and March 2018. Patients with at least one active CD-associated PAF at the time of ADA initiation, or who developed a PAF while being treated with ADA (thus requiring optimization of ADA), whose serum ADA concentrations were measured at least once during follow-up (induction or treatment maintenance) were considered eligible. The PAFs could be associated with upper, ileal, colonic, or rectal lesions. Patients who were eligible on the basis of the above criteria were included irrespective of the durations of their PAFs and ADA treatment, associated or previously received treatments, and surgical procedures performed, particularly the closure technique (glue, plug, or rectal advancement flap after removal of a seton). ADA treatment could have been optimized or not and an immunosuppressive drug may also be used in combination therapy. Pregnant or breastfeeding women and patients with a history of proctectomy or undrained abscesses were excluded.

Data collected and definitions

Data were collected between October 2017 and March 2018 using a standardized paper collection file.

The clinical data collected at each evaluation were as follows: remission of any fistula, perineal disease activity index (PDAI) score, presence of associated lesions in the rectum or anus, durations of PAFs and seton drainage, Harvey Bradshaw score, ADA dosage and duration of treatment, associated treatment, and adverse events. Patient characteristics collected and assessed were as follows: treatment center; age; sex; body mass index; co-morbidities; smoking status; Montreal classification; and medical and surgical history (abdominal surgery, number of fistula(e); simple, complex, or vaginal involvement; and surgical treatments for the PAF, including the type of closure performed). Pelvic magnetic resonance imaging (MRI) and colonoscopy findings obtained within 3 mo of clinical evaluation and in the absence of more recent changes in treatment were collected if performed, which was at the discretion of the physician. MRI images were reassessed by a single expert radiologist (Fernandez P). ADA and anti-ADA antibody serum concentrations were measured in the laboratories of each investigating center using a standardized drug-sensitive ELISA test (Lisa Tracker; Theradiag, France) or equivalent immunologic test[17]. If more than one serum ADA concentration was available, the trough concentration (Day 13 or 14 after ADA injection) was used, as recommended in a recent published study[18]. Serum ADA concentrations were measured systematically or at the times of clinical evaluations in accordance with the standard procedures at the participating centers. Some serum ADA concentrations were reported as greater than 16 μg/mL; therefore, all measures were recorded to a maximum of 16 μg/mL.

The primary study endpoint was clinical remission of PAF, defined as the absence of drainage (both spontaneous and after soft pressure on the orifice by the clinician, in accordance with Present's criteria [2]), with a PDAI ≤ 4, absence of a seton and assessment of the overall evaluation as favorable by the proctologist at the relevant center. This outcome was defined after agreement by experts from the Proctology Research Group of the French National Society of Colo-Proctology, the investigators being members of these groups. If one of the above criteria were absent, the PAF was described as active. Favorable evaluation by the proctologist was defined as no inflammation of external(s) and internal(s) orifice(s) and absence of current or new associated anal lesions (stenosis, ulceration, or fistulous branching) that had developed after introduction of ADA or the most recent change in modality of medical or surgical treatment. In patients with multiple CD-associated PAFs, all lesions had to have resolved to conclude the evaluation was favorable. The clinical remission criteria were reviewed at all evaluations.

The secondary study endpoint was healing, which was defined as clinical remission (as described above) plus radiological healing on pelvic MRI, this definition having been reached by agreement of the Proctology Research Group's experts and as reported by other research groups[19,20]. The full criteria were absence of hypersignal T2 or enhancement after gadolinium T1 injection, absence of any abscess, and absence of rectal inflammation.

ADA optimization was defined as shortening of the interval between injections to 7 d or increasing the dosage of injections to 80 mg (or both).

Combination therapy was defined as addition of an immunosuppressive drug (thiopurine or methotrexate) to ADA.

Endoscopic luminal remission was defined by a score of less than 6 on the CD Endoscopic Index of Severity.

Clinical luminal remission was defined by a Harvey Bradshaw score of less than 4.

ADA tolerance was assessed on the basis of occurrence of adverse events.

The following definitions apply to terms used in the remainder of this article. Immunization was defined as undetectable serum ADA concentrations and the presence of anti-ADA antibodies in concentrations above 20 ng/mL[21].

Complex PAFs were defined as PAF branched in two or more directions or presence of ramifications or diverticula[5,22].

PDAI scores are based on the presence of fistula drainage, pain, and its effects on activity, limitations on sexual activity, degree of induration, and type of fistula. A score greater than 4 indicates definite active disease.

Statistical analysis

First, patients' characteristics are reported according to occurrence of clinical remission. Quantitative variables are presented as median and interquartile range and categorical variables as frequencies and percentages. The overall number and proportion of patients achieving clinical remission, achieving healing, and having at least one adverse event were determined.

The distribution of durations of PAFs and drainage, ongoing treatment, and serum albumin and C-reactive protein concentrations was further explored according to the clinical remission status at the time of evaluation.

The relationship between serum ADA concentrations and each of the following was assessed: clinical remission, healing, endoscopic luminal remission, clinical luminal remission, treatment optimization, and treatment with combination therapy. These relationships were studied using univariate generalized estimating equations models, considering an evaluation as the statistical unit (some patients having undergone several evaluations).

Association between serum ADA concentrations at each visit and clinical remission was also adjust on the duration of treatment.

All analyses were performed using R version 3.2.0. All tests were two-tailed, and the level of statistical significance was set at $P = 0.05$.

RESULTS

Patient characteristics

Of the 45 patients who were screened for inclusion, 34 were found to be eligible (9 from Paris, 8 from Rennes, 13 from Nancy, and 4 from Saint-Etienne), 16 of whom were women (47%). The remaining 11 patients were excluded for the following reasons: three for wrong diagnoses (two anal stenosis, one ulcerative colitis), three because they had undergone proctectomy, one was a minor, one had an undrained abscess, and three had PAFs that had resolved. Patients had between one and four evaluations (total of 56 evaluations), 50% of them having only one evaluation. Three of the 56 evaluations were performed during induction of ADA treatment.

Overall, 44% of the patients ($n = 15$) achieved clinical remission. Pelvic MRI was available for 23 of the 34 patients (56%) and showed that 17% ($n = 4$) had radiological evidence of healing. Of the patients in clinical remission, 44% had radiological evidence of healing.

Table 1 summarizes the characteristics of the participants that were collected at the first evaluation. Most of these characteristics were comparable between patients who achieved and did not achieve clinical remission (Table 1), particularly smoking status (43% *vs* 47%) and previous treatment with biotherapies including infliximab (47% *vs* 53%). More patients' treatment was optimized at the first evaluation in patients who achieved clinical remission than in those who did not (60% *vs* 42%). Patients who achieved clinical remission tended to have fewer complex PAFs (73% *vs* 89%) and vaginal PAFs (7% *vs* 21%) and they had colonic lesions less frequently (33% *vs* 42%) Patients who achieved clinical remission tended to have undergone more fistula closure procedures than those who did not achieve remission (33% *vs* 16%).

None of the six patients who achieved remission and underwent more than one evaluation relapsed. These evaluations took place between 3 mo and 4 years after the first evaluation. All patients in clinical remission from PAF were in luminal remission (established by clinical and endoscopic evaluation), whereas half of the patients who did not achieve clinical remission from PAFs had luminal activity. Of the 19 patients with active disease, three had immunization status regarding ADA, five had undrained branches detected by MRI indicating insufficient surgical treatment, and six had low serum ADA concentrations (< 4 μg/mL) that could probably have benefited from optimization. The remaining five patients may have truly failed ADA treatment after 6 mo of well-conducted treatment.

Adverse events

Five patients (14.7%) had at least one adverse event leading to ADA discontinuation. Two of them were in the clinical remission group (pancytopenia and joint pain of no definite cause) and the remaining three in the activity group (axonal neuropathy attributed to ADA and digestive, pulmonary, and ganglionic tuberculosis; vertigo; and joint pain).

Serum ADA concentrations and clinical remission

Median serum ADA concentrations were significantly higher in assessment visits of patients in clinical remission than in those not in clinical remission [14 (10-16) *vs* 10 (2-15) μg/mL, $P = 0.02$ after adjustment on the duration of treatment] (Figures 1 and 2), with an area under the ROC curve of 65.6%. Clinical remission was not identified in the three evaluations of patients with immunization status regarding ADA.

The duration of treatment with ADA tended to be longer in the clinical remission than in the non-clinical remission group (37 *vs* 12 mo). The median duration of drainage with a seton was 8 mo in the clinical remission group compared with 9 mo in the non-clinical remission group (Table 2). The median duration of PAFs tended to be longer in the clinical remission than no clinical remission group (53 mo *vs* 12 mo) (Table 2).

Serum ADA concentrations and healing

Median serum ADA concentrations did not differ significantly between evaluations in which healing (clinical and MRI remission) was identified and those in which it was not [11 (7-14) *vs* 10 (4-16) μg/mL, $P = 0.69$].

Serum ADA concentrations and optimization or combination therapy

Serum ADA concentrations tended to be higher in patients whose treatment was optimized than in those whose treatment was not optimized [14 (5-16) μg/mL *vs* 10 (4-13) μg/mL, $P = 0.20$] and in patients receiving combination therapy than in those receiving ADA alone [12 (5-16) μg/mL *vs* 11 (5-14) μg/mL,

Table 1 Patients' characteristics according to clinical remission status

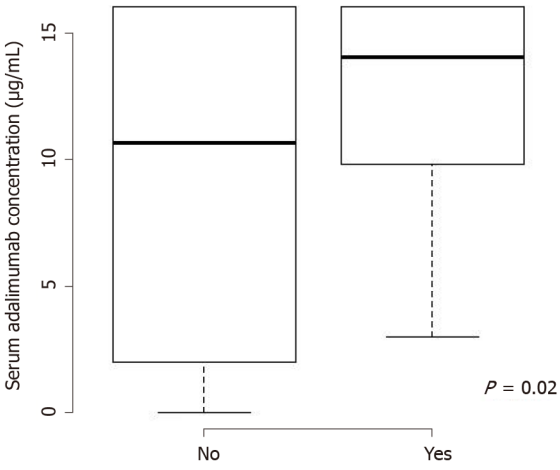
	Clinical remission, *n* (%) unless otherwise specified	
	No, *n* = 19	Yes, *n* = 15
Female sex	10 (53%)	6 (40%)
Age (yrs), Med [IQR]	34 [27-38]	35 [23-44]
BMI, Med [IQR]	28 [19-32]	22 [20-23]
Number of comorbidities[1], Med [IQR]	0 [0-1]	0 [0-1]
Active smoking, 1 MD	9 (47%)	6 (43%)
Crohn's disease phenotype (Montreal L)		
Terminal ileum	3 (16%)	4 (27%)
Colon	8 (42%)	5 (33%)
Ileo-colon	8 (42%)	5 (33%)
Upper digestive tract	0 (0%)	1 (7%)
Crohn's disease phenotype (Montreal B)		
Non-stricturing, non-penetrating	12 (63%)	8 (53%)
Stricturing	4 (21%)	2 (13%)
Penetrating	2 (11%)	2 (13%)
Stricturing + penetrating	1 (5%)	3 (20%)
Number of fistulas, Med [IQR]	2 [1-2]	2 [1-2]
Complex fistula	17 (89%)	11 (73%)
Vaginal fistula	4 (21%)	1 (7%)
Previous treatment with infliximab	10 (53%)	7 (47%)
Previous treatment with another anti-TNF-α[2]	10 (53%)	7 (47%)
Previous treatment with another form of biotherapy[3]	10 (53%)	7 (47%)
Previous treatment with combination therapy	12 (63%)	5 (33%)
Previous abdominal surgery		
No	14 (74%)	11 (73%)
Appendicectomy	0 (0%)	2 (13%)
Ileocecal resection	4 (21%)	2 (13%)
Colectomy	1 (6%)	0 (0%)
Previous fistulotomy	4 (21%)	6 (40%)
Previous seton	18 (95%)	13 (87%)
Previous flattening of abscesses	8 (42%)	10 (67%)
Previous closure techniques	3 (16%)	5 (33%)
Previous glue	2 (11%)	4 (27%)
Previous plug	0 (0%)	1 (7%)
Previous rectal advancement flap	1 (5%)	0 (0%)
Optimization at first visit	8 (42%)	9 (60%)
Combination therapy (with methotrexate or thiopurine) at first visit	12 (63%)	5 (33%)
Combination therapy and/or optimization at first evaluation	15 (79%)	9 (60%)

[1]Cardiological disorders, Anxiety-depressive disorders, Acute pancreatitis, Asthma, Joint involvement, Hypothyroidism.
[2]Infliximab, certolizumab, golimumab.
[3]Infliximab, certolizumab, golimumab, vedolizumab, ustekinumab. BMI: Body mass index; IQR: Interquartile range; Med: Median; MD: Missing data.

Table 2 Characteristics of perianal fistulas at each visit according to the presence of clinical remission

	Clinical remission, *n* (%) unless otherwise specified	
	No, *n* = 34	Yes, *n* = 22
PAF duration (Time from fistula diagnosis to date of visit) in mo, Med [IQR]	12 [8-23.5]	53.5 [32.75-81.75]
Drainage duration (Time from seton setting down to date of visit) in mo, Med [IQR]	9 [4.75-17]	8 [4-19]
Combination therapy (with methotrexate or thiopurine)	23 (68%)	7 (32%)
Optimization	19 (56%)	14 (64%)
Serum concentrations of ADA (μg/mL), Med [IQR]	10.7 [2.4-14.7]	14.1 [9.8-16]
C reactive protein (mg/L), Med [IQR]	2.7 [0.07-15.25]	0.9 [0-1.8]
Serum albumin (g/L), Med [IQR]	42 [38.25-47.3]	43 [41.85-44.25]
Length of treatment by ADA in mo, Med [IQR]	12 [6-34.75]	37.5 [23.75-46.75]

PAF: Perianal fistulas; Med: Median; ADA: Adalimumab.



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Figure 1 Serum adalimumab concentrations according to clinical remission status.

P = 0.11]. Neither of these differences was statistically significant.

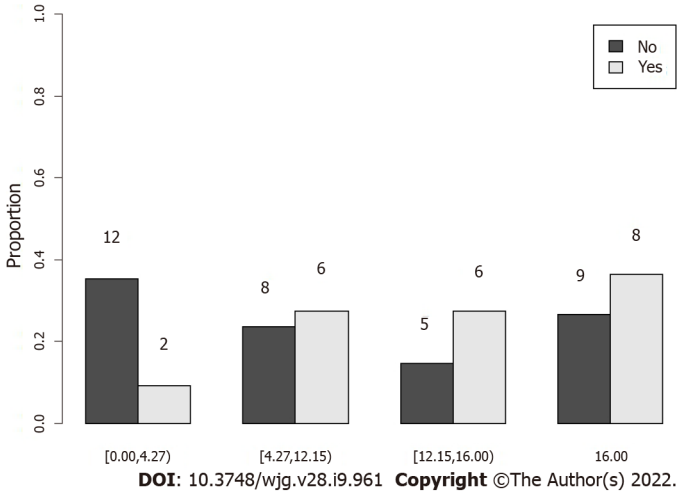
Serum ADA concentrations and luminal remission

Median serum ADA concentrations tended to be higher in patients with endoscopic luminal remission and in those with clinical luminal remission than in those without it [12 (6-16) μg/mL *vs* 2 (1-4) μg/mL, and 14 (10-16) μg/mL *vs* 4.2 (1-11) μg/mL, respectively].

DISCUSSION

In this study, we found higher median serum ADA concentrations in patients who were in clinical remission of CD-associated PAFs than in those with active disease, the higher concentration not being associated with a higher incidence of adverse events. To the best of our knowledge, this is the first multicenter study dedicated to investigating the relationship between serum ADA concentrations and remission of CD-associated PAFs. Our definition of healing of PAFs included clinical and MRI criteria, as recently recommended[5,20,22,25].

Our findings suggest that remission of CD-associated PAFs requires higher serum concentrations of ADA than those previously reported for resolution of luminal disease (between 4.5 and 12 μg/mL based on results of clinical trials)[9,10]. The high serum concentrations that we found to be associated with clinical remission seem to be more frequently achieved through optimization of ADA dosage or combination therapy (or both).



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Figure 2 Clinical remission according to quartiles of serum adalimumab concentrations.

Serum ADA concentrations have shown considerable variability and overlap between patients with and without clinical remission, as previously described[9]. It is likely that not all patients need to reach these high concentrations. For patients not achieving remission, we suggest measuring serum ADA concentrations and antibodies to exclude immunogenicity issue. Optimizing ADA dosage or adding combination therapy (or both) should be considered, even if we do not have yet a concentration to target.

In our study, patients with active lesions had more complex PAFs (89% *vs* 73%) and more vaginal fistulas, indicating the severity of the disease. No patients who achieved remission of PAFs had colorectal activity, highlighting the importance of achieving luminal remission when managing PAFs.

Four of the 23 patients (56% of the whole cohort) who had an MRI in our study achieved healing (17%).

Our low rate of healing of PAFs (clinical plus MRI remission) is likely attributable to our high rate of complex PAFs, the well-known delay between MRI remission compared with clinical remission (approximately 12 mo)[23], given that some follow up data were missing, and the fact that 44% of our patients did not undergo MRI evaluation.

We found no significant difference in serum ADA concentrations according to healing status [11 (7-14) μg/mL *vs* 10 (4-16) μg/mL, $P = 0.69$]. We think the lack of statistical significance is attributable to our small sample size, and our low rate of healing of PAFs, likely due to the accuracy of this robust criteria.

We found that the PAF duration was longer in patients with PAF activity than in those in remission (53.5 *vs* 12 mo), which likely reflects failure of medical or surgical (or both) treatment.

The three patients with anti-ADA antibodies and undetectable serum ADA had clinically active disease, which is consistent with the findings of a previous study that showed that the presence of anti-TNF-α antibodies is a risk factor for disease activity and recurrence[24].

Recently, two retrospective trials[15,16] were performed to assess the relationships between concentrations of anti-TNF medications, including ADA, and outcomes of CD-associated PAFs. Their results are consistent with ours: Strik *et al*[15] (19 patients treated with ADA) found significantly higher median serum ADA concentrations in patients with closed PAFs than in those with active PAFs [7 (6-11) μg/mL *vs* 5 (2-6) μg/mL, $P = 0.003$]. Additionally, Plevris *et al*[16] (35 patients treated with ADA) found significantly higher median ADA concentrations in individuals with healed fistulas than in those with unhealed fistulas (12.6 μg/mL vs. 2.7 μg/mL; $P < 0.01$).

Of note, our patients' serum ADA concentrations were much higher than in these two studies. Possible explanations for this discrepancy are that more of our patients were receiving combination therapy or optimization of ADA treatment (or both) and we had a high rate of complex PAFs (82%), including 15% with vaginal involvement. One strength of our study is the very precise description of our study cohort and their PAFs (PAF complexity, the presence or absence of setons, and endoscopic data). We chose a stricter clinical definition of remission, incorporating PDAI scores, and a greater proportion of our patients underwent radiological evaluation (no MRIs in Plevris *et al* study and 15% in the Strik *et al* study *vs* 56% in our study), which has been shown to be very important in assessing healing of PAFs[25].

In our study, high serum ADA concentrations was not associated with an increased incidence of adverse events, knowing that we only took into account the serious adverse events leading to a stop of ADA. In the literature, Drobne *et al*[26] and Greener *et al*[27] studies found that higher infliximab serum concentrations are not associated with a higher frequency of infections. Interestingly, Landemaine *et al* [28] study found that infection risk was individually correlated with cumulative increase in drug

exposure, but not infliximab trough level.

Our study had several important limitations. Being a cross-sectional, non-interventional, non-randomized study, management of patients was heterogeneous both in terms of surgical and medical treatment. Additionally, most data were collected retrospectively. However, it was an evaluation of management of CD-associated PAFs in real life in tertiary centers. We had a small sample size, as did the other two available studies, because of the competition of other biotherapies. Trough serum ADA concentrations were not always measured; thus, it is possible that ADA concentrations fluctuated between injections[18]. Timing of measurement of serum ADA concentrations was either systematic or clinically oriented, depending on the center, favoring non-responders or partial responders. With an area under the ROC curve that was close to 50%, we could not identify target serum ADA concentrations associated with clinical remission using the Youden index. Additionally, half of the patients had only one evaluation, limiting the availability of follow-up data.

CONCLUSION

In conclusion, there is an association between clinical remission of CD-associated PAFs and high serum ADA concentrations that is not associated with an increased incidence of adverse events. Our data suggest that higher ADA concentrations are associated with remission of CD-associated PAFs than in mucosal healing. Target serum ADA concentrations to guide physicians should be determined by a prospective trial.

ARTICLE HIGHLIGHTS

Research background

Perianal fistulas (PAFs) are a complication of Crohn's disease (CD) in approximately 20% of patients. Adalimumab (ADA) was shown to treat CD-associated PAFs. An association between serum anti-tumor necrosis factor (TNF)-α concentrations and clinical outcomes in patients with CD has been demonstrated; however, little information is available on serum ADA concentrations and PAFs remission.

Research motivation

Achieving permanent PAFs closure remains a challenge for physicians, especially for complex PAFs. Retrospective trials have suggested that higher target serum infliximab concentrations are required to reach remission in patients with CD-associated PAFs than in those with luminal disease only. Two recent retrospective trials also suggested that higher target serum ADA concentrations are associated with remission in patients with CD-associated PAFs. We lacked a study dedicated to the investigation of this relationship.

Research objectives

To study the relationship between serum ADA concentrations and clinical remission of CD-associated PAFs.

Research methods

We performed a multicenter cross-sectional study in France to assessed the relationship between serum ADA concentrations and clinical remission of CD-associated PAFs. We used a strict criteria to define clinical remission: absence of drainage (in accordance with Present's criteria), with a PDAI ≤ 4, absence of a seton and assessment of the overall evaluation as favorable by the proctologist at the relevant center. We also assessed healing defined by clinical and radiological (MRI) remission as a secondary endpoint.

Research results

We found higher median serum ADA concentrations in patients who were in clinical remission of CD-associated PAFs than in those with active disease, the higher concentration not being associated with a higher incidence of adverse events. We found no significant difference in serum ADA concentrations according to healing status, likely due to the accuracy of this robust criteria.

Research conclusions

Our findings suggest that remission of CD-associated PAFs requires higher serum concentrations of ADA than those previously reported for resolution of luminal disease.

Research perspectives

Target serum ADA concentrations to guide physicians should be determined by a prospective trial.

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FOOTNOTES

Author contributions: Sirmai L is the Guarantor of the article; Sirmai L conceived the study together with Pelletier AL and Abramowitz L; Fernandez P read the MRI images; Sirmai L, Pelletier AL, Zallot C, Bouguen G, Bouchard D, Roland Nicaise P, Peyneau M, Sironneau S, De Carvalho Bittencourt M, Petitcollin A, Roblin X, Siproudhis L, and Abramowitz L collected data; Gault N performed statistical analyses; all authors commented the article and approved the final version of the article, including the authorship list.

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Country/Territory of origin: France

ORCID number: Laura Sirmai 0000-0002-8627-8155; Anne-Laure Pelletier 0000-0003-4055-7615; Nathalie Gault 0000-0002-9913-9760; Camille Zallot 0000-0003-0993-6983; Guillaume Bouguen 0000-0002-7444-5905; Dominique Bouchard 0000-0003-0984-8545; Pascale Roland Nicaise 0000-0001-7137-135X; Marine Peyneau 0000-0002-5554-0835; Sandrine Sironneau 0000-0002-5656-7917; Marcelo De Carvalho Bittencourt 0000-0002-2698-2458; Antoine Petitcollin 0000-0003-4811-0439; Pedro Fernandez 0000-0001-8410-1623; Xavier Roblin 0000-0002-7929-4878; Laurent Siproudhis 0000-0002-2575-6790; Laurent Abramowitz 0000-0002-5106-4317.

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LETTER TO THE EDITOR

Radiomics-clinical nomogram for response to chemotherapy in synchronous liver metastasis of colorectal cancer: Good, but not good enough

Han Yan, Tu-Nan Yu

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Han Yan, Department of Colorectal Surgery, Sir Run Run Shaw Hospital, School of Medicine, Zhejiang University, Hangzhou 310016, Zhejiang Province, China

Tu-Nan Yu, Department of General Surgery, Sir Run Run Shaw Hospital, School of Medicine, Zhejiang University, Hangzhou 310016, Zhejiang Province, China

Corresponding author: Tu-Nan Yu, MD, PhD, Doctor, Statistician, Surgeon, Department of General Surgery, Sir Run Run Shaw Hospital, School of Medicine, Zhejiang University, No. 3 Qingchun East Road, Hangzhou 310016, Zhejiang Province, China. 3314006@zju.edu.cn

Abstract

There remains a persistent unmet need to detect the disease nonresponse (nonDR) subgroup before adjuvant therapy in synchronous liver metastasis patients with colorectal cancer. Ma's radiomics-clinical nomogram shows potential for the early detection of nonDR subgroups, but it is not good enough owing to at least three limitaions, which we address in this letter to the editor. First, the study did not explore RAS/BRAF mutations, HER2 amplifications, *etc.* to complement the current nomogram. Second, the nomogram was not validated in left- and right-sided tumors separately. Third, the most critical factor for determining the success of adjuvant therapy should be resectability rather than tumor size shrinkage, which was used in the study.

Key Words: Synchronous liver metastasis; Colorectal cancer; Radiomics

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Core Tip: There remains a persistent unmet need to detect the disease nonresponse subgroup before adjuvant therapy in synchronous liver metastasis patients with colorectal cancer. Ma's radiomics-clinical nomogram is currently not good enough, as the study did not explore the statuses of certain tumor genes, did not validate the nomogram in left- and right-sided tumors separately, and used tumor size shrinkage rather than resectability to judge the success of adjuvant therapy.

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TO THE EDITOR

Ma *et al*[1] recently published a novel study investigating the effect of magnetic resonance imaging-radiomics in predicting chemotherapeutic response in synchronous liver metastasis (SLM) patients with colorectal cancer (CRC). They proposed a radiomics-clinical nomogram (including the radiomics score, CA19-9, and lymphatic staging) with an area under the curve of 0.809, suggesting high predictive accuracy.

We congratulate the authors for their creative work, as in decision-making for adjuvant therapy in CRC patients with unresectable SLM, there remains a persistent unmet need to detect the disease nonresponse (nonDR) subgroup. Early detection of nonDR patients, aided by the radiomics-clinical nomogram of Ma's study, could result in substantial changes in subsequent therapeutic plans. For instance, in nonDR cases, more aggressive regimens could be applied instead of the frequently used FOLFOX or CAPOX, such as administration of bevacizumab to inhibit vascular endothelial growth factor or pembrolizumab for immunotherapy. Local regional therapies, including radiofrequency ablation and transcatheter arterial chemoembolization, could also be considered to treat SLM.

However, despite the aforementioned merit, there are at least three limitations to be discussed concerning this nomogram. First, although the authors explored tumor biomarkers, including CEA and CA19-9, to complement radiomics, the statuses of some critical tumor genes (*e.g.*, RAS/BRAF mutations, HER2 amplification, and MSI/MMR status) were not examined, despite the relevant recommendation in the latest National Comprehensive Cancer Network guideline[2]. Second, it is noteworthy that the biological behaviors of CRC differed depending on the anatomical location[3]. For instance, right-sided CRC patients with SLM were unlikely to respond to cetuximab and panitumumab as first-line therapy. Therefore, the performance of Ma's nomogram should be validated in right- and left-sided CRC separately. Last but not least, the most critical limitation was that the success of adjuvant therapy in CRC patients with SLM should be resectability, rather than tumor size shrinkage used in this study.

In conclusion, in CRC patients with SLM, Ma's radiomics-clinical nomogram shows potential for clinical utilization. However, it is currently not good enough.

FOOTNOTES

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Editorial Board Member of *World Journal of Gastroenterology*, Sombat Treeprasertsuk, MD, PhD, Professor of Internal Medicine, Assistant to President, for Cross Functions and Chief of Division of Gastroenterology, Chulalongkorn University, Rama 4 Road, Pathumwan, Bangkok 10330, Thailand. sombat.t@chula.ac.th

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OPINION REVIEW

Feasibility of therapeutic endoscopic ultrasound in the bridge-to-surgery scenario: The example of pancreatic adenocarcinoma

Giuseppe Vanella, Domenico Tamburrino, Gabriele Capurso, Michiel Bronswijk, Michele Reni, Giuseppe Dell'Anna, Stefano Crippa, Schalk Van der Merwe, Massimo Falconi, Paolo Giorgio Arcidiacono

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Giuseppe Vanella, Gabriele Capurso, Giuseppe Dell'Anna, Paolo Giorgio Arcidiacono, Pancreatobiliary Endoscopy and EUS Division, Pancreas Translational & Clinical Research Center, IRCCS San Raffaele Scientific Institute, Vita-Salute San Raffaele University, Milan 20132, Italy

Domenico Tamburrino, Stefano Crippa, Massimo Falconi, Pancreatic Surgery Unit, Pancreas Translational & Clinical Research Center, IRCCS San Raffaele Scientific Institute, Vita-Salute San Raffaele University, Milan 20132, Italy

Michiel Bronswijk, Schalk Van der Merwe, Department of Gastroenterology and Hepatology, University Hospital Gasthuisberg, University of Leuven, Leuven 3000, Belgium

Michiel Bronswijk, Department of Gastroenterology and Hepatology, Imelda Hospital, Bonheiden 2820, Belgium

Michele Reni, Department of Medical Oncology, Pancreas Translational & Clinical Research Center, IRCCS San Raffaele Scientific Institute, Milan 20132, Italy

Corresponding author: Paolo Giorgio Arcidiacono, FASGE, MD, Associate Professor, Director, Pancreatobiliary Endoscopy and EUS Division, Pancreas Translational & Clinical Research Center, IRCCS San Raffaele Scientific Institute, Vita-Salute San Raffaele University, Via Olgettina, 60, Milan 20132, Italy. arcidiacono.paologiorgio@hsr.it

Abstract

Upfront resection is becoming a rarer indication for pancreatic ductal adenocarcinoma, as biologic behavior and natural history of the disease has boosted indications for neoadjuvant treatments. Jaundice, gastric outlet obstruction and acute cholecystitis can frequently complicate this window of opportunity, resulting in potentially deleterious chemotherapy discontinuation, whose resumption relies on effective, prompt and long-lasting management of these complications. Although therapeutic endoscopic ultrasound (t-EUS) can potentially offer some advantages over comparators, its use in potentially resectable patients is primal and has unfairly been restricted for fear of potential technical difficulties during subsequent surgery. This is a narrative review of available evidence regarding EUS-guided choledochoduodenostomy, gastrojejunostomy and gallbladder drainage in the bridge-to-surgery scenario. Proof-of-concept evidence suggests no influence of t-EUS procedures on outcomes of eventual subsequent surgery. Moreover, the very high efficacy-invasiveness ratio

over comparators in managing pancreatic cancer-related symptoms or complications can provide a powerful weapon against chemotherapy discontinuation, potentially resulting in higher subsequent resectability. Available evidence is discussed in this short paper, together with technical notes that might be useful for endoscopists and surgeons operating in this scenario. No published evidence supports restricting t-EUS in potential surgical candidates, especially in the setting of pancreatic cancer patients undergoing neoadjuvant chemotherapy. Bridge-to-surgery t-EUS deserves further prospective evaluation.

Key Words: Endosonography; Gastrojejunostomy; Choledochoduodenostomy; Gallbladder drainage; Pancreatic cancer; Pancreatic surgery

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Core Tip: Despite the increase of a subset of patients with pancreatic adenocarcinoma undergoing neoadjuvant chemotherapy, therapeutic endoscopic ultrasound (EUS) has been unfairly restricted in potentially resectable patients. However, to date, no evidence suggests any influence of therapeutic EUS procedures on difficulty or outcomes of eventual subsequent surgery. Conversely, proof-of-concept papers have described uncomplicated surgery following EUS-guided gallbladder drainage, choledochoduodenostomy and gastrojejunostomy. Available evidence and technical notes are collected in this review. Due to the very high efficacy-invasiveness ratio of therapeutic EUS procedures, potentially resulting in less chemotherapy discontinuation, we believe that their use should not be restricted in the bridge-to-surgery scenario while implementing its prospective evaluation.

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INTRODUCTION

Jaundice, gastric outlet obstruction and acute cholecystitis (AC) can frequently complicate the clinical course of patients with pancreatic ductal adenocarcinoma (PDAC)[1]. Rapid, effective and long-lasting management of these events remains crucial to allow chemotherapy initiation or continuation. Traditional endoscopic or percutaneous palliation of these events may carry some limitations: Endoscopic retrograde cholangiopancreatography (ERCP) fails in up to 5%-10% of cases. Duodenal stenting is burdened by frequent symptoms recurrence. Percutaneous cholecystostomy (PT-GBD) is prone to tube dislodgement and cholecystitis recurrence[2-4]. Therapeutic EUS (t-EUS) is showing increasing potential in overcoming some of these limitations. However, EUS-guided procedures have been conventionally restricted to inoperable patients, for fear of interference with eventual surgery.

In an era in which upfront resection of PDAC is decreasing in favor of a more frequent use of neoadjuvant chemotherapy, this prudential limitation might result in a significant subset of patients being excluded from the potential advantages of t-EUS[5]. The aim of this narrative review was to discuss available evidence on outcomes of t-EUS in the bridge-to-surgery scenario.

METHODS

A literature search was performed regarding EUS-guided biliary drainage, EUS-guided gastrojejunostomy (EUS-GJ) and EUS-guided gallbladder drainage (EUS-GBD) in the bridge-to-surgery scenario up to July 2020. Available evidence was discussed in this narrative review, together with technical considerations and suggestions useful for endoscopists and surgeons operating in this setting. No original data are presented in the manuscript, and therefore Institutional Review Board approval was not required. Anonymized pictures are included from patients who have signed a specific written informed consent.

CLINICAL SCENARIO

In the combined treatment plan of PDAC, surgery is theoretically the only curative option, but only approximate 20% of patients are surgical candidates at diagnosis, the remaining being metastatic or locally unresectable[6].

Even in resectable disease, increasing knowledge of PDAC biology has expanded criteria for neoadjuvant chemotherapy to control potential micrometastases and select the best surgical candidates [5,7,8]. Therefore, half of PDAC patients, with resectable, borderline resectable or locally advanced tumor, will start a chemo (radio) therapeutic regimen, being eventually considered for subsequent surgery in case of stable disease or partial response. This "window of opportunity" where cancer behavior is ascertained can last 6 mo or more. During this time symptoms need to remain palliated through minimally invasive procedures, providing long-term efficacy with low risk of dysfunction/recurrence, to optimize tolerance of oncological treatments.

POTENTIAL ADVANTAGES OF THERAPEUTIC EUS PROCEDURES OVER ALTERNATIVES

EUS is gaining ground in palliation of cancer symptoms, where the least possible invasiveness allows non-delayed oncological treatments, potentially leading to increased survival and prolonged time to quality of life deterioration. Biliary drainage might be required in more than half of patients with PDAC [9]. The gold standard approach (ERCP) might fail in up to 5%-10% of cases, as the papilla might be unreachable or cannulation may fail[2].

EUS-guided biliary drainage has an established role when ERCP fails, avoiding morbidity of percutaneous drainage[10]. Electrocautery-enhanced lumen apposing metal stents have improved simplicity and safety of EUS-guided choledochoduodenostomy (EUS-CD; Figure 1) to such an extent that this procedure is being proposed as an upfront alternative to ERCP in distal malignant obstruction, with the potential to reduce the rate of post-ERCP acute pancreatitis[11,12].

Although surgical gastrojejunostomy has shown better long-term results for gastric outlet obstruction, endoscopic placement of duodenal self-expandable metal stents is conventionally used as a first-line treatment[3]. This minimally invasive technique harbors some limitations, as it may provide suboptimal relief with high risk of gastric outlet obstruction recurrence[6].

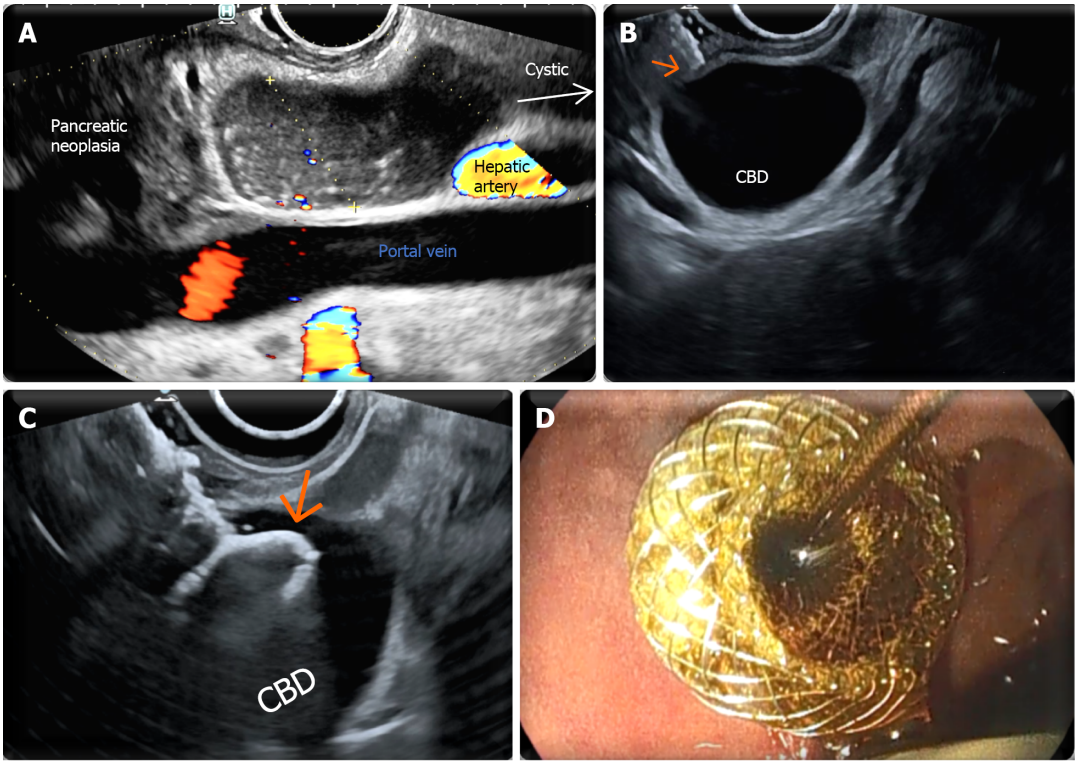
EUS-GJ is currently investigated as an alternative to both enteral stenting and surgical bypass. Placement of an electrocautery-enhanced lumen apposing metal stent between the stomach and proximal jejunum results in a large (2 cm) surgical-like anastomosis at a significant distance from the tumor. This minimally invasive procedure (Figure 2) may avoid both adverse events of surgery and the risk of primary failure, recurrence and re-interventions following enteral stenting[13-15].

Even if rarer, AC can also complicate the clinical history of PDAC patients, due to a neoplastic infiltration of the cystic duct, eventually worsened by other palliative maneuvers, such as the placement of a self-expandable biliary metal stent[16-18]. When surgical cholecystectomy is undesirable or unfeasible, EUS-GBD (Figure 3) has demonstrated its advantages over PT-GBD, resulting in equal technical success, paired with reduced 30-d and 1-year risk of adverse events, reintervention rates and AC recurrence[4]. Moreover, the large-diameter fistula between the gallbladder and the gastrointestinal tract allows for subsequent endoscopic clearance of gallstones, potentially offering a definitive solution for AC[19]. All these advantages might be even more valuable in the setting of patients undergoing neoadjuvant chemotherapy since a long-lasting, effective, minimally invasive palliation of cancer symptoms might result in reduced chemotherapy discontinuation and potentially to more frequent resectability.

EXISTING EVIDENCE ON SURGERY AFTER THERAPEUTIC EUS

To date there is limited experience on surgery following t-EUS, which is confined to cholecystectomy after EUS-GBD and a small series of pancreaticoduodenectomy after EUS-CD. As for cholecystectomy after EUS-GBD, a retrospective multicentric case-control study analyzed outcomes of subsequent cholecystectomy in 34 patients previously undergoing EUS-GBD with LAMS *vs* PT-GBD[20]. The study showed no influence of previous drainage modality in technical success, rate of conversion to open laparotomy or post-surgical adverse events, further showing a reduced operative time in the EUS-GBD cohort and a significantly higher number of interval procedures between PT-GBD and cholecystectomy due to plastic catheter maintenance or dislodgement.

Fabbri *et al*[21] described 5 patients undergoing pylorus-preserving pancreaticoduodenectomy after EUS-CD with 100% technical success and no biliary/duodenal fistula. In a recent study, Gaujoux *et al* [22] described 21 patients undergoing pancreaticoduodenectomy after EUS-CD with electrocautery-enhanced lumen apposing metal stents: all surgeries were successful, without any postoperative biliary fistula or stricture or tumor recurrence at the hepaticojejunostomy site.



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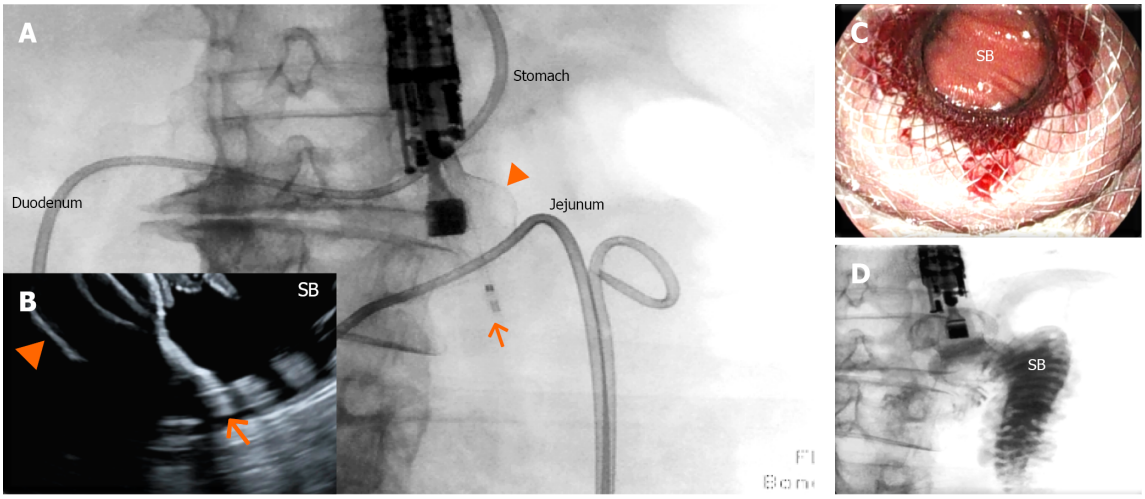
Figure 1 Endoscopic ultrasound-guided choledochoduodenostomy. A: Endosonographic identification of a window for endoscopic ultrasound-guided choledochoduodenostomy in a potentially resectable patient. The common bile duct (CBD) is evaluated from liver hilum to the neoplasia. A spot without intervening vessels is chosen as close as possible to the neoplasia; the caliber of the CBD is evaluated in the direction of the operative channel of the endoscope (yellow dotted line); B: The tip (arrow) of the electrocautery-enhanced lumen apposing metal stent is visibly in touch with the duodenal wall adjacent to a dilated CBD; C: The electrocautery-enhanced lumen apposing metal stent has passed through duodenal and biliary walls, and the distal flange (arrow) has been released inside the CBD; D: The proximal flange has been released inside the bulb with successful drainage of bile flow at the end of the procedure.

We recently published the first pancreaticoduodenectomy following EUS-guided double bypass. Surgical identification and disconnection of EUS-GJ was easy and fast and subsequent hepaticojejunostomy and gastrojejunostomy uncomplicated[23].

ENDOSCOPIC TRICKS FOR EUS-GUIDED PROCEDURES IN POTENTIALLY RESECTABLE PATIENTS

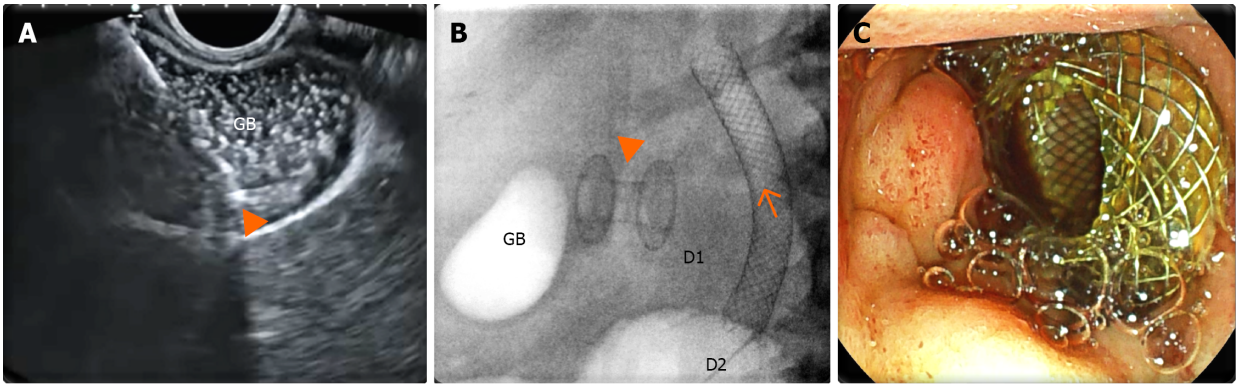
Endoscopists involved in t-EUS may wonder whether the technical approach to EUS-CD should change when treating potentially resectable patients. In our opinion, two principles must be kept in mind in this specific setting: EUS-CD should be performed: (1) Through the bulb; and (2) As far away from the hilum as possible. Although no evidence exists on the impact of these technical variables, a "distal" EUS-CD will allow more space for bile duct transection and hepaticojejunostomy (Figure 1A). Regarding EUS-CD location, a transbulbar (instead of transgastric) window nullifies the theoretical risk of tumor seeding since the bulb falls within surgical resection margins.

As for EUS-GJ, theoretical constraints are broader than the millimetric evaluation during EUS-CD. The EUS-GJ site must be kept as close as possible to the ligament of Treitz, in the first jejunal loop, to sacrifice a limited amount of jejunum during reconstruction. In our experience, this is obtained by careful evaluation of fluoroscopy during nasojejunal tube insertion. As previously described, we believe that puncture of the colon or too distant a loop can be avoided relying on EUS-guided transgastric visualization of the nasojejunal tube and fluid flow inside the jejunum[24]. For all these reasons, we never place the tip of the nasojejunal tube too far from the ligament of Treitz. Finally, in case both procedures are required, we have no concern in performing them during the same sedation, but we believe that sequence matters[24]. EUS-GJ must be performed first, as an effective gastroenteric conduit is fundamental for successful transluminal biliary drainage. Conversely, if EUS-GJ proves technically unfeasible, a correctly placed EUS-CD may be rendered dysfunctional, resulting in subsequent cholangitis[25].



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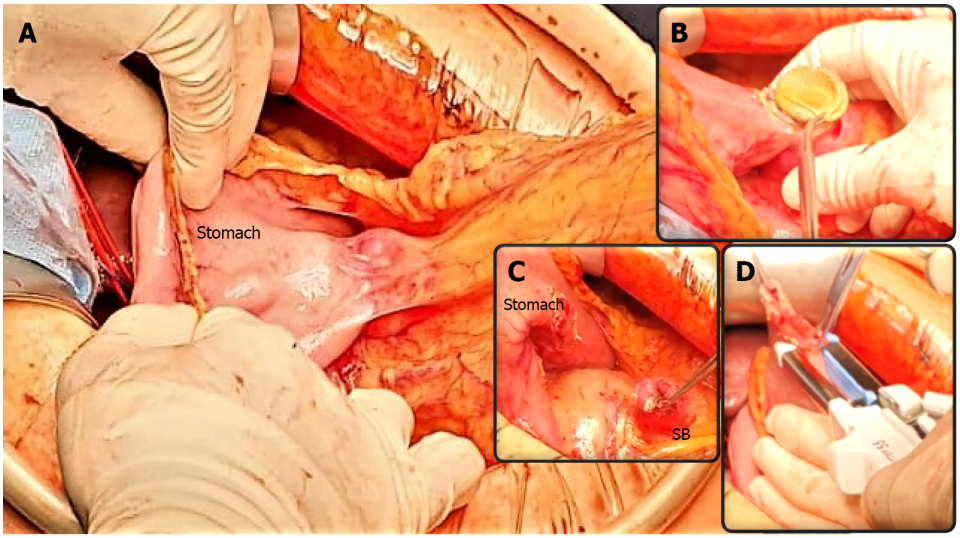
Figure 2 Endoscopic ultrasound-guided gastrojejunostomy. A and B: The small bowel has been distended with saline infusion through a nasojejunal tube. The dilated jejunal loop has been identified through the gastric wall by endosonography and has been accessed through a 20 mm enhanced lumen apposing metal stent (arrow: tip of the catheter; arrowhead: distal flange); under fluoroscopic (A) and endosonographic (B) guidance, demonstrating the opening of the distal flange (arrowhead) inside the small bowel (SB); C: The proximal flange of the electrocautery-enhanced lumen apposing metal stent has been released and dilated, and the SB can be visualized through the lumen apposing metal stent; D: Contrast injected through the nasojejunal tube can be aspirated through the lumen apposing metal stent inside the stomach.



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Figure 3 Endoscopic ultrasound-guided gallbladder drainage. A: Endosonographic view of the delivery system (arrowhead) of the enhanced lumen apposing metal stent inside a distended gallbladder (GB) full of sludge; B: Radioscopic view of the lumen apposing metal stent (arrowhead) released between the gallbladder and duodenal bulb [D1; biliary stent (arrow) released in the second duodenal portion (D2)]; C: Endoscopic view of the proximal flange of the lumen apposing metal stent inside the duodenal bulb.

As for EUS-GBD with LAMS, one important consideration might regard the site for drainage. If EUS-GBD is performed in a patient who is potentially a candidate to a pancreaticoduodenectomy, a transduodenal route for drainage makes the fistula lie within the resection margins of subsequent surgery. In all other kinds of surgeries (including simple cholecystectomy), a surgical revision of a cholecystogastrostomy is technically simpler than a cholecystoduodenostomy, and therefore the endoscopist should predilect a transgastric route when feasible. Moreover, all the attempts to reduce LAMS-related trauma must be pursued to avoid additional inflammatory reactions that might complicate surgery. We suggest the systematic placement of a short coaxial double-pigtail plastic stent inside the LAMS to avoid food impaction and to protect the contralateral wall from mechanical trauma, a lesson learnt from the management of peripancreatic fluid collections[26]. Furthermore, as described also in the setting of peripancreatic fluid collections, a longer stent indwelling might predispose to a higher risk of adverse events, and therefore we propose a systematic removal of the LAMS within 4 wk in case surgery must be postponed beyond this interval[27].



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Figure 4 Pancreaticoduodenectomy following endoscopic ultrasound-guided double bypass. A: The endoscopic ultrasound-guided gastrojejunostomy site is easily identified and a stable gastrojejunal anastomosis is visible (underlined by blue curves) between the stomach and the small bowel (SB); B-C: The endoscopic ultrasound-guided gastrojejunostomy site is opened with diathermic coagulation, the lumen apposing metal stent is removed (B), and the anastomosis cut (C); D: The SB is prepared for gastroenteric anastomosis, while the gastric defect will be closed using staplers. A classic pylorus-preserving pancreaticoduodenectomy is achievable.

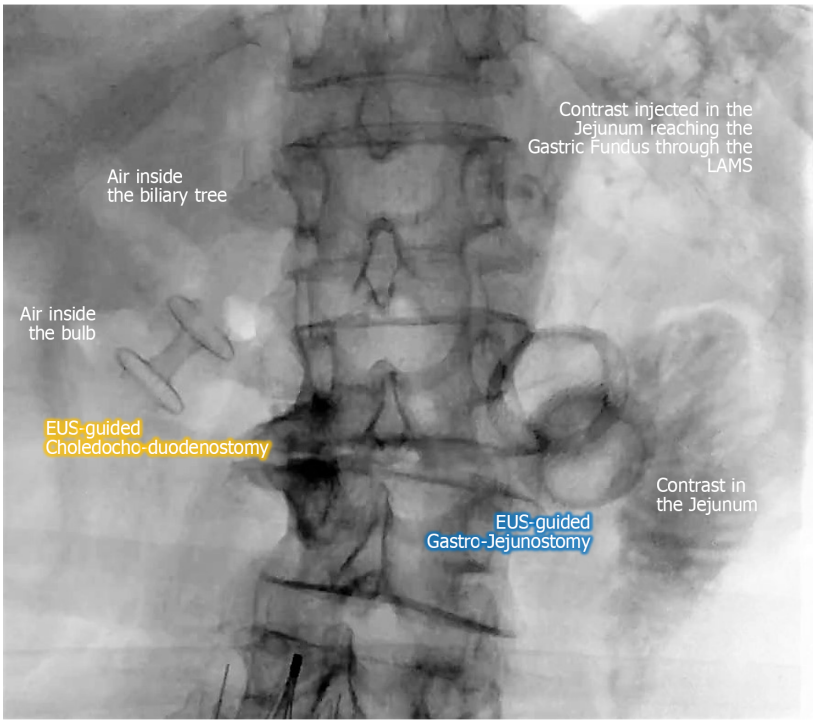
SURGICAL TRICKS FOR PANCREATICODUODENECTOMY FOLLOWING EUS-GUIDED DOUBLE BYPASS

T-EUS is not a contraindication for PD. Usually the GJ anastomosis is performed between the posterior gastric wall and the fourth duodenal portion/first jejunal loop. As the first part of the resection we suggest identifying the EUS-GJ anastomosis (Figure 4), to open it with diathermic coagulation and to remove the LAMS. At this stage, the gastric wall defect can be closed using staplers, whereas small bowel will be resected as part of the pancreaticoduodenectomy. With this technique, a classic pylorus-preserving pancreaticoduodenectomy is achievable. As a second stage, we suggest performing a retrograde cholecystectomy in order to reach the common bile duct at the liver hilum. At this point, a proper lymphadenectomy of the liver hilum is performed, and the hepatic artery, portal vein and common bile duct are isolated and encircled with vessel loops. The common bile duct should be cut above the cystic duct's confluence, and EUS-CD will therefore remain within resection margins and removed simultaneously.

In the case of former EUS-GBD, the approach will depend on the site of drainage and the type of surgery. If the patient is a candidate to a pancreaticoduodenectomy and the gallbladder was drained through the duodenum, the fistula remains within resection margins. In the case that the gallbladder was drained transgastric, the fistula can be transected as described for EUS-GJ. The gastric wall defect can be closed either using staplers or using suture with eventual omental protection.

CONCLUSION

Upfront resection is becoming a rarer indication in PDAC patients, as biologic behavior and natural history of the disease has boosted indications for neoadjuvant treatment. An effective chemotherapy, capable of carrying the patient to eventual surgery, greatly depends on an effective and long-lasting palliation of disease-related gastrointestinal symptoms and complications. While conventional endoscopic/percutaneous palliation carries intrinsic disadvantages, t-EUS may overcome these limitations, without apparently increasing technical difficulty or complicating subsequent surgery. For all these reasons we believe that t-EUS should not be restricted in the bridge-to-surgery scenario while deserving further prospective evaluation in this setting (Figure 5).



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Figure 5 Fluoroscopic final image of an endoscopic ultrasound-guided double bypass with choledochobulbostomy and gastrojejunostomy. EUS: Endoscopic ultrasound; LAMS: Lumen apposing metal stent.

FOOTNOTES

Author contributions: Vanella G, Capurso G and Arcidiacono PG conceived the manuscript; Vanella G, Bronswijk M and Tamburrino D performed the literature search; Vanella G, Bronswijk M, Tamburrino D, Dell'Anna G and Capurso G drafted the paper; Arcidiacono PG, Reni M, Crippa S, Van der Merwe S and Falconi M critically revised the manuscript for important intellectual content; Vanella G created the figures; all authors agreed on the final submitted version, accepting to be accountable for accuracy or integrity of any part of the work.

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Country/Territory of origin: Italy

ORCID number: Giuseppe Vanella 0000-0001-7280-1761; Domenico Tamburrino 0000-0003-1435-3651; Gabriele Capurso 0000-0002-0019-8753; Michiel Bronswijk 0000-0003-2039-5022; Michele Reni 0000-0002-6463-0293; Giuseppe Dell'Anna 0000-0002-9395-5468; Stefano Crippa 0000-0002-7370-1508; Schalk Van der Merwe 0000-0002-9891-2686; Massimo Falconi 0000-0001-9654-7243; Paolo Giorgio Arcidiacono 0000-0001-6692-7720.

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REVIEW

Malignant biliary obstruction due to metastatic non-hepato-pancreato-biliary cancer

Takeshi Okamoto

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Takeshi Okamoto, Department of Gastroenterology, St. Luke's International Hospital, Chuo-ku 104-8560, Tokyo, Japan

Corresponding author: Takeshi Okamoto, MD, Staff Physician, Department of Gastroenterology, St. Luke's International Hospital, 9-1 Akashicho, Chuo-ku 104-8560, Tokyo, Japan. tak@afia.jp

Abstract

Malignant biliary obstruction generally results from primary malignancies of the pancreatic head, bile duct, gallbladder, liver, and ampulla of Vater. Metastatic lesions from other primaries to these organs or nearby lymph nodes are rarer causes of biliary obstruction. The most common primaries include renal cancer, lung cancer, gastric cancer, colorectal cancer, breast cancer, lymphoma, and melanoma. They may be difficult to differentiate from primary hepato-pancreato-biliary cancer based on imaging studies, or even on biopsy. There is also no consensus on the optimal method of treatment, including the feasibility and effectiveness of endoscopic intervention or surgery. A thorough review of the literature on pancreato-biliary metastases and malignant biliary obstruction due to metastatic non-hepato-pancreato-biliary cancer is presented. The diagnostic modality and clinical characteristics may differ significantly depending on the type of primary cancer. Different primaries also cause malignant biliary obstruction in different ways, including direct invasion, pancreatic or biliary metastasis, hilar lymph node metastasis, liver metastasis, and peritoneal carcinomatosis. Metastasectomy may hold promise for some types of pancreato-biliary metastases. This review aims to elucidate the current knowledge in this area, which has received sparse attention in the past. The aging population, advances in diagnostic imaging, and improved treatment options may lead to an increase in these rare occurrences going forward.

Key Words: Bile duct obstruction; Obstructive jaundice; Pancreas; Metastasis; Endoscopic retrograde cholangiopancreatography

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Core Tip: Metastases from distant primary cancers are rare causes of biliary obstruction. The most common primaries include renal cancer, lung cancer, gastric cancer, colorectal cancer, breast cancer, lymphoma, and melanoma. Clinical presentation, appropriate immunohistochemical markers for pathological analysis, treatment options, and prognosis may differ depending on the primary cancer. A review of the existing literature and characteristics of metastases from each primary are presented.

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INTRODUCTION

Metastatic lesions from other primaries to the pancreatic head, biliary tree, liver, ampulla of Vater, and hilar or peripancreatic lymph nodes have the potential to cause biliary obstruction. The pancreas and biliary tree are rare destinations for metastases. However, such metastases can result from almost every primary, from brain tumor to melanoma of the toe.

In a multicenter analysis of 159 patients with pancreatic metastases, the most common primaries were renal cell carcinoma (38%), lung cancer (2%), colorectal cancer (11%), and sarcoma (6%)[1]. About 41% were isolated metastases, 78% were single masses, and were more or less uniformly distributed throughout the pancreas. About 24% were symptomatic and only ten cases presented with jaundice. Two-thirds were diagnosed by endoscopic ultrasound-fine-needle aspiration (EUS-FNA) and most of the remainder were diagnosed by surgery. Median overall survival was 43 mo, with extrapancreatic metastasis, tumor-related symptoms at diagnosis, and pathology of primary tumors independently predicting survival. In a literature review of 234 subjects, synchronous presentation with the primary tumor and surgical resection were important prognostic factors in addition to tumor-related symptoms at diagnosis and pathology of primary tumors[2]. There were marked differences in the studied subjects: 68% had renal cell carcinoma, 86% were isolated metastases, 79% involved the pancreatic head, and 62% were symptomatic at diagnosis, but extrapancreatic metastases were only observed in 14% of cases. As may be expected, metastases from lung cancer and melanoma had worse prognoses than those from renal cell carcinoma.

Autopsy studies report extremely high rates of pancreatic metastases. An autopsy database study found 81 pancreatic metastases among only 190 pancreatic tumors (43%). Major primaries included the lung (42%), gastrointestinal tract (25%), kidney (5%), and breast (4%)[3]. A Japanese autopsy study found 103 cases of pancreatic metastases among 690 cadavers with malignant tumors, implying that 15% of all metastatic malignancies involve the pancreas[4]. Specifically, pancreatic metastases were found in 35% of gastric cancers, 25% of bladder cancers, 21% of ovarian cancers, 15% of lung cancers, and 9% of renal cell carcinomas and breast cancers. One-third of pancreatic metastases were not visible macroscopically. About 17% involved the pancreas *via* direct invasion rather than distant metastasis.

Metastases to the bile duct are extremely rare. There are isolated case reports of from primaries including ovarian cancer, colon cancer, rectal cancer, esophageal cancer, gallbladder cancer, hepatocellular carcinoma, breast cancer, and malignant melanoma[5-13].

Herein, hepato-pancreato-biliary metastases from non-hepato-pancreato-biliary malignancies and resulting malignant biliary obstruction (MBO) are discussed. Characteristics of each primary are discussed after a brief overview of diagnosis and treatment of obstructive metastases.

DIAGONSIS

Diagnostic imaging

Computed tomography (CT) is the most common imaging modality used for cancer follow-up. One study found pancreatic metastases in 0.3% of 6623 patients followed up for various malignancies[14]. An analysis of 192 cases in seven imaging studies of pancreatic metastases from various primaries reveals that the most common primaries were renal cell carcinoma (30%), lung (26%), stomach/colon (13%), and breast (10%) (Table 1)[14-20]. Of these, 66% were solitary masses, 27% had multiple nodules, and 7% had diffuse involvement. Isolated pancreatic metastases were observed in 5%-33% of cases. Pancreatic metastases were found synchronously with the primary malignancy in 22%-44% of cases. A mass in the pancreatic head was found in 11%-29% of cases, but only 5%-6% had biliary dilation. Pancreatic duct dilatation was observed in 11%-22% of cases, with pancreatitis rarely observed. CA19-9 was elevated in 8%-28% of cases, but most primaries of such cases were gastrointestinal and may have

Table 1 Computed tomography findings of pancreatic metastases

			Primary Malignancy										Characteristics		
Ref.	Year	Cases	Renal	Lung	Breast	Stomach	Colon	Sarcoma	Melanoma	Ovary	Thyroid	Other	Solitary mass	Multiple nodules	Diffuse involvement
Ferrozzi *et al*[14]	1997	20	2	6	3	3	1		2	2	1		11	2	7
Klein *et al* [15]	1998	66	20	15	8	4		5	4	2	1	7	52	11	3
Tsitouridis *et al* [16]	2009	11	1	7	3								7	3	1
Angelleli *et al* [17]	2012	17	8	4	3			2					7	9	1
Shi *et al* [18]	2015	18	3	7		5 (stomach+colon)		2				1	12	6	
Choi *et al* [19]	2015	36	17	2		5	7	1	1	1		2	29	7	
Galia *et al* [20]	2018	24	6	8	2			1			4	3	9	13	2
Total		192	57	49	19	12	8	11	7	5	6	13	127	51	14
% of total			30%	26%	10%	7%	5%	6%	4%	3%	3%	7%	66%	27%	7%

been unrelated to pancreatic involvement.

CT characteristics of metastatic lesions were generally concordant with the primary lesions, except some cases of gastric cancer[19]. Metastases from renal cell carcinoma are generally hypervascular with well-defined margins, while most other metastases are hypoattenuating with unclear borders, similar to primary adenocarcinoma[14-19]. In a comparison with primary pancreatic adenocarcinoma, metastases had significantly less pancreatic duct dilatation, vascular involvement, parenchymal atrophy, or peripancreatic fluid[20]. Biliary dilatation was also less frequently observed, possibly due to the small number of metastases located in the pancreatic head in the study.

In summary, most metastases present as solitary masses or multiple nodules on CT and generally do not cause symptoms or biliary obstruction. Renal cell carcinoma metastases may be identified by hypervascular, well-defined lesions, while the absence of pancreatic duct dilatation or parenchymal atrophy may be clues for metastatic disease in general.

Studies on magnetic resonance imaging (MRI) and fluorodeoxyglucose-positron emission tomography/CT (FDG-PET/CT) for diagnosing pancreatic metastases are limited. MRI of metastases of renal cell carcinoma may also be distinguished from primary adenocarcinoma due to their vascularity [21]. One FDG-PET/CT study of 26 lesions in 19 patients found no difference in maximum standardized uptake values between pancreatic metastases and primary pancreatic adenocarcinoma[22]. However, several isodense intrapancreatic nodules missed on contrast CT were discovered by FDG-PET/CT. FDG-PET/CT may also be useful in detecting other unsuspected distant metastases.

Endoscopic ultrasound

Endoscopic ultrasound (EUS) may also provide clues to identify metastatic disease. In a study comparing 28 pancreatic metastases (23 solitary, four multiple, and one diffuse lesion) to 60 cases of

pancreatic adenocarcinoma, Hjiioka *et al*[23] reported that main pancreatic duct dilatation was also observed significantly more frequently in pancreatic adenocarcinoma than in metastatic lesions. Regular borders and absence of retention cysts predicted pancreatic metastases.

In a small study of contrast-enhanced harmonic EUS, pancreatic metastases from renal cell carcinoma and lymphoma were hyperenhancing, while most other metastases were hypoenhancing[24]. While both contrast-enhanced harmonic EUS and elastography may assist in differentiating malignant lesions from benign lesions, there is insufficient evidence in their ability to differentiate metastases from primary adenocarcinoma[25,26].

Fine-needle aspiration

Pancreatic metastatic accounted for 0.9%-2.5% of EUS-FNA samples of the pancreas and 4.7%-7.2% of pancreatic malignancies[27-29]. These figures may be higher than those of other modalities because lesions with typical findings of pancreatic adenocarcinoma may be resected without EUS-FNA. In the largest review of 582 pancreatic metastases diagnosed by EUS-FNA, renal cell carcinoma (34%), lung cancer (15%), colon cancer (10%), melanoma (7%), and breast cancer (6%) were the most common primaries[28].

EUS-FNA is a safe and well-accepted method of diagnosing such metastatic lesions. EUS-FNA for pancreatic metastases and primary adenocarcinoma have reported sensitivities of 75%-94% and 75%-84%, respectively, and specificities of 60%-100% and 97%-100%, respectively[30-33]. No significant complications including tumor seeding of the needle tract have been reported in EUS-FNA of pancreatic metastases[34]. Immunohistochemistry and *KRAS* mutation analysis can be crucial in reaching the final diagnosis[23].

TREATMENT

Endoscopic biliary drainage

In studies of endoscopic biliary drainage which include metastases from non-hepato-pancreato-biliary primaries, metastases are not necessarily a rare cause of MBO. Metastases were the cause of biliary obstruction in 14% of 1346 patients in 14 such studies[35-49]. The pooled average across six studies limited to distal MBO (n = 395) was 9%, while that of three studies limited to hilar MBO (n = 256) was 22%[40-48]. Gastric cancer (including lymph node metastases) was the cause of 80% of MBO across four studies focused on surgically altered anatomies[49-52]. MBO due to metastases from primaries with better prognoses than primary pancreato-biliary cancer may be able to better generally tolerate and benefit from endoscopic retrograde cholangiopancreatography (ERCP), although they may not be candidates for surgical resection. These figures may be overstated, as some reports which include no metastatic cases did not intentionally exclude them. Selection bias may also be a factor; most reports were from university-affiliated tertiary care centers or cancer institutes.

Studies focused exclusively on biliary drainage for metastatic cancer are mainly limited to those on percutaneous drainage[53,54]. One study on 93 patients achieved clinical success in 73% of patients and found that survival differed significantly depending on the primary tumor[54]. Subsequent chemotherapy prolonged median survival from 1 to 5 mo, with the greatest benefit observed in the 28 cases with colorectal cancer. Another study with 42 cases each of gastric and colorectal cancer found that performance status and absence of peritoneal metastases were associated with longer survival[55].

There is only one study on prognostic factors after ERCP stenting for biliary drainage in metastatic cancers of various origins[56]. Colorectal cancer was the most common primary (25%), and there was only one case of renal cell carcinoma. Technical success was achieved in 91.7% of patients and 67% of successfully cases received subsequent treatment. Performance status and treatment after drainage were independent predictors of overall survival.

One study on metallic stent placement in hilar MBO reported that metastatic disease from other primaries was an independent risk factor for technical failure[57]. The study only included five patients, of which one had pancreatic cancer. The authors reported that the extrinsic nature of the biliary stricture necessitated multiple procedures, including percutaneous drainage. Larger studies have failed to reproduce this result. EUS-guided intervention has also been reported with success in hilar lymph node or hepatic metastases from colorectal, breast, gastric, urogenital, and anal cancer[57]. With respect to endoscopic biliary drainage, it appears acceptable to approach MBO due to metastases in a similar manner as MBO from primary hepato-pancreato-biliary cancer.

Surgery

Metastasectomy accounted for 1.4% of 5745 pancreatic resections across six studies which provided total figures (range: 0.7%-3.1%)[58-63]. A review of 399 metastasectomies found that renal cell carcinoma accounted for 62.6% of cases[64]. Forty percent had symptoms at presentation and, in a separate review, 22% had jaundice[64,65]. About 10% were found synchronously with the primary tumor[64]. Median survival was 50.2 mo after surgery overall, but 71.7 mo for renal cell carcinoma. On the other hand, perioperative mortality was observed in 2.2% of all metastasectomies. Patients with isolated pancreatic

metastases had better prognoses than those with other metastases (45 mo *vs* 26 mo). In the separate review[65], renal cell carcinoma patients had a long post-surgical median survival of 105 mo, compared to 54 mo for colon cancer, 40 mo for sarcoma, 34 mo for ovarian cancer, 26 mo for breast cancer, 14 mo for melanoma, and 6 mo for lung cancer. On the other hand, pooled analysis showed long 5-year post-surgical survival of 61.1% for isolated pancreatic metastases (of which 74% were renal cell carcinomas) and 58.9% for local invasion from colon or gastric cancer[66].

A study of 98 metastasectomies found that old age, non-renal cell carcinomas, vascular invasion, and positive margins were independently associated with increased mortality risk[67]. Resection can generally be considered for isolated metastases. Many reports suggest long-term survival in renal cell carcinoma. Symptomatic relief may also be achieved; all colorectal cancer patients experienced symptomatic relief after metastasectomy in a surgical review[68]. While data is lacking for other primaries, metastasectomy should only be attempted when margin-negative resection can be expected. As melanoma and lung cancer patients have poor prognosis even after surgery, indication for metastasectomy should be considered with caution.

PRIMARY MALIGNANCIES

Renal cell carcinoma

Renal cell carcinoma is the most common primary for pancreatic metastases in CT, EUS-FNA, and surgical series. When such metastases occur, they are discovered synchronously with primary renal cell carcinoma in 7% of cases[69]. There is generally a long time lag of up to 32 years between nephrectomy and pancreatic metastases. Most have multiple extrapancreatic metastases. About half of isolated metastases occur in the pancreatic head. One study suggested a predilection for fatty pancreas[70].

Counterintuitively, many reports note that renal cell carcinoma patients with pancreatic metastases have better prognoses than those without, even when there are concomitant extrapancreatic metastases [69,71-73]. In one study, median overall survival with and without pancreatic metastases were 39 and 23 mo, respectively[73]. The size of isolated metastases, the number of metastatic pancreatic lesions, and time interval from nephrectomy to pancreatic metastasis had no impact on survival[69]. The laterality of the primary renal cancer has no impact on the portion of the pancreas affected. Affinity of indolent types of renal cell carcinomas to the pancreas has been suggested, with characteristic genetic mutations, high sensitivity to antiangiogenic treatment and resistance to immune check point inhibitors[69,74]. The extreme rarity of metastases to the biliary tree may also provide support for this affinity[75,76].

CT of pancreatic metastases are generally hypervascular with well-defined margins[14-16]. The hypointensity on T1-weighted and hyperintensity on T2-weighted imaging make them difficult to differentiate from primary adenocarcinoma on MRI[77]. They can also mimic neuroendocrine tumors on contrast MRI, due to their early contrast enhancement. Diagnosis can be made with high accuracy by EUS-FNA, with immunohistochemistry usually positive for pan-cytokeratin, CD10, EMA, and PAX-8 and negative for synaptophysin, chromogranin, and beta-catenin (nuclear staining)[78].

Despite the large number of reports on pancreatic metastases, there is a surprisingly small number of reports of biliary obstruction. Only two of 307 ERCP cases in eight studies involved metastases from renal cell carcinoma. There are isolated reports of MBO from ampullary or bile duct involvement[79,80]. Simultaneous gastric outlet obstruction can rarely occur[80].

About 80% of pancreatic metastases are resectable[81]. While there are no direct comparative studies, surgery for both single and multiple metastases to the pancreas is generally considered safe and associated with long-term survival[82-84]. A systematic review showed that extrapancreatic disease was a risk factor for recurrence after surgery but had no impact on survival[85]. However, it is not clear whether surgery is required. There was no significant difference in overall survival between tyrosine kinase inhibitors and surgery in one study[86]. Another study reported that the existence of pancreatic metastasis did not affect survival in patients treated with first-line tyrosine kinase inhibitors[87]. Radiotherapy was also effective in a small case series[88].

Lung cancer

Lung cancer is the leading cause of cancer death worldwide[89]. About 57% have metastatic disease at diagnosis[90]. About 3% of lung cancer patients develop pancreatic metastases[91]. The frequency of pancreatic metastases depends on the histological subtype, occurring most commonly in small cell carcinoma (10%), followed by adenocarcinoma (2.4%), large cell carcinoma (1.9%), and squamous cell carcinoma (1.1%)[92]. Most cases were asymptomatic[93]. A majority of pancreatic metastases present as solitary lesions (73%) but can also be multiple (12%) or diffuse (15%). Concomitant liver and adrenal metastases are observed in 73% and 69% of cases, respectively.

Small cell lung cancer presents initially with increased serum bilirubin in about 10% of cases, caused by hepatic (6%) or pancreatic (4%) metastases[94]. Obstructive jaundice due to hilar lymph node compression from metastatic small cell lung carcinoma has also been reported[95]. Most reports of metastasis-induced acute pancreatitis come from small cell lung cancer or gastric cancer metastasizing to the pancreatic head, with isolated reports of squamous cell lung cancer, adrenocortical carcinoma,

and breast cancer[96-99]. Twenty-six cases from small cell lung cancer have been reported to date[100]. While initially estimated to occur in 0.12% of small cell lung cancer cases, the figure may be higher as 14 cases were reported from a single institution[96,101]. Chemotherapy may provide a survival benefit[96].

At least 23 reports of primary bile duct small cell carcinoma have been reported[102]. While there are no reports of small cell lung cancer metastasizing to the bile duct, there are more than ten reports of biliary obstruction as the initial presentation of small cell lung cancer[103]. In one study, 4.0% of small cell lung cancers presented initially with extrahepatic obstruction due to pancreatic metastases, while another 5.6% had jaundice due to hepatic metastases[104]. While there was good overall response to chemotherapy, the latter group had a worse prognosis.

There are ten reports of squamous cell lung cancer metastasizing to the pancreas[105]. All were single lesions, 67% involved the pancreatic head, 50% had biliary or pancreatic duct dilation, and 30% had obstructive jaundice. There are two reports of lung adenocarcinoma metastasizing to the common bile duct, both causing biliary obstruction[106,107].

Seventeen cases of duodenal metastases from lung cancer have been reported, caused by adenocarcinoma (47%), squamous cell (29%), small cell (12%) or large cell (12%) cancers and arising in all four parts of the duodenum[108,109]. MBO occurred from two metastases occupying the second part (squamous cell and adenocarcinoma), both managed endoscopically[109,110].

CT and MRI may not be contributory in differentiating metastases from primary adenocarcinoma, or in hyperenhancing lesions, neuroendocrine tumors[105]. FDG-PET/CT found abnormal pancreatic accumulations in 1.6%-2.3% of lung cancer cases, discovering metastases from adenocarcinoma and small and large cell lung cancer as small as 6 mm[111]. All had metastases to at least one other organ. While one patient had acute pancreatitis, none developed obstructive jaundice.

As with metastases from other primaries, diagnosis can be made by EUS-FNA[112]. Thyroid transcription factor-1 can aid in differentiating pancreatic metastases from primary pancreatic adenocarcinoma and small cell lung cancer metastases from primary small cell biliary cancer[102,113,114]. *KRAS* G12C mutations and napsin A are also associated with lung adenocarcinoma, while *KRAS* G12R mutations, CK20, and CDX2 support the diagnosis of pancreatic adenocarcinoma[114].

In addition to ERCP for MBO and standard treatment for metastatic lung cancer, one study found a survival benefit in resecting pancreatic metastases, with a median survival of 29 mo for curative intent resection *vs* 8 mo for palliative surgery or medical management[115-117].

Gastric cancer

Most gastric cancers occur in the non-cardia and are commonly associated with *Helicobacter pylori* infection, leading to geographic differences in incidence. Liver metastases are observed in almost half of metastatic gastric cancer patients[118]. There are only 11 reports of pancreatic metastases, mostly resulting from moderately or poorly differentiated adenocarcinoma[119].

Obstructive jaundice arises most commonly from extrinsic lymph node compression, followed by intraductal metastases which present as band-like wall thickening with enhancement on contrast CT, much like primary cancer of the bile duct[120-122]. A minority arise from direct tumor invasion, extrinsic compression from liver metastases, or peritoneal carcinomatosis. Most result from Borrmann type 3 (63%-72%) or type 2 (21%-24%) adenocarcinomas with antral involvement (60%-98%), after total or partial gastrectomy (79%-89%)[121,123]. Histological composition of the primary tumors varies significantly across studies, with differentiated adenocarcinomas accounting for 9%-90% of total cases [121,123,124]. Obstructive jaundice occurs in 1.4%-2.3% of post-operative patients, with a median interval of 10-15 mo after surgery[125,126].

Drainage for MBO due to gastric cancer can involve two major issues: surgically altered anatomy and concomitant gastric outlet obstruction (GOO). As most affected patients have undergone total or partial gastrectomy, approaching the papilla can be extremely difficult (Figure 1). While percutaneous biliary drainage has largely been successful, poor prognostic factors after drainage include significant liver metastases, hilar strictures and high carbohydrate antigen (CA) 19-9[127,128]. The main cause of stent occlusion was sludge buildup (13%) in one study, with no cases of tumor ingrowth[122]. When ERCP is contraindicated, EUS-guided biliary drainage was comparable to percutaneous drainage in terms of both technical success rate and stent patency period[129]. As in the case of post-operative pancreatic cancer patients, ERCP and stent placement using a double-balloon enteroscope has also been reported for this purpose[49,52,130]. Technical success was reported in all 26 gastric cancer cases across two studies[49,52]. Median time to recurrent biliary obstruction for seven cases in one study was 7.4 mo, with two cases of tumor ingrowth and one case of mucosal hyperplasia[49].

After hepatobiliary cancers, gastric cancer is the most common cause of combined MBO and GOO, accounting for 4%-8% of such cases[131-133]. Other rare cases include colon, breast, and renal cancer [131]. The extrahepatic bile duct and first or second parts of the duodenum are most commonly involved. Regardless of the primary site, MBO tends to precede GOO. Double stenting of the duodenum and bile duct has success rates approaching 100%, with only rare reports of post-procedural pancreatitis [133,134]. Metallic stents tended to have longer patency than plastic stents, while more adverse events resulted from EUS-guided drainage when compared with ERCP[133].



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Figure 1 A 69-year-old man presented with obstructive jaundice due to recurrence 18 mo after distal gastrectomy and Roux-en-Y reconstruction for gastric cancer. A recurrent mass with central necrosis (white arrowheads) obstructed the extrahepatic bile duct (black arrow), causing dilatation of intrahepatic bile ducts and gallbladder (white arrows). While endoscopic ultrasound-guided hepaticogastrostomy led to symptomatic relief, the patient died 1 mo later.

Colorectal cancer

Almost half of colorectal cancer patients experience metastatic disease. Metastases to the liver ultimately occur in 25%-30% of affected patients, of which only about 25% can be resected[135-137]. Liver metastases tend to occur more commonly in left-sided cancers and in relatively young patients[137]. Other reported sites of metastases which may cause biliary obstruction include lymph nodes, pancreas, peritoneum, and the extrahepatic bile duct[138,139].

Cytokeratin (CK) 7 negativity and CK20 positivity in EUS-FNA specimens aid in differentiating from primary pancreato-biliary adenocarcinoma, which is generally CK positive and CK20 negative[140]. However, some types of primary pancreatic adenocarcinoma such as the colloid type may be CK7 negative/CK20 positive, complicating the differential diagnosis[30].

Biliary obstruction is associated with poor outcomes not only because it reflects widespread disease, but also because of chemotherapy cannot be performed at the desired dose. One study found jaundice in about 10% of metastatic colorectal cancer patients due to liver metastases (53%) or metastatic lymph nodes (47%)[141]. Endoscopic or percutaneous biliary drainage was only successful in 42% of cases with median overall survival of 1.5 mo, which improved to 9.6 mo when chemotherapy could be restarted. The study was unable to identify predictors of drainage failure, although drainage was attempted less often in cases with hilar involvement. In another study[142], biliary drainage mostly by ERCP was successful in about 65% of cases, allowing 70% of successful cases to restart chemotherapy and improving median survival from 33 to 262 d. A study on both colorectal (n = 32) and gastric cancers (n = 60) found that multiple hepatic metastases and hilar strictures were associated with unsuccessful percutaneous drainage, while poor performance status, multiple liver metastases, ascites, history of treatment with multiple chemotherapy regimens, undifferentiated carcinoma, and high CA 19-9 Levels were associated with poor prognosis[127].

A characteristic almost unique to colorectal cancer is the ability to spread along epithelial surfaces and grow intraductally, mimicking neoplasms of the lung, bladder, or intrahepatic bile ducts[143]. Liver metastases of colorectal cancer can exhibit intrabiliary extension in 3.6%-10.6% of cases, compared to 0.7%-1.9% of metastases from other primaries[144]. Such phenomena are most commonly observed in well-differentiated adenocarcinomas originating in the rectosigmoid[145,146]. Microscopic intrabiliary extension has been reported in up to 40.6% of liver metastases from colorectal cancer[145]. There are also reports of intrabiliary extension from hepatocellular carcinoma as well as liver metastases of neuroendocrine tumor, gastrointestinal tumor, and invasive lobular breast cancer[144,147]. Of the other reports on liver metastases, only the neuroendocrine tumor case presented signs of biliary obstruction [144].

Two patterns of intrabiliary growth were identified by Estrealla *et al*[144]: Bile duct colonization which replace the biliary epithelium and tumor plugs which may or may not affect the biliary epithelium. Such growth was limited to intrahepatic ducts in 72% of cases, while the remainder involved the hilum. Laboratory, imaging, and histological abnormalities suggesting biliary obstruction were all positively associated with the degree of biliary growth. While intrabiliary extension may mimic intrahepatic cholangiocarcinoma on imaging studies, they can generally be differentiated on immunohistochemistry with CK7 and CK20[140]. In contrast with liver metastases from other primaries, biliary biopsy may be useful in achieving a preoperative diagnosis[138]. Intrabiliary extension is paradoxically associated with a better prognosis, possibly because it occurs more frequently in well-differentiated

adenocarcinomas which have longer survival periods[145,146].

As isolated liver and lung metastasectomy have been established in colorectal cancer, resection of isolated pancreatic metastases may also be justifiable in selected patients. A review of 24 studies revealed that out of 37 colorectal cancer cases undergoing pancreatic resection, 19 had disease recurrence, with median survival of 21 mo[68]. In addition, all studied patients experienced symptomatic relief after surgery, which lasted until recurrence of cancer.

Breast cancer

Approximately 6% of breast cancer are metastatic at diagnosis in developed countries, and 20%-30% eventually develop metastases[148]. Most common sites of metastases are bone, lung, liver and brain [149,150]. Jaundice is found in 6%-25% of breast cancer patients with liver metastases, generally resulting from hepatic failure rather than MBO[151]. Some studies suggest worse prognosis when jaundice is present, most likely because chemotherapy must be reduced or discontinued as a result [152]. Liver metastases occur in about 5% of all breast cancer patients and 32% of metastatic cases[152, 153].

While about 80% of invasive breast cancers are invasive ductal carcinomas, lobular carcinoma is the most common histological subtype observed in gastrointestinal metastases[154,155]. Pancreatic metastases are found in 5%-13% of autopsies of breast cancer patients[156,157]. In a review of 24 cases of periampullary breast cancer metastases, MBO was observed at initial diagnosis in five cases, with metachronous MBO cases occurring 1-23 years later[158]. Our institution reported eleven cases of obstructive jaundice due to metastatic breast carcinoma, resulting from metastases to hilar or peripancreatic lymph nodes (36%), pancreas (27%), liver (18%), gallbladder (9%), and peritoneal carcinomatosis (9%)[99]. MBO due to metastases to the duodenum have also been reported[159].

Metastases can be difficult to differentiate from primary pancreatic cancer, both clinically and radiologically[28,147,155,160]. CA15-3 elevation may suggest breast cancer metastasis in some cases. As breast cancers are adenocarcinomas, diagnosis by EUS-FNA may require immunohistochemical analysis with monoclonal antibodies such as gross cystic disease fluid protein-15[161]. *KRAS* point mutation analysis has also been shown to be useful[23].

ERCP has acceptable technical success and adverse event rates, offering a chance for long-term survival if chemotherapy can be resumed[99,162]. Concomitant duodenal strictures may preclude ERCP in 18% of cases and may be treated using EUS-guided interventions (Figure 2)[99]. When resectable, metastatic breast cancer to the pancreas have a median survival of 26 mo[2]. While cases series have suggested potential for improved survival[163], there is insufficient data to determine whether solitary metastatic lesions to the pancreas should be resected. Survival in MBO with extensive metastases may average only 2 mo with palliative care alone[99].

Malignant melanoma and Merkel cell carcinoma

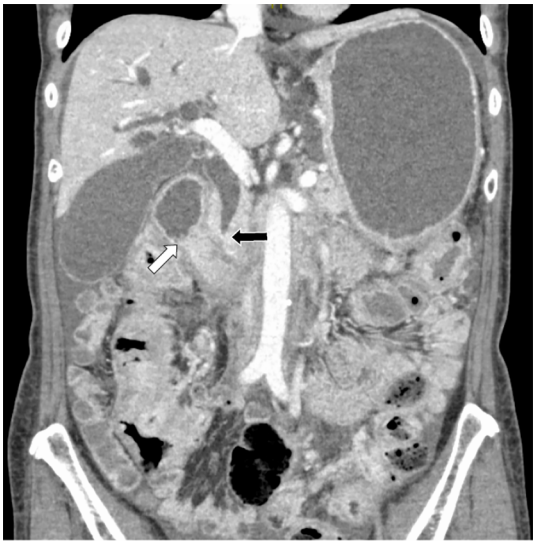
Metastatic melanoma has a poor prognosis with median survival of about 8 mo[164]. Visceral metastases, particularly when multiple, are associated with poor survival[165]. Pancreato-biliary involvement and obstructive jaundice from malignant melanoma can take various forms: primary malignant melanoma of the biliary tract, melanoma of unknown primary arising in the pancreas, pancreato-biliary metastasis, and bile duct compression from hepatic or lymph node metastases.

Autopsy studies on malignant melanoma have shown metastases to the liver in 54%-88%, intestines in 26%-58%, and pancreas in 38%-53% of cases, although discovery rates in the clinical setting are much lower[166]. It should be noted that primaries as well as metastases may not be black, as amelanotic melanoma accounts for up to 27.5% of melanoma cases[167].

Primary malignant melanoma of the biliary tract is a rare type of mucosal melanoma which can only be diagnosed after excluding primaries in other locations including the skin, eye, and gastrointestinal tract. There are 13 reports in the literature, of which 12 presented with jaundice[168]. It tends to present in relatively young males as black, polypoid lesions exhibiting endoluminal growth. Most cases arise in the common bile duct, but can involve intrahepatic bile ducts or the gallbladder. Surgery is the treatment of choice, with a good prognosis if complete resection is achieved. One case was successfully treated with immunotherapy.

Isolated metastases to the pancreas are relatively common. While findings on imaging are generally non-specific, isolated reports describe multiple hypoechoic nodules with hyperchoic septa and central necrosis on EUS[169,170]. T2-weighted MRI may help differentiate pancreatic melanoma, which tends to be hypointense, from pancreatic adenocarcinoma, which tends to be hyperintense[171]. If melanoma can be raised in the differential diagnosis, such lesions can be diagnosed by EUS-FNA with the help of immnohistochemical markers such as Human Melanoma Black 45 and Melan A[169,170]. Large analyses suggest a survival benefit for surgical resection[172,173]. Additional support comes from Wood *et al* [174], who resected eight cases of melanoma metastases to the pancreas and achieved curative resection in six, of which three survived for over five years.

Melanoma of unknown primary, characterized by metastases in lymph nodes or other areas where primary lesions are unlikely to arise without evidence of a separate primary lesion, accounts for 2.2%-3.2% of malignant melanomas[172,173,175]. There are ten reports of isolated pancreatic melanoma with no other lesions. There is no consensus on whether they can be considered primary pancreatic



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Figure 2 A 62-year-old woman presented with jaundice, nausea, and vomiting 13 years after partial mastectomy for breast cancer. Biopsy-proven duodenal metastases caused both bile duct (black arrow) and duodenal (white arrow) obstruction. Double stenting led to temporary symptomatic relief. The patient subsequently opted for palliative care.

melanoma. They are often resected surgically, justified based on the reports of isolated pancreatic metastases described above[172-175].

There are at least 18 reports of metastatic melanoma to the common bile duct[176]. Painless obstructive jaundice is the usual finding, and prognosis is dismal unless curative resection can be achieved. Five cases of ampullary metastasis causing obstructive jaundice have also been reported, all requiring endoscopic drainage or surgery[177-181].

Merkel cell carcinoma is another type of aggressive skin cancer which has a high recurrence rate after resection. About one-third of Merkel cell carcinoma patients eventually develop metastases, of which 8% develop pancreatic metastases[182]. It can be diagnosed by EUS-FNA and may cause obstructive jaundice when located in the pancreatic head[183]. While a relatively rare disease, the reported incidence is increasing. A high index of suspicion is required for patients with a history of resection and a thorough skin examination should be considered.

Soft tissue sarcoma

Soft tissue sarcomas are mesenchymal tumors which account for less than 1% of all cancers, but are extremely heterogeneous with over 75 subtypes[184]. Metastatic soft tissue sarcomas are generally refractory to chemotherapy and have median survival of less than one year. There are over 50 reports of pancreatic metastases from various types of soft tissue sarcoma, leiomyosarcoma being the most reported subtype[185-188]. Diagnosis was achieved by fine-needle aspiration in 20 cases, of which 11 were conducted by EUS-FNA[189].

In a CT study of 13 leiomyosarcoma cases with pancreatic metastases, 85% were women and primaries were mostly located in the uterus (39%), retroperitoneum (31%), or extremities (23%). Pancreatic metastases developed after a median interval of 24 mo after diagnosis, 38% were multiple, 46% contained necrosis, and 77% were hypovascular in the arterial phase[185]. While 69% had pancreatic head involvement, lesions were small (1.0-3.5 cm) and biliary and pancreatic duct obstruction were only observed in one patient each. Among 27 unique cases contained in two literature reviews, only two of twelve cases with pancreatic head metastases had jaundice[187,188].

There are over 50 cases of primary pancreatic leiomyosarcoma, which is the most common type of pancreatic stromal tumor and occurs most frequently in middle-aged females[189]. Cystic features may lead to misdiagnoses as pseudocysts or cystic neoplasms and to false-negative EUS-FNA results. There are also 23 reports of primary pancreatic carcinosarcoma as well as reports of various subtypes of sarcoma originating in the bile duct as well as the ampulla of Vater[190-194]. More than half of pancreatic carcinosarcomas occur in the pancreatic head, of which about half cause obstructive jaundice [190]. Thus, sarcoma should be included in the differential diagnosis of isolated pancreato-biliary tumors even in the absence of a coexisting primary tumor.

Surgery has been performed for both primary and metastatic sarcoma arising in the pancreas and duodenum[195,196]. The largest case series describes seven out of 17 existing reports of resection for pancreatic metastases, with one postoperative death and recurrence in all other six cases despite margin-free resection in four cases, with a median survival of 21 mo[195]. The benefits of resection therefore remain unclear.

Lymphoma

Biliary obstruction due to lymphoma can occur from primary hepato-pancreato-biliary lymphoma as well as secondary lymphoma directly extending from abdominal lymph nodes. Most involve B-cell non-Hodgkin's lymphomas (NHL), which cause 1%-2% of all MBOs, with a disproportionately high number of reports from diffuse large B-cell lymphoma[197,198]. MBO occurs in less than 2% of NHL and about 0.5% of Hodgkin's lymphoma[198,199]. Almost 1% of NHL patients present initially with MBO[200].

NHL involves the pancreas secondarily in over 30% of cases[201]. Primary pancreatic lymphoma is rare, accounting for less than 5% of extranodal NHL[202,203]. While pancreatic lymphoma causes jaundice in up to 42% of cases, biliary and pancreatic ducts may remain unaffected by even large lesions in the pancreatic head[201,204]. Clues favoring lymphoma over pancreatic cancer in imaging studies include absence of calcifications, patency of involved vessels and ducts, poor but homogenous contrast enhancement, and either a well-delineated mass or diffuse involvement[201].

There are about 30 cases of primary lymphoma of the common bile duct[205]. They generally present with obstructive jaundice but often have smooth strictures with negative findings in ERCP brushing cytology or biopsy. One report found 36 reports of primary gallbladder lymphomas, which are less likely to cause MBO than their bile duct counterparts[206]. There are at least 15 reports of primary duodenal lymphoma of various B-cell and T-cell subtypes which presented with obstructive jaundice [207,208]. Three were drained percutaneously and four were treated by ERCP.

MBO from lymphoma usually results from extrinsic compression, although several cases of direct bile duct invasion have been reported[209]. Results of biliary drainage vary significantly across studies, due to the small sample size in each. Ross *et al*[197] reported technical success in 84% of biliary drainage by ERCP (and 100% of cases without concomitant gastric outlet obstruction due to lymphoma). They recommend plastic stents as strictures in patients presenting initially with MBO resolve with treatment before stent exchange is necessary, while those who develop MBO later in the disease progression do not survive until their first stent exchange is due. Stent-free status was achieved in about one-third of all patients. On the other hand, none of eight patients achieved stent-free status in another study[198]. A third study reported that MBO resolved in all seven patients presenting initially with MBO, regardless of stent placement[200]. Most studies agree that initial presentation with MBO has a much better prognosis than those who develop MBO later on. Those initially presenting with jaundice had significantly improved survival after biliary drainage (21 mo *vs* 5 mo)[197]. While surgery is an option when a preoperative diagnosis cannot be reached or the lesion appears resectable, chemotherapy and/or involved site radiation therapy is generally considered the standard of care[210].

NHL and Kaposi's sarcoma should also be included in the differential for biliary obstruction in acquired immunodeficiency syndrome (AIDS) patients, alongside AIDS-related cholangiopathy. Both show hepatic or splenic involvement in about 15% of AIDS patients[211]. AIDS-related NHL more commonly causes MBO by extrinsic compression from lymph nodes or liver involvement rather than from a primary biliary lesion[212]. Primary pancreatic lymphoma is more common in AIDS patients than in the general population, accounting for about 5% of extranodal NHL cases[211]. Hepatic or hilar involvement of Kaposi's sarcoma, which can occur earlier in the disease course of AIDS, is an even rarer cause of MBO[213].

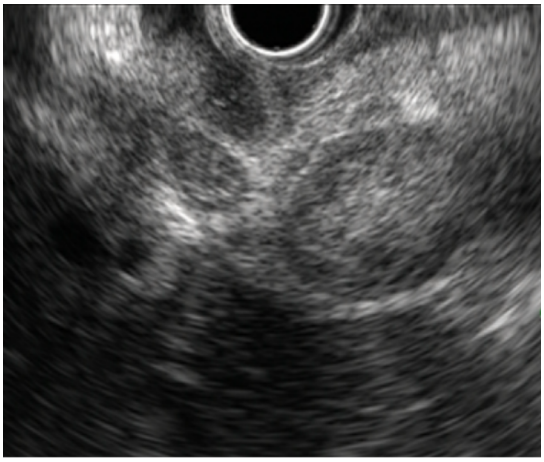
Leukemia

There are only several reports of leukemia presenting as obstructive jaundice[214-219]. The liver, bile duct, pancreas, or lymph nodes can be involved. Eight of 103 pancreatic metastases found in an autopsy study were cause by leukemia, suggesting that microscopic involvement may not be so rare[4]. Most reports in both adults and children involve acute lymphocytic leukemia. Obstructive jaundice was the presenting symptom in one case[215]. Care is required as obstructive lesions may not be visible on imaging[214]. While endoscopic and surgical treatment may be options to relieve biliary obstruction, aggressive chemotherapy is the standard of care.

Lymph node metastases

Hilar and peripancreatic lymph node metastases are a well-known cause of MBO. Such metastases have received little attention despite their frequency, perhaps because they are common occurrences in hepato-pancreato-biliary cancer. Hilar and distal biliary obstruction were caused by metastatic lymph nodes in 23% and 2%-17% (pooled average of 551 patients across eight studies: 11%) of cases treated mainly by ERCP stenting, respectively[220-228]. This figure may be higher for biliary obstruction in surgically altered anatomies; one report found six cases among 13 patients with surgically altered anatomies treated with metallic biliary stents (46%)[49].

In a study of biliary drainage mainly *via* ERCP, biliary obstruction due to hilar lymph node metastases resulted mainly from colon (46%), gastric (14%), and breast (14%) cancers[229]. Clinical success was achieved in 86% of cases but required a median of three procedures and percutaneous drainage in 20% of cases. The ability to resume chemotherapy was associated with improved survival in colon and breast cancer patients. A study of 65 patients with distal MBO due to lymph node metastases from gastric (31%), colorectal (18%), lung (11%), breast (8%), and other cancers found that covered metallic stents were had longer stent patency and less stent occlusion or tumor ingrowth than



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Figure 3 A 69-year-old woman presented with abdominal pain and jaundice 12 mo after surgery for high-grade serous ovarian cancer. Endoscopic ultrasound from the duodenal bulb revealed numerous metastatic lymph nodes obstructing the bile duct by extrinsic compression. Endoscopic biliary drainage was performed, but the patient died 1 mo later.

uncovered stents but had higher rates of acute pancreatitis[230]. Neither study noted any unique characteristics for any particular primary. Notably, there was only one case of lymph node metastasis from renal cell cancer, despite being the most common cause of metastases to the pancreas.

Another report found stent occlusion in 50% of lymph node metastasis cases *vs* 24% in primary biliary tract cancer[231]. While the authors of the study suggested a limited role for metallic stents in biliary obstruction caused by metastatic lymph nodes, no other studies demonstrated a significant difference from primary cancers. Currently, metallic stents are widely used for this purpose. Bilateral stenting in high-grade inoperable strictures leads to lower re-intervention rates without sacrificing technical success or increasing adverse events[232].

It should be noted that none of the studies reported pathological confirmation of lymph node metastases.

Thyroid

Thyroid cancer most commonly metastasizes to the lung and bone. Pancreatic metastases can occur in papillary, follicular, and medullary carcinoma[233]. In a review of 24 reported cases in English and Japanese, the average delay from thyroid cancer diagnosis to pancreatic metastasis is 7 years. Most were solitary lesions, with half arising in the pancreatic head. Biliary obstruction due to metastatic papillary thyroid cancer has been reported[234]. EUS-FNA is commonly used for diagnosis, and solitary metastases are generally resected with good results in selected cases[235].

Gynecological cancers

About 8% of ovarian cancer patients have metastases at diagnosis, but 22% ultimately develop metastases after a median interval of 44 mo after diagnosis[236]. There are 17 reported cases of pancreatic metastases[237]. As BRCA1 and BRCA2 mutations are common risk factors for both ovarian and pancreatic cancers, pancreatic lesions should be evaluated by EUS-FNA to rule out the possibility of double cancers[238]. While MBO generally results from metastatic lymph nodes (Figure 3), obstructive jaundice due to metastasis to the major papilla has been reported[239-241]. Distal pancreatectomy has been reported as an option for cytoreductive surgery in advanced ovarian cancer in a study of six patients with pancreatic metastases[242].

Less than ten cases of pancreatic metastasis from squamous cell carcinoma of the cervix have been reported[243]. On the other hand, biliary obstruction most often results from external bile duct compression, particularly in the porta hepatis[244]. Cases of periampullary and lymph node metastases requiring percutaneous drainage have been reported[120,245]. Endoscopic drainage has only been reported in one case to date[244].

There are only two reports of pancreatic metastasis of endometrial carcinoma, with no reported obstructive jaundice[246,247]. While endometrial carcinoma is generally discovered early, caution is required for the papillary serious and clear cell types due to their tendency for early metastasis[246].

Urological cancers

While there are no more than ten reports of prostate cancer metastasizing to the pancreas, an autopsy study found pancreatic metastases in 1.4% of cases[248-251]. Diagnosis with immunohistochemical staining for prostatic-specific antigen and successful ERCP have been reported[248].

Table 2 Relative frequency of pancreatic metastases by modality/procedure

Modality/procedure	Total cases	Renal	Lung	Breast	Stomach	Colon	Sarcoma	Melanoma	Ovary	Thyroid	Esophagus	Lymphoma	Other
CT	192	30%	26%	10%	7%	5%	6%	4%	3%	3%	0%	0%	7%
ERCP	307	1%	3%	4%	27%	38%	0%	2%	2%	0%	2%	1%	20%
EUS-FNA	515	40%	20%	6%	2%	9%	3%	10%	3%	1%	2%	0%	3%
Surgery	399	63%	3%	3%	3%	6%	7%	4%	5%	0%	0%	0%	6%
Autopsy	184	3%	28%	4%	22%	1%	2%	1%	2%	1%	0%	5%	31%
Weighted average[1]	1597	33%	14%	5%	10%	12%	4%	5%	3%	1%	1%	1%	11%

[1]Excludes duplicate data from the same institution during the same period using the same modality. Does not consider possible data duplication across studies of different modalities.
CT: Computed tomography; ERCP: Endoscopic retrograde cholangiopancreatography; EUS-FNA: Endoscopic ultrasound-guided fine-needle aspiration.

Pancreatic metastases were reported in four urothelial carcinoma cases, of which two presented with obstructive jaundice[252]. Lesions could generally be diagnosed by EUS-FNA, with the help of immunohistochemistry. Bile duct wall metastasis has been reported from the aggressive micropapillary variant of bladder carcinoma, which was successfully drained by ERCP[253].

Esophageal cancer

There are more than ten reports of esophageal squamous cell carcinoma metastasizing to the pancreas [54,254-259]. Such metastases can be synchronous or metachronous and may present as isolated metastases. Four cases underwent distal pancreatectomy among other treatments, with no short-term recurrence[255-258]. One case presented as a cystic lesion which was diagnosed by EUS-FNA[259]. Five caused MBO, while lesions of all single case reports were located in the pancreatic body or tail. There is one report of MBO from recurrent esophageal cancer after esophagectomy which was first treated by ERCP and subsequently retreated by EUS-guided hepatico-gastrictubestomy when duodenal invasion precluded repeat ERCP[260].

Pure primary squamous cell carcinoma is a rare but important differential diagnosis, of which at least 54 cases have been reported[261]. Slightly over half are located in the head and 55% have metastases at the time of diagnosis. MBO caused by metastatic squamous cell lung carcinoma has also been reported, requiring investigation for a lung primary[262].

Other primaries

There are isolated reports of pancreatic metastases from almost every malignancy, including meningioma, hemangiopericytoma, tonsillar squamous cell carcinoma, adenoid cystic carcinoma, hypopharyngeal carcinoma, thymoma, malignant pleural mesothelioma, pulmonary primitive neuroectodermal tumor, nephroblastoma, gastrointestinal stromal tumor, adrenocortical carcinoma, and testicular teratoma[263-275].

Table 3 Characteristics of malignant biliary obstruction caused by various primary malignancies

	Unique characteristics/diagnostic clues	Major causes of malignant biliary obstruction
Renal cell carcinoma	Enhancing lesion on imaging. Most pancreatic metastases resectable and associated with a good prognosis. Can arise up to 32 years after diagnosis of primary tumor	Pancreatic metastasis (biliary obstruction rare)
Lung cancer	Most reported in small cell lung cancer. Possible primary small cell biliary cancer	Pancreatic metastasis
Gastric cancer	Most cases present after surgery for Borrmann 3 antral lesions. Possible need for double stenting due to gastric outlet obstruction	Lymph nodes, liver metastasis, direct invasion
Colorectal cancer	Intraductal growth can mimic bile duct cancer	Liver metastasis, lymph nodes
Breast cancer	Most reported in invasive lobular carcinoma. Possible duodenal obstructionCan arise up to 32 years after diagnosis of primary tumor	Lymph nodes, pancreatic metastasis
Melanoma	Possible pancreato-biliary primary	Pancreatic metastasis
Lymphoma	More common in non-Hodgkin's lymphoma. Possible pancreato-biliary primary	Lymph nodes
Sarcoma	Possible pancreato-biliary primary	Pancreatic metastasis

CONCLUSION

Metastases from various primaries can cause MBO. The reported incidence pancreatic metastases vary across studies, depending on the selected modality (Table 2). While most can be diagnosed by EUS-FNA and treated by percutaneous or endoscopic drainage, factors specific to each primary should be kept in mind (Table 3). Surgery may be indicated in isolated metastases. There may be hope for long-term survival if systemic therapy can be resumed after biliary drainage or after margin-free resection of isolated metastases.

FOOTNOTES

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Country/Territory of origin: Japan

ORCID number: Takeshi Okamoto 0000-0001-9719-0282.

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REVIEW

An update on the diagnosis of gastroenteropancreatic neuroendocrine neoplasms

Jiayun M Fang, Jay Li, Jiaqi Shi

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Jiayun M Fang, Jiaqi Shi, Department of Pathology & Clinical Labs, University of Michigan, Ann Arbor, MI 48109, United States

Jay Li, Medical Scientist Training Program, University of Michigan, Ann Arbor, MI 48109, United States

Corresponding author: Jiaqi Shi, MD, PhD, Associate Professor, Department of Pathology & Clinical Labs, University of Michigan, 2800 Plymouth Rd. NCRC Bldg 35, Ann Arbor, MI 48109, United States. jiaqis@umich.edu

Abstract

Gastroenteropancreatic neuroendocrine neoplasms (GEP-NENs) arise from neuroendocrine cells found throughout the gastrointestinal tract and islet cells of the pancreas. The incidence and prevalence of GEP-NENs have been increasing each year due to higher awareness, improved diagnostic modalities, and increased incidental detection on cross-sectional imaging and endoscopy for cancer screening and other conditions and symptoms. GEP-NENs are a heterogeneous group of tumors and have a wide range in clinical presentation, histopathologic features, and molecular biology. Clinical presentation most commonly depends on whether the GEP-NEN secretes an active hormone. The World Health Organization recently updated the classification of GEP-NENs to introduce a distinction between high-grade neuroendocrine tumors and neuroendocrine carcinomas, which can be identified using histology and molecular studies and are more aggressive with a worse prognosis compared to high-grade neuroendocrine tumors. As our understanding of the biology of GEP-NENs has grown, new and improved diagnostic modalities can be developed and optimized. Here, we discuss clinical features and updates in diagnosis, including histopathological analysis, biomarkers, molecular techniques, and radiology of GEP-NENs. We review established diagnostic tests and discuss promising novel diagnostic tests that are currently in development or require further investigation and validation prior to broad utilization in patient care.

Key Words: Neuroendocrine tumor; Neuroendocrine carcinoma; Gastrointestinal; Pancreas; Pathology; Diagnosis

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Core Tip: Over the past decade, our understanding of the molecular biology underlying gastroenteropancreatic neuroendocrine neoplasms (GEP-NENs) has improved our available diagnostic tools. Although there have been many reviews on the diagnosis of GEP-NENs, the majority focus on one aspect of the diagnosis. This review provides a comprehensive discussion of the current and upcoming diagnostic tools available for GEP-NENs, including histopathology, new biomarkers, molecular techniques, and radiology.

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INTRODUCTION

Neuroendocrine neoplasms (NENs) account for 0.5% of all malignancies, and 62%-70% of these are found in the gastroenteropancreatic system (GEP-NENs)[1-3]. They arise from neuroendocrine cells, which are characterized by their ability to synthesize and secrete neuropeptides and hormones as well as the expression of neuroendocrine markers such as synaptophysin and chromogranin[2]. Neuroendocrine cells are most commonly found throughout the gastrointestinal tract, the islets of Langerhans of the pancreas, and in the lungs[2]. GEP-NENs are often slow-growing and indolent, so they can go undetected for years prior to diagnosis[4]. Though GEP-NENs were previously considered rare, the incidence has dramatically increased over the years as awareness of GEP-NENs grew and diagnostic modalities improved[1,4-7]. GEP-NENs are currently the second most prevalent gastrointestinal neoplasm, second only to colorectal adenocarcinoma[4]. Despite their indolent behavior, GEP-NENs can cause significant morbidity. Furthermore, their clinical presentation may mimic other classes of neoplasms, leading to inappropriate treatment and delays in appropriate therapy. Due to delays in diagnosis, metastases are present in 21% to 69% of patients at the time of diagnosis[6,7]. Therefore, it is imperative to come to an accurate diagnosis in a timely manner.

Oberndorfer first described GEP-NENs in 1907 as "Karzinoide" to describe benign-appearing tumors of the small intestine[2,6]. Now, the term "carcinoid" is no longer recommended as these neoplasms have been found to have the malignant potential[6]. The World Health Organization (WHO) classification of GEP-NENs has changed over the years. A major recent update is the division of NENs into neuroendocrine tumors (NETs) and neuroendocrine carcinomas (NECs). Previous editions classified GEP-NENs into grade 1 and grade 2 NETs, while grade 3 tumors were classified as NECs; however, molecular discoveries have aided in distinguishing grade 3 NETs from NECs and this distinction has been implemented as of the WHO 2019[8]. In addition to the changes in WHO classification, there are new developments in diagnosing GEP-NENs, including histopathology, biomarkers, and imaging. Here, we discuss updates on the diagnosis of GEP-NENs across these various modalities.

CLINICAL FEATURES

GEP-NENs can broadly be divided into functional and nonfunctional neoplasms. Though nonfunctional NENs can secrete calcitonin, chromogranins, ghrelin, neuron-specific enolase, or pancreatic polypeptide, they do not present with a hormone-related clinical syndrome[9]. By contrast, functional GEP-NENs secrete a hormone with an associated clinical syndrome caused by an excess of that hormone.

Nonfunctional GEP-NENs present with symptoms as primary tumor growth or metastases progress. For example, esophageal NENs are rare but present with dysphagia and vomiting due to physical obstruction[10]. Nonfunctional pancreatic NENs can present with symptoms of abdominal pain, early satiety, and obstructive jaundice[10,11]. Colorectal NENs present with hematochezia, change in bowel habits, abdominal pain, and anorectal symptoms[12-14].

With the increased usage of cross-sectional imaging and endoscopies to screen for cancer, many nonfunctional GEP-NENs are detected incidentally[15]. For example, gastric and colorectal NENs can be detected on upper and lower endoscopy, respectively[12,13,16,17]. Similarly, appendiceal NENs have been found incidentally in less than 1% of appendectomy specimens[14,18].

Functional GEP-NENs present with a clinical syndrome consistent with the hormone that they secrete. Due to the associated clinical syndrome, these often present earlier than nonfunctional GEP-NENs. Insulinomas are insulin-secreting tumors that present with symptoms of hypoglycemia such as palpitations, diaphoresis, and altered mental status[19]. Gastrinomas cause Zollinger-Ellison syndrome, in which excess gastrin leads to hypersecretion of gastric acid, resulting in severe peptic ulcer disease, gastroesophageal reflux disease, and chronic diarrhea[20]. Glucagonomas present with necrolytic

migratory erythema, diabetes mellitus, weight loss, and diarrhea[21-23]. VIPomas are characterized by autonomous secretion of VIP leading to watery diarrhea, hypokalemia, and achlorhydria syndrome[24, 25]. Secretion of serotonin and other active amines and peptides leads to carcinoid syndrome, which presents with episodic flushing, wheezing, and diarrhea[26,27].

WHO CLASSIFICATION UPDATE

The WHO classification of GEP-NENs has undergone numerous changes over the past couple of decades. The first WHO in 2000 described three categories: (1) Well-differentiated endocrine tumors (carcinoids) to describe neoplasms with low malignant potential and well-differentiated endocrine carcinomas for those with aggressive behavior and metastases; (2) Poorly-differentiated endocrine carcinomas; and (3) Mixed exocrine-endocrine tumors[28]. It was not until 2010 that the WHO updated the classification to "neuroendocrine" to describe the cell origin better and discouraged the use of "carcinoid"[28]. A critical change in 2010 was an introduction of a broader classification system for GEP-NENs that was not based on anatomic sites[8]. GEP-NENs were then categorized as grade 1 and grade 2 NETs, with grade 3 tumors being classified as NECs. This division was based on the Ki-67 proliferation index and mitotic count of the tumor (Table 1)[8,29].

Following the WHO 2010, it became apparent that there were two groups of grade 3 GEP-NENs with drastically different prognoses. One group was made up of neoplasms with a better prognosis that were proliferative but rarely displayed high-grade features (*e.g.*, nuclear pleomorphism and necrosis), while the other group included poorly differentiated NECs that were more aggressive with a poorer prognosis [29]. This distinction was best established in pancreatic NENs, and in 2017, the WHO introduced an additional classification distinguishing between well-differentiated grade 3 NETs and poorly-differentiated NECs in the pancreas[30]. The most recent WHO 2019 applied this distinction to all GEP-NENs (Table 1)[8].

MOLECULAR FINDINGS

Molecular discoveries have recently shown that NETs and NECs are distinct entities with different molecular profiles[31-33]. This has been best described in pancreatic NENs. Whole exome sequencing of pancreatic NETs led to the discovery that most pancreatic NETs are associated with multiple endocrine neoplasia type 1 (MEN1) inactivation (44%) and death domain-associated protein (DAXX)/alpha-thalassemia/mental retardation X-linked (ATRX) gene mutations (43%)[31]. Less commonly, alterations in the mTOR pathway (15%) have also been identified including in PTEN, TSC2, and PIK3CA[30,31].

These genetic alterations have been essential in distinguishing high-grade NETs from NECs. Up to 43% of high-grade pancreatic NETs demonstrate mutated *DAXX/ATRX*, whereas pancreatic NECs are not known to have this mutation[30-32]. Instead, up to 92% of pancreatic NECs have *TP53* or *RB1* mutations[30,32,34]. Other less commonly seen mutations in NECs include mutations in *KRAS*, *SMAD4*, *CDKN2A/p16*, and *BCL2*[8,30,32].

For non-pancreatic gastrointestinal NENs, genomic studies are still emerging but suggest that NECs similarly harbor *TP53* and *RB1* mutations[8]. On the other hand, genomic sequencing identified a very low mutation rate in extrapancreatic NETs, and no recurrent mutation has been identified[8,35]. Although no specific mutation has been validated in small intestinal NENs, analysis of chromosomal changes showed that approximately 50% of cases had a loss of chromosome 18 and 10%-30% of cases had a gain of chromosomes 4, 5, 7, 14, or 20[33]. Overall, due to the heterogeneity of gastrointestinal NETs, identifying recurrent mutations in extrapancreatic NETs has been challenging[8,33].

In addition to molecular classification, genome sequencing has been utilized to identify risk factors of genetic susceptibility to developing sporadic GEP-NENs. Autosomal dominantly inherited genetic syndromes account for a minority of GEP-NENs and include multiple endocrine neoplasia types 1 and 2, Von Hippel-Lindau syndrome, tuberous sclerosis, and neurofibromatosis type 1[36]. A 2011 study by Ter-Minassian *et al*[37] evaluated single-nucleotide polymorphisms (SNPs) in patients with sporadic NETs, including small bowel and pancreas primaries. They identified 2 SNPs that were associated with increased overall risk of NET, IL12A rs2243123, and DAD1 rs8005354, suggesting that inflammatory and apoptosis pathways play a role in tumorigenesis of NENs[37]. However, a larger follow-up genomic study in 2016 was not able to confirm these associations. Instead, Du *et al*[36] found a potential risk locus on 12q23 that may be associated with developing small bowel NENs. This locus is in proximity to ELK3, which is implicated in angiogenesis. A 2018 study by Obazee *et al*[38] analyzed susceptibility loci associated with pancreatic ductal adenocarcinoma for possible overlap association with pancreatic NENs and found that rs9543325, rs10919791, and rs1561927 may increase the risk of developing pancreatic NENs. Genome-wide association studies for GEP-NENs have been limited by sample size, but further studies along these lines may yield a greater understanding of the molecular pathways underlying the pathogenesis of GEP-NENs and potentially facilitate the identification of therapeutic targets.

Table 1 World Health Organization classification of gastroenteropancreatic neuroendocrine neoplasms

	Grade 1 NET		Grade 2 NET		Grade 3 NET		Grade 3 NEC	
	Mitoses/10 hpf	Ki-67	Mitoses/10 hpf	Ki-67	Mitoses/10 hpf	Ki-67	Mitoses/10 hpf	Ki-67
2010	< 2	< 3%	2-20	3%-20%	-	-	> 20	> 20%
2017[1]	< 2	< 3%	2-20	3%-20%	> 20	> 20%	> 20	> 20%
2019	< 2	< 3%	2-20	3%-20%	> 20	> 20%	> 20	> 20%

[1]Pancreas only.
NET: Neuroendocrine tumor; NEC: Neuroendocrine carcinoma; hpf: High power field.

HISTOPATHOLOGIC DIAGNOSIS

Histologic analysis of the tumor is necessary to establish the diagnosis of a GEP-NEN. The WHO 2019 classification divided GEP-NENs into NETs, grades 1 through 3, and NECs (Table 1). Low-grade NETs (grades 1 and 2) classically show an organoid architecture, but various patterns may be appreciated, including trabecular, glandular, tubuloacinar, and solid (Figure 1). Tumor cells are monotonous with round nuclei and finely granular cytoplasm. The chromatin is finely stippled and classically referred to as "salt and pepper." High-grade NETs (grade 3) have many overlapping morphologic features with low-grade NETs, with the key difference being that they show higher mitotic activity and higher proliferation indices (Ki-67). Rarely, high-grade NETs may show marked nuclear pleomorphism, diffuse infiltrative patterns, and necrosis. Such features can make it difficult to distinguish from NECs, which is why ancillary studies may be needed in NENs with high-grade features.

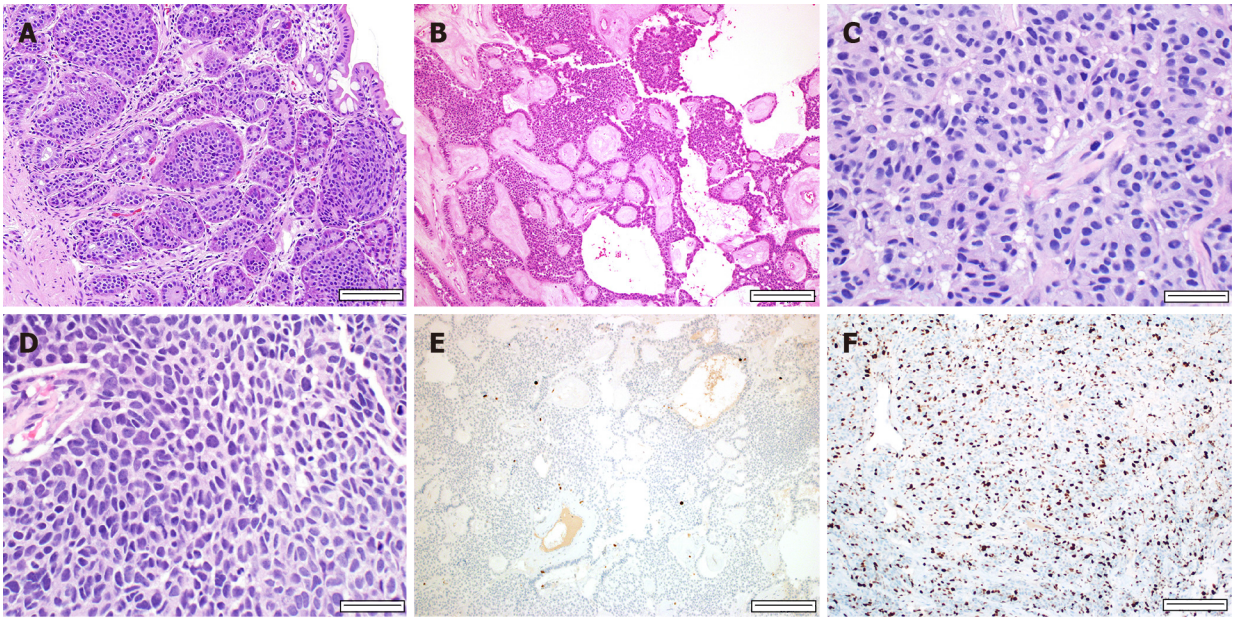
By contrast, NECs are poorly differentiated with significant atypia and frequently have geographic necrosis. They can be further subclassified into small cell NEC and large cell NEC. The small cell variant typically grows in a solid, diffuse, sheet-like pattern, and the tumor cells have scant cytoplasm and show nuclear molding with hyperchromatic nuclei and inconspicuous nucleoli. The large cell variant has moderate to abundant cytoplasm, vesicular nuclei, and prominent nucleoli. The classic "salt and pepper" chromatin is not appreciated.

Immunohistochemical stains for neuroendocrine differentiation are used to confirm the diagnosis. The most common markers are chromogranin and synaptophysin, with the former being more specific for neuroendocrine differentiation and the latter more sensitive. Other neuroendocrine markers include neuron-specific enolase and CD56. Insulinoma-associated protein 1 (INSM1) is a recently identified marker proposed to have high specificity for neuroendocrine differentiation[34,39]. In more poorly differentiated NENs, multiple markers may be needed to confirm the neuroendocrine etiology. Approximately 25% of NECs may lack chromogranin and synaptophysin expression, attributed to the decreased number of dense-core granules[30,34].

Tumor grading is dependent on mitotic activity and Ki-67. In situations where the Ki-67 index and mitotic index are discrepant, the higher grade is assigned, as studies have shown tumors tend to behave more like those of the higher grade[29,40]. Current recommendations include counting Ki-67 in at least 500 cells in "hot spots," areas with increased activity, and counting 50 high power fields for mitoses. "Eyeballing" the mitotic count and Ki-67 is discouraged due to the lack of reproducibility, and it is preferred to count on printed images of the "hot spots" manually. Automated systems for counting have been explored but are currently limited by high costs.

Distinguishing between high-grade NETs from NECs by morphology can be challenging. The molecular differences between NETs and NECs can assist in these situations, especially in pancreatic NENs[7,8,29,30,40]. As previously described, *DAXX/ATRX* mutations can be detected in approximately 43% of pancreatic NETs, including high-grade NETs. These mutations can be detected by loss of protein expression by DAXX and ATRX immunohistochemistry[32]. As for NECs, immunohistochemical stains for p53 and RB1 may be used, either showing aberrant p53 expression (diffuse positivity or null) or absent RB1 staining[32]. A subset of NECs also shows loss of p16 expression, which is not appreciated in NETs[33]. Overexpression of BCL2 has also been reported, especially in the small cell variant (up to 100%); however, approximately 18% of NETs may also demonstrate this[32]. Although most cases of NETs can be morphologically differentiated from NECs; immunohistochemistry is available when the morphology is not definitive.

Despite the advancements made in histopathology, it still has limitations[41]. Biopsies are invasive, prone to sampling error, and can only provide a snapshot of a single time point in the course of the disease. It is not capable of providing a real-time evaluation of disease progression, recurrence, or therapy response. Other diagnostic modalities such as anatomical and functional imaging and clinical symptoms and biomarkers are needed in the surveillance of disease[42].



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Figure 1 Morphologic features of low-grade well-differentiated neuroendocrine tumors and poorly differentiated neuroendocrine carcinoma. A-D: Gastroenteropancreatic neuroendocrine neoplasms have a variety of architectural patterns (hematoxylin and eosin staining); A and B: Low-grade well-differentiated neuroendocrine tumors (NETs) typically have monotonous cells with round nuclei and "salt and pepper" chromatin; C: High-grade well-differentiated NETs tend to have more nuclear pleomorphism with readily identifiable mitoses; D: Small cell carcinoma, a variant of neuroendocrine carcinoma, has significant atypia with nuclear molding and scant cytoplasm. Mitoses are also readily identified; E and F: In addition to mitotic count, the Ki-67 proliferation index is necessary for grading. Low-grade well-differentiated NETs have a low proliferation index, < 20% on Ki-67 immunohistochemical (IHC) stain (E), while high-grade well-differentiated NETs have a high proliferation index, > 20% on Ki-67 IHC stain (F). Scale bars: 100 μm (A), 200 μm (B, E and F), 50 μm (C and D).

BIOMARKERS

Due to the invasiveness of biopsy and limitations of histopathology, there is demand for non-invasive, reproducible biomarkers that can provide not only a diagnosis but also longitudinal data on prognosis, disease evolution, therapy response, and disease recurrence[41]. This has been challenging, and currently, there are no widely available biomarkers that can act as a standalone indicator[43]. However, with the emergence of multianalyte analysis, the field of biomarkers for GEP-NENs is expanding.

Monoanalytes

Monoanalytes are measured in plasma by enzyme-linked immunosorbent assays (ELISA). The primary targets were identified based on secretory products and include chromogranin A (CgA), pancreastatin, neuron-specific enolase, and neurokinin A[7,41,44]. While these biomarkers were initially regarded with much praise and are currently the only widely utilized biomarkers, they have limited sensitivity, specificity, and reproducibility[41,42,44,45]. Furthermore, they cannot identify early disease progression [41]. Overall, the greatest challenge is that a single analyte is incapable of providing information on the tumor molecular biology, such as cell proliferation and growth factor signaling[41,42,44].

Chromogranin A is a glycoprotein found in neuroendocrine cells and was first introduced as a biomarker over 3 decades ago[46,47]. It is currently the most commonly used biomarker for GEP-NENs [7]. It has a 10%-35% specificity, and its sensitivity ranges from 32% to 92%[44,48]. False elevations are common, especially in patients on proton pump inhibitors and those with chronic atrophic gastritis, renal insufficiency, arterial hypertension, and adenocarcinoma[6,7,42,48]. It is not recommended to use CgA as a screening tool, and it has greater utility in monitoring response to therapy and surveillance after a diagnosis has been made[7,44,49]. Studies originally showed that CgA correlated with tumor size and prognosis, though this is now considered controversial[41].

Another traditional biomarker for GEP-NENs, especially in patients with carcinoid syndrome, is 5-hydroxyindoleacetic acid (5-HIAA), a product of serotonin metabolism[50,51]. Although it may be measured in the plasma, it is more commonly measured in the urine. It is helpful in serotonin-secreting tumors, which account for only 15%-20% of GEP-NENs[41]. Elevation of urinary 5-HIAA has a sensitivity of 70% and a specificity of 90%-100% for NENs in the presence of carcinoid syndrome[41,52]. However, the sensitivity of this biomarker is as low as 35% in the absence of carcinoid syndrome[41,53,54]. Similar to other monoanalyte biomarkers, urinary 5-HIAA levels can be falsely elevated in many scenarios, including recent consumption of foods rich in serotonin, tryptophan, and dopamine, as well as malabsorptive diseases like celiac sprue and Whipple disease[51,53]. A study of 371 patients with

NENs showed that urine 5-HIAA level was not a useful prognosticator for overall survival[55]. Other specific hormone markers such as insulin, gastrin, glucagon, VIP, somatostatin, ACTH, and calcitonin are also available; however, these collectively only apply to < 2% of GEP-NENs[7,41].

In 2008, Leja *et al*[56] analyzed serotonin-producing metastatic small intestinal NENs and identified six possible novel marker genes, including paraneoplastic antigen Ma2. Cui *et al*[57] used ELISA to detect Ma2 autoantibodies in 124 patients with small intestinal NENs at different stages of the disease and showed a sensitivity that ranged from 46%-50% and a specificity of 98% compared to the healthy patients. Their findings suggested that Ma2 may be a better biomarker than CgA.

Angiogenic factors have also been suggested as potential biomarkers as GEP-NENs are highly vascularized tumors[58]. Angiopoietin-2 (Ang-2) and the receptor tyrosine kinase Tie-2 have gained the most attention as potential biomarkers out of the angiogenic factors[59]. Ang-2 binds to its receptor, Tie-2, promoting endothelial cell survival and influencing vascular remodeling[60]. In 2009, Srirajaskanthan *et al*[61] and Detjen *et al*[59] found that Ang-2 serum levels were increased in patients with NENs compared to healthy patients. In contrast, Melen-Mucha *et al*[58] compared multiple angiogenic factors, including Ang-2 and Tie-2, and CgA serum levels in patients with NENs to those without and found that only Tie-2 and CgA were elevated in patients with NENs compared to controls. However, they found that Ang-2 was increased in the subgroup of patients with metastatic disease compared to those with localized disease. Figueroa-Vega *et al*[60] also found that Ang-2 and Tie-2 Levels were elevated in patients with metastatic disease. Another key angiogenic factor is the vascular endothelial growth factor, which is largely studied for its prognostic role as a possible therapeutic target instead of diagnosing GEP-NENs[62-65]. Overall, unlike the current monoanalytes that rely on secretory products, angiogenic factors reflect the tumorigenesis of NENs and represent a potential future category of biomarkers. Isidori *et al*[66] have an ongoing clinical trial (NCT04464122) to evaluate how Tie-2 and other angiogenic factors change in GEP-NENs after treatment.

Multianalytes

Due to the limitations of monoanalyte analyses, multianalyte approaches have been studied over the last decade to improve the accuracy of biomarkers and correlate with tissue expression[41,44]. A panel of analytes, instead of a single biomarker as described above, is measured and interpreted to provide a more comprehensive picture of a tumor's biology[41,42,44]. For example, disease-specific analytes can be evaluated alongside markers associated with cell proliferation to provide a diagnosis and predict tumor behavior[44].

Neuroendocrine gene transcript assay (NETest) is the first neuroendocrine tumor liquid biopsy, using polymerase chain reaction (PCR) to detect 51 transcriptomic signatures of NENs[35,44,67-69]. These genes were identified to have significant differences in expression in patients with GEP-NENs and bronchopulmonary NENs *vs* those without NENs[41,70]. After measuring RNA expression in whole blood, an algorithm calculates a risk score that ranges from 0% to 100%[45]. Current cutoffs are < 20% normal, 21% to 40% stable/low risk disease, and 41%-100% progressive/high risk disease[41,45]. These cutoffs will likely be refined as more studies are performed. However, even with the current parameters, NETest has shown promising results with high sensitivity and specificity (> 95% and > 90%, respectively)[69].

In 2014, Modlin *et al*[44] compared NETest with the monoanlytes CgA, pancreastatin, and neurokinin A in 40 patients with grade 1 and grade 2 GEP-NETs and found that NETest was superior to the monoanalytes in sensitivity and specificity. The authors concluded that NETest could facilitate early detection of disease recurrence and predict therapy response[44]. In 2017, Pavel *et al*[42] followed patients with GEP-NENs for a median of 4 years to compare NETest and CgA and found that NETest more accurately correlated with the clinical status and was able to identify those with progressive disease approximately one year before being detectable by imaging. In 2016, Modlin *et al*[69,71] also found that the NETest risk score fell after tumor debulking, suggesting it can be used to identify residual disease after surgery[41]. A current clinical trial (NCT03012789) by Wren Laboratories investigates whether NETest accurately correlates with surgical excision, identifies residual tumor, and predicts early disease relapse[72].

Although NETest is highly sensitive and has shown value in disease monitoring, studies on its use as a screening tool are less promising. Van Treijen *et al*[70] compared patients with diagnosed GEP-NENs to healthy patients and found that NETest was less specific than CgA (56%-72% *vs* 83%, respectively). Al-Toubah *et al*[45] compared patients with metastatic GEP-NENs and bronchopulmonary NENs with a mixed group of healthy patients and patients with metastatic non-NEN gastrointestinal malignancies. Unlike the previous study, they found that NETest successfully ruled out 100% of healthy patients, but specificity was only 67% when compared to patients with non-NEN gastrointestinal malignancies[45]. This is likely because NETest includes genes whose expression is associated with proliferation and metabolism which may be upregulated in non-NEN malignancies as well as nonspecific environments of stress and inflammation[70]. Additionally, studies have shown that NETest does not correlate with tumor grade[70].

Overall, the sensitivity of NETest far exceeds that of other currently used biomarkers for GEP-NENs, while specificity has varied depending on the cohort[41,70]. Importantly, unlike current monoanalyte biomarkers, NETest is not affected by proton pump inhibitor use and diet[41]. Although it shows

promise as a valuable biomarker for GEP-NENs, further studies are still warranted in non-gastrointestinal NENs such as paragangliomas and malignancies with mixed epithelial or neuroendocrine phenotype, such as prostate cancer[43]. Wren Laboratories is conducting a clinical trial (NCT02270567) on patients with confirmed diagnoses of NENs to have a better overall understanding of NETest in clinical practice[73]. Another clinical trial (NCT02948946) by H. Lee Moffitt Cancer Center and Research Institute to evaluate the NETest sensitivity and specificity in GEP-NENs and lung NENs recently concluded[74]. Currently, studies show that NETest has great promise in identifying early disease progression, assessing therapy response, and evaluating if the surgical tumor resection is complete[43]. NETest is currently available at select accredited laboratories in the United States and Europe[75].

In addition to NETest, other multianalyte biomarkers are also being explored. MicroRNAs (miRNAs) are small non-coding RNAs that regulate gene expression at the post-transcriptional level and act as oncogenes or tumor suppressors[76]. They can be detected by PCR and have been investigated as a potential serum target. More than 100 miRNAs are differentially expressed in NENs[48,76]. About 10% of these are nonspecific in terms of tumor location, such as miRNA-375 and miRNA-7, while the remaining 90% appear to be specific to the anatomic site[76]. For GEP-NENs, potential biomarker targets have been identified in the small intestine (miRNA-7-75-p, miRNA-182, miRNA-183, and miRNA-96-5p), stomach (miRNA-375, miRNA-7, miR-96-5p, and miRNA-222), and pancreas (miRNA-193b, miRNA-144/451, miRNA-21, miR-1290, miRNA-103, miRNA-107, miRNA-155, miRNA-204, miR-328, miRNA-642, miRNA-3653, miRNA-23b, miRNA-137, miRNA-196a, and miRNA449a)[76,77]. Clinical applications of miRNAs have been challenging, and currently, there is no standardization of the process[41,78]. Existing studies have small sample sizes and inconsistent methodologies, making it difficult to draw conclusions[41,69].

Another area of development in multianalyte analyses is next-generation sequencing (NGS) and other genetic analyses. These include studies on samples consisting of primary tumor tissue and circulating tumor DNA (ctDNA), the fraction of cell-free DNA that is released by dying tumor cells and can be detected in plasma for analysis[79,80]. Recent studies have been guided by the current understanding of genes implicated in GEP-NENs, including *MEN1*, *DAXX*, *ATRX*, and mTOR pathway genes[31]. A 2017 study by Gleeson *et al*[81] used a 15 gene NGS panel to determine if any genes involved in commonly implicated pathways could be used as prognosticators in patients with pancreatic NENs. Only variants in *TSC2*, *KRAS*, and *TP53* were identified to have prognostic significance, and each of these variants was present in fewer than 10% of samples examined[81]. Further, 40% of tumors assessed were wild-type for all 15 genes assessed, and this set of patients did not demonstrate meaningful differences in tumor or clinical characteristics[81]. A 2018 study analyzed ctDNA in pancreatic NENs in 10 patients and showed a correlation with genetic characteristics of ctDNA and tumor tissue, suggesting a role for less invasive liquid biopsies in pancreatic NEN diagnosis and monitoring[82]. Zakka *et al*[83] performed a larger study in 2020 that further demonstrated the feasibility of NGS analysis of ctDNA in 320 patients with NENs, including those outside the gastrointestinal system. While their gene panel did not include implicated genes like *MEN1*, *ATRX*, or *DAXX*, and they lacked data for clinical or histopathological correlation in many patients, the study further reinforced the promise and necessity of future studies on liquid biopsies, ctDNA, and NGS[83]. Another 2021 study on NENs of various origins identified actionable mutations in over 50% of patients using NGS on liquid biopsies and formalin-fixed paraffin-embedded tissue[84]. Studies to date on GEP-NEN ctDNA analysis through NGS and other methods are promising and offer proof-of-concept of feasibility, clinical applicability, and potential prognostication of disease progression and survival. However, larger-scale prospective studies correlating genetic, histopathologic, and clinical data are needed before the widespread use of these tests. Additionally, an increased understanding of molecular pathways underlying the development and progression of GEP-NENs will refine genetic tests analyzing ctDNA and tumor tissue.

Multianalyte analysis of neuroendocrine metabolites has also been explored as a diagnostic strategy for GEP-NENs. A 2021 study from Jiménez *et al*[85] used nuclear magnetic resonance to compare urine samples from patients with GEP-NENs and healthy controls and generated a model that could accurately discriminate between the two groups. The study suggests that nuclear magnetic resonance could be a useful clinical tool for diagnosing GEP-NENs[85]. They identified several metabolites that were either increased or decreased in GEP-NENs, including kynurenine, hippurate, and phenylacetylglutamine[85]. These novel biomarkers represent areas of future study and suggest that a multianalyte test involving multiple metabolites that are altered in GEP-NENs could be more effective than monoanalyte tests.

IMAGING MODALITIES

Anatomic imaging

Conventional cross-sectional imaging such as computed tomography (CT) and magnetic resonance imaging (MRI) are critical diagnostic tools in localizing, characterizing, and staging GEP-NENs[9,86]. MRI is used less commonly than CT due to increased cost, acquisition time, and potential for motion

artifact[87]. Multiphasic CT with intravenous contrast is essential to increase diagnostic yield[87]. NENs are generally hypervascular and show enhancement in the late arterial phase, and NEN metastases are also hypervascular and best visualized in the arterial phase[87]. For detection of metastases to the liver, MRI is more sensitive than CT[88,89]. A 2003 study of different MRI techniques suggested that hepatic arterial phase and fast spin-echo T2 weighted images were most sensitive for hepatic metastases of NENs, further emphasizing the importance of multiphase imaging[89].

Recent advances in conventional cross-sectional imaging of GEP-NENs include studies assessing hepatic metastases of GEP-NENs with contrast-enhanced MRI utilizing hepatocellular phase-contrast agents[90]. The most studied agent is gadoxetate disodium, a gadolinium-based contrast with hepatobiliary excretion[91]. A 2018 study from Tirumani *et al*[92] compared the ability of 6 MRI phases after gadoxetate disodium injection to assess hepatic metastases of GEP-NENs and found that the hepatocellular phase was superior to all other phases examined. Another study demonstrated that combining diffusion-weighted and hepatobiliary phases of gadoxetate disodium-enhanced MRI in patients with suspected neuroendocrine liver metastases had the best diagnostic yield, with a sensitivity of 86% and a specificity of 94%, compared to other combinations of contrast-enhanced phases[93]. These studies highlight an essential role for contrast-enhanced MRI with gadoxetate disodium or other liver-targeted contrast agents in the assessment of GEP-NENs with potential liver metastases and long-term surveillance of disease with known liver involvement.

Functional imaging

Somatostatin receptor scintigraphy (SRS) has been used since the 1990s to assess GEP-NENs. Studies from 1995 and 1996 showed that ^{111}In-pentetreotide, a radiolabeled somatostatin analog, could safely and effectively detect GEP-NENs more effectively than conventional imaging[94,95]. A 2001 study examined ^{68}Ga-DOTATOC compared to older SRS techniques and found a higher diagnostic yield by 30%[96]. More tracers for GEP-NENs have been developed over the years, including Ga-DOTANOC[68] and Ga-DOTATATE[97-99]. Two 2016 studies compared ^{111}in-pentetreotide and ^{68}Ga-DOTATATE imaging for identification of primary tumor and metastatic lesions of NENs[98,100]. Both studies showed that ^{68}Ga-DOTATATE had identified more lesions than ^{111}In-pentetreotide, and altered management in 33%-36% of patients with GEP-NENs[98,100]. An established principle of functional imaging for GEP-NENs is the distinction in imaging characteristics between low-grade NENs *vs* high-grade NENs and NECs. Low-grade NENs express high levels of somatostatin receptors and are less metabolically active, and thus, ^{68}Ga-DOTATATE and other somatostatin analogs are superior to ^{18}F-fluorodeoxyglucose (^{18}F-FDG) as a tracer for functional imaging of grade 1 and 2 NENs[87,101,102]. High-grade NENs and NECs tend to have higher rates of glucose metabolism and lower expression of somatostatin receptors. As a result, ^{18}F-FDG is superior to somatostatin analogs for functional imaging of high-grade NENs[103,104].

Recent studies have made advances in comparing head-to-head imaging modalities and tracers to determine which are optimal for functional imaging of GEP-NENs. A 2017 study compared positron emission tomography (PET)/CT and PET/MRI using ^{68}Ga-DOTATOC and found that both imaging modalities performed comparably in identifying abdominal primary tumors and yield of lymph node metastases[105,106]. Sawicki *et al*[105] found that PET/CT performed better in identifying bone lesions, but PET/MRI with ^{68}Ga-DOTATOC outperformed PET/CT in identifying hepatic lesions. However, the further identification of metastases did not alter the management of patients in this study, as most already had advanced stages of the disease[105]. Given the prevalence of liver metastases in GEP-NENs, these data suggest that there may be a valuable role for PET/MRI over or in conjunction with PET/CT in the diagnosis and staging of GEP-NENs[107]. A similar 2021 study of 11 patients with GEP-NENs prospectively compared PET/MRI with ^{68}Ga-DOTATOC and PET/CT with ^{68}Ga-DOTATOC[88]. For detection of all lesions, PET/MRI with ^{68}Ga-DOTATOC outperformed PET/CT with ^{68}Ga-DOTATOC [88]. Consistent with the 2017 study, ^{68}Ga-DOTATOC PET/MRI was superior to ^{68}Ga-DOTATOC PET/CT in detecting liver metastases[88]. These studies suggest that PET/MRI with ^{68}Ga-DOTATOC may be superior to PET/CT in guiding the management of GEP-NENs.

Other recent studies have examined specific clinical scenarios when functional imaging is likely to influence the clinical management of patients with GEP-NENs. A 2017 study of 40 patients with metastatic NENs who had undergone CT or MRI but still had an unknown primary tumor location showed that ^{68}Ga-DOTATOC PET/CT could effectively localize the primary tumor to facilitate treatment[108]. A meta-analysis of studies on ^{68}Ga-DOTATOC PET/CT identified that this imaging modality changed management when the patient had a known NEN around half of the time[109]. By contrast, in patients with symptoms consistent with a NEN and elevated biomarkers but no proven NEN, there was only a 13% yield[109]. Notably, four studies included in this meta-analysis showed a 44% yield for detection of primary tumor site in patients with metastatic disease[109].

Finally, advances in the application of automation and artificial intelligence could improve diagnostic consistency and accuracy of functional imaging for GEP-NENs. This area has been more extensively studied for ^{18}F-FDG PET/CT[110,111]. A retrospective 2021 study retrospectively demonstrated that deep learning could facilitate the automation of detection of hepatic metastases, though future studies with larger sample sizes are required for further validation[112]. Continued optimization of imaging techniques and development of more selective tracers will continue to improve diagnostic yield and

ability of functional imaging to guide the management of GEP-NENs effectively.

Ultrasound

Endoscopic ultrasound (EUS) has been used for decades to assess gastrointestinal tract tumors, including GEP-NENs[113,114]. This technique combines endoscopy with ultrasound to image structures and can diagnose, stage, and sample malignancies[115]. EUS is especially useful for gastric, duodenal, pancreatic, and rectal NENs[116,117]. EUS is more sensitive than other modalities such as CT or MRI for pancreatic NENs and is the most sensitive method for detection of rectal NENs[118-122]. It provides additional information on the depth of invasion and can assess local lymph node involvement. Further advantages of EUS include the ability to perform a fine needle aspiration (FNA) to obtain tissue for cytologic and molecular analysis and to place a radiofrequency ablation probe for poor surgical candidates[114,116,123]. Cytologic analysis of pancreatic NENs facilitated by EUS-FNA including Ki-67 index correlates with tumor grade confirmed after resection, influences management, and predicts survival, especially when sampling is sufficient[124,125]. Disadvantages include operator dependence and limited assessment beyond the local area compared to broader imaging techniques[116,117].

Recent updates on EUS for the diagnosis of GEP-NENs include technical advances in ultrasonography and new histologic and molecular analyses of tissue obtained using EUS. A recent 2021 study compared the diagnostic accuracy of EUS-FNA and EUS with fine needle biopsy (EUS-FNB) and found that EUS-FNB may be superior to EUS-FNA for pancreatic NENs, including a better correlation of Ki-67 proliferation index between EUS-FNB and the surgical specimen[126]. However, there was no difference in the accuracy of grade estimation between EUS-FNB and EUS-FNA[118].

Immunocytochemical analysis can be performed on EUS-FNA samples for confirmation of the diagnosis of pancreatic NEN. A recent study immunocytochemically analyzed INSM1 expression in 14 EUS-FNA samples of pancreatic NENs and 15 pancreatic ductal adenocarcinomas (PDACs)[127]. All pancreatic NENs contained cells expressing INSM1, and INSM1 was expressed at a higher rate in pancreatic NEN samples than PDAC samples[127]. Advances in molecular analysis of EUS-FNA samples can also improve diagnostic accuracy for pancreatic lesions. A 2020 study used digital droplet PCR to detect *KRAS* mutations in EUS-FNA samples from PDAC, pancreatic NENs, and chronic pancreatitis. Combining molecular and cytologic analyses improved diagnostic accuracy from 74% with cytology alone to 91%[128]. Further technical advancement and refinement of molecular and cytologic analyses will continue to improve the efficacy of EUS and EUS-FNA.

CONCLUSION

There are many new developments in the pathologic, molecular, and imaging diagnosis of GEP-NENs. The WHO classification of GEP-NENs has changed over the years, with the most recent significant update being the distinction between high-grade NETs and NECs. Due to the heterogeneity of GEP-NENs, a multimodal approach to diagnosis and disease surveillance is necessary. A better understanding of the molecular biology of GEP-NENs has allowed for the distinction between high-grade NETs and NECs, the introduction of exciting new biomarker tests such as the NETest, and continued advances toward eventual validation and implementation of other multianalyte tests assessing biomarkers such as miRNA and ctDNA. Recent advances in imaging include the validation of improved PET tracers and determination of which imaging modalities are optimal for anatomic and functional imaging of primary GEP-NENs and metastases, especially to the liver. Updates to EUS and EUS-FNA include technological advances and improved molecular and cytological analysis of tissue obtained using EUS.

FOOTNOTES

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Country/Territory of origin: United States

ORCID number: Jiayun M Fang 0000-0001-9517-297X; Jay Li 0000-0002-8146-4450; Jiaqi Shi 0000-0003-4893-1587.

Corresponding Author's Membership in Professional Societies: United States and Canadian Academy of Pathology, 28212; American Association for Cancer Research (AACR), 76093; Pancreatobiliary Pathology Society; Rodger C. Haggitt Gastrointestinal Pathology Society.

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Basic Study

Loss of LAT1 sex-dependently delays recovery after caerulein-induced acute pancreatitis

Cristina M Hagen, Eva Roth, Theresia Reding Graf, François Verrey, Rolf Graf, Anurag Gupta, Giovanni Pellegrini, Nadège Poncet, Simone Mafalda Rodrigues Camargo

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Cristina M Hagen, Eva Roth, François Verrey, Nadège Poncet, Simone Mafalda Rodrigues Camargo, Institute of Physiology, University of Zurich, Zurich 8057, ZH, Switzerland

Theresia Reding Graf, Rolf Graf, Anurag Gupta, Swiss Hepato-Pancreato-Biliary Center, Department of Visceral and Transplantation Surgery, Zurich University Hospital, Zurich 8091, ZH, Switzerland

Giovanni Pellegrini, Institute of Veterinary Pathology, University of Zurich, Zurich 8057, ZH, Switzerland

Corresponding author: Simone Mafalda Rodrigues Camargo, PhD, Pharmacist, Senior Scientist, Institute of Physiology, University of Zurich, Winterthurerstrasse 190, Zurich 8057, ZH, Switzerland. simone.camargo@physiol.uzh.ch

Abstract

BACKGROUND

The expression of amino acid transporters is known to vary during acute pancreatitis (AP) except for LAT1 (*slc7a5*), the expression of which remains stable. LAT1 supports cell growth by importing leucine and thereby stimulates mammalian target of rapamycin (mTOR) activity, a phenomenon often observed in cancer cells. The mechanisms by which LAT1 influences physiological and pathophysiological processes and affects disease progression in the pancreas are not yet known.

AIM

To evaluate the role of LAT1 in the development of and recovery from AP.

METHODS

AP was induced with caerulein (cae) injections in female and male mice expressing LAT1 or after its knockout (LAT1 Cre/LoxP). The development of the initial AP injury and its recovery were followed for seven days after cae injections by daily measuring body weight, assessing microscopical tissue architecture, mRNA and protein expression, protein synthesis, and enzyme activity levels, as well as by testing the recruitment of immune cells by FACS and ELISA.

RESULTS

The initial injury, evaluated by measurements of plasma amylase, lipase, and

trypsin activity, as well as the gene expression of dedifferentiation markers, did not differ between the groups. However, early metabolic adaptations that support regeneration at later stages were blunted in LAT1 knockout mice. Especially in females, we observed less mTOR reactivation and dysfunctional autophagy. The later regeneration phase was clearly delayed in female LAT1 knockout mice, which did not regain normal expression of the pancreas-specific differentiation markers recombining binding protein suppressor of hairless-like protein (rbpjl) and basic helix-loop-helix family member A15 (mist1). Amylase mRNA and protein levels remained lower, and, strikingly, female LAT1 knockout mice presented signs of fibrosis lasting until day seven. In contrast, pancreas morphology had returned to normal in wild-type littermates.

CONCLUSION
LAT1 supports the regeneration of acinar cells after AP. Female mice lacking LAT1 exhibited more pronounced alterations than male mice, indicating a sexual dimorphism of amino acid metabolism.

Key Words: Acute pancreatitis; Amino acid transporter; LAT1; Metabolism; Regeneration; Fibrosis

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Core Tip: LAT1 (*slc7a5*) transports amino acids into cells and is an upstream regulator of metabolic signaling pathways. This transporter was shown to play an important role in highly proliferative cells during embryonic development and cancer, controlling protein synthesis and cell proliferation. In this study we provide evidence that LAT1 plays a role in the pancreatic acinar regeneration after acute pancreatitis. Additionally, knocking down LAT1 revealed a sex difference in the regenerative process, which may be supportive to understand gender differences in the clinical setting.

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INTRODUCTION

Acute pancreatitis (AP), an inflammatory disease of the exocrine pancreas, ranks among the most frequent gastrointestinal causes for hospital admission in the United States[1]. AP is mainly provoked by gallbladder stones, alcohol overconsumption, or other toxic substances disturbing the tightly regulated function of the exocrine pancreas[2]. The disease most often has a mild course with interstitial inflammation; however, it can also be severe with necrosis and detrimental complications. In severe, prolonged, or repetitive cases, sustained inflammation leads to expansion of extracellular matrix components, altering pancreatic architecture and replacing functional tissue. Significant fibrosis is accompanied by exocrine and endocrine insufficiency. It represents a risk factor for oncogenic transformation, seriously reducing life quality and expectancy[3,4].

Several theories have been suggested over the last decades to explain the pathogenesis of AP. Self-digestion following premature intrapancreatic activation of enzymes has become the most accepted one [5]. The disruption of various systems, leading to calcium signaling impairment, endoplasmic reticulum (ER) stress, and autophagy dysregulation[6], have been shown to lead to intrapancreatic trypsin activation. When activated prematurely upon internal or external influences, the digestive enzymes may provoke acinar cell death by autolysis, thereby triggering AP. This results not only in local but also systemic inflammatory responses. Activation of the immune system is a further crucial player in AP. Acinar cells themselves produce and release cytokines that attract neutrophils and macrophages, which influence the course of the inflammation[7].

Fundamental changes accompany the process of injury and regeneration of the tissue. They lead to an altered morphological appearance, reflecting a shift in the molecular expression profile that ultimately modifies the functional properties of the cells. Besides edema, inflammatory cell invasion, and cell death, reduction of digestive enzymes storage in zymogen granules has been observed. In addition, acinar-to-ductal metaplasia (ADM)[8] occurs, transforming the pancreas' phenotypical layout. Secretory acinar cells transiently dedifferentiate into pancreatic progenitor-like cells with embryonic properties, with a reduction of protein synthesis and a high proliferative capacity. They recover the capacity to

express embryonic transcription factors (*e.g.*, sox9, pdx1) and reduce the expression of markers characteristic of functionally differentiated cells (*e.g.*, PTFA1 complex, mist1/Bhlha15, digestive enzymes)[9]. This exerts a positive effect on the regenerative process, as shown by the fact that preventing ADM aggravates damage in AP[10].

Acinar cells are responsible for the synthesis, storage, and secretion of more than 200 different proteins. In these cells, approximately 70% of the total mRNA encodes for secreted proteins[11]. Translation is controlled by the mammalian target of rapamycin (mTOR) pathway[12] and induced by amino acid uptake[13,14]. Willet *et al*[15] proposed a stepwise adaptation of metabolic phases during AP, starting with an initial downregulation of the exocrine function and the activity of the mTOR pathway, followed by the activation of autophagy and dedifferentiation of acinar cells. The surviving cells would enter a quiescent phase and recycle cellular components to uphold their energy balance instead of producing secretory proteins. Subsequent proliferation and mTOR reactivation combined with the restoration of digestive capacity would reconstitute the healthy state.

Amino acids are actively transported into acinar cells to provide the necessary building blocks for protein synthesis. An influence on amino acid transporters (AAT) by diet[16] and early AP injury[17] has been shown in our previous work. We identified the localization of the glutamine and neutral amino acid transporters LAT1 (*slc7a5*), LAT2 (*slc7a2*), SNAT3 (*slc38a3*), and SNAT5 (*slc38a5*) at the basolateral membrane of acinar cells. In a healthy pancreas, Na^+-dependent SNAT3 and SNAT5 transport amino acids, such as glutamine, with a low affinity and high capacity into the cell[18]. These amino acids accumulate in the cells and serve as exchange substrates to uptake other amino acids such as leucine, transported by the antiporters LAT1 and LAT2[19]. During early injury of AP, SNAT3 and SNAT5 were strongly reduced. At the same time, LAT1 expression was preserved both at the mRNA and protein levels, suggesting that in LAT1 case, AAT may play a role in supporting the survival of remaining acinar cells rather than supporting secretory protein synthesis. Moreover, in a cell culture model mimicking pancreatitis, loss of LAT1 expression was associated with dedifferentiation and expression of senescence genes (*e.g.*, ptf1a, pdx, sox9, p21), further supporting its importance in maintaining acinar cell identity and survival[17,20].

LAT1 (*slc7a5*) and CD98 glycoprotein (*slc3a2*) form a heterodimeric antiporter selective for several essential neutral amino acids that are ubiquitously expressed, particularly in the brain, exocrine glands, testes, and immune cells[21]. Notably, LAT1 is highly active in proliferating cells with physiological importance during immune cell activation[22] or placental development, and its ablation causes early intrauterine death[23]. So far, this transporter has been studied mainly in cancer[24], where its overexpression promotes growth by delivering substrates and influencing various signaling pathways. Particularly, mTOR is activated by leucine transported through LAT1[25]. mTOR prompts survival and proliferation of the cells, explaining the role of LAT1 as a negative prognostic marker in cancers such as pancreatic ductal adenocarcinoma (PDAC)[26]. Moreover, enhanced endothelial LAT1 stimulates angiogenesis and thereby further boosts tumor progression[27].

The maintained presence of LAT1 during initial AP injury might support a pro-proliferative state favoring regeneration. In this study, we analyzed the role of AAT during the recovery of AP, especially focusing on LAT1. The results presented here illustrate the importance of sustained expression of LAT1 during the initial injury phase to ensure complete regeneration.

MATERIALS AND METHODS

Acute pancreatitis in wild-type and LAT1 knockout mice

The experiments were performed in C57Bl/6 (Jackson Laboratories) and LAT1-Cre/LoxP (obtained by crossing the Slc7a5$^{flox/flox}$ strain (B6.129P2-*Slc7a5tm1.1Daca*/J) with the B6.129-Gt(ROSA)26Sor-tm1(cre/ERT2)Tyj/J)[28,29] mice, as approved by the Zurich Cantonal Veterinary office (License ZH075/15) and handled in accordance with the Swiss Animal Welfare law and good laboratory practice. Mice were group-housed in standard conditions of controlled temperature (21-24 °C) and 12 h light-dark cycles and fed standard chow (#3436 Kliba Nafag, Kaiseraugst, Switzerland) and drinking water ad libitum.

At 2-3 mo of age, AP was induced *in vivo* by administering the secretagogue caerulein (cae) (Sigma, Louis, MO, United States) in twelve intraperitoneal injections of 50 μg/kg body weight at hourly intervals. LAT1 knockout was induced by tamoxifen (Sigma-Aldrich, Buchs, Switzerland) oral gavage 160 mg/kg for three consecutive days. LAT1 cre- wild-type (LAT1-wt) and LAT1 cre+ knockout (LAT1-ko) mice were treated with tamoxifen to rule out the possible effects of tamoxifen alone. AP in LAT1-wt and LAT1-ko mice was induced seven days after the first tamoxifen gavage, as control served animals injected with saline in the same regimen. Following treatment, blood samples were withdrawn for plasma analysis at the end of cae injections (12 h) with heparin coated capillary tubes from the tail, and body weight was measured daily until animals were sacrificed at specific time points between 24 h up to seven days after the first injection of cae or saline.

The sacrifice procedure was performed under inhalation anesthesia with isoflurane (Attane, Piramal Critical Care, Inc., Bethlehem, PA, United States). Tissue from the pancreas tail was obtained for RNA extraction immediately after laparotomy, snap-frozen in liquid nitrogen, and stored at -80 °C to avoid autodigestion. After heart blood drawing, a high dose of isoflurane was applied until all vital signs were terminated. The remaining pancreas was collected, snap-frozen in liquid nitrogen for protein analysis, or fixed in 3% paraformaldehyde (PFA) for 24 h and embedded in paraffin for subsequent microscopical analysis using the Microtom STP 120 (MICROTOM International, Walldorf, Germany) by immersion in progressive gradients of ethanol, Xylol, and paraffin, applying a standard protocol.

Measurement of plasma amylase and lipase

Twelve or 24 h after the first injection of cae or saline, plasma was collected by centrifugation (5 min at 8000 x g and 4 °C) of heparinized blood samples and kept at -80 °C until measurements were made. Measurement was done according to the manufacturer's instructions (Fuji Dri-Chem 4000i).

Hematoxylin/eosin and Masson's trichrome staining

The samples for the initial morphological analysis of wild-type mice (C57Bl/6) were collected from 24 h to seven days after the first injection of cae or saline. For LAT1 cre-inducible mice (LAT1-Cre/LoxP), samples were collected three and seven days after the first injection of cae or saline. The paraffin-embedded pancreas was sectioned (5 µm) and stained with hematoxylin/eosin (HE) or Masson's trichrome using a standard protocol. The morphological analysis for HE was performed on the scanned slides (Nanozoomer HT 2.O, Hamamatsu, Japan) from the Laboratory for Animal Model Pathology (LAMP) at the University of Zurich. A 40 x magnification was used (objective lens magnification of x 40, eyepiece magnification of x 10), and the analysis to grade the different stages of AP was done in a blinded fashion. Tissue injury was morphometrically graded according to edema, inflammatory cell invasion, and ADM using the slide viewer NDP.view 2 (HAMAMATSU PHOTONICS K.K., Japan). Edema was scored from 0 to 3 (0 for no edema present, 3 for most pronounced edema) depending on subjective interlobular space. The presence of inflammatory cells was quantified in ten randomly chosen microscopic fields of 0.1 mm^2 in 40 x magnification. ADM was quantified as percentage of metaplasia in relation to total exocrine pancreatic tissue. The morphological analysis of Masson's trichrome staining was done using a Nikon Eclipse TE300 epifluorescence microscope (Nikon Instruments Inc., Melville, NY) equipped with a DS-5 M Standard charge-coupled device camera (Nikon Instruments Inc.). The score was performed blind by two independent observers.

Immunohistochemistry and immunofluorescence

Immunohistochemistry was performed on 5 µm paraffin-embedded tissue slides using the Dako Autostainer Universal Staining System Model# LV-1 and the Leica BOND RX according to the supplier's instructions. The analysis was performed on the scanned slides using the slide viewer NDP.view 2. Stained acinar cells were quantified in ten randomly chosen microscopic fields of 0.1 mm^2 in 40 x magnification. For immunofluorescence, the slides were deparaffinized in a Microm spin tissue processor STP-120, reversing the steps of immersion. Antigen retrieval was performed in the pressure cook (HistosPRO (SW 2.0.0), Rapid Microwave Histoprocessor) as described in Table 1. After antigen retrieval, sections were blocked (2% BSA, 0.04% Triton X-100 in PBS, pH 7.4) and incubated with the primary antibody. The appropriate secondary antibodies were added, and nuclei were stained with 4′,6-diamidino-2-phenylindole (Dapi, Thermo Fischer Scientific, Waltham, MA, United States). Sections were mounted using a Dako Glycergel mounting medium (DakoCytomation, Glostrup, Denmark).

The sections were analyzed with a Nikon Eclipse TE300 epifluorescence microscope (Nikon Instruments Inc., Melville, NY) equipped with a DS-5 M Standard charge-coupled device camera (Nikon Instruments Inc.). Pictures were acquired with NIS Elements (Nikon Instruments Inc.) and processed in Photoshop 9.

Western blotting

The samples for protein analysis by Western blotting were collected 24 h and three days after the first injection of cae or saline. Pancreas samples were fractioned into membrane and cytosol components for AAT analysis as previously described[17]. Briefly, tissue was homogenized in an ice-cold resuspension buffer (200 mmol/L mannitol, 80 mmol/L HEPES, 41 mmol/L KOH, protease inhibitor, pH 7.5) using beads (MagNALyser Green Beads, Roche, Switzerland) for 2 × 30 s 6000 rpm in a tissue homogenizer (Precellys® 24, Bertin Technologies, France). After an initial centrifugation step (15 min at 800 × g, 4 °C), cytosol and membrane were obtained by ultracentrifugation (1 h at 100000 × g, 4 °C). To prepare a total lysate, tissue was homogenized in an ice-cold RIPA buffer (20 mmol/L Tris-HCl pH 7.5, 150 mmol/L NaCl, 1 mmol/L Na_2EDTA, 1 mmol/L EGTA, 1% NP-40, 1% sodium deoxycholate, 2.5 mmol/L sodium pyrophosphate, 1 mmol/L β-glycerophosphate, 1 mmol/L Na_3VO_4, 1 µg/mL leupeptin, protease inhibitor, pH 7.5). The tissue was homogenized (2 × 20 s 5500 rpm) and sonicated, and samples were centrifuged (20 min at 16000 × g, 4 °C). The supernatant representing total lysate was used to analyze amylase, eukaryotic translation initiation factor 2α (eIF2α), microtubule-associated proteins 1A/1B light chain 3B (LC3), ubiquin-binding protein p62, S6 kinase (S6K), smooth muscle actin (SMA), and

Table 1 Antibodies and conditions applied in immunohistochemistry and immunofluorescence experiments

Antigen	Antigen retrieval	Primary antibody	Antibody dilution
Amylase	SDS 0.1%	LifeSpan BioSciences, Inc. (LS-B8239)	1:1000
Caspase 3	Sodium citrate pH 6	Cell Signaling (9664)	1:400
E-cadherin	Tris-EDTA pH 9	Invitrogen (13-1900)	1:200
LAT1	Tris-EDTA pH 9	Cosmo Bio (KE026)	1:100
pH3	Sodium citrate pH 6	Merk-Sigma (06-570)	1:1000
SMA	Sodium citrate pH 6	Abcam (AB5694)	1:500
SNAT5	Tris-EDTA pH 9	Pineda (599341)	1:2000

pH3: Phosphohistone 3; SMA: Smooth muscle actin.

vimentin. Protein content was measured by a modified Lowry method (Biorad, Hercules, CA) according to the manufacturer's instructions. Samples were separated on sodium dodecyl sulfate polyacrylamide gels (SDS-PAGE) and transferred electrophoretically to a polyvinylidene difluoride (PVDF) membrane (Immobilon-P, Millipore, Bedford, MA, United States). The membranes were blocked and incubated with antibodies, as described in Table 2.

Protein was identified by a chemiluminescent method (Merck Millipore, Darmstadt, Germany) and detected with the luminescent image analyzer FujiFilm LAS-4000 camera (GE Healthcare, Glattbrugg, Switzerland) according to the manufacturer's instructions. Densitometric quantification of the bands was performed using the software ImageJ (National Institutes of Health, Bethesda, MD, United States).

RNA extraction and qPCR

The samples for RNA extraction were collected 24 h, three days, and seven days after the first injection of cae or saline. Total RNA was extracted using RNeasy Mini Kit (Qiagen, Basel, Switzerland), and tissue was homogenized using beads (Precellys® 24, Bertin Technologies, France). The RNA quality control was performed by microchip analysis Agilent 2100 Bioanalyzer (NanoChip, Agilent Technologies, Santa Clara, CA, United States) with a minimal required RIN of 7.0. Reverse transcription was performed according to the manufacturer's instructions, and 10 ng cDNA was added to Taq-Man Universal PCR master mix (Applied Biosystems) using a Prism 7700 cycler (Applied Biosystems, Foster City, CA, United States). The primers and probes used in this study not yet published[16,17,30,31] are listed in Table 3.

The mRNA expression of the gene of interest (GOI) relative to that of the housekeeping gene (HKG, 18S) was calculated by applying the Delta CT method ($r = 2^{[Ct(HKG)-Ct(GOI)]}$).

Assays for trypsin activated, MPO, and MCP1

The samples collected 24 h after the first injection of cae or saline were prepared according to Lau and Bhatia [32]. Briefly, one-fourth of the pancreas was homogenized with MagNALyser Green Beads (Roche, Switzerland) and ice-cold 10 mmol/L sodium phosphate buffer pH 7.4 using a Precellys® 24 tissue homogenizer (Bertin Technologies, France) for 2 × 30 s 6800 rpm. DNA was determined in the total lysate, and the supernatant was used for trypsin, myeloperoxidase (MPO), and monocyte chemotactic protein 1 (MCP1) measurements. As previously described[33], trypsin activity was measured using Boc-Q-A-R-MCA (BACHEM, Bubendorf, Switzerland) as substrate. The activity of trypsin was normalized to DNA and expressed as nmol/μg DNA. MPO measurement was determined with 3,3′,5,5′-Tetramethylbenzidine (TMB, Sigma) after 2 minutes incubation. The final MPO activity was normalized to DNA and expressed as fold change to control samples. MCP1 was measured by ELISA (R&D Systems, Inc., Minneapolis, MN, United States) according to the manufacturer's instructions, and the final concentration of MCP1 was expressed as pg/μg DNA. DNA was quantified using a fluorescence Quantitation Kit (Sigma-Aldrich, St. Louis, MO) according to the manufacturer's instructions.

FACS

The pancreas of mice 24 h after the first injection of cae were excised and immediately digested with collagenase (1 mg/mL) and DNAaseI (100 U/mL) and filtered through a 40 μm filter to remove debris. The single-cell suspension was incubated for 30 min at 4 °C with a master mix of antibodies targeting cell surface markers for lymphocytes (CD8a, NK1.1, CD4, CD45.2) or myeloid cells (CD11b, Ly6G, Ly6C, CD62L, CD45.2). After washing and fixing with 2% PFA, samples were acquired using FACS Canto II (BD Biosciences). Flow cytometry data were analyzed using FlowJo v10.7.1 software (Tree Star Inc.).

Table 2 Antibodies and conditions applied in Western blotting experiments

Antigen	Total protein loaded (μg)	Primary antibody	Antibody dilution
Amylase	2.5	LifeSpan BioSciences, Inc. (LS-B8239)	1:10000
β-tubulin	Varying	Sigma (T8328)	1:5000
LAT1	20	Cosmobio (KE026)	1:500
LC3	100	MLB (PM036)	1:250
NA/K-ATPase	Varying	Santa Cruz (sc-48345)	1:10000
p62	50	MBL (PM045)	1:1000
peIF2α	25	Cell Signaling (3398)	1:1000
pS6K	50	Cell Signaling (9205S)	1:1000
SMA	25	Sigma (C6198)	1:1000
teIF2α	25	Cell Signaling (9722)	1:1000
tS6K	50	Cell Signaling (2708P)	1:1000
Vimentin	30	Invitrogen (MA5-11883)	1:1000

LC3: Microtubule-associated proteins 1A/1B light chain 3B; peIF2: Phosphorylated eukaryotic translation initiation factor 2; pS6K: Phosphorylated S6 kinase; SMA: Smooth muscle actin; teIF2α: Total eukaryotic translation initiation factor 2α; tS6K: Total S6 kinase.

Incorporation of leucine in protein to determine protein synthesis

Protein synthesis was analyzed 24 h and three days after the first injection of cae or saline. Food was removed at least one hour before mice received gavage containing 1.5 mmol/L leucine and H^3-leucine (0.7 μCi/10 g of body weight). Mice were anesthetized, and 15 min after the gavage, the blood and pancreas were collected. The organ was cut into four pieces, weighed, and snap-frozen. Serum was used to calculate the specific radioactivity by measuring total leucine by ultra performance liquid chromatography[16] and the radioactive leucine. The tissue was homogenized in 0.6 M perchloric acid using beads and a Precellys system (2 × 30 s 6800 rpm) and incubated for one hour on ice. The pellet was obtained by centrifugation (15 min at 10000 × g), washed, and resuspended in 0.3 M sodium hydroxide. The resuspended pellet was used for the measurement of total protein and incorporated radiolabeled leucine. The rate of protein synthesis was expressed as pmol of leucine incorporated into protein per hour (pmol leu /mg protein/hour) as previously described[12].

Statistics

Data were analyzed and represented in GraphPad Prism 5 (GraphPad Software, San Diego, CA, United States) as means ± standard error of the mean (SEM). Statistical comparison between LAT1-wt and LAT1-ko groups at various time points of pancreatitis was done by unpaired two-tailed student's *t*-test or one-way ANOVA with Bonferroni post hoc test. *P* values of 0.05 or less were regarded as statistically significant and labeled as $^aP \leq 0.05$, $^bP \leq 0.01$, $^cP \leq 0.001$.

RESULTS

LAT1 amino acid transporter expression differs from other transporters during AP injury and regeneration

Expression of AAT was analyzed in a model for AP and correlated with morphology, dedifferentiation, and functional markers. AP initial injury was histologically evidenced by the invasion of inflammatory cells and interstitial edema (Figure 1A), peaking one to two days after AP induction (Figure 1B and C). On days three and four after the first injection of cae, the most dedifferentiated structures appearing as ADM were observed throughout the tissue (Figure 1D). Besides the formation of ADM crucial for regeneration[34], dedifferentiation and reduction of specific organ function were also characterized by the expression of known indicator genes. As depicted in Figure 1E, cytokeratin 19 (ck19) expression as a very early marker for dedifferentiation rose and was sustained throughout the period during which ADM was observed. Pancraes associated transcription factor 1a (ptf1a), which with recombining binding protein suppressor of hairless-like protein (rbpjl) forms the pancreas transcription factor 1 (PTF1) complex determining pancreatic fate and supporting acinar cell function[35], transiently increased two-fold. Additionally, an increased expression of SRY-box transcription factor 9 (sox9) coincided with the presence of ADM structures, as previously noted[36,37]. On days three and four,

Table 3 Primers and probes for mouse genes used in qPCR

Gene	Primers	Probe (Universal Probe Library Roche)	Accession No.
Amylase 2A4			
S	TGATGACTGGGCTTTGTCAG	4	NM_001160150.1
AS	GCCATCGACCTTATCTCCAG		
ccnb2			
S	CAACCGTACCAAGTTCATCG	3	NM_007630.2
AS	GATGGCAGTCCAGTGTCTAGG		
CD206			
S	CCTTGAACCCATTTATCATTCC	49	NM_008625.2
AS	CTGTAGCAGTGGCCTGCAT		
ck19			
S	AGTCCCAGCTCAGCATGAA	97	NM_008471.3
AS	TAACGGGCCTCCGTCTCT		
Clusterin			
S	AAGTACTACCTTCGGGTCTCCA	6	NM_013492.3
AS	AGCTTCACCACCACCTCAGT		
col4a1			
S	GGCCCTTCATTAGCAGGTG	9	NM_009931.2
AS	TCTGAATGGTCTGACTGTGTACC		
cpa1			
S	CCCCTATGGCTACACATCAGA	63	NM_025350.4
AS	TCCCGTGTAGAGATGTCAAGG		
ctsb			
S	AAGCTGTGTGGCACTGTCCT	72	NM_007798.3
AS	GATCTATGTCCTCACCGAACG		
ctsd			
S	CCCTCCATTCATTGCAAGATAC	3	NM_009983.2
AS	TGCTGGACTTGTCACTGTTGT		
ctsl			
S	ACAGAAGACTGTATGGCACGAA	25	NM_009984.4
AS	GGATCATTCTCATGTTCTTCTCC		
Elastase			
S	ACGGAGCACCCTGTTACATC	22	NM_026419.2
AS	CTGCTGCAGCTTGTCTGG		
F4/80			
S	AGGAGGACTTCTCCAAGCCTA	67	NM_010130.4
AS	AGGCCTCTCAGACTTCTGCTT		
icam-1			
S	GCTACCATCACCGTGTATTCG	53	NM_010493.3
AS	AGGTCCTTGCCTACTTGCTG		
IL-1β			

S	AGTTGACGGACCCCAAAAG	38	NM_008361.4
AS	AGCTGGATGCTCTCATCAGG		
IL-6			
S	GCTACCAAACTGGATATAATCAGGA	6	NM_031168
AS	CCAGGTAGCTATGGTACTCCCGAA		
IL-10			
S	CAGAGCCACATGCTCCTAGA	41	NM_010548.2
AS	TGTCCAGCTGGTCCTTTGTT		
lipase			
S	GCCACGATGCTAATGCTGT	3	NM_026925.3
AS	ATCACTGAAGCAGCCGAGTT		
mist1			
S	GGCTAAAGCTACGTGTCCTTG	77	NM_010800.3
AS	GCTCCAGGCTGGTTTTCC		
nox2			
S	TGCCAACTTCCTCAGCTACA	20	NM_007807.5
AS	GTGCACAGCAAAGTGATTGG		
p21			
S	TTGCCAGCAGAATAAAAGGTG	9	NM_001111099.2
AS	TTTGCTCCTGTGCGGAAC		
p27			
S	GTTAGCGGAGCAGTGTCCA	62	NM_009875.4
AS	TCTGTTCTGTTGGCCCTTTT		
p62			
S	AACTGGAGCCCACGTCCT	22	NM_001290769.1
AS	CTCCCCCACATTCTTCAGG		
prss1+3			
S	CTGCTGGCACTCAGTGTCTC	20	NM_053243.2
AS	GCAGGTCTGGTTCACTGACA		
prss2			
S	CCAGAGTGGCCTCTGTACCT	20	NM_009430.2
AS	AGCAGGTCTGGGTTGTTCAC		
ptf1a			
S	GCTTGGCCATAGGCTACATT	73	NM_018809.2
AS	GGACTGTCCTCTCCGAGGT		
rbpjl			
S	TCAGAAATCCTATGGAAATGAGAA	20	NM_009036.1
AS	GGTCTTGCATTGGCTTCAC		
sma/Acta2			
S	TAACCCTTCAGCGTTCAGC	20	NM_007392.3
AS	ACATAGCTGGAGCAGCGTCT		
sox9			
S	CAGCAAGACTCTGGGCAAG	25	NM_011448.4

AS	ATCGGGGTGGTCTTTCTTGT		
spink1			
S	CACCCAGATCTTCGACAATG	83	NM_009258.5
AS	TAAACTCAGCAGGGCCAAAG		
tnfα			
S	CTGTAGCCCACGTCGTAGC	25	ENSMUST00000025263
AS	TTTGAGATCCATGCCGTTG		
tgfβ			
S	CTCAGAGCAAGAGAAAGCACTG	3	NM_001164074.1
AS	CGTTGATGAACCAGTTACAGACC		
Vimentin			
S	CGAGGAGAGCAGGATTTCTC	53	NM_011701.4
AS	GGAAGTGACTCCAGGTTAGTTTCTC		

ccnb2: Cyclin b2; CD206/Mrc1: macrophage mannose receptor C type 1,ck19: Cytokeratin 19; col4a1: Collagen type IV alpha 1 chain; cpa1: Carpoxypeptidase 1; ctsb: Cathepsin b; ctsd: Cathepsin d; ctsl: Cathepsin l; icam-1: Intercellular adhesion molecule 1; IL-1: Interleukin 1; IL-6 and 10: Interleukin 6 and 10; mist1: Basic helix-loop-helix family member A15; nox2: NADPH oxidase 2; p21/cip/Cdkn1a: Cyclin Dependent Kinase Inhibitor 1A; p27/kip/Cdkn1b: cyclin-dependent kinase inhibitor 1B, prss1+3: Serine protease 1+3; prss2: Serine protease 2; ptf1a: Pancreas associated transcription factor 1a; rbpjl: Recombining binding protein suppressor of hairless-like protein; sma/Acta2: α smooth muscle actin/alpha-actin-2; sox9: SRY-box transcription factor 9; spink1: Serine peptidase inhibitor kazal type 1; tnfα: Tumor necrosis factor α; tgfβ: Transforming growth factor β.

when dedifferentiation was highest, we observed a peak expression of the M-phase mitosis indicator, cyclin b2. In contrast, the expression of differentiation and functional acinar cell genes, rbpjl and basic helix-loop-helix family member A15 (mist1), declined quickly in the initial injury phase along with mRNA levels of amylase and carboxypeptidase 1 (cpa1) (Figure 1F). Diverging from rbpjl and mist1, mRNA levels of amylase and cpa1 lagged at still lower to normal levels after seven days, indicating incomplete functional recovery despite the near-normal morphological appearance of the tissue. The decrease of enzyme expression as functional acinar cell markers and a decrease in protein synthesis would be expected to correlate with the decreased expression of AAT. Such a parallel regulation could indeed be nicely observed with the expression of the sodium-dependent transporter for neutral amino acids SNAT5 (*slc38a5*) and, to some extent, for SNAT3 (*slc38a3*) (Figure 1G). The expression of SNAT5, however, did not return to normal levels during seven days, similar to amylase and cpa1. On the other hand, the mRNA expression of the amino acid exchangers for neutral amino acids LAT1 (*slc7a5*) and LAT2 (*slc7a8*) was increased (Figure 1H). It should be noted that, at the protein level, LAT1 decreased (Figure 1I) but persisted along the course of the disease. Immunofluorescence showed that LAT1 was still expressed in the remaining acinar (Figure 1J) and ADM cells alike. During regeneration (three days after AP induction), LAT1 was localized in ADM, where the expression of the epithelial cell marker E-cadherin was lost (Figure 1K). This suggests that LAT1 impacts survival and recovery of acinar cells during AP, maintaining a relatively steady expression, in contrast to SNAT5 that declined after AP induction (Figure 1J).

A pancreas lacking LAT1 expression showed similar acute pancreatitis-induced early injury to a wild-type pancreas

A LAT1 inducible whole-body knockout (LAT1 cre- wild-type "LAT1-wt" and LAT1 cre+ knockout "LAT1-ko") model was used to analyze whether LAT1 plays a role in the regeneration of the pancreas during AP, and the timeline of the experiments is depicted in Figure 2A. The knockout of LAT1 induced with tamoxifen was demonstrated to be efficient at the mRNA level and was maintained throughout the experimental observation (Figure 2B). Moreover, the mRNA results corresponded with the disappearance of protein in the tissue (Figure 2C and D). The ablation of LAT1 did not induce a compensatory overexpression of other AAT, and the levels of LAT2, xCT, SNAT2, SNAT3, and SNAT5 did not differ between genotypes or sexes (Figure 2E).

The severity of AP was analyzed by plasma amylase and lipase levels, neutrophils' and macrophages' recruitment and activity and trypsin activation. Pathways of metabolic adaptation and autophagy capacity were examined together with AAT expression and protein synthesis of the exocrine pancreas.

After inducing AP in LAT1-wt and LAT1-ko mice, the levels of plasma amylase and lipase measured 12 h after the first cae injection were elevated, confirming the development of AP (Figure 2F). Additionally, we observed a sex difference concerning the enzyme levels. Amylase ($P < 0.05$ for LAT1-ko) and lipase ($P < 0.01$ for LAT1-wt) were higher in male than in female mice 12 h after the first cae

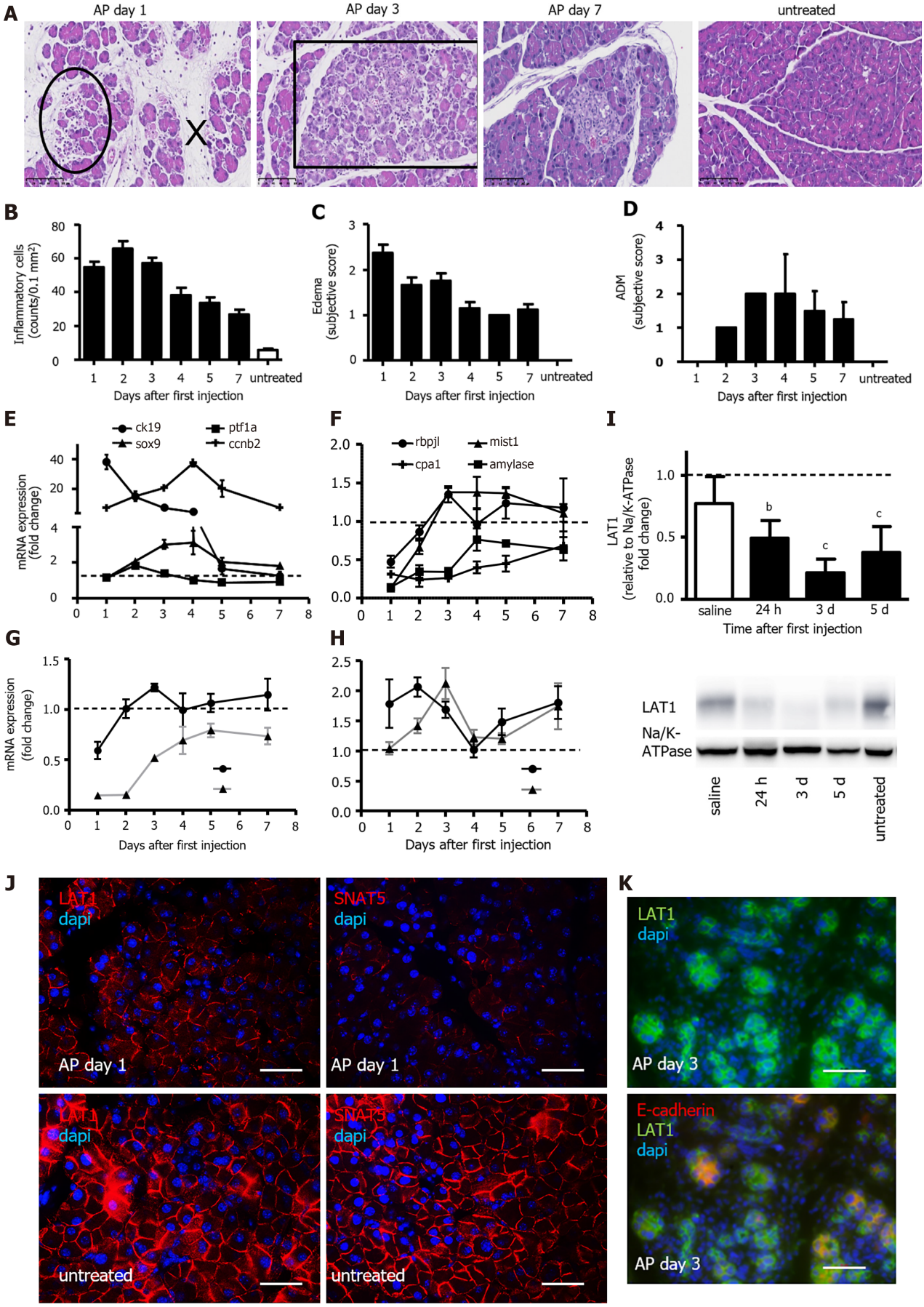


Figure 1 During recovery from acute pancreatitis, the expression of the amino acid transporter LAT1 was changed in a different way alongside dedifferentiation and the appearance of maturity markers for acinar cells. A-D: Morphological analysis of the pancreas after the induction

of acute pancreatitis (AP). A: HE stain depicting changes during the observed time period. Circle: inflammatory cells, x: edema, square: acinar-to-ductal metaplasia (ADM); B: Quantification of inflammatory cells as counts per 0.1 mm^2 field; C: Edema; D: ADM score from 0 to 3; E-F: Variation of dedifferentiation and maturity markers over seven days; mRNA expression for genes of E: dedifferentiation (ck19, SRY-box transcription factor 9 (sox9), pancreas associated transcription factor 1a (ptf1a)) and proliferation (cyclin b2 (ccnb2)) as well as F: organ-specific maturity (recombining binding protein suppressor of hairless-like protein (rbpjl), basic helix-loop-helix family member A15 (mist1), amylase, and carboxypeptidase 1 (cpa1)) in pancreas was analyzed *via* qPCR and expressed as a fold change that was compared to untreated controls, $n = 4$. The line indicates the expression level found in the control animals; G-H: Variations of amino acid transporter (AAT) gene expression alongside injury and regeneration. mRNA expression of G: SNAT3 (*slc38a3*) and SNAT5 (*slc38a5*) and H: LAT1 (*slc7a5*) and LAT2 (*slc7a8*) was examined *via* qPCR and expressed as a fold change that was compared to untreated controls ($n = 4$). The line indicates the expression level found in the control animals; I-K: LAT1 expression decreased but did not disappear during injury and regeneration; I: LAT1 protein was analyzed with Western blotting (10% SDS-PAGE gel and membrane fraction of cell lysates), and it showed a band at approximately 39 kDa. The results were expressed relative to Na/K-ATPase and were normalized in line with untreated animals. The line represents the level in untreated animals. Mean ± SEM, $n = 4$. Statistical analysis was conducted with ANOVA and Bonferroni and then compared with the saline-injected negative control. $aP < 0.05$, $bP < 0.01$, $cP < 0.001$; J: LAT1 and SNAT5 were located at the basolateral membrane of acinar cells. The paraffin-embedded mouse pancreas was stained red for LAT1 or SNAT5, and the nuclei were stained in blue (4',6-diamidino-2-phenylindole or DAPI). The panels on the left depict LAT1 24 h after AP induction (upper), and the untreated controls are below them. The panels on the right depict SNAT5 24 h after AP (upper) and the untreated controls are shown underneath. The bar length corresponds to 50 μm. K: LAT1 was present in dedifferentiated acinar cells, which were characterized by the absence of E-cadherin co-expression. The paraffin-embedded mouse pancreas was stained for LAT1 (green) and E-cadherin (red), and the nuclei were stained in blue (dapi). The top panel only depicts LAT1, while the bottom panel is overlayed with E-cadherin; in both instances, this is three days after AP induction. The bar length corresponds to 50 μm.

injection. As we showed for wild-type mice in Figure 1, LAT1-wt and LAT1-ko mice from both sexes presented elevated ck19 expression. However, LAT1-ko mice showed more pronounced expression of ck19 than their LAT1-wt littermates, and levels in male mice rose more pronouncedly than in female mice. This indicates a more dedifferentiated state in males, as ck19 is a prominent marker of ADM. The expression of clusterin, another known early injury marker in AP, was also upregulated but did not differ between genotypes or sexes (Figure 2G). Expression of the enzymes amylase, elastase, cpa1, and lipase in the pancreas of control mice (treated with saline) or with AP (24 h after the first cae injection) did not differ between the groups (Figure 2H). This suggests that male and female LAT1-wt and LAT1-ko mice started with the same mRNA levels of these genes, with no sex or genotype differences.

A very early change observed during AP injury is the activation of trypsinogen to trypsin in the pancreas[38]. The activation of trypsin increased after AP induction in both genotypes. Despite the tendency of higher values in LAT1-ko mice, they were spread over a wider range and did not reach significance (Figure 3A). Earlier timepoint measurements (six hours after the first cae or saline injection) confirmed the higher variability in trypsin activation for LAT1-ko mice. They did not statistically differ from LAT1-wt mice (data not shown). The expression at the mRNA level of trypsinogens serine protease 1+3, serine protease 2 (prss1+3, prss2), and trypsin inhibitor serine peptidase inhibitor kazal type 1 (spink1) (Figure 3B) or cathepsins B, D, and L (ctsb, ctsd, and ctsl) (Figure 3C) showed no significant differences in saline-injected control mice for both genotypes and sexes. The transcription of cationic (prss1) and meso (prss3) trypsinogen increased during AP (Figure 3B), differing from other digestive enzymes that decreased (Figure 2H). The basal expression of prss1 and prss3 in LAT1-ko mice was more variable and tended to be higher than in LAT1-wt mice, concomitant with trypsin activity tending to be higher in LAT1-ko mice. The expression of the trypsin inhibitor spink1 remained stable during AP and was only discretely elevated in female LAT1-ko mice with AP (Figure 3B). In cae injected mice, the expression of all cathepsins increased compared to control mice, and mRNA of cathepsins B and D, which modify trypsin activation[39-41], tended to be more abundant in males than females (Figure 3C). These findings do not support a more severe injury but suggest LAT1 ablation interfered with differentiation and caused variable trypsin activation.

The pancreas of mice lacking LAT1 expression showed lower neutrophil recruitment in response to acute pancreatitis

We further analyzed whether LAT1-ko mice developed the same inflammatory reaction regarding neutrophils and macrophages recruitment. Twenty-four hours after AP induction, mRNA for intercellular adhesion molecule 1 (icam-1), which mediates endothelial transmigration of leukocytes, was higher in male LAT1-ko than in female and wild-type mice. The pro-inflammatory cytokine tumor necrosis factor α (tnfα) was found to be lower in female LAT1-ko mice than LAT1-wt mice (Figure 4A). The expression of NADPH oxidase 2 (nox2), an enzyme in phagocytes that produces superoxide, was significantly reduced in LAT1-ko mice (Figure 4A). Myeloperoxidase (MPO) activity (Figure 4B) was also slightly reduced in LAT1-ko mice, implying low neutrophil recruitment. This finding was confirmed by FACS analysis, as Ly6G+ neutrophils were found to be significantly reduced in the LAT1-ko pancreas (Figure 4C). Besides neutrophil recruitment, we analyzed the release of monocyte chemotactic protein 1 (MCP1) by acinar cells and the expression of macrophage marker F4/80. The chemoattractant for macrophages MCP1 was equally increased between genotypes and sexes after AP induction, suggesting similar recruitment of macrophages (Figure 4D). The expression of F4/80 mRNA was elevated during AP and did not differ between genotypes; however, LAT1-ko males showed higher F4/80 mRNA levels than females (Figure 4E). Additionally, the inflammatory cytokines released by

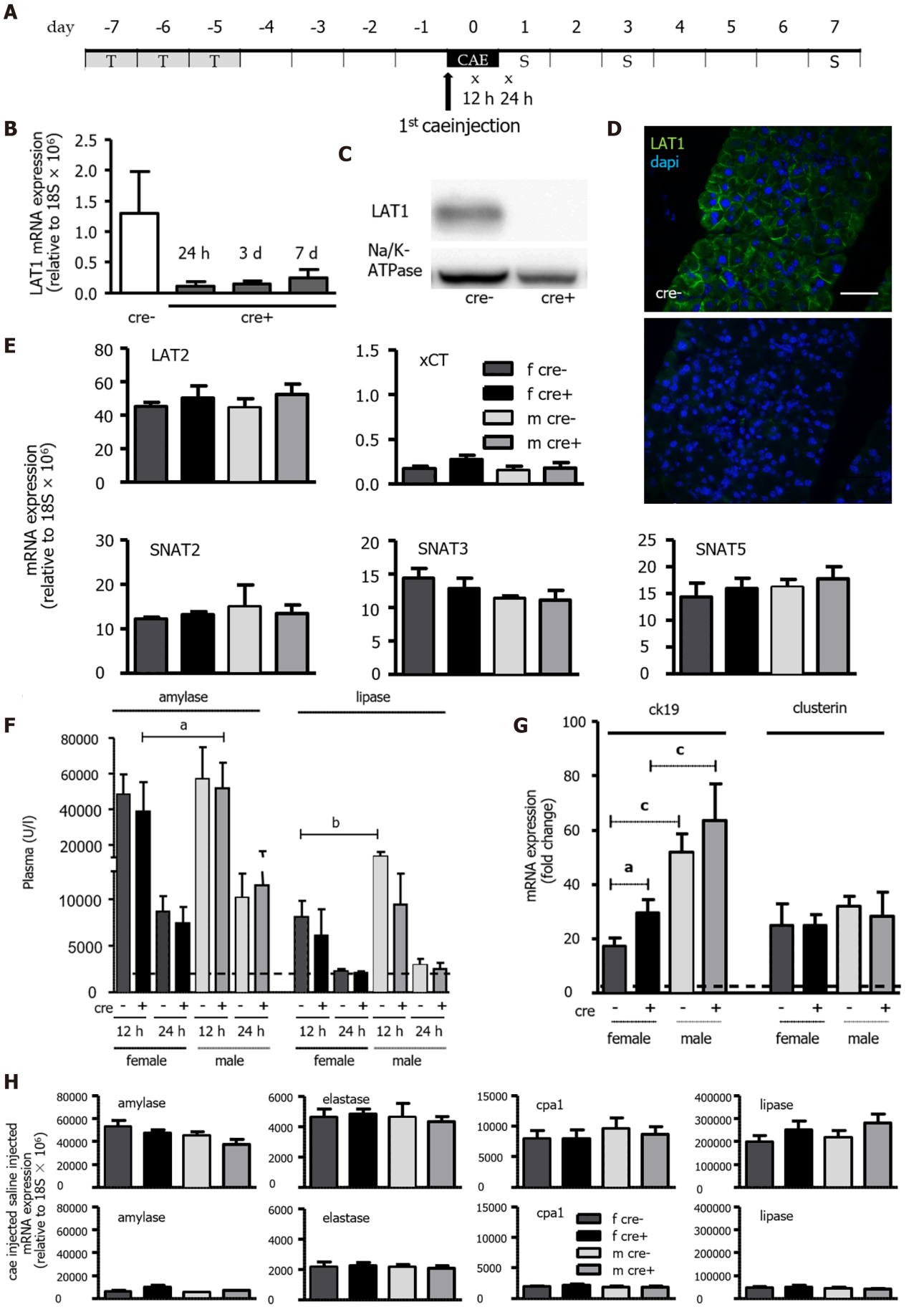


Figure 2 Tamoxifen-induced knockout of LAT1 was efficient in the pancreas, and the mice developed acute pancreatitis in an equivocal

manner. A: Experimental timeline. Three consecutive days of Tamoxifen gavage began seven days prior to acute pancreatitis (AP) induction by caerulein (cae). The first injection of cae marks day zero. Blood for amylase and lipase analysis was drawn 12 and 24 h afterwards (indicated with x). The mice were sacrificed on days one, three, and seven following the first cae injection (indicated with S); B-D: The knockout of LAT1 (*slc7a5*) at the mRNA and protein level was efficient during the period the experiments were conducted. B: mRNA expression for LAT1 was analyzed *via* qPCR and relative to a housekeeping gene (18S × 10^6) in LAT1-ko mice one, three and seven days after the onset of AP, and it was compared to the LAT1-wt mice. C: The LAT1 protein was analyzed with Western blotting (10% SDS-PAGE gel, membrane fraction of cell lysates) three days after the onset of AP, and it displayed a band at approximately 39 kDa. The results were expressed in a manner relative to Na/K-ATPase, and they were normalized in line with untreated animals; D: The paraffin-embedded mouse pancreas was stained for LAT1 (green), and the nuclei were stained in blue (dapi). The top panel depicts LAT1 in LAT1-wt mice, and the bottom is in relation to LAT1-ko mice. The bar length corresponds to 50 μm. E: Neither the knocking out of LAT1 nor the administration of tamoxifen treatment modified the expression of other amino acid transporters (AAT). The mRNA expression of LAT2 (*slc7a8*), xCT (*slc7a11*), SNAT2 (*slc38a2*), SNAT3 (*slc38a3*), and SNAT5 (*slc38a5*) was analyzed *via* qPCR relative to a housekeeping gene (18S × 10^6) in LAT1-wt and LAT1-ko mice treated with tamoxifen and saline. Mean ± SEM, n = 3-8; F: Plasma amylase and lipase increased in LAT1-wt and LAT1-ko mice after the onset of AP. The line indicates the plasma value when it is at a healthy level. Mean ± SEM, n = 7-25; G: LAT1-ko mice exhibited an enhanced presence of dedifferentiation markers. The mRNA expression of cytokeratin 19 (ck19) and clusterin was analyzed *via* qPCR and expressed as a fold change relative to saline-injected controls 24 h after the onset of AP. The line indicates the expression level of the control animals. Mean ± SEM, n = 5-7. H: mRNA expression of amylase, elastase, carboxypeptidase 1 (cpa1), and lipase was analyzed *via* qPCR relative to a housekeeping gene (18S × 10^6), and it was not modified by LAT1 knockout or tamoxifen treatment, yet it decreased after the onset of AP. The upper panel depicts the mice injected with saline, and the lower panel highlights the mice 24 h after AP induction. Mean ± SEM, n = 3-8. The statistical analysis was conducted by ANOVA and Bonferroni, and then it was compared, as indicated by the capped line. $^aP < 0.05$, $^bP < 0.01$, $^cP < 0.001$.

macrophages, interleukin 1β (IL-1β) and interleukin 6 (IL-6), were less expressed in female mice, suggesting a slightly less efficient macrophage recruitment in the female LAT1-ko pancreas following AP induction.

Altogether, our results suggest that despite recruiting less activated neutrophils and macrophages, LAT1-ko mice sustained the same initial injury based on plasma lipase, amylase, and trypsin activation levels.

LAT1 ablation hindered the activation of early regenerative metabolic adaptation

Another characteristic of AP is the fast reduction of function upon injury, with transcription and translation of proteins notably dropping[42,43]. As depicted in Figure 5A, there was a marked reduction in total protein synthesis in mice treated with cae but without difference between genotypes and sexes. Reduction in protein synthesis, inhibition of the mammalian target of rapamycin (mTOR) pathway, autophagy activation, and reactivation of mTOR are important events during the earlier metabolic adaptations and regeneration from AP[15]. As LAT1 is an upstream regulator in the mTOR pathway[25,44], the phosphorylation of its downstream target S6 kinase (S6K) was analyzed. S6K phosphorylation was found significantly reduced in female LAT1-ko mice ($P < 0.05$) during AP (Figure 5B). The phosphorylation of the general control nonderepressible 2 (GCN2) pathway marker eukaryotic translation initiation factor 2α (eIF2α), sensing intracellular shortage of amino acids, was increased in both genotypes during AP (Figure 5C). It was significantly higher in female LAT1-wt mice compared to their male littermates ($P < 0.05$), putatively suggesting accentuated sex-specific nutritional stress. Regarding expression of other AAT, only slight variations were observed. Most notably, female mice transcribed xCT (*slc7a11*) to a lesser extent than males ($P < 0.01$) (Figure 5D) but contrarily upregulated the expression of LAT2 ($P < 0.05$). This increase in LAT2 was possibly induced by the GCN2 pathway, as has been previously detected in cell culture[45]. AAT for glutamine did not vary between genotypes or sexes. SNAT3 and SNAT5 expression showed a general decrease during AP, SNAT2 a slight increase. Female LAT1-ko mice not only activated the mTOR pathway to a lesser extent and displayed pronounced sensitivity to nutritional stress, but they tended to show signs of defective autophagy. The well-established marker for autophagy, given as the ratio of microtubule-associated proteins 1A/1B light chain 3B II:I (LC3II:I)[46], was higher in female LAT1-ko compared to LAT1-wt mice ($P < 0.05$) (Figure 5E). The expression of mRNA coding for p62, a regulatory protein of autophagy[47], was increased during AP with higher levels in male mice (Figure 5F), but this did not translate to a change of p62 protein, which was equal among genotypes and sexes (Figure 5G) and suggested accumulation of the cargo protein in females.

Our results suggest that LAT1-ko mice activated the mTOR downstream target SK6 less effectively during the initial AP injury phase compared to LAT1-wt mice. Females more pronouncedly activated the starvation pathway GCN2, implied by increased phosphorylation of its downstream target eIF2α. They also showed evidence for dysfunctional autophagy, suggestive of an early metabolic adaptation depending on sex and the presence of LAT1.

LAT1-ko mice showed delayed recovery from acute pancreatitis with a more accentuated effect in females

As for wild-type mice, morphology, differentiation, and dedifferentiation markers, indicating the functional state of acinar cells, were analyzed in female and male LAT1-wt and LAT1-ko mice at the time points for which we observed maximum regenerative changes (day three) and almost normal

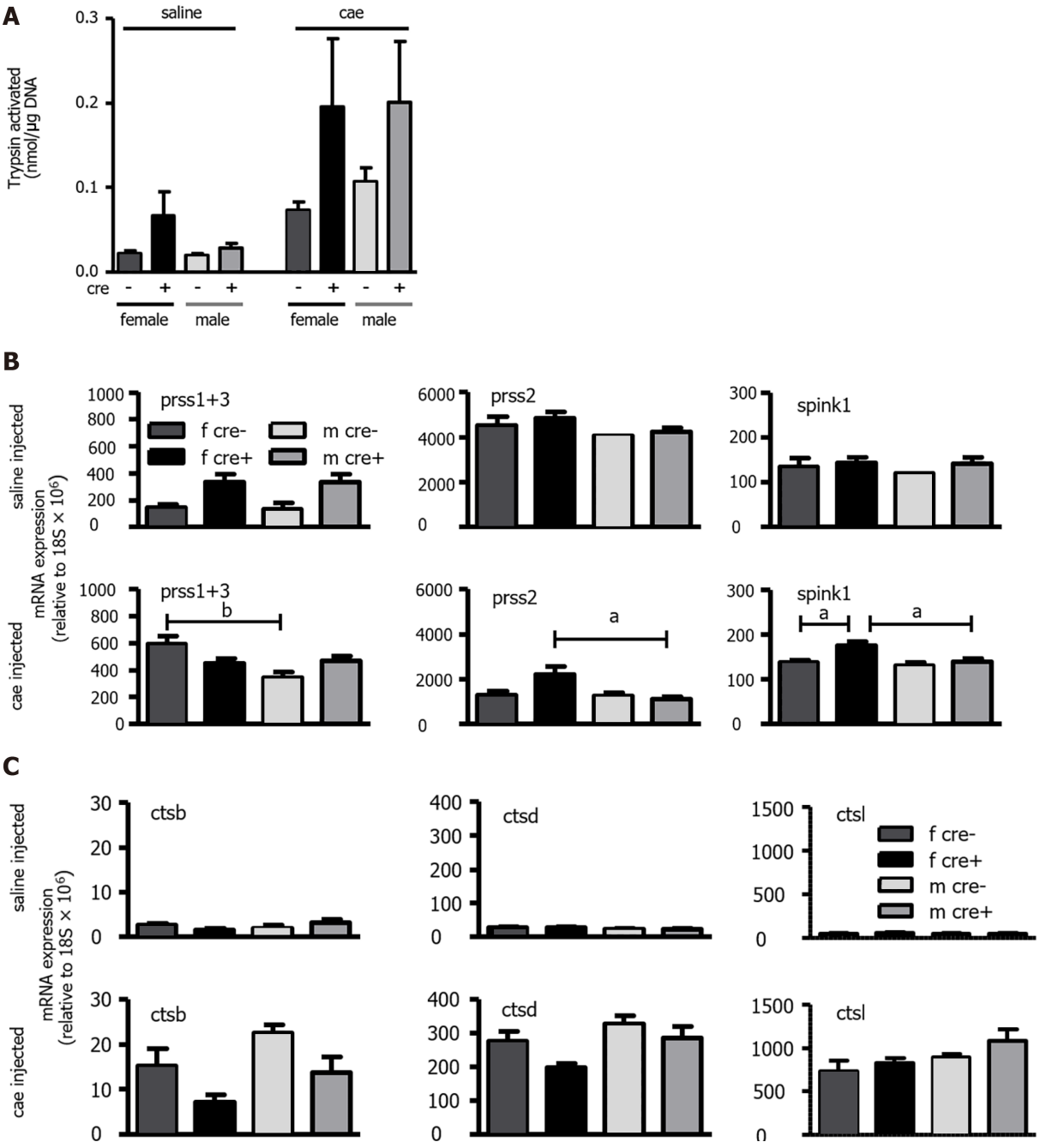


Figure 3 The trypsin activation observed in LAT1-ko mice had higher variability, and the expression of trypsinogens and cathepsins differed from LAT1-wt mice. A: Trypsin activation was observed in LAT1-wt and LAT1-ko mice 24 h after acute pancreatitis (AP) induction. The activity was measured using Boc-Q-A-R-MCA and expressed as nmol/µg DNA. Mean ± SEM, n = 3-7; B-C: The mRNA expression of trypsinogens, trypsin inhibitor, and cathepsins. The mRNA expressions of serine protease 1+3 (prss1+3) and serine protease 2 (prss2) along with serine peptidase inhibitor kazal type 1 (spink1) (B) and cathepsin B, D, and L (ctsb, ctsd, and ctsl) (C) were analyzed *via* qPCR and expressed relative to a housekeeping gene (18S × 10^6) in LAT1-wt and LAT1-ko mice. The upper panel depicts the mice that were injected with saline, and the lower panel shows the mice 24 h after AP induction. Mean ± SEM, n = 7-14. Statistical analysis was conducted with ANOVA and Bonferroni and compared, as indicated by the capped line. $^aP < 0.05$, $^bP < 0.01$, $^cP < 0.001$.

morphology (day seven) after induction of AP. Additionally, the body weights of the mice were controlled daily in this period. On day one of AP, the genotypes showed the same relative change of body weight but thereafter, LAT1-ko and especially female LAT1-ko lost more weight and did so more prolongedly (Figure 6A). Female LAT1-ko mice reached the highest weight loss on days three to five and still lagged their littermates on day seven.

As observed for wild-type mice three days after the induction of AP (Figure 1D), the presence of ADM could be detected in the tissue. Male LAT1-ko did not differ from male LAT1-wt mice, but female LAT1-ko had more extensive ADM areas than their LAT1-wt littermates (Figure 6B). By day seven, pancreatic tissue morphology in males of both genotypes and female LAT1-wt mice was almost restored, while female LAT1-ko mice still presented more areas with ADM.

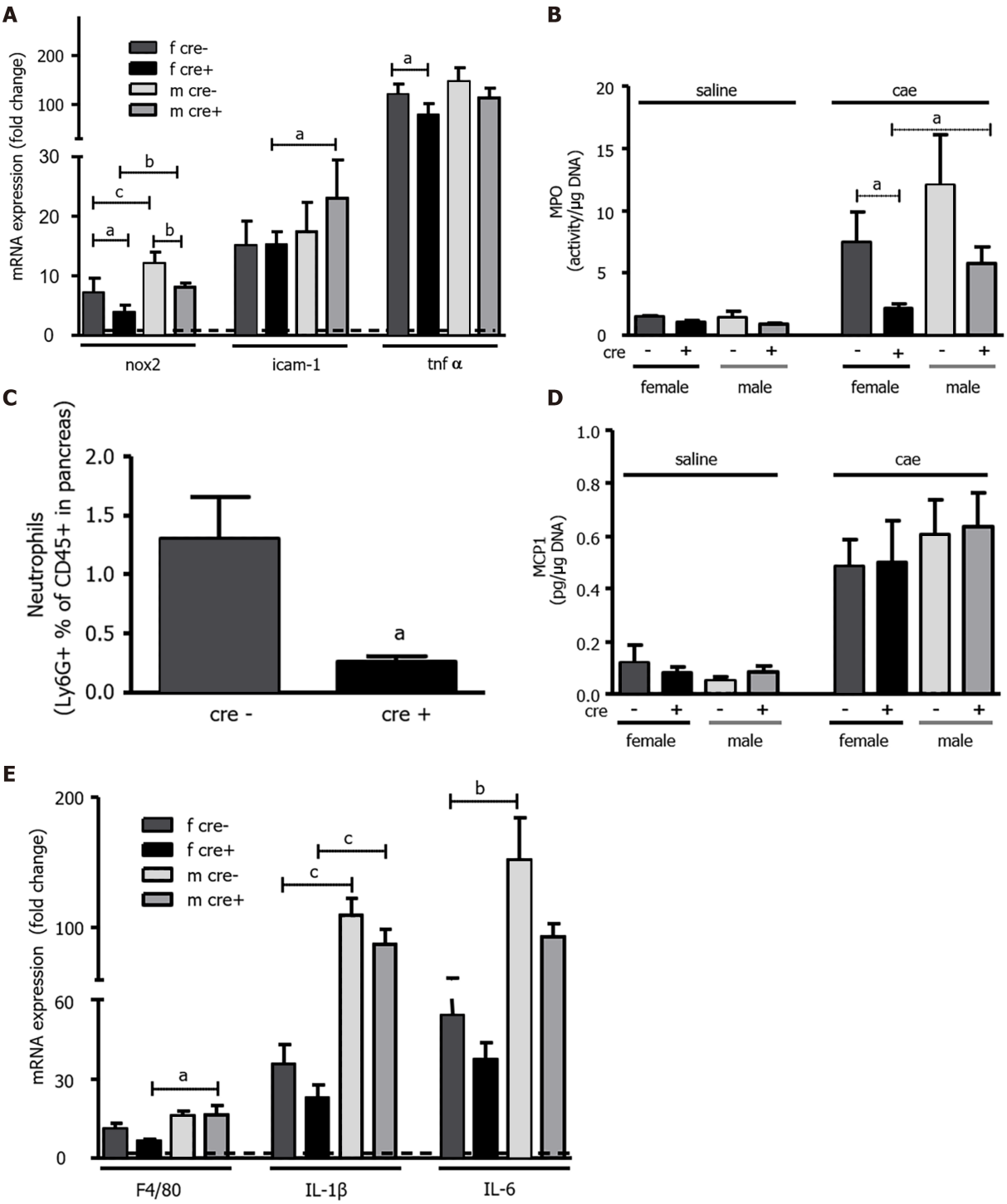


Figure 4 The number and activity of neutrophils in LAT1-ko mice was reduced, but the capacity to recruit macrophages was maintained. A-C: LAT1-ko mice recruited less neutrophils than LAT1-wt cre-mice. A: The mRNA expressions of NADPH oxidase 2 (nox2), tumor necrosis factor α (tnfα), and intercellular adhesion molecule 1 (icam-1) were analyzed *via* qPCR and expressed as a fold change relative to saline-injected controls 24 h after the onset of acute pancreatitis (AP). The line indicates the expression level of the control animals. Mean ± SEM, *n* = 4-7; B: Myeloperoxidase activity in LAT1-ko mice 24 h after saline or caerulein (cae) injection was expressed as activity/µg DNA. Mean ± SEM, *n* = 3-8; C: The lower number of neutrophils recruited was assessed by FACS analysis of the pancreas 24 h after the onset of AP. The results illustrate the ratio of Ly6G positive to CD45 positive cells. Mean ± SEM, *n* = 4-5. Statistical analysis was conducted with an unpaired, two-tailed t-test. $^{a}P < 0.05$; D-E: Recruitment of macrophages was equal in both genotypes, but female LAT1-ko mice exhibited a lower expression of macrophage marker and interleukins; D: Monocyte chemotactic protein 1 was equal between genotypes and sexes; this was measured by ELISA in the pancreas of saline or cae-injected mice and expressed as pg/µg DNA. Mean ± SEM, *n* = 3-8; E: Macrophage marker F4/80, interleukin 1β (IL-1β), and IL-6 were lower in female LAT1-ko when analyzed *via* qPCR and expressed as a fold change relative to saline-injected controls 24 h after the onset of AP. The line indicates the expression level of control animals. Mean ± SEM, *n* = 4-8. Statistical analysis was conducted with ANOVA and Bonferroni and then compared, as indicated by the capped line. $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$.

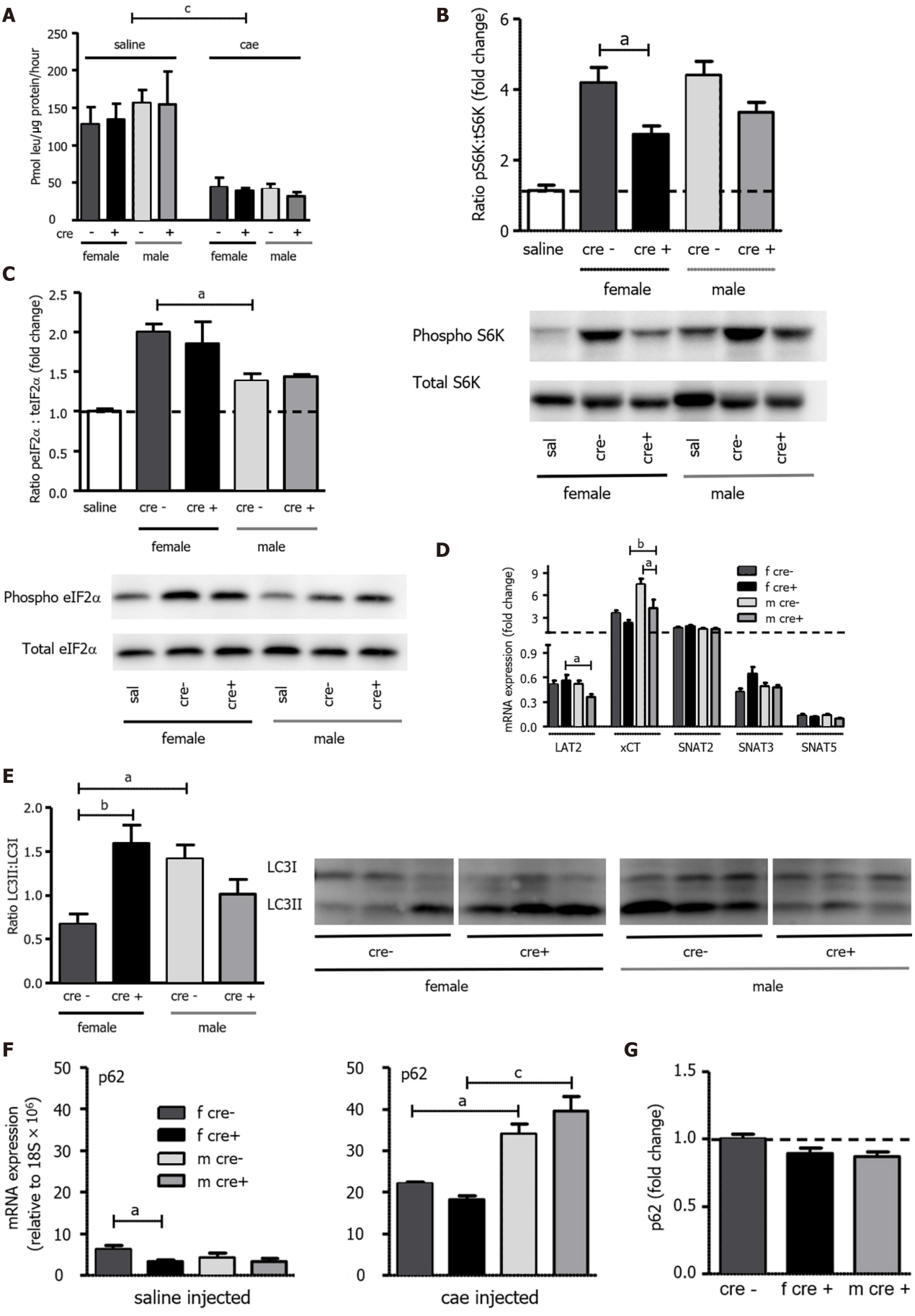


Figure 5 Protein synthesis, mammalian target of rapamycin, and general control nonderepressible 2 pathways as well as autophagy were

differently regulated upon knockout of LAT1. A: As measured by the incorporation of ^{3}H-leucine into total protein and expressed as pmol leu/mg protein/hour, protein synthesis was seen to decrease in both genotypes 24 h after the onset of acute pancreatitis (AP). Mean ± SEM, *n* = 3-8; B: The mammalian target of rapamycin (mTOR) downstream target S6 kinase (S6K) was activated to a lesser degree in female LAT1-ko mice. Phosphorylated and total S6K levels were determined with Western blotting (8% SDS-PAGE gel, cells' total lysates) 24 h after the onset of AP, and they showed a band at approximately 70 kDa. The results were expressed as a ratio of phosphorylated to total protein relative to the saline-injected controls; C: General control nonderepressible (GCN2) was more activated in females. The GCN2 downstream target eukaryotic-translation-initiating factor 2α (eIF2α) was analyzed *via* Western blotting (12% SDS-PAGE gel, cells' total lysates) 24 h after the onset of AP, and it exhibited a band at approximately 38 kDa. The results were expressed as ratio of phosphorylated to total protein, which was relative to the saline-injected controls. Mean ± SEM, *n* = 4; D: Variation of amino acid transporter expression after AP induction. The mRNA expression of LAT2 (*slc7a8*), xCT (*slc7a11*), SNAT2 (*slc38a2*), SNAT3 (*slc38a3*), and SNAT5 (*slc38a5*) was analyzed *via* qPCR and expressed as a fold change that was compared to saline-injected controls 24 h after the onset of AP. The line indicates the expression level of the control animals. Mean ± SEM, *n* = 5-8; E-G: Female LAT1-ko mice presented with enhanced markers for dysfunctional autophagy. E: The ratio of microtubule-associated protein 1A/1B-light chain 3 II (LC3II) to LC3I was higher in female LAT1-ko compared to female LAT1-wt. Moreover, the LC3II-to-LC3I ratio was examined using Western blotting (13.5% SDS-PAGE gel, cells' total lysates) 24 h after the onset of AP; a double band appeared at approximately 15 kDa. The upper band represents LC3I, and the lower band indicates LC3II. Mean ± SEM, *n* = 3-6; F-G: Male mice express more p62 mRNA, yet no more protein, than their female counterparts; F: The mRNA expression of p62 was analyzed *via* qPCR and expressed relative to a housekeeping gene (18S × 10^{6}) in LAT1-wt and LAT1-ko mice. The upper panel depicts the mice that were injected with saline, and the lower panel highlights the mice 24 h after AP induction. Mean ± SEM, *n* = 3-7; G: The p62 protein was analyzed with Western blotting (12% SDS-PAGE gel, cells' total lysates) 24 h after the onset of AP, and it exhibited a band at approximately 62 kDa. The results were expressed relative to β-tubulin and normalized in line with the LAT1-wt average indicated by the line. Mean ± SEM, *n* = 5-8. The statistical analysis was conducted by ANOVA and Bonferroni and then compared, as indicated by the capped line. *a* *P* < 0.05, *bP* < 0.01, *cP* < 0.001.

Overall, mRNA expression of genes characteristic for mature acinar cells analyzed on day three, rbpjl and mist1, was lower in female and male LAT1-ko mice than their LAT1-wt littermates. By day seven, expression in female LAT1-ko mice remained low, while female LAT1-wt mice and males from both genotypes had already restored expression levels (Figure 6C). This slower regeneration process in LAT1-ko mice, especially in females, was not caused by a difference in initial acinar cell death or following proliferation. Cleaved caspase, indicating apoptosis, was higher in acinar cells on day three for all genotypes and sexes compared to day seven, and the groups did not statistically differ between each other (Figure 6D). The phosphohistone 3 marker suggests that the proliferation of acinar cells was ongoing during the later time point of day seven, with only a tendency for female and male LAT1-ko mice to proliferate less than LAT1-wt animals early on day three (Figure 6E). Simultaneously, dedifferentiation markers sox9 and ck19 were elevated on days three and seven in both genotypes and sexes compared to saline-injected controls (Figure 6F). The early marker of dedifferentiation ck19 had already been elevated in both female and male LAT1-ko mice 24 h after AP induction (Figure 2G). On day three, male LAT1-wt and LAT1-ko mice behaved similarly, while female LAT1-ko reached higher ck19 levels than their LAT1-wt littermates, and these higher levels were maintained until day seven (Figure 6F). The described findings imply that pancreatic remodeling lasted beyond histologically determined normalization of the tissue and that LAT1 ablation in females worsened the condition.

Acinar cell function was assessed by amylase mRNA and protein expression as well as by total protein synthesis. Amylase levels were strongly reduced in LAT1-ko mice on day three, and female LAT1-ko showed still lower levels on day seven relative to female LAT1-wt mice (Figure 7A). Amylase levels were also decreased in LAT1-ko mice, as evidenced by immunofluorescence of the pancreas from mice of both sexes (Figure 7B). The quantification done by Western blotting confirmed the qualitative analysis through immunofluorescence, where we observed that female LAT1-ko mice expressed less amylase compared to LAT1-wt mice (Figure 7C). The observation that total protein synthesis in the tissue was still reduced three days after AP induction in both genotypes and female LAT1-ko mice remained significantly lower than saline-treated mice ($P < 0.05$) (Figure 7D) suggests that these lower amylase levels were due to a decrease in protein synthesis. Interestingly, the key regulators of protein synthesis mTOR (pS6) and GCN2 (eIF2α) pathways were equally activated in LAT1-wt and LAT1-ko mice. Ratios of phosphorylated to total S6K were not different from LAT1-wt (Figure 7E), and the same was observed for eIF2α, which had returned to control levels for both genotypes by day three (Figure 7F). The transport of amino acids into the cell is also crucial for protein synthesis. The expression of AAT was similar between the genotypes but differed between the sexes. In contrast to the early phase of AP (Figure 5D), SNAT3 expression remained lower in female compared to male mice on days three and seven (Figure 7G). The expression of xCT and SNAT2 were still elevated on day three, as we had observed after 24 h, but at this later time, xCT expression was similar in females and males. At the same time, LAT2 mRNA expression had almost returned to normal in all groups. SNAT5 expression, similar to amylase, remained reduced until the end of the experiment. Since we did not observe a difference in proliferation or acinar cell death, we analyzed the expression of markers for senescence and cell cycle arrest [48]. The gene encoding p21 (CDKN1A/CIP1), an inhibitor of cyclin-dependent kinases, was higher in female LAT1-ko mice ($P < 0.01$) three days after AP induction (Figure 7H). Another inhibitor of cyclin-dependent kinases, p27 (CDKN1B/KIP1), only tended to be elevated in female LAT1-ko mice seven days after AP induction. Lasting cell cycle inhibition might further indicate stalling recovery accentuated in female LAT1-ko mice.

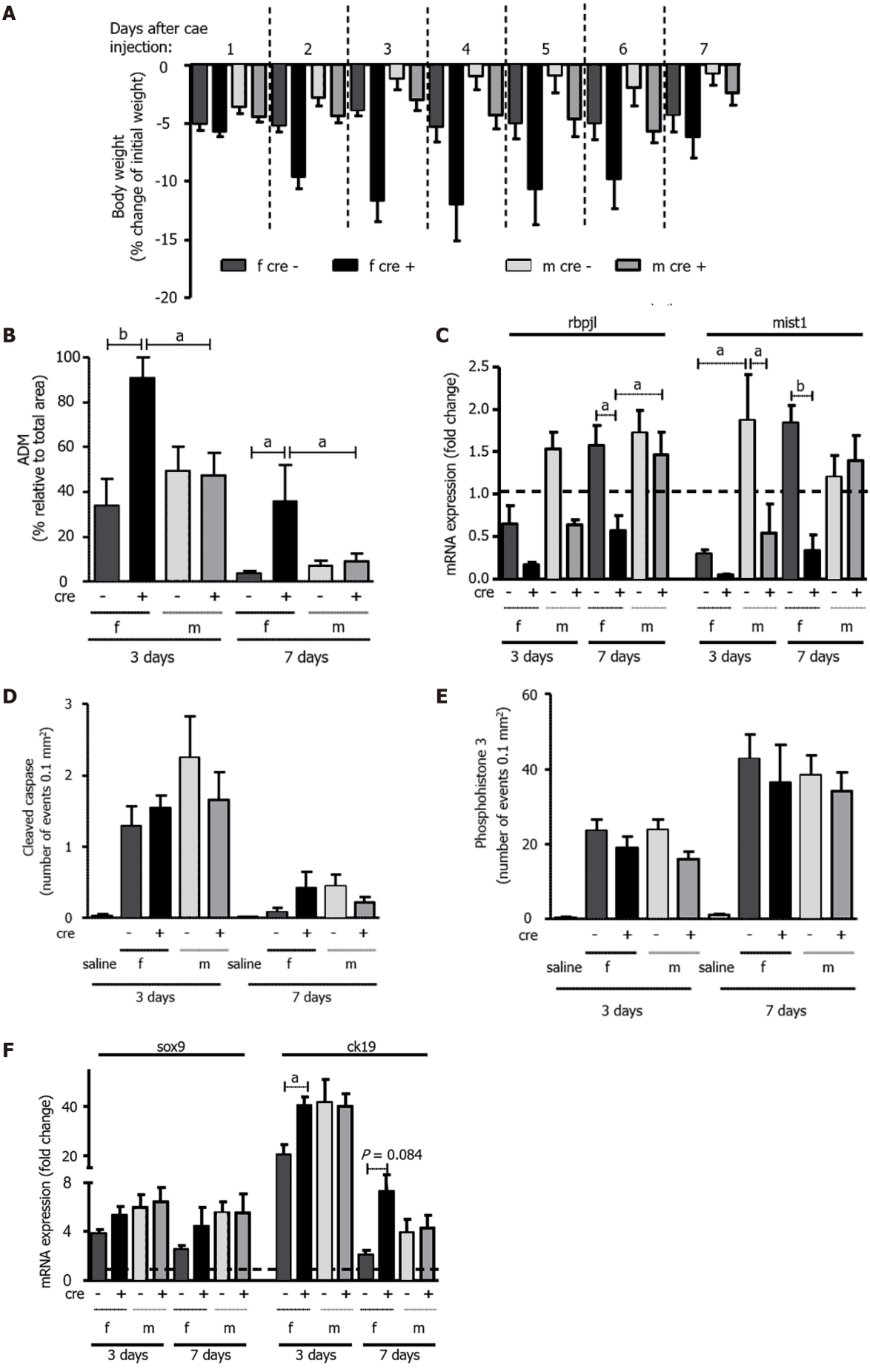


Figure 6 LAT1-ko mice showed a delayed recovery from acute pancreatitis, and the effect was more accentuated in females. A: Weight loss

in female LAT1-ko mice was more accentuated and prolonged compared to their LAT1-wt littermates. Their body weight was measured daily over a period of seven days after the caerulein injection, and it was then expressed as weight loss relative to their initial body weight. Mean ± SEM, n = 5-17; B: Acinar-to-ductal metaplasia (ADM) areas in female LAT1-ko mice were larger than in their littermates three and seven days after the onset of acute pancreatitis (AP). ADM structures were identified microscopically in the HE stains and expressed as a percentage that was relative to the total area of the pancreas. Mean ± SEM, n = 5-9; C: Markers of pancreas-specific differentiation were lower in female LAT1-ko mice up to seven days after the onset of AP. Meanwhile, the mRNA expression of recombining the binding protein suppressor of hairless-like protein (rbpjl) and basic helix-loop-helix family member A15 (mist1) was analyzed *via* qPCR, expressed as a fold change, and then compared to saline-injected controls three and seven days after the onset of AP. The line indicates the expression level found in the control animals. Mean ± SEM, n = 3-5; D-E: Apoptosis and proliferation did not differ between the groups with AP, as demonstrated by immunohistochemistry analyses of D: cleaved caspase and E: phosphohistone 3, respectively. The results were expressed according to the number of events in acinar cells per 0.1 mm^2. Mean ± SEM, n = 5-9; F: The expression level of genes that are characteristic of dedifferentiation was elevated in female LAT1-ko mice up to day seven. Meanwhile, the mRNA expression of SRY-box transcription factor 9 (sox9) and cytokeratin 19 (ck19) was determined *via* qPCR and expressed as a fold change, which was compared to saline-injected controls three and seven days after the onset of AP. The line indicates the expression level of the control animals. Mean ± SEM, n = 3-5. Statistical analysis was conducted with ANOVA and Bonferroni and then compared, as indicated by the capped line. $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$.

This set of experiments suggests that LAT1-ko mice were delayed in initiating differentiation, recovering protein synthesis capacity, and restoring body weight compared to their LAT1-wt littermates. Also, at three to seven days, female LAT1-ko mice presented disrupted regeneration steps compared to male littermates.

Female LAT1-ko mice presented higher fibrosis marker expression and microscopical evidence of fibrosis compared to their wild-type littermates

The presence of fibrosis alters the morphological characteristics of the tissue but can also influence cellular interaction and slower regeneration processes after AP[8]. To verify if the ablation of LAT1 in LAT1-ko mice could modify regeneration by influencing the generation of fibrosis, we assessed the presence of fibrosis indicators directly by histological and immunohistological analysis as well as by mRNA expression and protein levels of fibrosis markers. The Masson's trichrome histological staining showed noticeable aggregation of fibrotic tissue throughout the pancreas of LAT1-ko females (Figure 8A). The activation of pancreatic stellate cells (PSC) was evidenced by the deposition of SMA with immunofluorescence (Figure 8B) and Western blotting analysis (Figure 8C) as well as a decrease in vimentin ($P < 0.05$) (Figure 8D). Both confirmed the higher presence of markers characteristic for activated PSC in female LAT1-ko mice. Female LAT1-ko mice exhibited a higher presence of collagen mRNA on day three and tended to express more transforming growth factor β (tgfβ) than their male littermates on days three and seven (Figure 8E). Macrophages have been previously identified to influence the restoration of homeostasis[49]. In females, the expression of macrophage marker F4/80 and CD206/MRC1 indicative of type 2 macrophages did not differ from males three days after AP (Figure 8E) but had a slight tendency to be lower, while the anti-inflammatory marker IL-10 was not enhanced in females or males. Interestingly, seven days after AP, all the genes related to fibrosis remained elevated in female LAT1-ko compared to male mice. Altogether, these results propose that LAT1-ko mice developed more fibrosis and regenerated more slowly, with accentuated manifestation in females.

DISCUSSION

A change in the expression of AAT during the early phase of AP was shown in our previous work[17]. To investigate the significance of this observation, we analyzed the course of AP from the early phase to regeneration in the present study, focusing on the role of the AAT LAT1 (*slc7a5*). We examined the expression of different AAT, protein synthesis and upstream regulatory mechanisms, markers of injury, proliferation, and fibrosis. We observed a delay in the recovery phase of LAT1-ko mice compared to LAT1-wt after AP induction. We here discuss the possible influences of LAT1 expression on these mechanisms in particular and in AP disease progression in general and the limitations and possible follow-ups of this study.

LAT1 ablation was not compensated by other amino acid transporters in a global LAT1 knockout mouse model

AATs are responsible for the continuous influx of amino acids used for protein synthesis in acinar cells [50] and can also act upstream of cellular pathways controlling cell survival and proliferation[51]. LAT1 expression persisted during the first 24 h of AP when the differentiation markers recombining binding protein suppressor of hairless-like protein (rbpjl) and basic helix-loop-helix family member A15 (mist1) were reduced, and the early dedifferentiation marker ck19 was increased. LAT1 was detected on membranes of acinar cells and ADM, suggesting its presence in the remaining dedifferentiated acinar cells may support cell survival. The knockout of LAT1 was efficient until the end of the experiment and

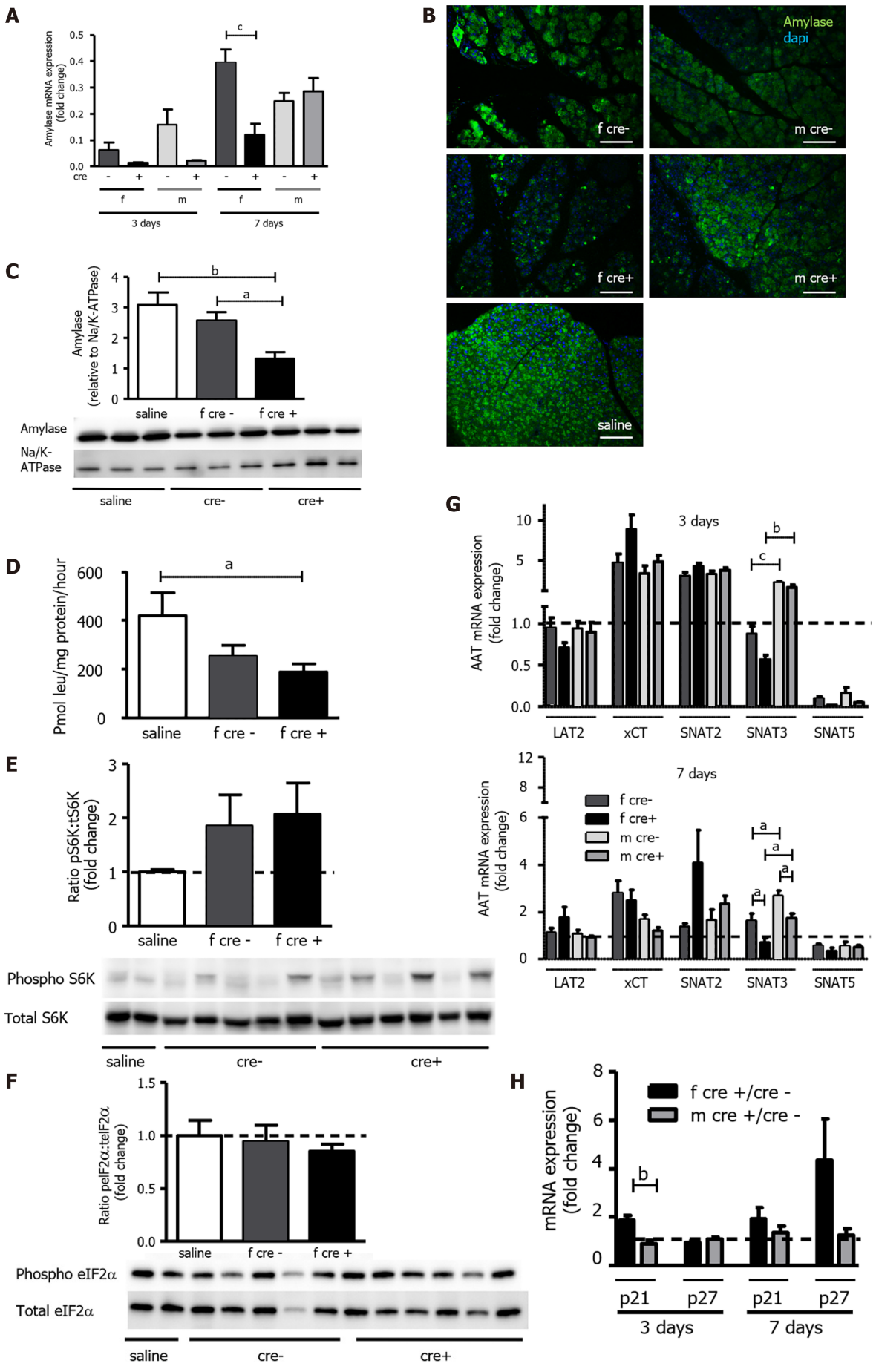


Figure 7 Functional recovery of acinar cells was delayed in LAT1-ko mice after injury. A-C: Amylase mRNA and protein levels were reduced in

female LAT1-ko mice. A: The mRNA of amylase was examined *via* qPCR and expressed as a fold change; subsequently, this value was compared to those relating to the saline-injected controls three and seven days after the onset of acute pancreatitis (AP). Mean ± SEM, *n* = 45. Subsequently, statistical analysis was conducted with ANOVA and Bonferroni and then compared, as indicated by the capped line. *cP* < 0.001; B: Decreased amylase expression could also be evidenced by reduced signal intensity in immunofluorescence three days after AP induction. The paraffin-embedded mouse pancreas was stained for amylase (green), and the nuclei were stained in blue (dapi). The bar length corresponds to 100 μm; C: Amylase protein was analyzed with Western blotting (10% SDS-PAGE gel, cells' total lysates) three days after the onset of AP; it exhibited a band at approximately 60 kDa. The results were expressed relative to Na/K-ATPase; D: LAT1-ko mice showed lower protein synthesis compared to the saline-injected controls, as measured by the incorporation of ^{3}H-leucine into total protein and expressed as pmol leu/mg protein/hour three days after the onset of AP in female mice. Mean ± SEM, *n* = 3-8. Statistical analysis was conducted with ANOVA and Bonferroni and then compared, as indicated by the capped line. *aP* < 0.05; E-F: Mammalian target of rapamycin was increased, and the general control nonderepressible 2 (GCN2) pathway was not activated in LAT1-ko mice three days after AP; E: Phosphorylated and total S6K levels were determined with Western blotting (8% SDS-PAGE gel, cells' total lysates) three days after the onset of AP; there was a band at approximately 70 kDa. The results were expressed as a ratio of phosphorylated to total protein relative to the saline-injected controls; F: GCN2's downstream target eukaryotic-translation-initiation factor 2α was analyzed using Western blotting (12% SDS-PAGE gel, cells' total lysates) three days after the onset of AP, and a band appeared at approximately 38 kDa. The results were expressed as a ratio of phosphorylated to total protein relative to the saline-injected controls. Mean ± SEM, *n* = 5-6; G: The expression of SNAT3 was lower in female mice until seven days after the onset of AP. The mRNA expression of LAT2 (*slc7a8*), xCT (*slc7a11*), SNAT2 (*slc38a2*), SNAT3 (*slc38a3*), and SNAT5 (*slc38a5*) was analyzed *via* qPCR and expressed as a fold change in comparison with saline-injected controls three and seven days after the onset of AP. The line indicates the expression level of the control animals. Mean ± SEM, *n* = 5-10. Statistical analysis was conducted with ANOVA and Bonferroni and then compared, as indicated by the capped line. *aP* < 0.05, *bP* < 0.01, *cP* < 0.001; H: Expression of senescence markers was more accentuated in female LAT1-ko mice up until seven days after the onset of AP. The mRNA levels of p21 and p27 were analyzed *via* qPCR and expressed as a fold change, which was compared to the LAT1-wt group three and seven days after the onset of AP. The line indicates the expression level of the LAT1-wt animals. Mean ± SEM, *n* = 5. Statistical analysis was conducted *via* an unpaired, two-tailed t-test. As indicated by the capped line, a comparison was then made. *bP* < 0.01.

did not cause compensatory upregulation of other AAT. LAT2 (*slc7a8*), especially, which has a similar substrate specificity as LAT1[52] and the capacity to activate mammalian target of rapamycin (mTOR) signaling[53], was not upregulated in acinar cells. This stands in contrast to constitutive muscle-specific ablation of LAT1 (*MCK-cre slc7a5*), where LAT2 mRNA was upregulated in muscle fibers[29]. We also investigated the potential effect of LAT2 deficiency on LAT1 expression in the pancreas, but no direct compensation was detected (data not shown). The expression of other AAT, which we previously described to be modulated during early AP induction[17], was neither influenced by LAT1 ablation nor the treatment with tamoxifen. To conclude, the inducible LAT1 knockout mouse model appeared to be adequate to study the role of LAT1 in the different phases of AP without compensation of other transporters in the pancreas.

LAT1 knockout mice sustained a similar injury to the wild-type 24 h after acute pancreatitis induction

The intrapancreatic activation of trypsinogen to trypsin has been suggested as an early event during AP and is used as a marker for the extent of injury[5]. In our study, both genotypes presented a similar mean increase of intrapancreatic trypsin activity, but the values in knockout mice were more variable than in wild-types. Intrapancreatic activation of trypsinogen is controlled by the interplay of cathepsin B, cathepsin D, and cathepsin L[40,54,55] localized in the lysosomes of acinar cells and also released by leukocytes[41]. The specific ablation of cathepsin D in leukocytes has a pronounced effect on trypsinogen activation during AP, suggesting the reduced neutrophil recruitment we observed in LAT1-ko mice might have contributed to the variability in trypsin activation. Nonetheless, mice with deficient neutrophil recruitment can develop AP and only present a stalled increase of plasma lipase[56]. In our model, the reduced number of neutrophils did not delay or depress plasma injury markers. Plasma levels of lipase and amylase were equally changed in both genotypes and confirm that LAT1 ablation did not culminate in less severe injury. We describe for the first time that LAT1 expression affects leukocyte recruitment, while the importance of LAT1 expression for the activation of macrophages[57, 58] and T-cells[22] has already been recognized.

Further, we did not observe a variation of macrophage markers between the genotypes, but a sex difference appeared. Female mice presented diminished expression of macrophage markers in the early injury phase. Moreover, mRNA for IL-1β and IL-6 in female LAT1-ko mice was reduced 24 h after AP induction. This possibly corresponded with a lower number of monocytes and the missing transporter activity on these cells. Yet, LAT1 genotypes displayed the same indicators of AP, implying that the disease developed in the presence of fewer neutrophils and macrophages. As we applied a whole-body knockout, the effects of immune cells' LAT1 expression on inflammatory processes during AP overlap with the significance of this AAT in acinar cells. We envisage future studies to focus on the impact of LAT1 in pancreatic acinar cells with a conditional inducible knockout for LAT1, specifically in these secretory cells.

LAT1 ablation interfered with the mTOR pathway and regeneration of acinar cells in the early injury phase 24 h after acute pancreatitis induction

Another established characteristic of AP is the reduced pancreatic function evidenced by a decreased protein synthesis[43]. In acinar cells, the synthesis of digestive enzymes is regulated through the mTOR pathway[59] and amino acid availability[60]. The initial inhibition of the mTOR pathway, decreased

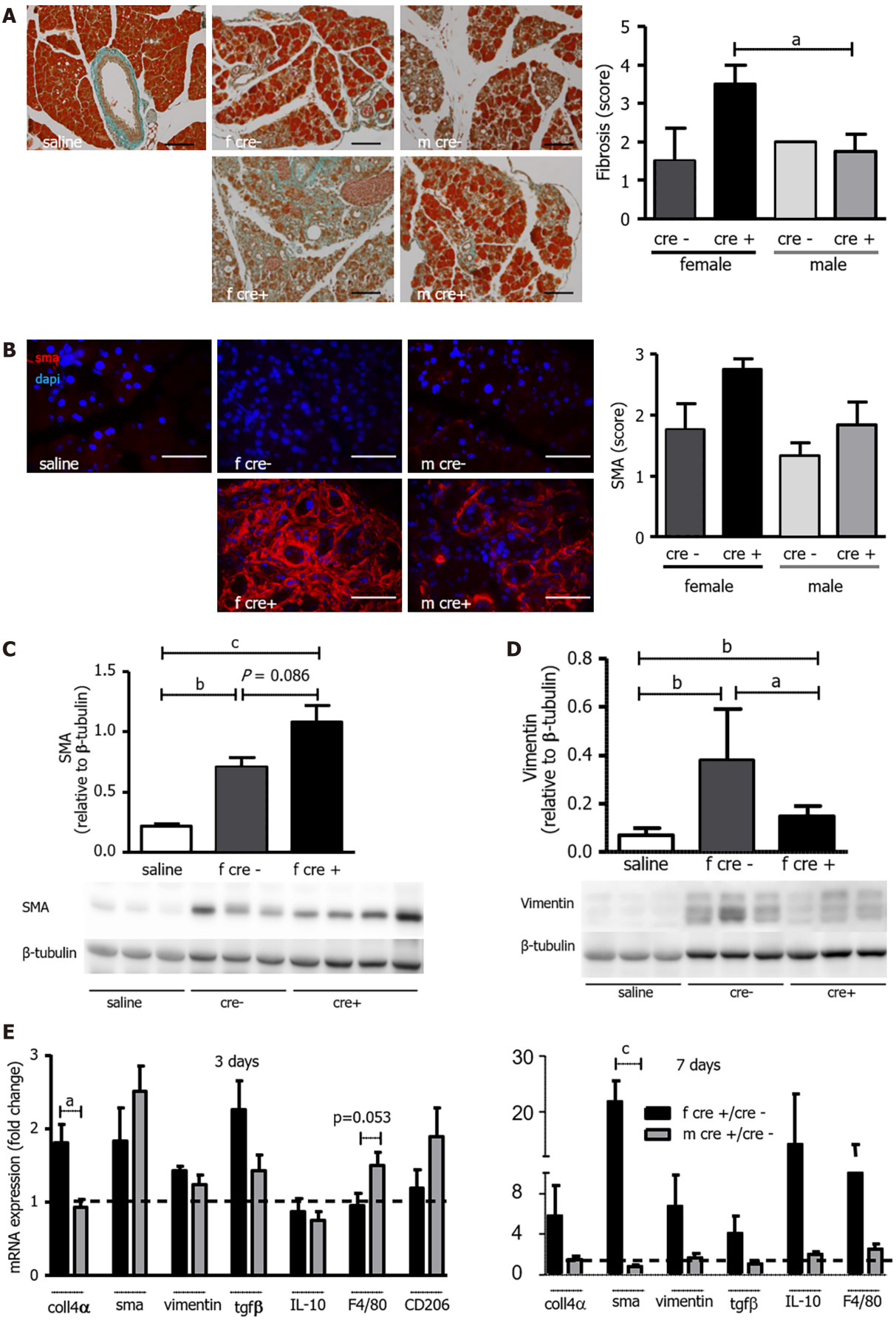


Figure 8 Female LAT1-ko mice presented with higher levels of fibrosis and expression of markers thereof. A: Female LAT1-ko mice presented with larger areas of fibrosis. Masson's trichrome staining (left) and quantification (right) of fibrosis in the pancreas three days after the onset of acute pancreatitis (AP).

A score from 0 to 5 was attributed to each section in a blinded fashion, which was dependent on the extent of the fibrosis. The Bar length corresponds to 100 μm. Mean ± SEM, *n* = 4-6. Statistical analysis was conducted with ANOVA and Bonferroni and then compared, as indicated by the capped line. *aP* < 0.05; B-D: Female LAT1-ko mice exhibited enhanced activation of pancreatic stellate cells (PSC) compared to their littermates; B: Immunohistochemistry (left) and quantification (right) for smooth muscle actin (SMA) in the pancreas three days after the onset of AP. A score from 0 to 3 was attributed to each section in a blinded fashion depending on the signal intensity of the SMA staining. The paraffin-embedded mouse pancreas was stained for SMA (red) and the nuclei were stained in blue (dapi). The bar length corresponds to 50 μm. Mean ± SEM, *n* = 5; C: SMA that was indicative of activated PSC was also analyzed with Western blotting (12% SDS-PAGE gel, cells' total lysates from female mice) three days after the onset of AP; this process led to a band being exhibited at approximately 42 kDa. The results were expressed relative to β-tubulin. Mean ± SEM, *n* = 3-6. Statistical analysis was conducted by ANOVA and Bonferroni and then compared, as indicated by the capped line. *bP* < 0.01, *cP* < 0.001; D: The marker for non-activated stellate cells of vimentin was less expressed in the female LAT1-ko mice. Vimentin was also analyzed by Western blotting (10% SDS-PAGE gel, cells' total lysates from female mice) three days after the onset of AP and showed a band at approximately 57 kDa. The results were expressed relative to β-tubulin. Mean ± SEM, *n* = 3. Statistical analysis was conducted with ANOVA and Bonferroni and then compared, as indicated by the capped line. *aP* < 0.05; E: Female LAT1-ko mice showed an elevated expression of markers for fibrosis and similar markers for macrophages when compared to male mice on day three. The mRNA levels of collagen 4α (coll4α), SMA, vimentin, transforming growth factor β, interleukin 10, F4/80, and CD206 were analyzed *via* qPCR and expressed as a fold change in comparison with LAT1-wt mice three and seven days after the onset of AP. The line indicates the expression level of LAT1-wt animals. Mean ± SEM, *n* = 5. Statistical analysis was conducted *via* an unpaired, two-tailed *t*-test, and a comparison was then made, as indicated by the capped line. *aP* < 0.05, *cP* < 0.001.

protein synthesis, and activation of autophagy have been shown as crucial for the dedifferentiation process. The following reactivation of the mTOR pathway allows cells to proliferate and differentiate and supports tissue repair[15]. In our model, mTOR reactivation 24 h after AP induction was less pronounced in LAT1-ko mice. As shown in other cell types, LAT1 works as a sensor for extracellular amino acid availability and upstream regulator of mTOR[25,58]. LAT1 ablation can inhibit this pathway [61], and our results suggest that LAT1 knockout partially blocked the reactivation of mTOR. Moreover, the GCN2 intracellular sensing pathway for amino acid availability was found enhanced after AP. GCN2 decreases general protein synthesis to reduce energy expenditure and induces upregulation of genes such as AAT (LAT2, xCT, SNAT3) and stress markers (activating transcription factor 4 (ATF4), CHOP, CHAC1)[45]. The induction of the starvation pathway *via* GCN2 was activated in both genotypes and was higher in female mice. More than that, we found certain sex differences between the regulation of GCN2 downstream genes during the early injury phase 24 h following the onset of AP. While SNAT3 and ATF4, C/EBP homologous protein (CHOP), and Glutathione Specific Gamma-Glutamylcyclotransferase 1 (CHAC1) (data not shown) behaved equally for all genotypes and sexes, LAT2 and xCT expression varied between female and male mice. These results imply that GCN2 downstream regulation in acinar cells is sex-dependent, as previously observed for mTOR[62]. Besides sex, distinctive regulation of the mTOR pathway depending on age and tissue type has been found in mice. Notably, in the liver and heart, mTOR is more enhanced in fasted female than male mice, while activation in muscle and fat revealed no sex difference. A better understanding of mTOR and GCN2 pathway regulation in the pancreas according to sex and age may be helpful to comprehend regeneration processes in different situations and influences of other components such as AAT.

Another source of amino acids during starvation arises through the activation of autophagy. Autophagy plays an important role during regeneration[15], and inhibited or defective autophagy can eventually delay this process[63]. We observed indicators of abnormally large autolysosomes (high LC3II/LC3I ratio) in female LAT1-ko mice and accumulation of cargo protein (p62) characteristic of defective autophagy in AP[64,65]. These results suggest that female LAT1-ko mice especially did not manage to compensate for the less activated mTOR pathway by increasing the catabolism of pre-existing proteins. To summarize, LAT1-ko mice showed blunted activation of the mTOR pathway and enhanced amino acid starvation accentuated in female mice with indicators of dysfunctional autophagy. Even though AP injury itself was not more severe, the ablation of LAT1 weakened the initial mechanisms that sustain regeneration in acinar cells.

Ablation of LAT1 leads to slower regeneration identified three to seven days after acute pancreatitis induction

We followed up for seven days to evaluate if the initial blunted metabolic adaptation observed in LAT1-ko mice influenced later regeneration steps. LAT1-ko mice, particularly females, presented accentuated weight loss and recovered more slowly than their LAT1-wt littermates. The knockout of LAT1 was induced by tamoxifen, which is known to change fat composition in female but not in male mice[66]. The enhanced weight loss in female LAT1-ko mice cannot be explained by tamoxifen alone since female LAT1-wt were likewise treated with the drug and did not present the same sustained weight loss. In addition to tamoxifen effects, the reduction in digestibility and absorption of nutrients generally taking place during AP[67] might have accounted for the more severe weight loss in females.

When it comes to functional pancreatic integrity, the transcription factor mist1 warrants further attention. Mist1 is highly expressed in a mature pancreas and governs digestive enzymes[68]. In rodents, transcription of digestive enzymes and differentiation marker mist1 decreases after AP induction[9,20]. Upon AP, mist1 expression is temporarily silenced to allow functional reduction, dedifferentiation, proliferation, and regeneration to occur[10]. Indeed, female LAT1-ko mice did not

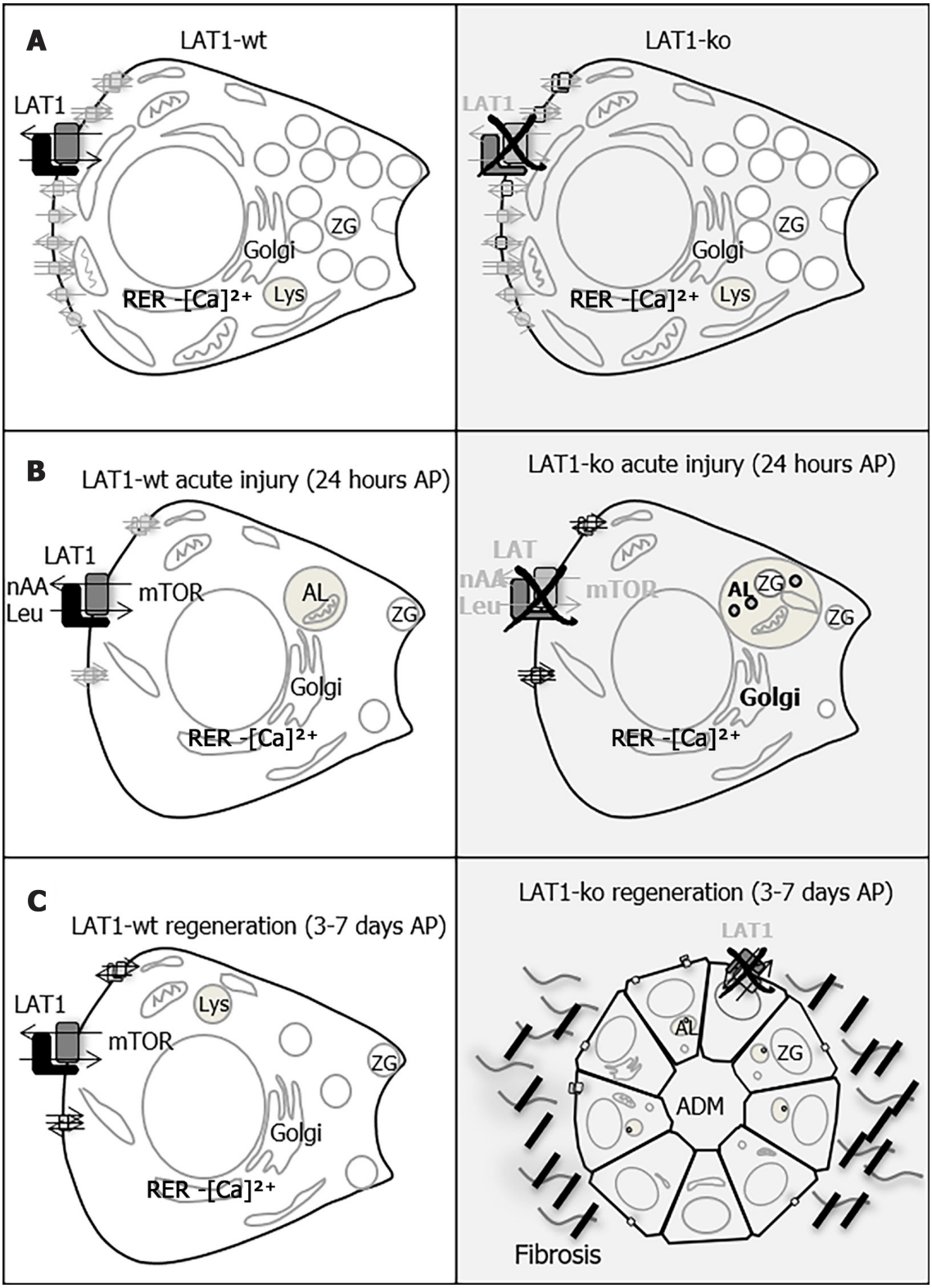


Figure 9 LAT1 ablation affects the initial metabolic adaptation that is necessary for the efficient repair after acute pancreatitis. Acinar cells have a pyramidal shape and display a typical secretory machinery with a well-developed rough endoplasmic reticulum (RER) and Golgi apparatus (Golgi), which are localized to the perinuclear region, as well as lysosomes (Lys). In the apical region, digestive enzymes are stored in zymogen granules (ZG). A: Amino acid transporters (AAT) are expressed on the basolateral membrane of acinar cells and are responsible for the transportation of amino acids into the cells to maintain the synthesis of secreted enzymes; this is so they can be used as an energy source and to guarantee cell growth and regeneration after injury. LAT1 exchanges leucine (Leu) with other neutral amino acids (nAA), and the amino acid flow acts as an upstream regulator of the mammalian target of rapamycin (mTOR) pathway, which regulates protein synthesis and enables cell differentiation/survival. Morphology, enzyme expression, and other AAT expressions did not differ between healthy LAT1 wild-type (LAT1-wt, white, left-hand panel) and healthy LAT1 knockout (LAT1-ko, gray, right-hand panel) mice over the period observed in this study; B: During the early phase of AP, the remaining acinar cells entered a metabolic arrest, dedifferentiated, and temporarily reduced their secretory phenotype. An increase in autophagy (AL, autolysosome), followed by the re-activation of the mTOR pathway is decisive for the later steps of regeneration. LAT1 expression is maintained on

acinar cells of LAT1-wt mice in this early phase, and it plays a role in the reactivation of mTOR. In LAT1-ko mice, the massive reduction of secretory enzyme expression was accompanied by markers of defective autophagy and a less-efficient activation of mTOR; C: Three days after the initial injury, LAT1-ko acinar cells were less differentiated and presented more areas of acinar-to-ductal metaplasia (ADM), expressed less secretory enzymes, and showed increased fibrosis. The delayed regeneration observed in acinar cells lacking LAT1 suggests that this occurred as a result of the improperly activated early metabolic response after injury.

recover mist1 expression until the end of the experiment. In agreement with the lower expression of differentiation markers, the expression of amylase indicative of pancreatic function was reduced compared to their female and male littermates. In humans, exocrine pancreatic insufficiency can develop even after milder episodes of AP, increasing the risk of malnutrition and malabsorption for prolonged periods after AP[69]. This process might have been amplified in female mice, where the ablation of LAT1 slowed the recovery of exocrine function after AP induction.

Mist1 is also an upstream regulator of the AAT SNAT5[9,70]. SNAT5 behaved as amylase and did not regain its normal expression level until the end of the experiment. This finding suggests that SNAT5 may play a role in protein synthesis, although knockout of this AAT did not induce changes in pancreas function or morphology of healthy mice[71]. In contrast to SNAT5, the AAT xCT and SNAT2 were still overexpressed three days after AP induction in all groups. They are known to be influenced by different cellular stress pathways. xCT expression is modulated by the master regulator of oxidative stress nuclear factor erythroid 2-related factor 2 (nrf2)[72], which was likewise elevated in all groups (data not shown). SNAT2 expression is controlled by the GCN2-ATF4 starvation pathway[73], which was increased in the earlier stages during AP. Interestingly, SNAT3 was overexpressed three days after AP induction in our model as well, but only in male mice. Since SNAT3 is influenced by androgens[74], the increased expression of this transporter might have an endocrine cause. Our results show that the expression of AAT during the regeneration phase varied, as observed for the early injury after AP induction. Thus, the regulation of AAT seems to be multifactorial, and a certain degree of sexual dimorphism in amino acid metabolism is present, as observed in the liver[75].

Further, mist1 directly controls the senescence marker p21 in acinar cells[76], which was enhanced during AP, especially in female LAT1-ko up to seven days. Overexpression of p21 has been correlated with the development of fibrosis[77], which can occur transiently during tissue regeneration and prolong recovery from AP[78,79]. Fibrosis is usually accompanied by the activation of PSC and the deposition of extracellular matrix (ECM) proteins[80]. Accordingly, we observed that female LAT1-ko mice presented higher deposition of ECM and showed higher activation of PSC, despite tamoxifen treatment. Tamoxifen is known to reduce PSC activation in the liver[81] and pancreas[82], especially in males[83]. Cae- and saline-injected mice from both genotypes and sexes were treated with tamoxifen to minimize the outcome in our setup. Thus, tamoxifen alone did not account for the difference in PSC activation and ECM synthesis. This implies that the delayed recovery in female mice was mainly caused by LAT1 ablation.

The maintenance or resolution of fibrosis is also regulated by metalloproteases released by specific populations of macrophages[49]. Throughout the phases of acute inflammation to recovery, the macrophage profile changes from M1- to M2-like, with M1 active during the early injury and M2 playing a role during regeneration. The ablation of macrophages in the early injury phase protects the tissue, while the ablation of macrophages three days after injury delays regeneration[84]. Interestingly, female LAT1-ko mice exhibited indicators of fewer macrophages in the tissue during the initial injury phase but partially recovered macrophage markers by day three. Moreover, the marker for M2 macrophages did not differ between LAT1-ko females and males. This implies that macrophages were not solely responsible for the increased ECM deposition in female LAT1-ko.

Finally, although we know that acinar cells can activate PSC[85], it remains unexplored whether changes in acinar cells' amino acid metabolism can influence the microenvironment and promote PSC activation or ECM deposition. Insight into these mechanisms might advance the understanding of pancreatic disease with future clinical implications.

CONCLUSION

Altogether, we showed that the ablation of LAT1 (*slc7a5*) affects the initial metabolic adaptation necessary for the efficient repair after AP, finally delaying regeneration and enhancing fibrosis (Figure 9A-C). This study revealed important elements for a better understanding of repair processes in pancreatic tissue after AP. Equally significant, with gender medicine evolving in diverse fields[86], including in the clinical setting of AP[87,88], we suggest that a higher consideration of sex differences with separate evaluation of female and male specimens should also be established in pre-clinical studies of AP.

ARTICLE HIGHLIGHTS

Research background

As a highly metabolically active organ, the pancreas depends on a constant supply of amino acids to synthesize digestive enzymes and for use as an energy source. This multifunctionality is reflected in the broad range of expressed amino acid transporters (AAT). Furthermore, after acute injury, the organ is capable of regeneration. Following acute pancreatitis (AP), acinar cells change their metabolic and functional state, initially dedifferentiating, increasing proliferation, and finally restoring their secretory function. AAT that are expressed in pancreatic acinar cells might play an additional role, which involves controlling the transcription of secreted enzymes and thus influencing the course of pancreatic disease and regeneration. LAT1 works as a sensor for extracellular amino acid availability and is an upstream regulator of the mammalian target of rapamycin pathway. This transporter is overexpressed in highly proliferative cells. Additionally, our previous study showed that LAT1 is expressed in acinar cells and that LAT1 expression in the early phase of AP is maintained in a different way to other AAT.

Research motivation

Lasting pancreatic disease is often detrimental to the quality and duration of life. Despite the high incidence of AP, there are no specific treatments, yet understanding pathophysiological processes is a crucial part of enabling future studies on preventative and therapeutic options. The role of AAT in this process is not comprehensively understood. Their pattern of expression may indicate that necessary metabolic adjustments are needed to meet the requirements for regeneration and reconstitution of function, which could be used as a supportive intervention during treatment.

Research objectives

The focus of the present study was to analyze metabolic alterations in the pancreas during acute inflammation by concentrating on the role of LAT1. The degree of initial injury as well as the course of regeneration were studied to investigate the significance of LAT1.

Research methods

To investigate the role of LAT1, an inducible knockout mouse model was applied, and the course of caerulein- (cae-) induced AP was studied in line with the phases of injury through to regeneration. Moreover, we analyzed the function of acinar cells and several markers of dedifferentiation and metabolic pathways.

Research results

The absence of LAT1 did not cause more severe injury during AP, but it did alter early metabolic adaptation. Consequently, differentiation and the function of acinar cells in LAT1 knockout mice were reduced, which influenced the regeneration phase. A sex-related difference was also revealed: female mice sustained more pronounced cell dedifferentiation and fibrosis.

Research conclusions

LAT1 supports the regeneration of pancreatic acinar cells after AP *via* metabolic adaptation, influencing their differentiation as well as their recovery of function and phenotype. The knocking out of LAT1 had a more accentuated effect in female mice, suggesting that a sex-dependent dimorphism in AAT is present in the pancreas.

Research perspectives

Future studies should focus on the role of AAT and metabolic pathways in acinar cells by using conditional knockout models. An acinar-cell conditional knockout or overexpressing mouse model will allow us to analyze the effect of AAT, specifically in pancreatic cells, without affecting their expression in immune cells, which play an important role in the development of AP. Additionally, induction methods other than tamoxifen should be tried, and sex-dependent differences as well as the influence of age should be further considered. These models will enable a better understanding of the metabolic mechanisms, guaranteeing the survival of acinar cells after different insults.

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FOOTNOTES

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Country/Territory of origin: Switzerland

ORCID number: Cristina M Hagen 0000-0002-0393-4154; Eva Roth 0000-0001-9707-1355; Theresia Reding Graf 0000-0003-4613-0874; François Verrey 0000-0003-3250-9824; Rolf Graf 0000-0001-7449-7302; Anurag Gupta 0000-0001-6850-3489; Giovanni Pellegrini 0000-0001-9593-5578; Nadège Poncet 0000-0002-0199-994X; Simone Mafalda Rodrigues Camargo 0000-0003-0091-7380.

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ORIGINAL ARTICLE

Retrospective Study

Diagnostic performance of endoscopic classifications for neoplastic lesions in patients with ulcerative colitis: A retrospective case-control study

Yuichi Kida, Takeshi Yamamura, Keiko Maeda, Tsunaki Sawada, Eri Ishikawa, Yasuyuki Mizutani, Naomi Kakushima, Kazuhiro Furukawa, Takuya Ishikawa, Eizaburo Ohno, Hiroki Kawashima, Masanao Nakamura, Masatoshi Ishigami, Mitsuhiro Fujishiro

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Yuichi Kida, Takeshi Yamamura, Eri Ishikawa, Yasuyuki Mizutani, Naomi Kakushima, Kazuhiro Furukawa, Takuya Ishikawa, Eizaburo Ohno, Masanao Nakamura, Masatoshi Ishigami, Mitsuhiro Fujishiro, Department of Gastroenterology and Hepatology, Nagoya University Graduate School of Medicine, Nagoya 466-8550, Aichi, Japan

Keiko Maeda, Tsunaki Sawada, Hiroki Kawashima, Department of Endoscopy, Nagoya University Hospital, Nagoya 466-8560, Aichi, Japan

Mitsuhiro Fujishiro, Department of Gastroenterology, Graduate School of Medicine, The University of Tokyo, Tokyo 113-8655, Japan

Corresponding author: Takeshi Yamamura, MD, PhD, Assistant Professor, Department of Gastroenterology and Hepatology, Nagoya University Graduate School of Medicine, 65 Tsuruma-cho, Showa-ku, Nagoya 466-8550, Aichi, Japan. tyamamu@med.nagoya-u.ac.jp

Abstract

BACKGROUND

It is unclear whether the Japan Narrow-Band Imaging Expert Team (JNET) classification and pit pattern classification are applicable for diagnosing neoplastic lesions in patients with ulcerative colitis (UC).

AIM

To clarify the diagnostic performance of these classifications for neoplastic lesions in patients with UC.

METHODS

This study was conducted as a single-center, retrospective case-control study. Twenty-one lesions in 19 patients with UC-associated neoplasms (UCAN) and 23 lesions in 22 UC patients with sporadic neoplasms (SN), evaluated by magnifying image-enhanced endoscopy, were retrospectively and separately assessed by six endoscopists (three experts, three non-experts), using the JNET and pit pattern classifications. The results were compared with the pathological diagnoses to evaluate the diagnostic performance. Inter- and intra-observer agreements were

calculated.

RESULTS

In this study, JNET type 2A and pit pattern type III/IV were used as indicators of low-grade dysplasia, JNET type 2B and pit pattern type V_I low irregularity were used as indicators of high-grade dysplasia to shallow submucosal invasive carcinoma, JNET type 3 and pit pattern type V_I high irregularity/V_N were used as indicators of deep submucosal invasive carcinoma. In the UCAN group, JNET type 2A and pit pattern type III/IV had a low positive predictive value (PPV; 50.0% and 40.0%, respectively); however, they had a high negative predictive value (NPV; 94.7% and 100%, respectively). Conversely, in the SN group, JNET type 2A and pit pattern type III/IV had a high PPV (100% for both) but a low NPV (63.6% and 77.8%, respectively). In both groups, JNET type 3 and pit pattern type V_I-high irregularity/V_N showed high specificity. The inter-observer agreement of JNET classification and pit pattern classification for UCAN among experts were 0.401 and 0.364, in the same manner for SN, 0.666 and 0.597, respectively. The intra-observer agreements of JNET classification and pit pattern classification for UCAN among experts were 0.387, 0.454, for SN, 0.803 and 0.567, respectively.

CONCLUSION

The accuracy of endoscopic diagnosis using both classifications was lower for UCAN than for SN. Endoscopic diagnosis of UCAN tended to be underestimated compared with the pathological results.

Key Words: Diagnostic performance; Japan Narrow-Band Imaging Expert Team classification; Pit pattern classification; Sporadic neoplasms; Ulcerative colitis; Ulcerative colitis-associated neoplasms

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Core Tip: This retrospective case-control study evaluated the diagnostic performance of the Japan Narrow-Band Imaging Expert Team (JNET) and pit pattern classifications for neoplastic lesions in patients with ulcerative colitis (UC). The JNET and pit pattern classifications did not show high accuracy in diagnosing the pathology and invasion depth of neoplastic lesions in patients with UC. Endoscopic diagnosis of UC-associated neoplasms tended to be underestimated when compared with pathological results. Endoscopic diagnosis of neoplastic lesions in patients with UC is still difficult, and treatment strategies need to be carefully determined.

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INTRODUCTION

Patients with long-standing ulcerative colitis (UC) are at risk for colorectal tumors due to chronic inflammation. The cumulative risk of colorectal cancer at 10, 20, and 30 years after UC onset are reportedly 1.6%, 8.3%, and 18.4%, respectively[1]. Consequent to improvements in UC treatment, long-standing UC cases have gradually increased, and surveillance colonoscopy has become more important. UC patients are exposed to the risk of not only UC-associated neoplasms (UCAN) but also sporadic neoplasms (SN). As the treatment strategy for UCAN greatly differs from that for SN, distinguishing UCAN from SN is important[2]. In line with the Surveillance for Colorectal Endoscopic Neoplasia Detection and Management in Inflammatory Bowel Disease Patients: International Consensus Recommendations (SCENIC) consensus statement, endoscopic resection now tends to be accepted as a treatment for endoscopically visible dysplasia[3]. With the support from the SCENIC consensus statement, endoscopic treatments for visible dysplasia have gradually increased and have attracted attention recently[4-6].

UCAN differentiation by endoscopic findings had been described previously. Prior studies revealed that features of surface structure and vascular pattern obtained by magnifying Narrow-Band Imaging (NBI) and chromoendoscopy are useful in diagnosing UCAN[7-11]. Additionally, multimodal endo-

Table 1 Clinical characteristics of ulcerative colitis patients with neoplastic lesions (mean ± SD)

	UCAN group (*n* = 19)	SN group (*n* = 22)	*P* value
Age at UC onset (yr)	35.7 ± 10.9	48.8 ± 14.6	0.003[a]
Disease duration (yr)	17.8 ± 9.4	12.9 ± 10.7	0.120[a]
Sex			0.829[b]
Male	11	12	
Female	8	10	
Extent of disease			0.231[b]
Total colitis	16	14	
Left-sided colitis	3	6	
Proctitis	0	2	
Clinical type			0. 139[b]
Relapse and remission	10	15	
Chronic persistent	9	5	
First attack	0	2	
Pathological type			< 0.001[b]
LGD	2	16	
HGD	11	5	
Shallow submucosal invasive carcinoma	3	0	
Deep submucosal invasive carcinoma	5	2	
Primary sclerosing cholangitis	1	0	0.463[c]
Family history of colorectal cancer	1	2	0.639[c]

[a]Student *t*-test.
[b]Chi-square test.
[c]Fisher's exact test.
P < 0.05 was considered statistically significant. SD: Standard deviation; HGD: High-grade dysplasia; LGD: Low-grade dysplasia; SN: Sporadic neoplasms; UC: Ulcerative colitis; UCAN: Ulcerative colitis-associated neoplasms.

scopic classification without the use of magnifying endoscopy has been reported[12]. Most of these studies focused on differentiating neoplastic from non-neoplastic lesions or on detecting these lesions, not on qualitatively diagnosing neoplastic lesions in UC patients. While several studies have focused on differentiating UCAN from non-neoplastic lesions, few reports have explored the differentiation of UCAN from SN[2].

The Japan NBI Expert Team (JNET) and pit pattern classifications are useful for determining the pathology and invasion depth of colorectal tumors[13,14]. Both classifications have high reproducibility and good diagnostic accuracy in terms of pathology and invasion depth[15-19], as well as good intra- and inter-observer agreement rates for diagnosing colorectal tumors[18,20]. Dysplastic pit patterns are sometimes observed even in non-dysplastic lesions due to inflammation and regenerative changes in UC patients[21]. Surface and vascular patterns are modified by inflammation in UCAN[22]. These patterns are likely to be modified by inflammation not only in UCAN but also in SN located in the inflamed mucosa. Therefore, whether these endoscopic classifications apply to the diagnosis of neoplastic lesions in UC patients remains unclear. Only a few reports have described the usefulness of both classifications in diagnosing UCAN[22], and there have been no reports on their use for classifying SN in UC patients. Hence, the present retrospective case-control study aimed to evaluate the diagnostic performance of the JNET and pit pattern classifications for neoplastic lesions in UC patients.

MATERIALS AND METHODS

Patients

A total of 89 UC patients who had neoplastic lesions that could be pathologically evaluated by biopsy, endoscopic resection, or surgery and who underwent colonoscopy at Nagoya University Hospital from

Table 2 Characteristics of endoscopic and macroscopic findings

	UCAN group (*n* = 21)	SN group (*n* = 23)	*P* value
Tumor location			0.044[a]
Proctosigmoid colon	17	12	
Others	4	11	
Tumor color			0.032[a]
Red	15	9	
Pale or the same as the surrounding mucosa	6	14	
Lesion border			< 0.001[a]
Clear	5	23	
Unclear	16	0	
Tumor morphology			0.173[a]
Pedunculated	0	0	
Sessile	8	7	
Superficial elevated	7	14	
Flat	2	0	
Depressed	4	2	
UCEIS (median, range)	2 (0–4)	0 (0–5)	< 0.001[b]

[a]Chi-square test.
[b]Mann–Whitney *U* test.
P < 0.05 was considered statistically significant. SN: Sporadic neoplasms; UCAN: Ulcerative colitis-associated neoplasms; UCEIS: Ulcerative Colitis Endoscopic Index of Severity.

August 2005 to April 2020 were consecutively registered. Neoplastic lesions located in the colonic mucosa outside the previously or currently inflamed mucosa were excluded. Additionally, lesions magnified using both NBI or Blue LASER imaging (BLI) and chromoendoscopy with indigo carmine or crystal violet were included. The present study ultimately enrolled 41 UC patients with 44 lesions that could be assessed using both the JNET and pit pattern classifications. According to pathological findings, these patients were divided into two groups—namely, the UCAN group, which comprised 19 patients with 21 lesions, and the SN group, which consisted of 22 UC patients with 23 lesions.

Endoscopic evaluation

Endoscopists conducted routine white-light imaging observation. When neoplastic lesions were identified, magnifying NBI or BLI and magnifying chromoendoscopy using indigo carmine or crystal violet were performed. All lesions were endoscopically detectable, visually identified, and subsequently diagnosed using target biopsy. The morphological type of neoplasms was categorized in accordance with the SCENIC consensus statement[3]. The severity of inflammation in the mucosa surrounding neoplasms was assessed using the Ulcerative Colitis Endoscopic Index of Severity (UCEIS)[23]. The JNET and pit pattern classifications were employed to evaluate the pathology and invasion depth of neoplasms by endoscopy. With the JNET classification, lesions were categorized based on surface and vascular patterns into types 1, 2A, 2B, and 3. The pit pattern classification was used under indigo carmine or crystal violet observation; lesions were categorized based on form of crypt orifices into types I, II, III, IV, V_I low irregularity, V_I high irregularity, and V_N. This study used JNET type 2A and pit pattern type III/IV as indicators of low-grade dysplasia (LGD), based on previous reports[22]. Furthermore, in the same manner, as LGD, JNET type 2B and pit pattern type V_I low irregularity were utilized as indicators of high-grade dysplasia (HGD) to shallow submucosal invasive carcinoma (sSM), whereas JNET type 3 and pit pattern type V_I high irregularity/V_N were used as indicators of deep submucosal invasive carcinoma (dSM). Endoscopic images corresponding to the part that could be evaluated pathologically were extracted, and six endoscopists (three experts, three non-experts) each evaluated the endoscopic findings. Experts were defined as those with ≥ 5-year experience in magnifying image-enhanced endoscopy and who had managed more than 1000 cases[18]. Endoscopists independently evaluated the images obtained from 44 lesions; when individual diagnostic interpretations differed, they discussed the case until a consensus was reached. Diagnostic performance was assessed by consensus of the first diagnosis of three endoscopists. Inter- and intra-observer agreements

were calculated for the diagnostic results of each endoscopist. The second diagnosis was performed by randomly switching the order of images at ≥ 1 mo after the first round of diagnosis to calculate for intra-observer agreement.

Pathological assessment

Two pathologists specializing in the gastrointestinal tract conducted pathological diagnosis of UCAN and SN according to the Riddell *et al*[24]'s pathological system. UCAN and SN were differentiated based on pathological results. If necessary, p53 and Ki-67 immunostaining were performed. UCAN was diagnosed for cases with diffuse and strong expression or complete absence of p53 immunostaining [25]. Differentiation of Ki-67-positive cells from the basal mucosal side toward the superficial mucosal side[25,26], called "bottom-up," was also useful in diagnosing UCAN. Contrary to the UCAN, expression of p53 is low in SN. Moreover, Ki-67-positive cells are mainly distributed at the superficial zone of the mucosal layer, and tumor cells differentiate towards the basal side of the mucosa in the SN [26], also known as "top-down". Dysplasia was classified into LGD and HGD according to the degree of cellular and nuclear dysplastic change. Submucosal invasive carcinoma was divided into dSM and sSM depending on whether the vertical invasion depth exceeded 1000 μm. When two pathologists had different diagnoses, they discussed the case until a conclusion was reached.

Data collection

Clinical data, including age at UC onset, disease duration, sex, disease distribution (total colitis, left-sided colitis, proctitis), clinical type (relapse and remission, chronic persistent, first attack), primary sclerosing cholangitis (PSC), and family history of colorectal cancer, were retrospectively collected from medical records and investigated. Endoscopic findings, including location, color, lesion border, morphology, and UCEIS, were obtained from medical reports and evaluated.

Statistical analysis

Continuous variables were expressed as mean ± SD or as median with range and were compared using Student's *t*-test or Mann–Whitney *U* test, depending on the normality of data distribution, as determined by the Shapiro–Wilk test. Categorial variables were compared using Fisher's exact test or chi-square test. Sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and accuracy were calculated for both the JNET and pit pattern classifications. Inter- and intra-observer agreements were calculated using κ coefficient and arbitrarily interpreted as follows: 0–0.20, poor; 0.21–0.40, fair; 0.41–0.60, moderate; 0.61–0.80, substantial; and 0.81–1.00, excellent. Statistical analysis was conducted using SPSS Statistics version 27 (IBM Corp., Armonk, NY, United States) and R3.6.3 (CRAN, freeware, https://personal.hs. hirosaki-u.ac.jp/pteiki/reserch/stat/R/), with $P < 0.05$ being indicative of statistical significance.

RESULTS

Clinical characteristics

All patients were divided into two groups according to pathological findings. Inter-observer agreement in the diagnosis between UCAN and SN by the two pathologists was 0.531. The clinical characteristics of both groups are summarized in Table 1. The UCAN group had a significantly lower mean age at UC onset than the SN group (35.7 *vs* 48.8 years, $P = 0.003$). Pathological findings indicated 2 LGD lesions, 11 HGD lesions, 3 sSM lesions, and 5 dSM lesions in the UCAN group and 16 LGD lesions, 5 HGD lesions, and 2 dSM lesions in the SN group. No significant differences in the disease duration, sex, extent of disease, disease distribution, presence of PSC, and family history of colorectal cancer were identified between the two groups.

Endoscopic and clinical findings

The endoscopic findings for both groups are presented in Table 2. A total of 17 (81.0%) and 12 (52.2%) lesions were detected in the proctosigmoid colon in the UCAN and SN groups, respectively ($P = 0.044$). The UCAN group had a higher percentage of reddish lesions than the SN group (71.4% *vs* 39.1%, $P = 0.032$). All lesions in the SN group exhibited a clear border, whereas 16 lesions (76.2%) in the UCAN group showed an unclear border ($P < 0.001$). The UCAN group had a higher proportion of flat or depressed lesions than the SN group (28.6% *vs* 8.7%, $P = 0.094$). Inflammation in the mucosa surrounding neoplasms was more severe in the UCAN group than in the SN group [UCEIS (median): 2 *vs* 0, $P < 0.001$].

Diagnostic performance of the JNET and pit pattern classifications

Diagnostic performance for each type in the JNET and pit pattern classifications is shown in Tables 3 and 4, respectively. Sensitivity, specificity, PPV, NPV, and accuracy were calculated for experts and non-experts separately.

Table 3 Diagnostic performance (95% confidence interval) for each type in the Japan Narrow-Band Imaging Expert Team classification

	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Accuracy (%)
UCAN JNET type 2A					
Experts	50.0 (10.2–85.6)	94.7 (90.5–98.5)	50.0 (10.2–85.6)	94.7 (90.5–98.5)	90.5 (82.9–97.3)
Non-experts	100 (36.9–100)	78.9 (72.3–78.9)	33.3 (12.3–33.3)	100 (91.6–100)	81.0 (68.9–81.0)
UCAN JNET type 2B					
Experts	78.6 (64.6–89.5)	57.1 (29.2–79.1)	78.6 (64.6–89.5)	57.1 (29.2–79.1)	71.4 (52.8–86.1)
Non-experts	78.6 (63.8–87.9)	71.4 (41.9–90.0)	84.6 (68.7–94.6)	62.5 (36.6–78.8)	76.2 (56.5–88.6)
UCAN JNET type 3					
Experts	60.0 (27.5–75.9)	93.8 (83.6–98.7)	75.0 (34.4–94.9)	88.2 (78.7–92.9)	85.7 (70.3–93.3)
Non-experts	40.0 (14.9–40.0)	100 (92.2–100)	100 (37.3–100)	84.2 (77.6–84.2)	85.7 (73.8–85.7)
SN JNET type 2A					
Experts	75.0 (62.6–75.0)	100 (71.6–100)	100 (83.4–100)	63.6 (45.5–63.6)	82.6 (65.3–82.6)
Non-experts	87.5 (74.7–92.5)	85.7 (56.4–97.1)	93.3 (79.6–98.7)	75.0 (49.3–85.0)	87.0 (69.1–93.9)
SN JNET type 2B					
Experts	100 (63.4–100)	83.3 (73.2–83.3)	62.5 (39.6–62.5)	100 (87.8–100)	87.0 (71.0–87.0)
Non-experts	80.0 (42.6–96.1)	83.3 (72.9–87.8)	57.1 (30.4–68.7)	93.8 (82.1–98.8)	82.6 (66.4–89.6)
SN JNET type 3					
Experts	50.0 (11.2–50.0)	100 (96.3–100)	100 (22.4–100)	95.5 (91.9–95.5)	95.7 (88.9–95.7)
Non-experts	50.0 (11.2–50.0)	100 (96.3–100)	100 (22.4–100)	95.5 (91.9–95.5)	95.7 (88.9–95.7)

CI: Confidence interval; NPV: Negative predictive value; PPV: Positive predictive value; SN: Sporadic neoplasms; UCAN: Ulcerative colitis-associated neoplasms; JNET: Japan Narrow-Band Imaging Expert Team.

In the UCAN group, JNET type 2A had a low PPV [experts *vs* non-experts: 50.0% (10.2–85.6) *vs* 33.3% (12.3–33.3)] and a high NPV [experts *vs* non-experts: 94.7% (90.5–98.5) *vs* 100% (91.6–100)] for both experts and non-experts. Conversely, in the SN group, JNET type 2A had a high PPV [experts *vs* non-experts: 100% (83.4–100) *vs* 93.3% (79.6–98.7)] and a low NPV [experts *vs* non-experts: 63.6% (45.5–63.6) *vs* 75.0% (49.3–85.0)]. In the UCAN group, the accuracy of diagnosis for JNET types 2A, 2B, and 3 by experts was 90.5%, 71.4%, and 85.7%, respectively, and that by non-experts was 81.0%, 76.2%, and 85.7%, respectively.

In the UCAN group, pit pattern type III/IV had a low PPV [experts *vs* non-experts: 40.0% (14.9–40.0) *vs* 20.0% (7.3–20.0)] and a high NPV [experts *vs* non-experts: 100% (92.2–100) *vs* 100% (88.4–100)] for both experts and non-experts. Conversely, in the SN group, pit pattern type III/IV had a high PPV [experts *vs* non-experts: 100% (86.4–100) *vs* 92.9% (77.8–98.6)] and a low NPV [77.8% (56.6–77.8) *vs* 66.7% (43.3–75.6)]. In the UCAN group, the accuracy of diagnosis for pit pattern type III/IV, type V_I low irregularity, and type V_I high irregularity/V_N by experts was 85.7%, 57.1%, and 76.2%, respectively, and that by non-experts was 61.9%, 57.1%, and 85.7%, respectively. The accuracy of diagnosis for JNET type 3 and pit pattern type V_I high irregularity/V_N by both experts and non-experts was higher in the SN group than in the UCAN group. Figure 1 shows a representative case of UCAN misdiagnosed by all endoscopists.

Intra-observer and inter-observer agreements for the JNET and pit pattern classifications

Intra-observer agreement was separately calculated for experts and non-experts (Table 5). The intra-observer agreement among experts for the JNET classification of UCAN, pit pattern classification of UCAN, JNET classification of SN, and pit pattern classification of SN was 0.387, 0.454, 0.803, and 0.567, respectively. The corresponding values for non-experts were 0.640, 0.569, 0.828, and 0.628, respectively. The intra-observer agreement for SN was higher than that for UCAN. Among non-experts, the intra-observer agreement for both UCAN and SN was higher with the JNET classification than with the pit pattern classification.

Inter-observer agreement was calculated similarly (Table 6). The inter-observer agreement among experts for the JNET classification of UCAN, pit pattern classification of UCAN, JNET classification of SN, and pit pattern classification of SN was 0.401, 0.364, 0.666, and 0.597, respectively. The corresponding values for non-experts were 0.237, 0.378, 0.503, and 0.437, respectively. Overall, the inter-

Table 4 Diagnostic performance (95% confidence interval) for each type in the pit pattern classification

	Sensitivity, (%)	Specificity, (%)	PPV, (%)	NPV, (%)	Accuracy, (%)
UCAN pit pattern type III/IV					
Experts	100 (37.3–100)	84.2 (77.6–84.2)	40.0 (14.9–40.0)	100 (92.2–100)	85.7 (73.8–85.7)
Non-experts	100 (36.4–100)	57.9 (51.2–57.9)	20.0 (7.3–20.0)	100 (88.4–100)	61.9 (49.8–61.9)
UCAN pit pattern type VI low irregularity					
Experts	50.0 (35.2–59.8)	71.4 (41.9–91.0)	77.8 (54.8–93.0)	41.7 (24.4–53.1)	57.1 (37.5–70.2)
Non-experts	42.9 (28.4–48.7)	85.7 (56.7–97.3)	85.7 (56.7–97.3)	42.9 (28.4–48.7)	57.1 (37.8–64.9)
UCAN pit pattern type VI high irregularity/VN					
Experts	60.0 (26.1–85.8)	81.3 (70.7–89.3)	50.0 (21.8–71.5)	86.7 (75.4–95.3)	76.2 (60.1–88.5)
Non-experts	60.0 (27.5–75.9)	93.8 (83.6–98.7)	75.0 (34.4–94.9)	88.2 (78.7–92.9)	85.7 (70.3–93.3)
SN pit pattern type III/IV					
Experts	87.5 (75.6–87.5)	100 (72.8–100)	100 (86.4–100)	77.8 (56.6–77.8)	91.3 (74.8–91.3)
Non-experts	81.3 (68.1–86.3)	85.7 (55.6–97.3)	92.9 (77.8–98.6)	66.7 (43.3–75.6)	82.6 (64.3–89.6)
SN pit pattern type VI low irregularity					
Experts	100 (63.4–100)	83.3 (73.2–83.3)	62.5 (39.6–62.5)	100 (87.8–100)	87.0 (71.0–87.0)
Non-experts	60.0 (26.0–85.9)	83.3 (73.9–90.5)	50.0 (21.7–71.6)	88.2 (78.2–95.8)	78.3 (63.5–89.5)
SN pit pattern type VI high irregularity/VN					
Experts	50.0 (11.2–50.0)	100 (96.3–100)	100 (22.4–100)	95.5 (91.9–95.5)	95.7 (88.9–95.7)
Non-experts	50.0 (10.2–85.6)	95.2 (91.4–98.6)	50.0 (10.2–85.6)	95.2 (91.4–98.6)	91.3 (84.4–97.5)

CI: Confidence interval; NPV: Negative predictive value; PPV: Positive predictive value; SN: Sporadic neoplasms; UCAN: Ulcerative colitis-associated neoplasms.

Table 5 Intra-observer agreement

	Experts	Non-experts
UCAN		
JNET classification	0.387 (0.369–0.521)	0.640 (0.566–0.708)
Pit pattern classification	0.454 (0.391–0.509)	0.569 (0.422–0.599)
SN		
JNET classification	0.803 (0.581–0.832)	0.828 (0.686–0.849)
Pit pattern classification	0.567 (0.477–0.595)	0.628 (0.422–0.766)

JNET: Japan Narrow-Band Imaging Expert Team; SN: Sporadic neoplasms; UCAN: Ulcerative colitis-associated neoplasms.

observer agreement for SN was higher than that for UCAN among both experts and non-experts, irrespective of the classification system used.

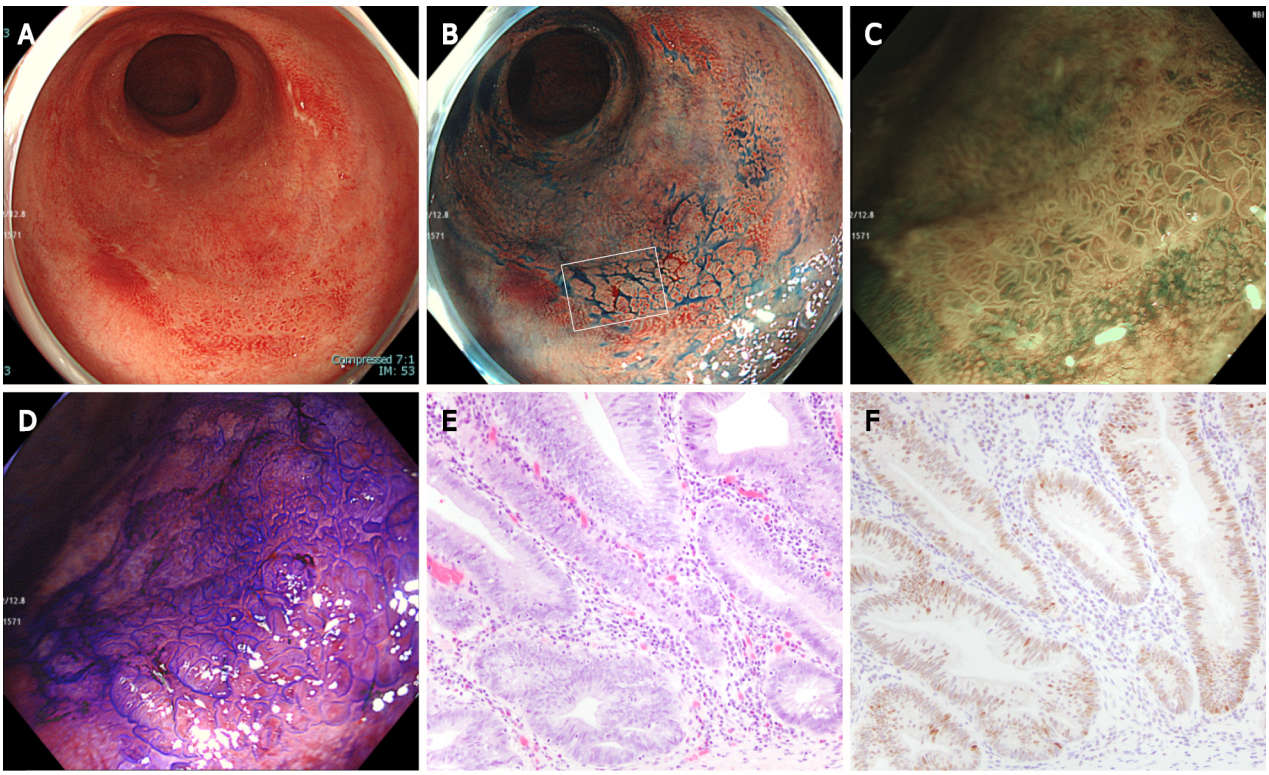
DISCUSSION

In the present study, we evaluated the performance of the JNET and pit pattern classifications in patients with UC. The JNET classification evaluates the tumors' surface and vascular patterns, whereas the pit pattern classification assesses the form of pits on the tumor surface. Colonic mucosal inflammation in UC patients modifies the tumors' surface and vascular patterns and is considered to reduce the diagnostic accuracy of both classifications. Here, we revealed that the accuracy of diagnosing colorectal tumors using JNET and pit pattern classifications was lower in UC patients, particularly those with UCAN, than in non-UC patients[15-18]. The agreement rates were lower for both UCAN and SN

Table 6 Inter-observer agreement

	Experts	Non-experts
UCAN		
JNET classification	0.401	0.237
Pit pattern classification	0.364	0.378
SN		
JNET classification	0.666	0.503
Pit-pattern classification	0.597	0.437

JNET: Japan Narrow-Band Imaging Expert Team; SN: Sporadic neoplasms; UCAN: Ulcerative colitis-associated neoplasms.



DOI: 10.3748/wjg.v28.i10.1055 **Coptright** © The Author(s) 2022.

Figure 1 Endoscopic features of ulcerative colitis-associated neoplasms misdiagnosed by all endoscopists. A: White-light imaging reveals a flat elevated lesion in the rectum; B: Chromoendoscopy with indigo carmine shows a clear lesion border; C: Magnifying endoscopy with narrow-band imaging of box in (B) shows regular surface and vascular patterns, which were classified by all endoscopists as Japan Narrow-Band Imaging Expert Team classification type 2A; D: Magnifying endoscopy with crystal violet chromoendoscopy of box in (B) reveals relatively uniform villous structures, which were classified by all endoscopists as pit pattern type IV; E: Pathological examination of the resected specimen by endoscopic submucosal dissection shows architectural atypia. This lesion was pathologically diagnosed as high-grade dysplasia (hematoxylin and eosin staining, original magnification × 50); F: Immunohistochemistry for p53 on serial section of (E).

patients than for non-UC patients. The diagnostic performance of both classifications in UC patients is substantially lower than their previously reported diagnostic performance in non-UC patients[15-18].

Previous reports revealed that, compared to SN, UCAN is more common in the proctosigmoid colon and features more redness, unclear border, flat and depressed lesions, and a higher degree of surrounding inflammation[2]. On magnifying chromoendoscopy, pit pattern types III, IV, and V, which are also caused by regenerative changes, are useful in diagnosing UCAN[9,21]. In the present study, the UCAN group had a significantly higher proportion of lesions with endoscopically unclear border and severe inflammation in the mucosa surrounding neoplasms than the SN group. As colorectal tumors can considerably impact the quality of life of UC patients, it is essential for endoscopists to understand these endoscopic features of UCAN.

Table 7 Diagnostic results of Japan Narrow-Band Imaging Expert Team classification type 2A and pit pattern classification type III/IV in ulcerative colitis-associated neoplasms

	Number	Pathological result		
		LGD (*n* = 2)	HGD-sSM (*n* = 14)	dSM (*n* = 5)
Endoscopist 1				
JNET type 2A	8	1	7	0
Pit type III/IV	7	2	3	2
Endoscopist 2				
JNET type 2A	2	1	1	0
Pit type III/IV	5	2	3	0
Endoscopist 3				
JNET type 2A	2	0	1	1
Pit type III/IV	7	2	5	0
Endoscopist 4				
JNET type 2A	9	2	6	1
Pit type III/IV	8	2	5	1
Endoscopist 5				
JNET type 2A	5	2	2	1
Pit type III/IV	8	2	6	0
Endoscopist 6				
JNET type 2A	7	2	5	0
Pit type III/IV	9	2	6	1

LGD: Low-grade dysplasia; HGD: High-grade dysplasia; sSM: Shallow submucosal invasive carcinoma; dSM: Deep submucosal invasive carcinoma; JNET: Japan Narrow-Band Imaging Expert Team; UCAN: Ulcerative colitis-associated neoplasms.

In the UCAN group, JNET type 2A and pit pattern type III/IV had a low PPV but with a high NPV in LGD diagnosis, and JNET type 2B and pit pattern type V_I low irregularity had a low NPV in the diagnosis of HGD to sSM. This was because several lesions in UCAN were diagnosed as JNET type 2A or pit pattern type III/IV, even though they were actually HGD to sSM. Additional detailed analysis revealed that most endoscopists diagnosed about one-quarter of HGD to sSM lesions as JNET type 2A and one-third of HGD to sSM lesions as pit pattern type III/IV. Furthermore, a small number of dSM lesions were diagnosed as JNET type 2A or pit pattern type III/IV (Table 7).

In the SN group, JNET type 2A and pit pattern type III/IV had a high PPV but with a low NPV in LGD diagnosis, and JNET type 2B and pit pattern type V_I low irregularity had a low PPV in the diagnosis of HGD to sSM. Because JNET type 2B and pit pattern type V_I low irregularity include lesions from LGD to dSM, these types have low PPV even in non-UC patients[17,19,27]. Several LGD and dSM lesions in the SN group were diagnosed as JNET type 2B and pit pattern type V_I low irregularity. JNET type 3 and pit pattern type V_I high irregularity/V_N in both UCAN and SN groups showed low sensitivity but with high specificity and accuracy. Previous studies showed that JNET type 3 and pit pattern type V_I high irregularity/V_N have high specificity for dSM diagnosis in both UC and non-UC patients[17-19,22]. Regardless of whether the surface and vascular patterns are modified by inflammation, JNET type 3 and pit pattern type V_I high irregularity/V_N have been confirmed to be useful in diagnosing dSM among UC patients. In UCAN, the tumors' surface structure sometimes could not represent the dysplastic change due to the bottom-up growth pattern; hence, it is considered that several lesions are underestimated by endoscopic classifications. Additionally, SN located in the inflamed mucosa, especially SN in the severely inflamed mucosa, tends to be misdiagnosed due to the influence of inflammation.

Intra-observer agreement was higher among non-experts than among experts; however, the difference was not statistically significant. Inter-observer agreement did not also significantly differ but was higher in experts than in non-experts. Irrespective of the endoscopists' experience, a consistent endoscopic diagnosis of neoplastic lesions in UC patients was difficult to achieve. In particular, the intra- and inter-observer agreements were lower for UCAN than for SN.

The present study has some limitations. First, our study was conducted on a small number of cases; however, given its retrospective nature, the same size could not be set a priori. We believe that the small number of typical LGDs in this study was responsible for the unsatisfactory diagnostic accuracy. Second, only neoplastic lesions evaluated using both the JNET and pit pattern classifications were included. While inflammation and regenerative changes might be evaluated as neoplastic patterns by both JNET and pit pattern classifications, our study could not include non-neoplastic lesions. Non-neoplastic lesions should be included in future studies. Third, differentiation between UCAN and SN was based on pathology; nevertheless, even in pathology, distinguishing UCAN from SN can be difficult.

CONCLUSION

The JNET and pit pattern classifications did not show high accuracy in diagnosing the pathology and invasion depth of neoplastic lesions in UC patients. Overall, the endoscopic diagnosis of UCAN tended to be underestimated as compared to the pathological results. Endoscopic diagnosis of neoplastic lesions in UC patients is still difficult, and treatment strategies need to be carefully determined.

ARTICLE HIGHLIGHTS

Research background

Patients with long-standing ulcerative colitis (UC) have a risk of colorectal tumors due to chronic inflammation. Endoscopic treatments for patients with UC have gradually increased and have attracted attention recently.

Research motivation

Surface and vascular patterns of tumors located in the inflamed mucosa are likely to be modified by inflammation. For that reasons, it is unclear whether the Japan Narrow-Band Imaging Expert Team (JNET) classification and pit pattern classification are applicable to the diagnosis of neoplastic lesions in patients with UC.

Research objectives

The present study aimed to clarify the diagnostic performance of JNET and pit pattern classifications for neoplastic lesions in patients with UC.

Research methods

We analyzed 41 UC patients with 44 lesions that could be assessed using both the JNET and pit pattern classifications. We devided them into the UC-associated neoplasms (UCAN) group (21 lesions) and sporadic neoplasms (SN) group (23 lesions) according to the pathological results. Six endoscopists each evaluated the endoscopic findings by using both endoscopic classifications.

Research results

In the UCAN group, the accuracy of diagnosis for JNET types 2A, 2B, and 3 by experts was 90.5%, 71.4%, and 85.7%, respectively. In the same manner, the accuracy of diagnosis for pit pattern type III/IV, type V_I low irregularity, and type V_I high irregularity/V_N by experts was 85.7%, 57.1%, and 76.2%, respectively.

Research conclusions

The JNET and pit pattern classifications did not show high accuracy in diagnosing the pathology and invasion depth of neoplastic lesions in patients with UC. Endoscopic diagnosis of UCAN tended to be underestimated, as compared to the pathological results.

Research perspectives

Future prospective studies with a large number of UC patients are needed in clinical practice.

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FOOTNOTES

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Country/Territory of origin: Japan

ORCID number: Yuichi Kida 0000-0002-1345-1922; Takeshi Yamamura 0000-0003-4994-016X; Keiko Maeda 0000-0001-7615-0476; Tsunaki Sawada 0000-0002-4779-9708; Eri Ishikawa 0000-0003-1623-7996; Yasuyuki Mizutani 0000-0002-4363-3161; Naomi Kakushima 0000-0002-9635-2099; Kazuhiro Furukawa 0000-0003-0980-9095; Takuya Ishikawa 0000-0001-5814-3555; Eizaburo Ohno 0000-0002-7730-4630; Hiroki Kawashima 0000-0002-3720-781X; Masanao Nakamura 0000-0002-5444-143X; Masatoshi Ishigami 0000-0003-0938-631X; Mitsuhiro Fujishiro 0000-0002-4074-1140.

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ORIGINAL ARTICLE

Observational Study

Gut dysbiosis and small intestinal bacterial overgrowth as independent forms of gut microbiota disorders in cirrhosis

Roman Maslennikov, Vladimir Ivashkin, Irina Efremova, Elena Poluektova, Anna Kudryavtseva, George Krasnov

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Roman Maslennikov, Vladimir Ivashkin, Irina Efremova, Elena Poluektova, Department of Internal Medicine, Gastroenterology and Hepatology, Sechenov University, Moscow 119435, Russia

Roman Maslennikov, Vladimir Ivashkin, Elena Poluektova, Anna Kudryavtseva, Scientific Community for Human Microbiome Research, Moscow 119435, Russia

Roman Maslennikov, Department of Internal Medicine 1, Consultative and Diagnostic Center 2 of the Moscow City Health Department, Moscow 107564, Russia

Anna Kudryavtseva, George Krasnov, Laboratory of Postgenomic Research, Engelhardt Institute of Molecular Biology, Russian Academy of Sciences, Moscow 119991, Russia

Corresponding author: Roman Maslennikov, MD, PhD, Assistant Professor, Professor, Department of Internal Medicine, Gastroenterology and Hepatology, Sechenov University, Pogodinskaya str., 1, bld. 1, Moscow 119435, Russia. mmmm00@yandex.ru

Abstract

BACKGROUND

Gut dysbiosis and small intestinal bacterial overgrowth (SIBO) are commonly observed in patients with cirrhosis. Despite the substantial number of articles describing the relations between disorders of gut microbiota and various manifestations of cirrhosis, dysbiosis and SIBO were always studied separately.

AIM

To study the relationship of gut dysbiosis and SIBO in cirrhosis.

METHODS

This observational study included 47 in-patients with cirrhosis. Stool microbiome was assessed using 16S rRNA gene sequencing. SIBO was assessed using the lactulose hydrogen breath test.

RESULTS

SIBO was found in 24/47 (51.1%) patients. Patients with SIBO had a higher abundance of Firmicutes (P = 0.017) and Fusobacteria (P = 0.011), and a lower abundance of Bacteroidetes (P = 0.013) than patients without SIBO. This increase in the abundance of Firmicutes occurred mainly due to an increase in the abundance of bacteria from the genus *Blautia* (P = 0.020) of the *Lachnospiraceae*

family (P = 0.047), while the abundance of other major families of this phylum [*Ruminococcaceae* (P = 0.856), *Peptostreptococcaceae* (P = 0.066), *Clostridiaceae* (P = 0.463), *Eubacteriaceae* (P = 0.463), *Lactobacillaceae* (P = 0.413), and *Veillonellaceae* (P = 0.632)] did not differ significantly between the patients with and without SIBO. Reduced level of Bacteroidetes in samples from patients with SIBO was a result of the decrease in bacterial numbers from all the major families of this phylum [*Bacteroidaceae* (P = 0.014), *Porphyromonadaceae* (P = 0.002), and *Rikenellaceae* (P = 0.047)], with the exception of *Prevotellaceae* (P = 0.941). There were no significant differences in the abundance of taxa that were the main biomarkers of cirrhosis-associated gut dysbiosis [Proteobacteria (P = 0.790), Bacilli (P = 0.573), *Enterobacteriaceae* (P = 0.632), *Streptococcaceae* (P = 0.170), *Staphylococcaceae* (P = 0.450), and *Enterococcaceae* (P = 0.873)] between patients with and without SIBO.

CONCLUSION

Despite the differences observed in the gut microbiome between patients with and without SIBO, gut dysbiosis and SIBO are most likely independent disorders of gut microbiota in cirrhosis.

Key Words: Dysbiosis; Gut-liver axis; Microbiome; Small intestinal bacterial overgrowth; Cirrhosis; Microbiota

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Core Tip: Patients with small intestinal bacterial overgrowth (SIBO) had a higher abundance of Firmicutes and Fusobacteria, and a lower abundance of Bacteroidetes than patients without SIBO. This increase in the abundance of Firmicutes occurred mainly due to an increase in the abundance of bacteria from the genus Blautia of the Lachnospiraceae family. There were no significant differences in the abundance of taxa that were the main biomarkers of cirrhosis-associated gut dysbiosis.

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INTRODUCTION

Cirrhosis is one of the most serious problems in hepatology and is not only a disease of the liver, but it also affects other organs. The damage that occurs in these other organs can contribute to further progression of cirrhosis, forming a vicious circle. The examples of such organs are the gut and the community of microorganisms that reside within it (the gut microbiota). Two forms of disorders of gut microbiota have been described in cirrhosis: changes in its composition[1-5] (gut dysbiosis[6-10]) and an increase of bacterial number in the small intestine (small intestinal bacterial overgrowth, SIBO)[11-14]. It is believed that these disorders, in combination with increased intestinal permeability, lead to increased penetration of bacteria and their components into the intestinal wall, mesenteric lymph nodes, ascitic fluid, and portal and systemic bloodstreams[15]. This, in turn, leads to systemic inflammation, vasodilation, compensatory fluid retention, and worsening portal hypertension[16,17].

Despite the substantial number of articles describing the relations between disorders of gut microbiota and various manifestations of cirrhosis, dysbiosis and SIBO were always studied separately. Our research aims to study the relationship of gut dysbiosis and SIBO in cirrhosis.

MATERIALS AND METHODS

Patients

In this observational study, 95 consecutive patients with cirrhosis were admitted to the Department of Hepatology of Clinic for Internal Diseases, Gastroenterology and Hepatology at Sechenov University (Moscow, Russia) and screened for inclusion. The study procedures were explained to potential participants, and written informed consent was obtained before enrollment. The present study was approved by the Ethics Committee of Sechenov University.

The inclusion criteria were as follows: diagnosis of cirrhosis verified by histology or clinical, biochemical, and ultrasound findings; and age between 18 and 70 years. The exclusion criteria were as follows: use of lactulose, lactitol, or other prebiotics, probiotics, antibiotics, or metformin in the past 6 wk; alcohol consumption in the past 6 wk; or inflammatory bowel disease, cancer, or any other serious disease. We used these criteria to exclude the influence of these factors on the composition of the gut microbiota. Of the original 95 patients screened for inclusion, 47 were enrolled in the study while 48 were excluded (Figure 1).

SIBO assessment

The participants of this study were 47 in-patients who were diagnosed with cirrhosis at admission. The morning after admission, SIBO was assessed using a lactulose hydrogen breath test (Gastrolyzer, Bedfont, The United Kingdom), following the manufacturer's instructions and in accordance with the North American Consensus[18]. Participants were considered to have SIBO if breath hydrogen concentration increased by at least 20 ppm above the baseline value within 90 min[18].

Gut microbiome analysis

The morning after admission, a stool sample was taken into a sterile disposable container and immediately frozen at -80 °C[19].

DNA from the stool was isolated using the MagNa Pure Compact Nucleic Acid Isolation Kit I (Roche, Basel, Switzerland) according to the manufacturer's instructions. Libraries for sequencing were prepared by two rounds of polymerase chain reaction (PCR) amplification. In the first round, specific primers for the v3-v4 region of the 16S ribosomal RNA gene were used: 16S-F TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG and 16S-R GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC.

After amplification, the PCR product was purified using AMPure XP magnetic beads (Beckman Coulter, Brea, CA, United States). Then, a second round of PCR was performed to attach specific adapters and enable multiplexing of the samples. To begin, 5 μL of the first PCR product was added to the reaction after ball cleaning with primers containing Illumina indices (Nextera XT Index v2 Primers; San Diego, CA, United States) and adapter sequences as well as 2× KAPA HiFi HotStart ReadyMix. The amplification products were also purified using AMPure XP beads (Beckman Coulter). The concentrations of the prepared libraries were then measured using a Qubit 2.0 fluorimeter (London, United Kingdom) and quantitative PCR. The quality of the libraries was assessed using the Agilent 2100 Bioanalyzer (Santa Clara, CA, United States). The libraries were mixed in equal proportions and diluted to the required concentration to be run on a MiSeq (Illumina) device. Pair-end readings of 300 + 300 nucleotides were obtained. Reads were trimmed from the 3'-tail with Trimmomatic (Illumina) and then merged into a single amplicon with the MeFiT tool[20,21]. We did not perform operational taxonomic unit picking; instead, we classified amplicon sequences with the Ribosomal Database Project (RDP) classifier and RDP database[22].

Statistical analysis

Statistical analysis was performed with STATISTICA 10 (StatSoft Inc., Tulsa, OK, United States). The data are presented as medians (interquartile ranges). The abundance of taxa of the gut microbiome is presented as a percentage. Differences between continuous variables were assessed with the Mann-Whitney test. Fisher's exact test was used to assess the differences between categorical variables. P values ≤ 0.05 were considered as statistically significant.

RESULTS

Out of the 47 participants in our study, the results from the lactulose hydrogen breath test revealed that 24 (51.1%) of patients met the criteria for SIBO. No significant differences in etiology (Figure 2), demographics, drugs used before inclusion, and the main manifestations of cirrhosis were observed between patients with and without SIBO, other than the value of C-reactive protein, which was higher in patients with SIBO. Patients with SIBO also displayed a trend toward having a larger spleen and higher incidence of ascites (Table 1).

In terms of phyla, samples from patients with SIBO revealed a higher abundance of Firmicutes and Fusobacteria, and lower abundance of Bacteroidetes compared to those from patients without SIBO. There were no significant differences in the numbers of Proteobacteria and Actinobacteria as well as the main Firmicutes classes (Clostridia, Bacilli and Negativicutes, Table 2).

Our results reveal that the increase in the abundance of Firmicutes observed in patients with SIBO occurs mainly due to a larger population of bacterial species from the *Lachnospiraceae* family. The number of bacteria from the other major families of this phylum does not differ significantly between the samples taken from the two groups of patients (Table 3). After the exclusion of *Lachnospiraceae* from Firmicutes, the difference in the abundance of bacteria under this phylum was no longer significant [39.8 (32.3-53.3) *vs* 42.6 (25.1-54.3); P = 0.773].

Table 1 Descriptive statistics of patients with and without small intestinal bacterial overgrowth

	Patients with SIBO (*n* = 24)	Patients without SIBO (*n* = 23)	*P* value
Age, yr	50 (39-59)	51 (37-58)	0.949
Male/female	12/12	11/12	0.557
Body mass index, kg/m^2	25.0 (22.9-27.8)	24.1 (22.3-26.1)	0.302
Red blood cells, 10^{12}/L	3.7 (3.3-4.2)	4.0 (3.6-4.3)	0.322
White blood cells, 10^9/L	3.6 (2.6-4.9)	4.0 (3.2-5.6)	0.437
Platelets, 10^9/L	78 (49-108)	90 (59-115)	0.229
C-reactive protein, mg/L	11.4 (1.6-17.1)	2.1 (0.1-9.2)	0.025
Serum albumen, g/L	35 (31-40)	36 (31-41)	0.617
Serum total bilirubin, μmol/L	36 (27-54)	38 (23-64)	0.848
Prothrombin (Quick test), %	57 (50-68)	64 (53-70)	0.233
Ascites, *n* (%)	17 (70.8)	10 (43.4)	0.054
Esophageal varices, *n* (%)	19 (79.2)	19 (82.6)	0.528
Spleen length, cm	16.2 (14.0-20.0)	14.6 (13.3-16.2)	0.080
Portal vein diameter, mm	13.0 (12.0-13.7)	12.0 (11.0-13.5)	0.263
Overt hepatic encephalopathy, *n* (%)	9 (37.5)	8 (34.8)	0.544
Child-Pugh score	8 (6-10)	8 (6-9)	0.551
Drugs used by patients within 6 mo prior to study enrollment			
Proton pump inhibitors, *n* (%)	9 (37.5)	8 (34.8)	0.544
Beta blockers, *n* (%)	5 (20.8)	3 (13.0)	0.375
L-ornithine-L-aspartate, *n* (%)	4 (16.7)	4 (17.4)	0.625
Diuretics, *n* (%)	13 (54.2)	8 (34.8)	0.149
Ursodeoxycholic acid, *n* (%)	4 (16.7)	5 (21.7)	0.471
Glucocorticoids, *n* (%)	2 (8.3)	6 (26.1)	0.109
Antiviral drugs, *n* (%)	7 (29.2)	4 (17.4)	0.273

SIBO: Small intestinal bacterial overgrowth.

The reduced population of Bacteroidetes in patients with SIBO results from a reduction in all major families of this phylum, with the exception of *Prevotellaceae*. Although there was no significant difference in the overall levels of Proteobacteria between the samples from the two patient groups, differences were detected in the minor families of the phylum. Specifically, samples from patients with SIBO revealed that the proportion of *Pasteurellaceae* and *Moraxellaceae* were higher, and *Desulfovibrionaceae* was lower, compared to those from patients without SIBO. Similarly, there was also no significant difference in the overall levels of bacteria in the Actinobacteria phylum, but the proportion of bacteria from the *Actinomycetaceae* and *Micrococcaceae* minor families were higher in the samples taken from patients with SIBO than in samples from patients without (Table 3).

At the genus level, analysis of samples from patients with SIBO revealed increased the abundance of *Blautia*, *Fusicatenibacter*, *Acinetobacter*, *Oribacterium*, and *Haemophilus* bacteria compared to the samples of patients without SIBO. In contrast, *Bacteroides*, *Oscillibacter*, *Alistipes*, *Parabacteroides*, *Barnesiella*, and *Intestinimonas* levels were lower (Table 4).

Further analysis demonstrated that *Brautia* was the main contributor to the increase in the abundances of the *Lachnospiraceae* family and the Firmicutes phylum in patients with SIBO. After subtracting the contribution of these bacteria, the statistical differences between the patient groups were no longer significant [*Lachnospiraceae*: 28.6 (19.6-35.0) *vs* 23.2 (10.7-33.2), $P = 0.177$; Firmicutes: 74.0 (62.2-79.5) *vs* 65.7 (55.9-78.6); $P = 0.425$].

The Firmicutes/Bacteroides ratio was significantly higher in patients with SIBO than in patients without SIBO, while the modified dysbiosis ratio that determines the prognosis in cirrhosis[10] did not differ significantly between the two groups of patients (Figure 3).

Table 2 Abundance of bacteria at the phylum and class levels in gut microbiome of patients with and without small intestinal bacterial overgrowth

Taxon	Patients with SIBO (*n* = 24)	Patients without SIBO (*n* = 23)	*P* value
Phyla			
Firmicutes	90.2 (77.7-94.3)	80.0 (67.5-87.2)	0.017
Bacteroidetes	5.3 (1.5-7.7)	6.8 (5.6-1.3)	0.013
Proteobacteria	1.3 (0.2-3.0)	1.7 (0.2-4.1)	0.790
Actinobacteria	0.5 (0.3-1.6)	0.8 (0.4-3.8)	0.343
Fusobacteria	0.01 (0.00-0.03)	0.00 (0.00-0.00)	0.011
Main classes of *Firmicutes*			
Clostridia	77.9 (67.1-84.6)	70.9 (61.6-83.1)	0.151
Bacilli	2.4 (0.4-7.1)	1.1 (0.4-7.3)	0.573
Negativicutes	0.5 (0.2-0.8)	0.4 (0.2-1.4)	0.956

SIBO: Small intestinal bacterial overgrowth.

DISCUSSION

We detect SIBO in half of the patients with cirrhosis, which is consistent with the data of previous studies[13]. SIBO is thought be caused by a slowdown in the motility of the small intestine, use of proton pump inhibitors, exocrine pancreatic insufficiency, immune and other disorders[23,24]. Furthermore, an association between delayed orocecal transit and SIBO has been reported in patients with cirrhosis[25]. Despite the presence of immune disorders[26], there are no studies on the association of them with the development of SIBO in cirrhosis. There was no difference in the frequency of using proton pump inhibitors between the patients with and without SIBO in our study.

Cirrhosis-associated gut dysbiosis is represented by an increase in the abundance of Proteobactria that produce active endoctoxin[1-5], and the facultative anaerobic bacteria Bacilli[4,6-8] that is capable of bacterial translocation[27]. This is accompanied by the decrease in the levels of beneficial bacteria from the major families under the Clostridia class[4,7,8,10]. Since the number of Clostridia and Bacilli bacteria can change in opposite directions, the abundance of Firmicutes, which include Clostridia and Bacilli, has been demonstrated to be either increased[9] or decreased[5] in cirrhosis. The change in levels of Bacteriodetes in cirrhosis has been reported to decrease[1,9], increase[2], not change[10], and be increased in compensated cirrhosis, while reduced to normal in decompensated cirrhosis[7].

The mechanisms that lead to gut dysbiosis in cirrhosis are not clear; it is suggested that they may be similar to those for SIBO. If SIBO and gut dysbiosis result from the same factors, it would be logical to assume that dysbiosis is more pronounced in patients with SIBO. However, the abundance of the main biomarkers of cirrhosis-associated dysbiosis (Proteobacteria, Bacilli and Clostridia) was not significantly different between patients with and without SIBO in our study. Moreover, the abundance of *Lachnospiraceae*, a decrease in which is also one of the biomarkers of cirrhosis-associated dysbiosis[6-8,10], on the contrary, was significantly higher in SIBO.

The gut microbiome has been evaluated depending on the presence of SIBO in patients who do not have cirrhosis in a small number of studies. It was shown that the composition of the small intestine[28,29] and fecal[30] microbiome does not depend significantly on the presence of SIBO. However, among patients with diarrheal variant of irritable bowel syndrome, the abundance of *Prevotella* were higher, while the abundance of *Bacteroides* were reduced in persons with SIBO compared with persons without SIBO[31]. Another study revealed that Firmicutes was increased in fecal microbiota of patients with SIBO[32]. Our research also showed an increase in the abundance of Firmicutes and a decrease in the abundance of *Bacteroides* but without a significant change in the abundance of *Prevotella* in fecal microbiota of patients with SIBO. Thus, changes in the gut microbiome of SIBO patients with cirrhosis are partly consistent with changes in it in SIBO patients with other diseases and do not coincide with the changes that are characteristic of cirrhosis-associated dysbiosis.

An increase in Firmicutes/Bacteroides ratio which was observed in our study in patients with SIBO is reported in patients with a significant slowdown in orosecal transit in cirrhosis[9]. Nevertheless, there were no differences in the abundance of Proteobacteria and Bacilli between cirrhotic patients with normal and delayed orocecal in that study. It can therefore be assumed that the slowing down of orocecal transit is a main factor in the development of SIBO, but it has a minimal effect on the development of cirrhosis-associated gut dysbiosis.

Table 3 Abundance of bacteria at the family level in gut microbiome of patients with and without small intestinal bacterial overgrowth

Family	Patients with SIBO (*n* = 24)	Patients without SIBO (*n* = 23)	*P* value
Families of *Clostridia*			
Lachnospiraceae	41.00 (29.71-55.82)	31.30 (17.55-43.85)	0.047
Ruminococcaceae	26.15 (13.79-35.50)	21.41 (14.89-34.43)	0.856
Peptostreptococcaceae	0.50 (0.18-2.72)	0.17 (0.04-1.13)	0.066
Clostridiaceae	0.30 (0.01-1.12)	0.10 (0.01-0.47)	0.463
Eubacteriaceae	0.00 (0.00-0.00)	0.00 (0.00-0.01)	0.463
Families of *Bacilli*			
Streptococcaceae	1.46 (0.29-6.03)	0.41 (0.10-3.42)	0.170
Lactobacillaceae	0.23 (0.03-0.56)	0.10 (0.01-0.38)	0.413
Staphylococcaceae	0.02 (0.01-0.03)	0.01 (0.01-0.02)	0.450
Enterococcaceae	0.01 (0.00-0.04)	0.01 (0.00-0.23)	0.873
Family of *Negativicutes*			
Veillonellaceae	0.43 (0.07-0.73)	0.16 (0.05-1.15)	0.632
Families of *Bacteroidetes*			
Bacteroidaceae	1.13 (0.35-3.19)	3.77 (1.05-4.96)	0.014
Prevotellaceae	0.56 (0.01-3.12)	0.27 (0.04-3.07)	0.941
Porphyromonadaceae	0.11 (0.02-0.29)	0.40 (0.23-0.66)	0.002
Rikenellaceae	0.07 (0.01-0.74)	0.45 (0.06-1.35)	0.047
Families of *Proteobacteria*			
Enterobacteriaceae	0.72 (0.02-2.13)	1.51 (0.05-2.81)	0.632
Moraxellaceae	0.04 (0.02-0.05)	0.02 (0.01-0.04)	0.014
Pasteurellaceae	0.01 (0.00-0.15)	0.00 (0.00-0.01)	0.025
Sutterellaceae	0.01 (0.00-0.06)	0.02 (0.00-0.05)	0.400
Desulfovibrionaceae	0.00 (0.00-0.01)	0.01 (0.00-0.03)	0.042
Families of *Actinobacteria*			
Bifidobacteriaceae	0.46 (0.14-1.50)	0.65 (0.12-3.22)	0.366
Coriobacteriaceae	0.09 (0.07-0.14)	0.08 (0.04-0.20)	0.790
Actinomycetaceae	0.02 (0.01-0.04)	0.01 (0.00-0.03)	0.039
Micrococcaceae	0.02 (0.01-0.06)	0.01 (0.00-0.01)	0.045

SIBO: Small intestinal bacterial overgrowth.

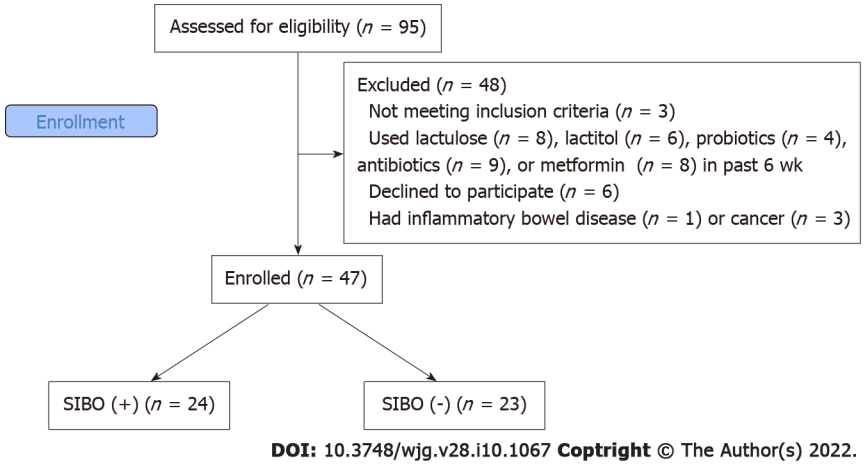
In our study, bacterial species that mainly increase in patients with SIBO belong to the *Blautia* genus. These bacteria have the ability to convert primary bile acids into secondary bile acids[33], for example, chenodeoxycholic acid to lithocholic acid. The ratio of lithocholic to chenodeoxycholic acid in feces strongly correlates with the content of *Blautia* in the gut microbiome of cirrhosis patients[8]. Furthermore, an increase in the content of bile acids in the feces is accompanied by an increase in the abundance of *Blautia* in persons on a cholerectic diet[34].

The changes in bile metabolism may explain the differences in the gut microbiome of cirrhosis patients with SIBO. The increase in the number of bacteria leads to the increased deconjugation of primary bile acids in the small intestine in patients with SIBO[35]. As deconjugated primary bile acids have a lower affinity for the proteins that carry them through the epithelium of the terminal ileum[36], more of these acids enter the large intestine. These bile acids may be a growth factor for *Blautia* and similar bacteria. The reduction in the abundance of Bacteroidetes may be the result of suppression their growth by bile acids or due to antagonism with *Blautia*. A larger study examining the amount and composition of bile acids in feces, the time of orocecal transit, the gut microbiome, and SIBO in cirrhosis should be performed to test this hypothesis.

Table 4 Abundance of bacteria at the genus level in gut microbiome of patients with and without small intestinal bacterial overgrowth

Genus	Family	Patients with SIBO (*n* = 24)	Patients without SIBO (*n* = 23)	*P* value
Blautia	*Lachnospiraceae*	13.38 (6.78-20.52)	6.99 (3.22-13.47)	0.020
Faecalibacterium	*Ruminococcaceae*	8.64 (3.65-19.55)	7.82 (3.02-12.74)	0.516
Gemmiger	*Ruminococcaceae*	2.33 (0.95-4.69)	1.65 (0.03-2.86)	0.237
Ruminococcus	*Ruminococcaceae*	1.80 (0.01-3.51)	0.58 (0.12-1.83)	0.617
Roseburia	*Lachnospiraceae*	1.66 (0.51-4.20)	0.92 (0.13-3.42)	0.377
Streptococcus	*Streptococcaceae*	1.36 (0.22-5.78)	0.37 (0.08-3.42)	0.151
Dorea	*Lachnospiraceae*	1.18 (0.63-2.51)	0.69 (0.42-1.35)	0.099
Bacteroides	*Bacteroidaceae*	1.15 (0.34-3.04)	3.59 (1.08-5.05)	0.012
Fusicatenibacter	*Lachnospiraceae*	0.79 (0.27-1.06)	0.13 (0.04-0.31)	0.003
Prevotella	*Prevotellaceae*	0.43 (0.01-2.27)	0.12 (0.01-2.19)	0.856
Escherichia	*Enterobacteriaceae*	0.38 (0.01-1.64)	0.28 (0.01-1.31)	0.890
Bifidobacterium	*Bifidobacteriaceae*	0.32 (0.11-1.15)	0.47 (0.07-2.39)	0.413
Oscillibacter	*Oscillospiraceae*	0.07 (0.01-0.15)	0.21 (0.09-0.38)	0.025
Alistipes	*Rikenellaceae*	0.06 (0.01-0.52)	0.41 (0.06-1.25)	0.025
Anaerostipes	*Lachnospiraceae*	0.62 (0.06-0.50)	0.49 (0.08-0.67)	0.170
Parabacteroides	*Porphyromonadaceae*	0.03 (0.00-0.11)	0.16 (0.05-0.29)	0.009
Acinetobacter	*Moraxellaceae*	0.03 (0.02-0.05)	0.02 (0.01-0.03)	0.004
Akkermansia	*Akkermansiaceae*	0.01 (0.00-0.71)	0.01 (0.00-1.33)	0.983
Oribacterium	*Lachnospiraceae*	0.01 (0.00-0.02)	0.00 (0.00-0.00)	0.040
Barnesiella	*Porphyromonadaceae*	0.00 (0.00-0.03)	0.03 (0.00-0.06)	0.025
Intestinimonas	*Oscillospiraceae*	0.00 (0.00-0.02)	0.03 (0.01-0.06)	0.015
Haemophilus	*Pasteurellaceae*	0.00 (0.00-0.10)	0.00 (0.00-0.01)	0.031

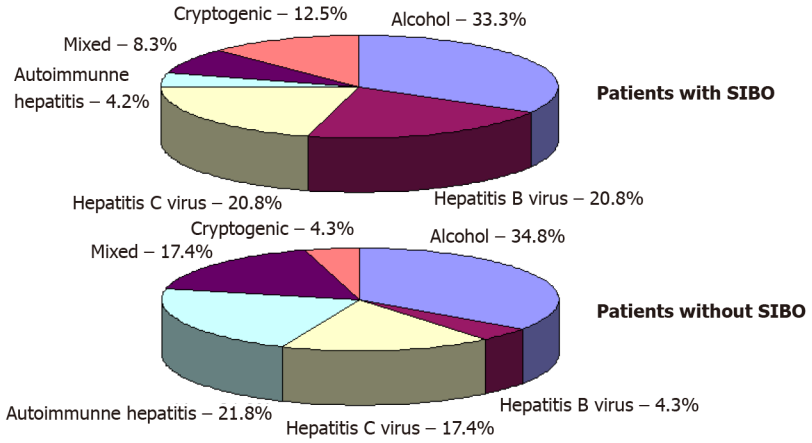
Genera with an abundance of < 1% and not significantly different between patient groups are not presented. SIBO: Small intestinal bacterial overgrowth.



DOI: 10.3748/wjg.v28.i10.1067 **Coptright** © The Author(s) 2022.

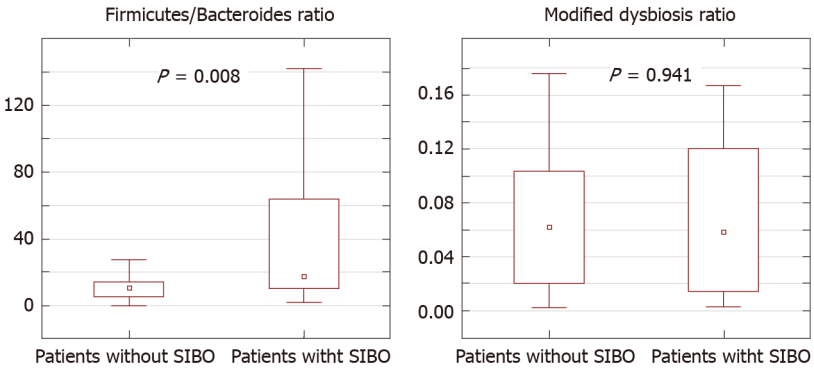
Figure 1 CONSORT 2010 flow diagram. SIBO: Small intestinal bacterial overgrowth.

The strength of our study is represented by the facts that it is the first that assesses the relationship between SIBO and gut dysbiosis in cirrhosis, as well as that we suggested the mechanism of development of changes in the gut microbiota in cirrhosis patients with SIBO and the idea for the next studies that can confirm or refute this hypothesis.



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Figure 2 Etiology of cirrhosis in patients with and without small intestinal bacterial overgrowth. SIBO: Small intestinal bacterial overgrowth.



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Figure 3 Complex indices of gut microbiome in patients with and without small intestinal bacterial overgrowth. SIBO: Small intestinal bacterial overgrowth.

The limitation of our study is its small size, but this did not prevent significant results from being obtained.

CONCLUSION

In conclusion, we showed that despite the difference in the gut microbiome between patients with and without SIBO, gut dysbiosis and SIBO are most likely relatively independent forms of disorders of the gut microbiota in cirrhosis.

ARTICLE HIGHLIGHTS

Research background

Gut dysbiosis and small intestinal bacterial overgrowth (SIBO) are commonly observed in patients with cirrhosis.

Research motivation

Despite the substantial number of articles describing the relations between disorders of gut microbiota and various manifestations of cirrhosis, dysbiosis and SIBO were always studied separately.

Research objectives

To study the relationship of gut dysbiosis and SIBO in cirrhosis.

Research methods

This observational study included 47 in-patients with cirrhosis. Stool microbiome was assessed using 16S rRNA gene sequencing. SIBO was assessed using the lactulose hydrogen breath test.

Research results

Patients with SIBO had a higher abundance of Firmicutes and Fusobacteria, and a lower abundance of Bacteroidetes than patients without SIBO. This increase in the abundance of Firmicutes occurred mainly due to an increase in the abundance of bacteria from the genus *Blautia* of the *Lachnospiraceae* family, while the abundance of other major families of this phylum did not differ significantly between the patients with and without SIBO. There were no significant differences in the abundance of taxa that were the main biomarkers of cirrhosis-associated gut dysbiosis between patients with and without SIBO.

Research conclusions

Despite the differences observed in the gut microbiome between patients with and without SIBO, gut dysbiosis and SIBO are most likely independent disorders of gut microbiota in cirrhosis.

Research perspectives

Research perspectives are to study the mechanisms of development of SIBO and gut dysbiosis in patients with cirrhosis.

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FOOTNOTES

Author contributions: Ivashkin V contributed research idea; Ivashkin V and Maslennikov R contributed study design; all authors contributed research and data analysis; Kudryavtseva A performed gut microbiota sequencing; Krasnov G performed bioinfomatics analysis of gut microbiota sequencing data; Maslennikov R contributed draft writing; all authors contributed draft editing; Maslennikov R is the guarantor.

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Informed consent statement: The study procedures were explained to potential participants, and written informed consent was obtained before enrollment.

Conflict-of-interest statement: No conflicts of interest.

Data sharing statement: The data can be provided upon request to the corresponding author.

STROBE statement: The authors have read the STROBE Statement-checklist of items, and the manuscript was prepared and revised according to the STROBE Statement-checklist of items.

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Country/Territory of origin: Russia

ORCID number: Roman Maslennikov 0000-0001-7513-1636; Vladimir Ivashkin 0000-0002-6815-6015; Irina Efremova 0000-0002-4112-0426; Elena Poluektova 0000-0002-9038-3732; Anna Kudryavtseva 0000-0002-3722-8207; George Krasnov 0000-0002-6493-8378.

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CASE REPORT

Successful treatment of an enormous rectal mucosa-associated lymphoid tissue lymphoma by endoscopic full-thickness resection: A case report

Fang-Yuan Li, Xiao-Long Zhang, Qi-De Zhang, Yao-Hui Wang

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Fang-Yuan Li, Xiao-Long Zhang, Qi-De Zhang, Digestive Endoscopy Center, Affiliated Hospital of Nanjing University of Chinese Medicine, Jiangsu Province Hospital of TCM, Nanjing 210046, Jiangsu Province, China

Yao-Hui Wang, Department of Pathology, Affiliated Hospital of Nanjing University of Chinese Medicine, Jiangsu Province Hospital of TCM, Nanjing 210046, Jiangsu Province, China

Corresponding author: Xiao-Long Zhang, PhD, Lecturer, Digestive Endoscopy Center, Affiliated Hospital of Nanjing University of Chinese Medicine, Jiangsu Province Hospital of TCM, No. 155 Hanzhong Road, Qinhuai District, Nanjing 210046, Jiangsu Province, China. chufangzai1234@163.com

Abstract

BACKGROUND

Colorectal mucosa-associated lymphoid tissue lymphoma (MALToma), a rare kind of nongastric MALToma, lacks consensus on its endoscopic features and standard therapies. According to previous studies on the clinical characteristics and outcomes of colorectal MALToma, endoscopic resection remains a good therapeutic strategy.

CASE SUMMARY

A 71-year-old woman suffered intermittent hematochezia for 1 mo, accompanied with abdominal pains but without weight loss, fever, chills or fatigue. Colonoscopy showed a massive hemispheric mass with rough and hyperemic mucosa in the lower rectum. Narrow-band imaging magnifying endoscopy detected some branching abnormal blood vessels and disappearance of glandular structure, which was similar with the tree-like appearance sign in gastric MALToma. Endoscopic ultrasonography revealed the lesion to be hypoechoic, boundary-defined, and echo uniform inside, originating from the muscularis propria. Abdominal enhanced computed tomography (CT) demonstrated a soft tissue mass with defined boundary. No enlarged superficial lymph nodes were detected by B-mode ultrasound. C^{13}-urea breath test and serum *Helicobacter pylori* antibody were both negative. The patient underwent endoscopic full-thickness resection. Postoperative pathological analysis indicated colorectal MALToma. The patient remained asymptomatic after discharge, and follow-up positron emission tomography–CT and colonoscopy showed no residual lesion, remnants or lymph

node metastasis.

CONCLUSION
This case provides new information on the specific endoscopic features of colorectal MALToma and an alternative treatment for patients.

Key Words: Mucosa-associated lymphoid tissue lymphoma; Endoscopic full-thickness resection; Endoscopic minimally invasive surgery; Endoscopy; Case report

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Core tip: Colorectal mucosa-associated lymphoid tissue lymphoma (MALToma) is considered a rare type of nongastric MALToma with no specific endoscopic features and no standard therapeutic strategies. We present a case of a large rectal MALToma that exhibited the tree-like appearance (TLA) sign and was successfully treated by modified endoscopic full-thickness resection (EFTR). This case highlights that TLA sign tends to be a specific feature of colorectal MALToma and EFTR seems to be a feasible and economical choice for the treatment of large colorectal MALToma.

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INTRODUCTION

Mucosa-associated lymphoid tissue lymphoma (MALToma) is a subtype of non-Hodgkin's lymphoma derived from B cells in the peripheral follicular area of lymph nodes. As MALToma progresses, it may transform into diffuse large B-cell lymphoma, which has high malignancy. Despite the increase in detection rate, colorectal MALToma, accounting for only 2.5% of gastrointestinal MALTomas, is still a rare disease worldwide[1]. As a result, the clinical characteristics, especially the endoscopic features, and standard therapy of colorectal MALToma have not been clearly established. In this case report, we describe a large rectal MALToma that was treated successfully with endoscopic full-thickness resection (EFTR), which may provide a new solution for colorectal MALToma.

CASE PRESENTATION

Chief complaints

A 71-year-old woman visited our institution to address intermittent hematochezia.

History of present illness

The patient's symptoms started 1 mo prior, accompanied with abdominal pains but without weight loss, fever, chills or fatigue.

History of past illness

The patient had a free previous medical history.

Physical examination

No other obvious sign was detected, except by digital rectal examination, which revealed a large protrusive lesion but left no blood on the gloves.

Laboratory examinations

No positive findings were obtained from the standard laboratory tests (including blood routine and markers of liver function, renal function and tumor).

Imaging examinations

Colonoscopy showed a 60 mm × 50 mm hemispheric mass in the lower rectum at 20 mm proximal to the

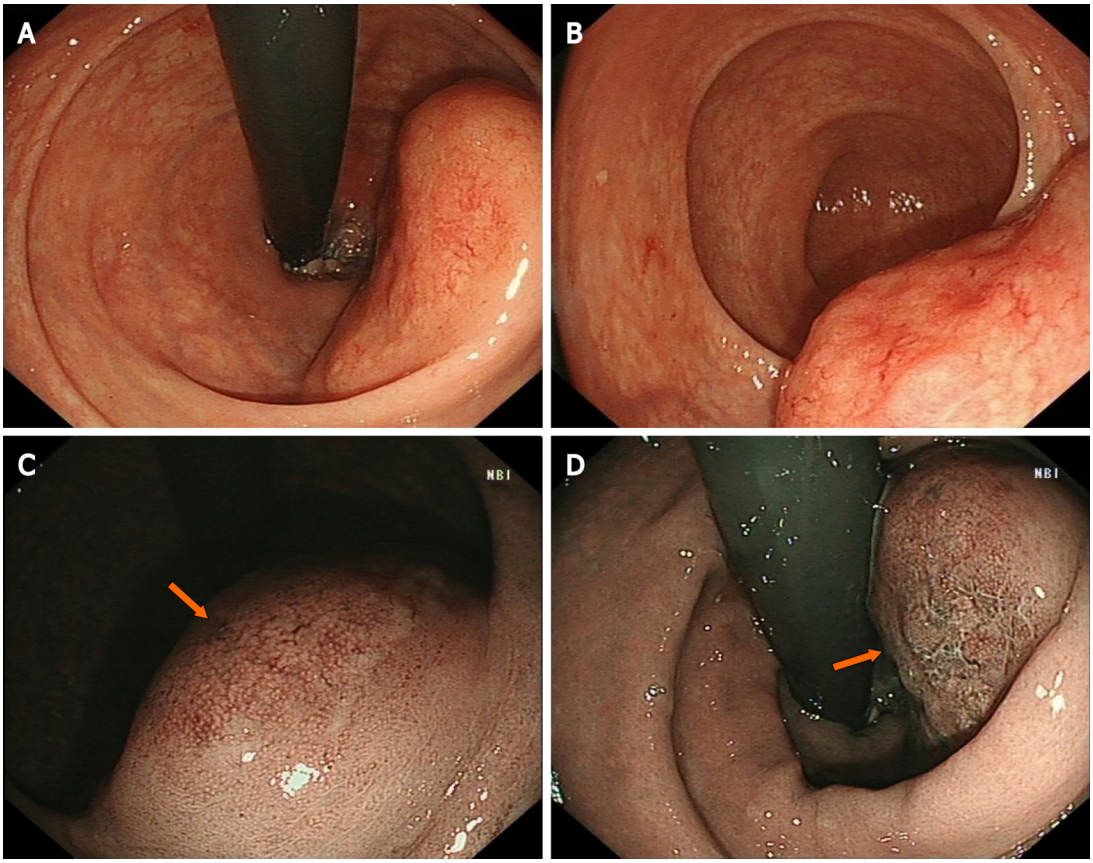


Figure 1 Endoscopic features of the lesion. A: White light endoscopy discovered a huge volume of subepithelial tumor in the lower rectum; B: Uneven and hyperemic coverage of mucosa; C: Narrow-band imaging magnifying endoscopy showing the irregular branching vascular (arrow); D: Disappeared glandular structure (arrow), which is called tree-like appearance.

anal verge, showing rough and hyperemic mucosa (Figure 1A and B). The narrow-band imaging magnifying (NBI-M) endoscopy detected some branching abnormal blood vessels and disappearance of glandular structure. This specific manifestation was consistent with the tree-like appearance (TLA) sign as is described in gastric MALToma (Figure 1C and D). Endoscopic ultrasonography (EUS) revealed the lesion to be hypoechoic, boundary-defined, and echo uniform inside, originating from the muscularis propria (Figure 2A). Abdominal enhanced computed tomography (CT) also demonstrated a 50 mm × 30 mm soft tissue mass with defined boundary in the lower rectum (Figure 2B). No enlarged superficial lymph nodes were detected by B-mode ultrasound. Additionally, C^{13}-urea breath test and serum *Helicobacter pylori* antibody were both negative.

FINAL DIAGNOSIS

Histopathological analysis revealed lymphoid proliferation with dysplasia, accompanied by variable large cells. The tumor cells were positive for CD20, Pax-5 and mum-1, and negative for CD3, CD5, CD10, c-myc, SOX11 and cyclin-D1. The Ki67 index was 5%. Light-chain restriction determination showed kappa restriction. Taken together, the final diagnosis of the presented case was compatible with MALToma (Figure 3).

TREATMENT

Considering the location and volume of the lesion, surgical operation would not be able to preserve the anal structures. Therefore, after sufficient communication with the patient, we performed EFTR under intravenous anesthesia (Video). During the operation, a special T-type endoknife (ESD knife; Micro-Tech, Nanjing, China) was used to cut through the mucosal surface into the submucosa layer by layer and the tumor body was exposed clearly. Then, we further stripped the connective tissue until the tumor was completely removed (Figure 4A). Finally, 14 SureClips™ (SureClip™ Hemoclips; Micro-Tech) combined with an endoloop were used for purse suture of the wound (Figure 4B and C). The tumor

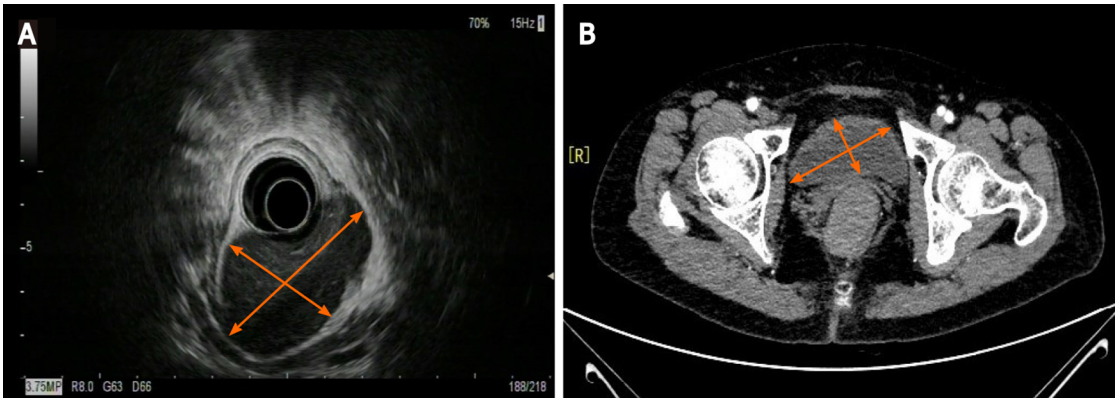


Figure 2 Endoscopic ultrasonography (EUS) and computed tomography (CT) of the lesion. A: EUS showing a 50 mm, low echo, submucosal lesion in the low rectum; B: Abdominal enhanced CT imaging revealing a 50 mm × 30 mm soft tissue mass with defined boundary, uniform density and moderate enhancement.

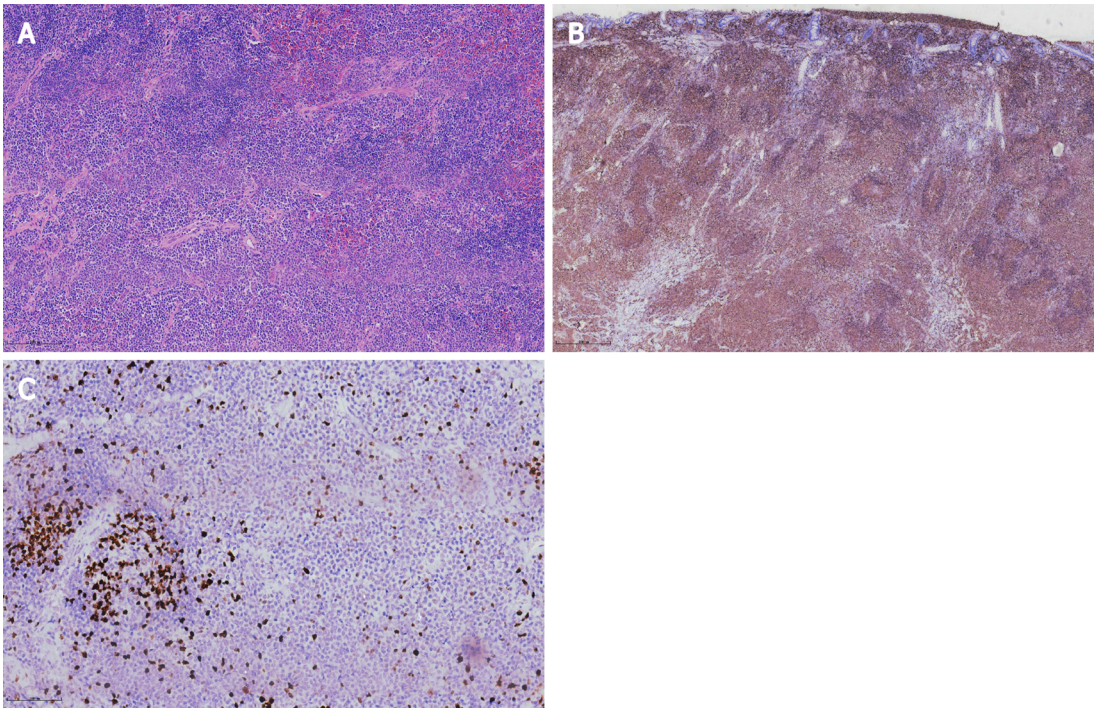


Figure 3 Histopathological and immunohistochemical analysis of the specimen. A: Hematoxylin–eosin staining, × 100; B: CD20 staining, × 40; C: Ki67, × 200.

tissue with intact capsule was removed by a nylon rope and the measured size was about 65 mm × 55 mm (Figure 4D). Preventive antibiotic was prescribed for 3 d after the operation.

OUTCOME AND FOLLOW-UP

The patient recovered steadily and was discharged 5 d after our endoscopic surgery. Six months later, positron emission tomography–CT examination showed no sign of residual lesions or lymph node metastasis. Follow-up colonoscopy after 12 mo revealed a healed lesion without remnants.

DISCUSSION

Given that colorectal MALToma only accounts for a small proportion of all colorectal malignancies, its clinical features are largely unknown[1,2]. The population most susceptible to colorectal MALToma are middle-age and old-age women, and a few of the patients may exhibit some latent symptoms, such as

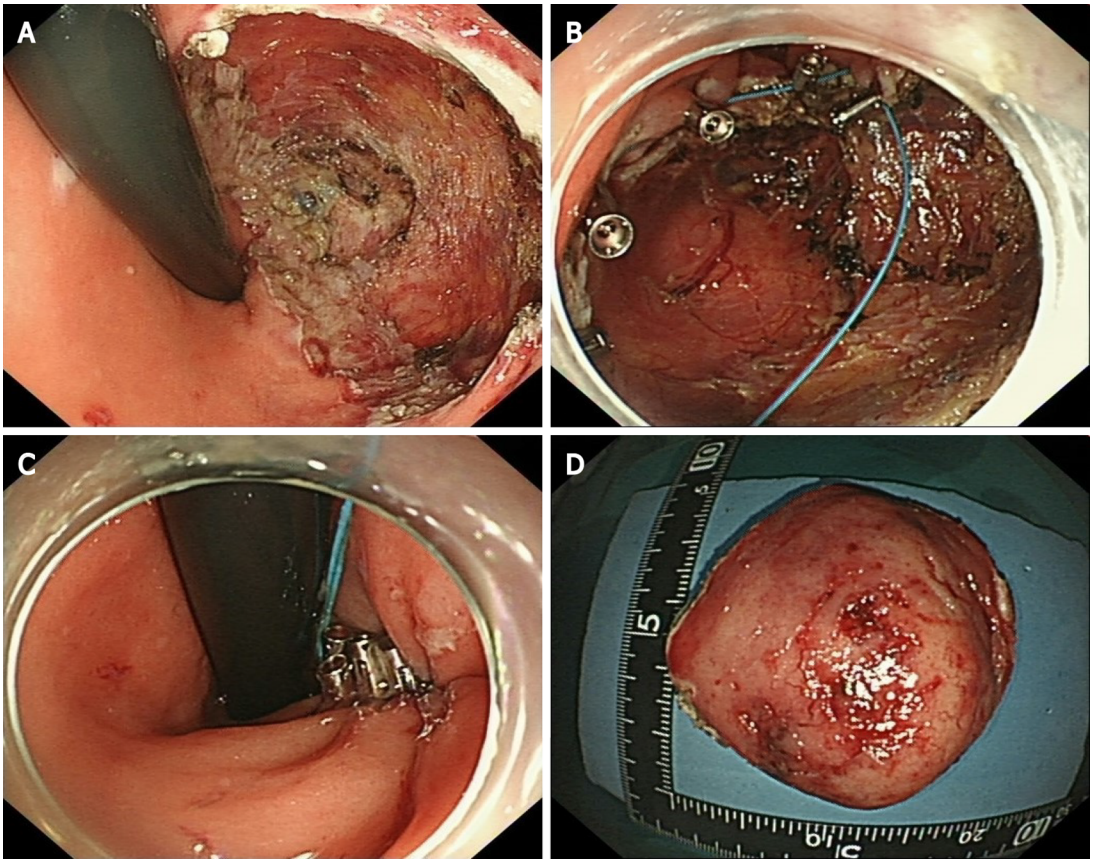


Figure 4 Procedures of the modified endoscopic full-thickness resection and purse suture. A: Circumferential ulcer after endoscopic full-thickness resection; B: SureClips™ and an endoloop were used for purse suture; C: Fraping the endoloop and closing the wound; D: The resected specimen.

fecal occult blood and black stool[2,3]. Colonoscopy remains an effective diagnostic method for MALToma. However, there is still no consensus on the relevant endoscopic characteristics and classification of colorectal MALTomas[2]. Recent studies have suggested some characteristics of colorectal MALToma. For example, the most common site is the rectum and the lesion usually appears as a singlet [1,2]. In addition, the most common endoscopic phenotype is subepithelial tumor (commonly referred to as SET) type, followed by polyposis type, ileitis type, and epithelioid mass type[2]. All these characteristics were in accordance with that in our case. However, in contrast to that in the present case, tumors > 50 mm in size, mostly within 20 mm according to a previous report[1], have been seldom reported. Conventionally, submucosal tumor with low echo lesion feature as evaluated by EUS has been most often considered as gastrointestinal stromal tumor, lipomyoma or neuroendocrine tumor. In the present case, the uneven and broken surface detected in white light mode caught our attention. We decided to perform NBI-M endoscopy to observe this lesion and delineate the border. A TLA, similar to the description of gastric MALToma in some reviews[4], was obvious (Figure 2). We reviewed the lesion pictures and descriptions in some previous reports and we recognized that all these statements were indicating the same endoscopic characteristics[5,6]. This finding suggested that the TLA sign may also be a specific feature of colorectal MALToma.

Today, the standard therapy of colorectal MALToma is still under debate. Different from gastric MALToma, colorectal MALToma showed no significant association with *H. pylori* infection and no satisfactory response to *H. pylori* eradication in most cases[7,8]. Although some experts still consider *H. pylori* eradication as the first-line treatment because of noninvasiveness[9], anti-*H. pylori* therapy seems unnecessary and unreasonable for those patients who test negative for *H. pylori*; this was similar to the situation in the present case. From another perspective, colorectal MALTomas have their own features. Many clinical retrospective studies have indicated that most colorectal MALTomas could be classified as Lugano stage I and II, which are considered of lower malignancy and more slowly progressive[3,10]. Meanwhile, it has been indicated that the long-range outcome exhibits no significant difference between the patients who received monotherapy and those who received combined therapy (surgery or radiation)[2]. These features offer the possibility of locoregional treatments, such as endoscopic resection, surgical resection, and radiotherapy[11]. Therefore, endoscopic resection, as a mini-invasive therapy, seems to be an appropriate therapeutic option for early-stage colorectal MALTomas.

Interestingly, we noticed that nearly half of the cases treated by conventional endoscopic techniques tested positive on surgical margins, due to the diversity of endoscopic morphology and the lesion size [2]. In the present case, allowing for the large size of this lesion and the patient's strong desire for anal

preservation, EFTR seemed to be a good treatment strategy. This allowed us to resect a deep tumor with a small piece of the complete gastrointestinal wall without any assisted device[12]. During this operation, the highest priority is the complete removal of the tumor without interruption of its capsule. Meanwhile, the skillful use of the endoknife helped reduce the switch between instruments and improved efficiency. The purse suture, consisting of SureClips™ and endoloop, can decrease the tension on the wound and reduce the occurrence of delayed perforation. Although some reports have indicated that over-the-scope clips have good overall technical efficacy in stitching wounds of lesions ≤ 2 cm[12], the purse suture has the potential to be a cost-effective approach for giant lesions.

CONCLUSION

Colorectal MALToma, which lacks clinical and endoscopic features, is easily missed and misdiagnosed. Prior to histological diagnosis, a predictable identification of suspicious lesion under endoscopy is important to define the border of the lesion and prevent margin-positive resection. Meanwhile, accurate pathological diagnosis is also challenging, and endoscopists and pathologists must renew the research progress on this rare disease in time and join efforts for improving rates of final diagnosis. According to our successful experience with the present case, EFTR combined with purse suture seems to be a feasible and economical choice for the treatment of enormous colorectal MALToma, especially of the SET type. However, the combined application of EFTR and another therapy scheme needs further clinical practice.

FOOTNOTES

Author contributions: Zhang QD conceived and performed the operation; Wang YH analyzed and interpreted the histopathological and immunohistochemical results; Li FY collected the clinical information and wrote the manuscript; Zhang XL revised the manuscript.

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Country/Territory of origin: China

ORCID number: Fang-Yuan Li 0000-0003-4802-4514; Xiao-Long Zhang 0000-0003-2803-6366; Qi-De Zhang 0000-0002-9222-6036; Yao-Hui Wang 0000-0001-6001-9457.

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LETTER TO THE EDITOR

Comment "Asymptomatic small intestinal ulcerative lesions: Obesity and *Helicobacter pylori* are likely to be risk factors"

Wei Wang, Zhi-Peng Li, Juan Zhang, Zi-Jun Lin, Mei-Lin Ma, Si-Si Chen

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Wei Wang, Department of Interventional Oncology, Taizhou Municipal Hospital, Taizhou 318000, Zhejiang Province, China

Zhi-Peng Li, Juan Zhang, Zi-Jun Lin, Mei-Lin Ma, Medical College, Taizhou University, Taizhou 318000, Zhejiang Province, China

Si-Si Chen, Department of Obstetrics and Gynecology, Taizhou Women and Children's Hospital of Wenzhou Medical University, Taizhou 318000, Zhejiang Province, China

Corresponding author: Si-Si Chen, MD, Occupational Physician, Department of Obstetrics and Gynecology, Taizhou Women and Children's Hospital of Wenzhou Medical University, No. 188, Qianjin Village, Jiajia Street, Jiaojiang District, Taizhou 318000, Zhejiang, China. chensisiobstetrics@126.com

Abstract

There are many causes of anemia. It is unreasonable to simply associate anemia with asymptomatic ulcers in the small intestine.

Key Words: Intrinsic factor; Iron deficiency anemia; Megaloblastic anemia; Nonsteroidal anti-inflammatory drugs; Statistical bias

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Core Tip: Asymptomatic small intestinal ulcers may cause anemia due to vitamin B12 absorption or iron utilization, but anemia can also be caused by other digestive diseases such as gastric ulcer or gastrectomy or other chronic wasting diseases. Establishing a link between anemia symptoms and asymptomatic ulcers in the small intestine requires more extensive data support and other auxiliary examinations. Case reports and small sample investigations are not enough to support this link.

Citation: Wang W, Li ZP, Zhang J, Lin ZJ, Ma ML, Chen SS. Comment "Asymptomatic small intestinal ulcerative lesions: Obesity and *Helicobacter pylori* are likely to be risk factors". *World J Gastroenterol* 2022; 28(10): 1085-1087
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TO THE EDITOR

We are glad to read the original article by Fujimori[1] on asymptomatic ulcers in the small intestine. The authors found that small intestinal ulcers were usually not accompanied by obvious symptoms, and the patients' various examinations, such as physical examination and blood routine, were usually normal. However, they found in the limited pathological report of small intestinal ulcers that small intestinal ulcers were usually accompanied by the detection of *Helicobacter pylori* (*H. pylori*) and high body mass index (BMI), and the detection rate of small intestinal ulcers in men was higher than in women. This article provided more detailed data and research background and obtained reasonable results with the author's research and analysis. However, in our opinion, this article has problems worthy of further discussion. We are willing to contribute to the debate and want the author to reply.

In the Abstract section, we noticed that 10% of the healthy subjects mentioned by the author had a ruptured small intestinal mucosa, and the number of ruptures was 1–3. However, in the following, "What should I do if the small intestinal mucosa ruptures?" is written as 1–2. Is this the author's clerical error or is the concept not clearly defined? In the Introduction part, the author cited a recent research report to illustrate the relationship between Crohn's disease and ileal ulcers. However, the number of samples in this report is too small, and there is no rigorous statistical verification and analysis to directly infer the ileal ulcer. The connection with Crohn's disease is considered less rigorously.

Nonsteroidal anti-inflammatory drugs (NSAIDs)[2] and aspirin were used simultaneously to compare the utility of this combination, as mentioned in the analysis of drug administration studies. I think aspirin and NSAIDs should not be a paratactic relation because aspirin is included in NSAIDs. Maybe it is a good idea to modify NSAIDs to other types of NSAIDs.

The author repeatedly mentioned in the article that anemia was an important reminder for further examination. However, there are many reasons for anemia. For example, insufficient intake of folic acid and vitamin B12 can lead to megaloblastic anemia. Although daily diet can meet the needs of folic acid and vitamin B12 for erythropoiesis, the absorption of vitamin B12 requires the participation of intrinsic factors[3,4]. Intrinsic factors are produced by parietal cells in the stomach. After the intrinsic factor is combined with vitamin B12, it promotes the reabsorption of vitamin B12 in the distal ileum through specific receptors on the ileal mucosa. Although the terminal ileum plays an irreplaceable role in maintaining vitamin B12 Levels, disruption of any of these processes can lead to anemia. For example, gastrectomy can affect the secretion of internal factors and cause vitamin B12 reabsorption disorder[5]. In addition, iron is another important factor in the synthesis of red blood cells[6]. Iron deficiency anemia may be caused by insufficient iron intake or excessive iron loss due to chronic blood loss. Therefore, we believe that simply linking anemia with asymptomatic ulcers in the small intestine is unreasonable.

In the analysis, we found some data that were not suitable, in our opinion. For example, in Table 1 (https://www.wjgnet.com/1007-9327/full/v28/i3/332-T1.htm), in addition to the increased cases reported in the United States in 2005 and 2007 and Japan in 2016, the number of sample cases provided in other years and regions is too small. Because statistical analyses in cases of too few samples may lead to bias or bias in the results, the results obtained in this case are not representative. In addition, the data provided by the author has a single source and cannot represent the small intestinal ulcer situation of the global population. Therefore, the representativeness of the results of this experiment needs further study. In addition, we can see in Table 2 (https://www.wjgnet.com/1007-9327/full/v28/i3/332-T2.htm) that the data provided by Fujimori[1] in 2011 did not include female samples. The author bluntly pointed out in the results that small intestinal ulcers were more common in men. We consider whether such results are a deviation or error due to statistical reasons. Besides, mucosal breaks were strongly correlated with BMI and were significantly more common in *H. pylori*-infected subjects mentioned in the analysis section. I do not understand how this conclusion was drawn. Table 3 (https://www.wjgnet.com/1007-9327/full/v28/i3/332-T3.htm) shows the *P*-value for sex, age, and smoking. Among them, the *P*-values for sex, BMI, and *H. pylori* infection were statistically significant. The *P*-value for BMI was the smallest. However, it does not mean that BMI had a strong correlation with mucosal breaks. A *P*-value less than 0.05 only indicate statistical significance. Similarly, the study does not provide convincing evidence to confirm mucosal breaks were significantly more common in *H. pylori*-infected subjects.

FOOTNOTES

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ORCID number: Wei Wang 0000-0001-5630-3287; Zhi-Peng Li 0000-0002-0355-7889; Juan Zhang 0000-0001-7225-362X; Zi-Jun Lin 0000-0003-1478-3440; Mei-Lin Ma 0000-0002-5689-7756; Si-Si Chen 0000-0003-0125-8022.

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Editorial Board Member of World Journal of Gastroenterology, Nikolaos Papadopoulos, MD, PhD, Chief Physician, Consultant, 1st Department of Internal Medicine, 417 Army Share Fund Hospital, Athens 11521, Greece. nipapmed@gmail.com

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REVIEW

B cells in pancreatic cancer stroma

Francesca Romana Delvecchio, Michelle R Goulart, Rachel Elizabeth Ann Fincham, Michele Bombadieri, Hemant M Kocher

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Francesca Romana Delvecchio, Michele Bombadieri, William Harvey Research Institute, Queen Mary University of London, London EC1M 6BQ, United Kingdom

Francesca Romana Delvecchio, Michelle R Goulart, Rachel Elizabeth Ann Fincham, Hemant M Kocher, Barts Cancer Institute, Queen Mary University of London, London EC1M 6BQ, United Kingdom

Hemant M Kocher, Barts and the London HPB Centre, Barts Health NHS Trust, London E1 1BB, United Kingdom

Corresponding author: Hemant M Kocher, FRCS (Gen Surg), MBBS, MD, MS, Professor, Barts Cancer Institute, Queen Mary University of London, Charterhouse Square, London EC1M 6BQ, London, United Kingdom. h.kocher@qmul.ac.uk

Abstract

Pancreatic cancer is a disease with high unmet clinical need. Pancreatic cancer is also characterised by an intense fibrotic stroma, which harbours many immune cells. Studies in both human and animal models have demonstrated that the immune system plays a crucial role in modulating tumour onset and progression. In human pancreatic ductal adenocarcinoma, high B-cell infiltration correlates with better patient survival. Hence, B cells have received recent interest in pancreatic cancer as potential therapeutic targets. However, the data on the role of B cells in murine models is unclear as it is dependent on the pancreatic cancer model used to study. Nevertheless, it appears that B cells do organise along with other immune cells such as a network of follicular dendritic cells (DCs), surrounded by T cells and DCs to form tertiary lymphoid structures (TLS). TLS are increasingly recognised as sites for antigen presentation, T-cell activation, B-cell maturation and differentiation in plasma cells. In this review we dissect the role of B cells and provide directions for future studies to harness the role of B cells in treatment of human pancreatic cancer.

Key Words: B cells; Pancreatic cancer; Cancer immunology; Tertiary lymphoid structures; Anti-tumour immunoglobulins; Plasma cells

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Core Tip: The role of B cells in pancreatic ductal adenocarcinoma tumorigenesis is controversial. Human studies show clusters of B cells, interacting with other immune cells, forming active sites of the immune response, called tertiary lymphoid structures. *In vitro* experiments and *in vivo* studies using B-cell deficient mice suggest the role of an immuno-suppressive B cell phenotype to induce tumour-progression. These discordant findings highlight the need of further studies using better murine models to recapitulate pancreatic cancer and its immune infiltrate.

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INTRODUCTION

Pancreatic cancer and its immune infiltration

The majority (about 95%) of pancreatic cancers (adenocarcinomas) arise from the exocrine pancreas, most likely from the epithelial cells lining the pancreatic duct, to form gland-like structures, and hence, are commonly referred to as pancreatic ductal adenocarcinoma (PDAC), though mucinous tumours are the second most common histological type of pancreatic cancer[1]. PDAC is the gastrointestinal tumour with the poorest prognosis, with 80% of the patients presenting with advanced disease. A mere 15%-20% of the patients are suitable for surgical resection, which currently represents the only curative option for pancreatic cancer. For advanced PDAC, the most common systemic treatment is single-agent gemcitabine which is increasingly being replaced with a combination of chemotherapeutics (*e.g.*, FOLFIRINOX or gemcitabine-nab-paclitaxel), at least in patients with good performance status as first-line treatment[2]. Although immunotherapies have gained success in other cancers, there are no approved immunotherapies for PDAC[2].

Many immuno-therapeutic approaches are under investigation for PDAC. Immune-checkpoint inhibition has shown clinical benefit in 2% of PDAC patients harbouring a DNA mismatch repair (MMR) deficiency[3,4]. Vaccination strategies are also being tested including "personalised" dendritic cell (DC)-vaccines loaded with the antigen[5,6]. GVAX [granulocyte-macrophage colony-stimulating factor (GM-CSF)-secreting, allogeneic PDAC vaccine, NCT01417000, NCT00727441, NCT00084383] are being investigated further[7,8]. Furthermore specific use of immune cells is being explored by adoptive transfer of T cells carrying chimeric antigen receptors[9-12], or recover the immuno-suppression and chemo-sensitivity using Ibrutinib, the inhibitor of Bruton's tyrosine kinase (BTK), a member of the B-cell receptors (BCR) signalling pathway (NCT02436668), targeting regulatory B cells and macrophages.

PDAC is conventionally known as a "cold tumour", due to low inflammatory cytokine profile and hypoxia, low mutational load and exclusion of infiltrating lymphocytes[13,14]. Recent research has identified an "immunogenic subtype" enriched in genes associated to B-cell signalling, $CD4^+$ and $CD8^+$ T cells, and antigen presentation[13]. Furthermore, the combination of genetic, stromal, and immunological features of PDAC can lead to further definition of novel immune-subtypes which may have prognostic value and the possibility of identifying tumours with immuno-therapeutic potential[15]. Whilst spatial distribution and infiltration of T cells and the formation of clusters with B cells is associated with better outcome in human and murine models of PDAC[16,17], *in vivo* studies of B cell depletion in murine models of PDAC describe a pro-tumorigenic role of B cells[18-21]. These discordant findings can be ascribed to the different tumour sub-types analysed and to the use of dissimilar murine models. For example, mice that are genetically lacking in B cells might behave differently to those where depletion of B cells is conducted by a depleting antibody[22]. In this review, we critically discuss the evidence for the perceived dichotomous role of B cells in pancreatic cancer.

TUMOUR-SUPPRESSING ROLE OF B CELLS IN SOLID CANCERS

Immuno-histochemical analysis using CD20, and metagene analysis for B-cell signature, showed a positive correlation between B-cell infiltration and patient prognosis in many different cancer types. For example, work in primary cutaneous melanoma (n = 106, immunostaining, multivariate analysis) demonstrated that intra- and peri-tumoral B cells are important, in particular $CD20^+/OX40^+$ cell density [23]. In high-grade serous ovarian cancer (n = 70, immunostaining of tissues and FACS of peripheral blood), suggested a role for CD27-memory B cells[24]. In basal-like breast cancer (n = 728, breast cancer, TCGA dataset, B-cell mRNA signature) and non-small cell lung cancer (NSCLC) (n = 74, untreated patients with early-stage NSCLC and 122 patients with treated advanced-stage NSCLC; immunos-

taining and FACS analysis) demonstrated a prognostic value for follicular B cells[25,26]. In sarcoma (n = 608, soft-tissue sarcomas; gene expression profiles) led to the identification of different immunophenotypes, and the B-cell enriched demonstrated improved survival and response to immunotherapy [27]. Some studies included the organisation of tumour-infiltrating B cells into tertiary lymphoid structures (TLS) in addition to the B-cell density[28,29]. B cells are known to act as antigen-presenting cells (APCs) or antibody-producing cells[30]. Thus, presence of B cells or at least their subsets or organisation within cancer tissues seem to confer prognostic benefit suggesting a role for humoral immunity in the anti-tumour response mounted by the host[31].

Tumours can express antigens recognised as non-self by the immune system to induce a specific anti-tumour immune response, collectively referred to as the "cancer immunome"[32]. In this context, B cells with high affinity for a specific tumour-associated antigen (TAA), engulf and process the antigen to display it on their cell surface; thus, acting as APCs. This complex is recognised by activated T helper cells, which induce B-cell proliferation and clonal expansion. Some B cells may serve as memory cells whilst others act as effector cells that differentiate into antibody-producing plasma cells[33]. The antibody–TAA binding also initiates the destruction of the tumour cells expressing the TAA by several mechanisms, such as opsonisation and macrophage recognition and phagocytosis, or blocking of the receptors associated with tumour cell proliferation and survival, or uptake *via* Fcγ receptors, leading to antigen cross-presentation and vigorous $CD4^+$ and $CD8^+$ T cell responses, complement-dependent cytotoxicity (CDC), or antibody-dependent cellular cytotoxicity (ADCC).

Antibody-production

B cell affinity maturation and differentiation to plasma cells have been described within TLS in several cancers, in addition to the usual places of maturation such as lymph nodes[34]. Tumour-specific B cells may acquire somatic hyper-mutations (SHMs) in TLS and extra-follicular B cells maturation has been described[35-37]. Furthermore expansion of tumour-specific B cells without SHMs may reflect a mechanism of T cell-independent or T cell-dependent but germinal centre-independent B cell activation [38]. For example, in gastric cancer, tumour-infiltrating B cells showed broad variations in the degrees of SHMs, with some producing functional antibodies directed against sulfated glycosaminoglycan with, at least, tumour growth-suppressive properties *in vitro*[39].

Since cancer is driven by mutations in "self-proteins", cancer-associated auto-antibodies are detectable[40]. These antibodies may be in response to "self-antigens" which are either over-expressed [*e.g.*, human epidermal growth factor receptor 2 (HER2/neu)] or aberrantly expressed (*e.g.*, cancer-testis antigen) during tumorigenesis. Mechanisms for secretion of cancer-related auto-antibodies include changes in the expression levels, altered protein structures, presentation of dying tumour cells (due to chemo/radiotherapy for example) to the immune system leading an abnormal exposure of autologous intracellular antigens[40]. Antigen load and duration of exposure may induce humoral immune responses since antibodies against several TAA (such as p53, New York esophageal squamous cell carcinoma-1 (NY-ESO-1), surviving, tyrosinase) were more frequently found in advanced tumour stages [41]. Antibodies produced by tumour infiltrating B cells may induce lysis of cancer cells by ADCC or CDC, leading to the direct killing of the cancer cells[42]. Murine models demonstrate binding of tumour B-cell antibodies to mouse tumours in an antigen-specific manner and complement-dependent lysis[43-45]. Binding of C3 components to CD21 (the complement receptor 2) induces B-cell activation to promote anti-cancer responses[46].

Promoting T cell response

B cells may represent the most abundant APC since DCs are scarce in the tissue[47]. Tumour infiltrating B cells can also provide antigen-independent help to cytolytic T cells (CTLs) within the tumour, by interaction between CD27 expressed on helper B cells and CD70 on CTLs, promoting their antigen-independent survival and proliferation of T cells[48].

Activation of bystander B cells

B cells can also be stimulated by transactivation of bystander B cells not in direct contact with the antigen, *via* transfer of human leukocyte antigen-peptide complexes or BCRs contained in exosomes or cytonemes[49]. These activated bystander B cells can *per se* produce antibodies and/or serve as APC, but also release T-cell activating cytokines, thus amplifying the cellular and humoral immune response, even with a limited antigen load[49].

Interaction with T follicular helper cells, in intra-tumour TLS

Presence of TLS within the tumour parenchyma correlates with better patient survival[50,51]. Within TLS, B cells in close proximity to T cells and interact with T follicular helper cells and follicular DCs and promote germinal centre (GC) reaction, which results in B-cell differentiation into memory B cells and long-term surviving plasma cells. Within TLS, B cells can act as APCs and produce anti-tumour antibodies, exhibiting tumour-specific humoral responses *in situ*[26,36,37]. NSCLC-infiltrating B cells were shown to produce *in vitro* immunoglobulin (Ig) G and IgA directed against tumour antigens (MAGE, LAGE-1, NY-ESO-1, P53)[26]. Micro-dissected TLS-derived B cells from breast cancer showed

poly-clonality and high mutation rate, suggestive of an affinity maturation occurring within TLS[36]. Moreover colorectal cancer-infiltrating B cells were shown to produce IgG which bound epitopes on the cell membrane of different tumour cell lines[37,52]. TLS may also be artificially induced by neo-adjuvant treatment such as with anti-programmed cell death protein (PD) 1 in NSCLC, or vaccination against human papilloma virus (HPV) in cervical cancer patients[53,54]. Furthermore, presence of TLS is associated with response to immuno-therapy in NSCLC, melanoma and sarcoma patients[27,50,55,56].

TUMOUR-PROMOTING ROLE OF B CELLS IN SOLID CANCERS

Immuno-histochemical characterisation of the tumoral immune infiltrate has shown a negative correlation between B cell/plasma cell infiltration with patient survival in melanoma, prostate cancer, lung cancer and ovarian cancer[57-60]. Furthermore, the detection of tumour specific (auto)-antibodies in the sera of cancer patients was associated with poor prognosis[61]. Depending on the tumour type studied and murine model investigated, a number of mechanisms for the pro-tumorigenic nature of B cells have been suggested.

Antibody production

Whilst several human studies show a positive correlation between antibodies directed against Her2/neu or mucin 1 (MUC-1) with favourable patient prognosis, high serum anti-p53 antibody levels are associated with poor prognosis[61-64]. It has been speculated that this may be due to high antigen load and exposure rather than a reflection of poor immune activity. Antibodies activate the complement system once they have bound the antigen in the immune-complexes[65]. However, murine studies showed that, counter-intuitively, some antibodies might contribute to the progression of tumours by formation of circulating immune-complexes (CICs). These CICs can bind to myeloid cells within tumours, and activate their Fcγ receptors to induce myeloid suppressor cell activity which promotes tumorigenesis[65,66]. Immune-complexes formation can lead also to the activation of complement cascades resulting in formation of C3 and C5a anaphylatoxins, which can induce the recruitment of inflammatory cells which, in turn, may provide a rich pro-angiogenic and pro-tumoral environment [66]. Deposition of complement components per se does not induce chronic inflammation during tumorigenesis in HPV16/recombination activating gene $2^{-/-}$ murine model of skin cancers. However, transfer of competent B cells as well as serum from immuno-competent animals could enhance pre-malignant to malignant transformation for skin cancer, raising the speculation that B-cell derived antibodies home into the neoplastic tissue and activate the complement cascade, mediating recruitment of innate immune cells; thus, modulating a tumour-promoting chronic inflammation[66].

Furthermore, different IgG subclasses have distinct biological function[67]. IgG4 is associated with chronic antigen exposure, typical of cancer disease, and *in vitro* and *in vivo* studies have demonstrated that this subclass counteracts anti-tumour immunity by antagonising IgG1-mediated immunity[68]. The presence of IgG4 in tumour microenvironment (TME) not only prevents IgG1-FcR-mediated effector functions, contributing to tumour evasion to humoral immunity, but could also impair therapeutic antibody effector function[69].

Cytokine production by B cells

B cells have been shown to directly inhibit cytotoxic T-cell responses in several tumour models *via* the production of B-cell-derived factor[70,71]. The negative correlation between high tumour-infiltrating B cells and prognosis in prostate cancer was ascribed to the production of lymphotoxin by tumoral B cells recruited by chemokine (C-X-C motif) ligand 13 (CXCL13) signalling, after androgen ablation by castration in a mouse prostate cancer model[58,72]. Lymphotoxin activates non-canonical and canonical nuclear factor kappa-B signalling and signal transducer and activator of transcription 3 in the remaining cancer cells, resulting in androgen-refractory growth and tumour progression[72].

B-regulatory functions

Akin to T-cell subtypes, phenotypically and functionally distinct B-cell subpopulations have been identified. In presence of chronic exposure to the antigen and chronic inflammation, such auto-immune encephalomyelitis or colitis, and cancer, B cells may acquire a regulatory phenotype[73-75]. This subset of B cells have been shown to have immunosuppressive properties, alongside with myeloid-derived suppressor cells or T-regulatory cells (Tregs), thus expanding the team of the suppressive immune players within the TME[76,77]. These B-regulatory cells act as tumour promoters by affecting the function of other immune cells, through immunosuppressive factors, such as transforming-growth factor (TGF)-β, interleukin (IL)-4, and IL-10, which are associated with Th2 skewing of T cells, IL-13 and IL-35, that support tumour-cell growth as well as M2 polarisation of tumour-associated macrophages (TAMs). Moreover, immunosuppression is further induced through PD1 expression, which, by binding to PD-L1 on the surface of tumour cells, can abrogate tumour recognition and killing. In addition to these indirect mechanisms, B regulatory cells can be directly pro-tumourigenic, for example, B-cell derived TGF-β promotes epithelial-mesenchymal transition in colorectal cancer, or through

CD40/CD154 signalling pathway drives primary liver cancer[78,79]. These distinct B-cell phenotypes and mechanisms may account for the paradoxical tumour-promoting role of B cells observed in human studies and murine models.

However, the depletion of B cells using a B-cell depleting antibody, for treatment of renal cell carcinoma, melanoma or colorectal cancer, did not show any clinical benefit[80,81]. In particular, in an old early phase clinical trial involving patients with advanced colorectal cancer (n = 14), a reduction of the tumour size was observed after treatment with Rituximab, a humanised monoclonal antibody directed against human CD20, and was associated with a reduction of hyper-positive CD21 B cells in peripheral blood[81]. Surprisingly, this observation has not been further explored in later phase clinical trials. Nevertheless this observation is substantiated by *in vivo* studies using syngeneic tumour implantation models[82]. The vast majority of these studies, using genetically deficient murine models for B cells, show that B-cell infiltration within the TME produces worse outcomes in mouse models[71]. In contrast, acute B-cell depletion using anti-CD20 antibody did not recapitulate these findings[83]. It is important to note that B-cell deficient mice manifest several secondary immune abnormalities that may contribute to their tumour-suppressive phenotype[83].

B cell exhaustion

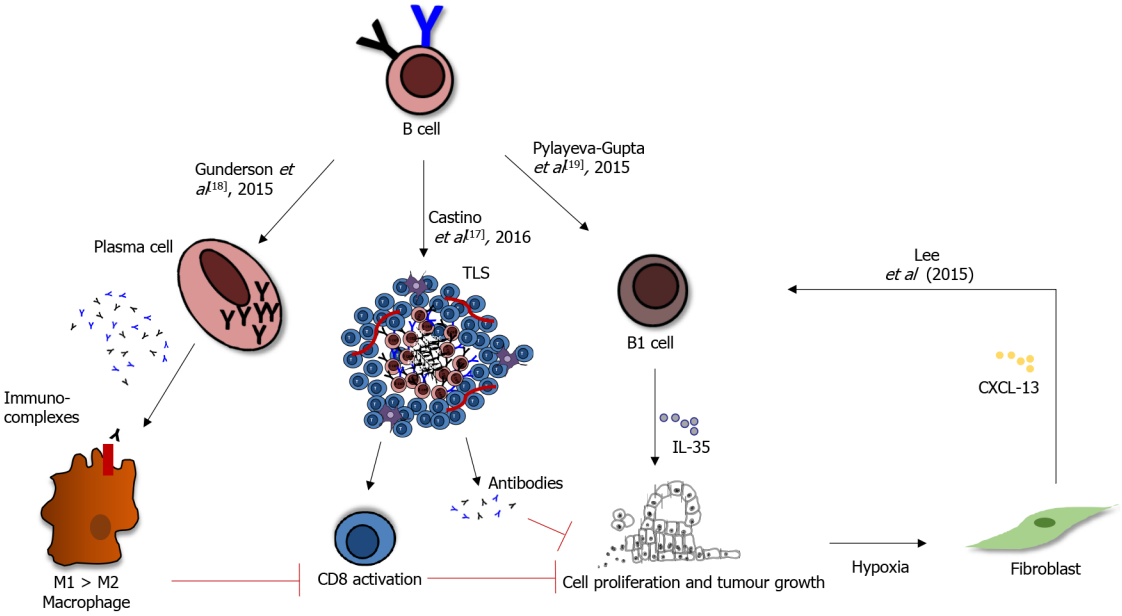
Akin to T cell exhaustion, recent reports describe a reversible state of B cell dysfunction, different from anergy and senescence, named B-cell exhaustion. Exhausted B cells, identified in viremic HIV patient blood[84], and described in older and auto-immune patients[85], are phenotypically characterised by low CD21 and CD27 expression, high expression of inhibitory receptors, and deficient effector functions [86]. In NSCLC and breast cancer, exhausted B cells, also named tissue-like memory B cells, were found to correlate with T regulatory cells and exhausted PD1$^+$ CD4$^+$/CD8$^+$ T cells[87,88].

B CELLS IN PDAC

Similar to other cancers, the role of B cells in pancreatic cancer is controversial, perhaps due to model selection in various studies. B cells are generally associated with an improved outcome in PDAC patients[14], and yet often, their presence correlates with tumour growth and shorter survival in murine models of pancreatic cancer, with various mechanisms offered for this pro-tumorigenic role[18-21]. For example, immunoglobulins produced by splenic B cells may form immune complexes, that can bind TAMs and induce M2 polarisation, consequently suppressing the CD8$^+$ T-cell cytotoxic activity; thus, driving tumour progression (Figure 1). Both B cells and macrophages were shown to express activated Bruton's tyrosine kinase (BTK). *In vitro* the use of the BTK inhibitor Ibrutinib blocked the M2 polarisation of macrophages that occurred following co-culture with B cells, suggesting that B cells promote the pro-tumorigenic macrophage phenotype, and that BTK signalling is tumour-promoting in both these immune cell types. The use of the BTK inhibitor Ibrutinib in orthotopic pancreatic tumours *in vivo* reduced tumour growth[18]. A subset of regulatory B cells, called B1 (identified as CD1d^{hi} , CD5$^+$, 10% of all B cells within the murine tumours), express relatively high levels IL-12a and Ebi3 transcripts, which encode for IL-35: An immunosuppressive cytokine (Figure 1)[19]. The injection of IL12a$^{-/-}$ B cells was unable to restore tumour growth, implying that B-cell-derived IL-35 drives tumour cell proliferation[19]. Moreover in the presence of hypoxia, induced by HIF1α deletion, fibroblasts secrete CXCL13, which recruits B cells to the tumour site; in particular the B1 regulatory B cells, which promote tumour growth (Figure 1)[20].

However, these immuno-suppressive B cells represent a mere 10% of the entire B-cell population in PDAC. Therefore, their pro-tumorigenic role might be overcome by the presence of a much larger proportion of pro-inflammatory B cells. The genetic analysis of bulk intra-tumoral B-cell population showed a pro-inflammatory and immuno-stimulatory phenotype in both orthotopic and the KPC (KrasG12D-Pdx1-Cre) genetic models of PDAC[22]. Indeed, the phenotype of splenic B cells differs from the intra-tumour B-cell phenotype[22]. Since Gunderson and colleagues used splenic and not intra-tumour B cells, in co-culture experiments with bone-marrow-derived macrophages, the immune-suppressive role of B-cells described by them might be irrelevant within the tumour microenvironment [18]. Furthermore, in independent experiments, it appears that the regulatory phenotype is not acquired in the tumour microenvironment. CD1d^{hi} CD5$^+$ B cells isolated from a healthy spleen and injected into a μMT mice (genetic depletion of B cells from birth) before orthotopic cancer cell injection rapidly restored tumour cell growth[19].

Interestingly, most studies investigating the role of B cells in cancer immunity were conducted in B-cell-deficient mice, where the absence of B cells restricted tumour growth in a variety of tumour models, generally suggesting that B cells inhibit rather than enhance spontaneous anti-tumour immunity[18-20, 82,83,89]. On the other hand, the majority of models using an acute B-cell depletion in an established tumour (for example, achieved by treatment with a B-cell depleting antibody, anti-CD20) enhanced tumour growth, suggesting that B cells may have an anti-tumoral role[89-91]. Since, this anti-tumoral aspect is not confirmed if B-cell depletion occurs before initiation of tumour growth, we can speculate that B cells play an initial immunosuppressive/pro-tumoral role; perhaps a role played by circulating or



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Figure 1 B-cell role in pancreatic cancer. B cells mature in plasma cells, which can produce immunoglobulin G, and are able to reprogram the M1 macrophage phenotype to M2 *via* Bruton's tyrosine kinase activation. B regulatory cells are able to produce immune-suppressive cytokines, which inhibit the anti-tumour immune response, leading to tumour growth. Furthermore, in presence of hypoxia, stromal fibroblasts can secrete chemokine (C-X-C motif) ligand 13, which recruit B regulatory cells ($CD1d^{hi}CD5^{+}$) and B1 B cells, resulting in faster tumour growth. Clusters of B cells, with follicular dendritic cells and T cells, are sites for T cell priming and B cell maturation and differentiation into antibody-producing cells, with anti-tumoral effect. TLS: Tertiary lymphoid structures; IL-35: Interleukin-35; CXCL-13: Chemokine (C-X-C motif) ligand 13.

peripheral B cells. However, over the course of tumour development, as B cells infiltrate tumours, they form TLS and acquire a pro-inflammatory phenotype that sustains DC recruitment and activation and antigen presentation, resulting in an anti-tumoral role[14,89,92]. Of note, depletion of B cells earlier in PDAC development in a more relevant pre-clinical model of PDAC, KPC (KrasG12D-Pdx1-Cre) mice, did not impact disease progression[22]. In contrast, B-cell compartment is competent before and during human PDAC tumourigenesis. Lastly, it is now well understood that B-cell-deficient murine models harbour several immune abnormalities, such as defects in myeloid subsets, which may render those mice tumour-resistant[93]. Therefore, acute B-cell depletion in tumour-bearing mice may represent a more reliant model to study the effect of B cells in cancer (Figure 1) [14,22,92].

Based on these considerations, B-cell depletion may prevent TLS formation, suggesting that removing B cells in PDAC patients may be detrimental, as the tumours are deprived of sites of DC localisation and anti-tumour immune response[34,94]. Presence of TLS has been shown to be associated with improved patient survival in PDAC[16,17,95]. The location of TLS (peri-tumoral and intra-tumoral) may be important since those with intra-tumoral TLS had better outcome[16]. PDAC tissues with intra-tumoral TLS showed significantly higher infiltration of T and B cells and lower infiltration of immunosuppressive cells, as well as significantly higher expression of Th1- and Th17-related genes.

It is possible that the dual behaviour of B cells in non-metastatic PDAC patients is dependent on their spatial organisation[17]. Favourable clinical outcome was observed when B cells were organised in TLS, whilst worse patient survival was observed when B cells were scattered at the tumour-stroma edge. The two studies show a different TLS distribution, probably due at the different approaches used for the identification. Hiraoka *et al*[16] demonstrate a near-universal presence of TLS within human PDAC tissue based on H&E staining, whilst Castino *et al*[17] identify the aggregate pattern only in a subset of patients. This apparent discrepancy, described also in other cancers, can be resolved thorough TLS functional characterisation, such as activation status and composition, through use of key phenotypic markers; thus, rendering them more useful in predicting patients' outcome[95-97].

In the KPC transgenic murine model, more closely mimicking human cancer, sporadic presence of TLS was observed, but in the orthotopic model of PDAC, lacking the characteristic desmoplastic stroma, TLS were not observed[17]. Not only TLS developed spontaneously within the tumour parenchyma of the KPC mice, but also their formation could be enhanced by injection with the immunotherapeutic DNA-vaccine encoding the glycolytic enzyme ENO1. The vaccination induced a higher number of TLS, $PD1^{+}$ GC formation and increased antigen-specific T-cells infiltration[17]. Furthermore injection of chemokine (C-C motif) ligand 21 (CCL21) in a subcutaneous PDAC murine model showed a beneficial effect, by inhibiting tumour growth, decreasing distant metastasis, and recruiting T and DCs within the TME[98]. In keeping with these observations, NSCLC patients are receiving intra-tumoral injections of CCL21-transduced autologous DCs in a phase I clinical trial (NCT00601094, NCT01574222)[99].

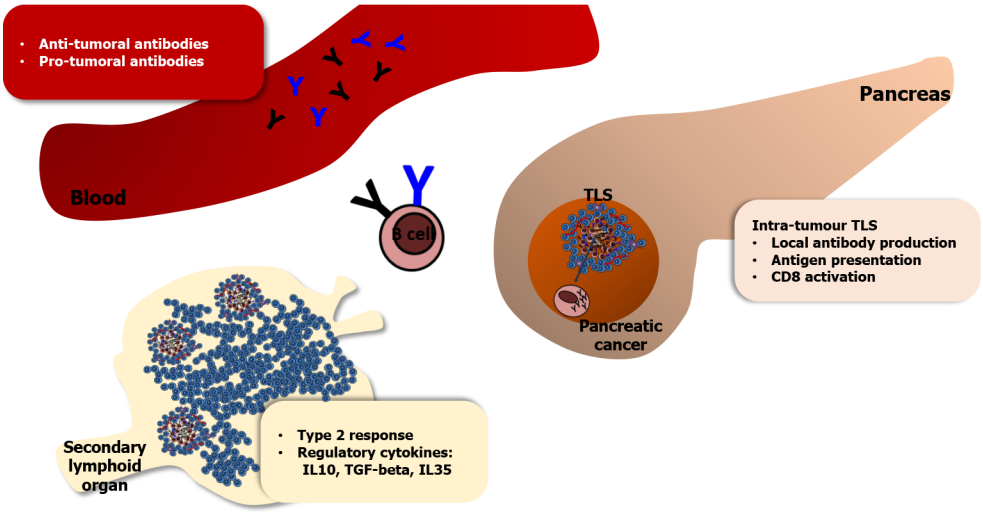
Several studies report the development of TLS after anti-tumour vaccination protocols, including pancreatic cancer[6,95,99]. Lutz *et al*[95] used an irradiated, GVAX given as a single agent or in combination with low-dose cyclophosphamide to deplete regulatory T cells, showing a way to convert a "non-immunogenic" neoplasm such as PDAC, into an "immunogenic" neoplasm, by inducing infiltration of T cells and development of TLS in the TME. The study describes the presence of TLS as defined by a core of B cells and follicular DCs, Ki67 positivity, suggesting the presence of a germinal centre, and $CD3^+$ T cells. Among these, there were $CD4^+$ cells in close vicinity to mature DCs ($CD83^+$ and $DC\text{-}LAMP^+$), and monocyte/macrophages, suggesting that these aggregates exhibited adaptive immunity [95]. A better characterisation of the T-cell subsets suggested the presence of negative regulatory signals in the aggregates: most of the aggregates presented $FoxP3^+$ cells and upregulated PD-L1 expression. Thus, the activities of GVAX included both the recruitment of effector T cells into the TME and the upregulation of immunosuppressive regulatory mechanisms, specifically the expression of PD-L1 and T-regs infiltration. But the net impact of the infiltration of both T-effector (T-eff) and Tregs, expressed as ratios of interferon γ-producing Teff/Tregs, were higher in vaccinated patients, suggesting that GVAX can alter the balance of T-eff and T-regs, in favour of an anti-tumour response. The number of TLS resulted increased after combination of GVAX with cyclophosphamide[95].

TLS are also known to be site for the formation of antigen-specific B cells and development of memory response and represent an "antibody factory" within non-lymphoid tissues. Intra-tumoral B cells have been shown to produce high-affinity anti-tumour antibodies, mostly IgG, in several human and murine model studies, providing evidence that tumour-specific humoral responses can be generated *in situ*, within TLS[26,36,37,52]. Such evidence of humoral response is provided by the presence of germinal centres and follicular DC network in PDAC-associated TLS[16]. Furthermore there is evidence of antibody production by intra-tumour derived B cells in PDAC[100]. IgG production against wild type and mutant KRAS targets (a common occurrence in human PDAC) was assessed to study the antigen specificity of PDAC infiltrating B cells[100]. Incubation of tumour infiltrating B-cell supernatant suggests that B-cell responses targeting mutant and not wild-type KRAS are present in the parenchyma of PDAC, yet not detectable in the serum[100].

The identification of TAA-directed immunoglobulins in PDAC would be of great use in cancer therapy. For example, IgG1 antibody PAM4, identified by vaccination of mice with mucin purified from human pancreatic cancer cells, has been applied in radio-immunotherapy and diagnosis[101]. In PDAC, serum titre of MUC-1 specific immunoglobulins correlates with improved patients survival[62]. Examples of anti-MUC1 antibody-based therapeutics developed against pancreatic cancer and that are in clinical trials are huPAM4, PankoMab-GEX (Gatipotuzumab), AR20.5[102-104]. Many other pancreatic cancer specific antigens could serve as valid clinical targets[11,105-108].

CONCLUSION

B cells play a different role in human and murine cancers. In PDAC, high B-cell infiltrate is associated with better prognosis, especially when those B-cells cluster in TLS (Figure 2). Yet this is discordant with data obtained using orthotopic models of PDAC, where B-cell depletion suggests an early, pro-tumour function of B cells (Figure 2). This apparent paradox can be explained with B cells playing different roles as the tumour progresses and evolves. Firstly, there are differences between intra-tumour and peripheral immune-responses, as demonstrated by *in vivo* studies in both PDAC[22] and other cancers [109,110]. The more complex cell-cell interactions within the TME may influence B cell phenotype. There are inherent difficulties to recapitulate these features in murine models where the desmoplasia, a characteristic feature of human PDAC may not always be present[111-113]. Furthermore, as with T cells, multiple B-cell subsets have been extensively described in murine models of cancer, but not in human PDAC; and this would be the new frontier of investigation. Despite the current failure of immuno-therapy in PDAC, exploring new successful immuno-therapeutic avenues may still be possible. For example, immuno-therapy with immune-checkpoint inhibitors appears effective in the small percentage of PDAC patients harbouring MMR deficiency. Target immunotherapy should be considered for the different PDAC (immune)-subtypes, and should aim to enhance the potential in situ anti-tumour response, which arises within some tumours (TLS+ve patients), with a possibility to revert the immune-suppressive TME. Current immuno-therapeutics under investigation in PDAC in relation to B-cell modulation, include promoting the anti-tumour response [the GVAX vaccine induces the *in situ* formation of active clusters of T and B cells (TLS)] or inducing the immuno-tolerance (Ibrutinib, BTK inhibitor). Combination with other stromal modulating approaches may yield substantial benefits[111, 114,115]. An extensive immuno-genetic and immuno-phenotypic profiling of tumour infiltrating B cells may pave the way towards the understanding of integrated tumoral immune system in PDAC and generate crucial new therapeutic insights.



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Figure 2 Mechanisms by which B cells regulate tumour growth. Different conclusions drawn from human and mouse studies can be accommodated within this model which takes into account the different intra-tumour and peripheral immune-responses. *In vitro* and *in vivo* studies usually measure the functional immune response in secondary lymphoid organs or blood, rather than studying the infiltration and the spatial organisation of different immune cells within the tumour microenvironment. In the pancreas, B cells can form clusters with T cells, named tertiary lymphoid structures, which are sites of antigen presentation, CD78 activation and antibody production. However, in secondary lymphoid organ the presence of B cells during T cell priming can skew the immune response towards Th2, attenuating Type 1 response. Furthermore, B-regulatory cells can produce immune-suppressive cytokines, which inhibit the anti-tumour immune-response. Finally, a positive correlation is found between serum immunoglobulin G (IgG) 1 and increased survival. However, repeated isotype switching within IgG subclasses generates in human IgG4, an isotype that has been linked to regulatory functions, in mouse models IgG2a, with pro-inflammatory functions. TLS: Tertiary lymphoid structures; IL: Interleukin; TGF-beta: Transforming-growth factor-β.

FOOTNOTES

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Country/Territory of origin: United Kingdom

ORCID number: Francesca Romana Delvecchio 0000-0002-3661-1212; Michelle R Goulart 0000-0001-8333-3908; Rachel Elizabeth Ann Fincham 0000-0002-4699-5002; Michele Bombadieri 0000-0002-3878-5216; Hemant M Kocher 0000-0001-6771-1905.

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REVIEW

Impaired coagulation, liver dysfunction and COVID-19: Discovering an intriguing relationship

Damiano D'Ardes, Andrea Boccatonda, Giulio Cocco, Stefano Fabiani, Ilaria Rossi, Marco Bucci, Maria Teresa Guagnano, Cosima Schiavone, Francesco Cipollone

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Damiano D'Ardes, Ilaria Rossi, Marco Bucci, Maria Teresa Guagnano, Francesco Cipollone, "Clinica Medica" Institute, Department of Medicine and Aging Sciences, "G. D'Annunzio" University of Chieti-Pescara, Chieti 66100, Italy

Andrea Boccatonda, Giulio Cocco, Stefano Fabiani, Cosima Schiavone, Unit of Ultrasound, "G. D'Annunzio" University of Chieti-Pescara, Chieti 66100, Italy

Corresponding author: Damiano D'Ardes, MD, Doctor, Research Fellow, "Clinica Medica" Institute, Department of Medicine and Aging Sciences, "G. D'Annunzio" University of Chieti-Pescara, Chieti 66100, Italy. 89damiano@gmail.com

Abstract

Coronavirus disease 2019 (COVID-19) is, at present, one of the most relevant global health problems. In the literature hepatic alterations have been described in COVID-19 patients, and they are mainly represented by worsening of underlying chronic liver disease leading to hepatic decompensation and liver failure with higher mortality. Several potential mechanisms used by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) to cause liver damage have been hypothesized. COVID-19 primary liver injury is less common than secondary liver injury. Most of the available data demonstrate how liver damage in SARS-CoV-2 infection is likely due to systemic inflammation, and it is less likely mediated by a cytopathic effect directed on liver cells. Moreover, liver alterations could be caused by hypoxic injury and drugs (antibiotics and non-steroidal anti-inflammatory drugs, remdesivir, tocilizumab, tofacitinib and dexamethasone). SARS-CoV-2 infection can induce multiple vascular district atherothrombosis by affecting simultaneously cerebral, coronary and peripheral vascular beds. Data in the literature highlight how the virus triggers an exaggerated immune response, which added to the cytopathic effect of the virus can induce endothelial damage and a prothrombotic dysregulation of hemostasis. This leads to a higher incidence of symptomatic and confirmed venous thrombosis and of pulmonary embolisms, especially in central, lobar or segmental pulmonary arteries, in COVID-19. There are currently fewer data for arterial thrombosis, while myocardial injury was identified in 7%-17% of patients hospitalized with SARS-CoV-2 infection and 22%-31% in the intensive care unit setting. Available data also revealed a higher occurrence of stroke and more serious forms of peripheral arterial disease in COVID-19 patients. Hemostasis dysregulation is observed during the COVID-19 course. Lower platelet count, mildly increased prothrombin time and increased D-

dimer are typical laboratory features of patients with severe SARS-CoV-2 infection, described as "COVID-19 associated coagulopathy." These alterations are correlated to poor outcomes. Moreover, patients with severe SARS-CoV-2 infection are characterized by high levels of von Willebrand factor with subsequent ADAMTS13 deficiency and impaired fibrinolysis. Platelet hyperreactivity, hypercoagulability and hypofibrinolysis during SARS-CoV-2 infection induce a pathological state named as "immuno-thromboinflammation." Finally, liver dysfunction and coagulopathy are often observed at the same time in patients with COVID-19. The hypothesis that liver dysfunction could be mediated by microvascular thrombosis has been supported by post-mortem findings and extensive vascular portal and sinusoidal thrombosis observation. Other evidence has shown a correlation between coagulation and liver damage in COVID-19, underlined by the transaminase association with coagulopathy, identified through laboratory markers such as prothrombin time, international normalized ratio, fibrinogen, D-dimer, fibrin/fibrinogen degradation products and platelet count. Other possible mechanisms like immunogenesis of COVID-19 damage or massive pericyte activation with consequent vessel wall fibrosis have been suggested.

Key Words: COVID-19; SARS-CoV-2; Liver; Coagulation

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Core Tip: Evidence in the literature suggests a contribution of the severe acute respiratory syndrome coronavirus 2 to the pathophysiology of liver injury, atherothrombosis and coagulation disorders. This minireview explores the possible mechanisms by which these alterations are generated during coronavirus disease 2019 according to current knowledge.

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INTRODUCTION

Coronavirus disease 2019 (COVID-19) is, at present, one of the most relevant global health problems, declared a pandemic on March 11, 2020 by the World Health Organization[1]. It is caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), a novel positive-sense single-stranded RNA betacoronavirus[2,3]. The pulmonary manifestation of COVID-19, including pneumonia and acute respiratory distress syndrome, are well known, but it is necessary to emphasize that the coronavirus deleterious effects are also exerted on many other organ systems and are responsible for extrapulmonary manifestations[4].

SARS-CoV-2 can cause both direct and indirect cardiovascular sequelae (myocardial injury, acute coronary syndromes, cardiomyopathy, acute cor pulmonale, arrhythmias and cardiogenic shock), acute kidney injury and gastrointestinal symptoms, such as diarrhea, nausea, vomiting, abdominal pain and anorexia[4]. Neurological complications include headaches, dizziness, ageusia, myalgia, anosmia up to stroke, Guillain-Barré and encephalopathy, and dermatological signs have also been described (petechiae, urticaria, vesicles, erythematous rash, livedo reticularis)[4]. Hepatobiliary manifestations can be observed especially in patients with severe presentations of COVID-19 and occur mainly with increased plasma levels of transaminases and bilirubin[4]. Thromboembolic events have also been described in COVID-19, such as acute limb ischemia, which can occur in patients without existing peripheral arterial disease and in those receiving thromboprophylaxis. Acute abdominal-thoracic aortic thrombosis and mesenteric ischemia are less common but associated with significant morbidity and mortality[5]. Several studies have also demonstrated increased rates of deep vein thrombosis and pulmonary embolism in COVID-19 patient[5-7]. Acute cerebrovascular disease, including ischemic stroke, and disseminated intravascular coagulation are also severe thrombotic complications of COVID-19 that must not be forgotten[5].

This article aims to explore the possible mechanisms underlying hepatic and hemocoagulative alterations in COVID-19 through the search of published, readily accessible, peer-reviewed, full articles related to this topic written in English and found on PubMed.

COVID-19 AND LIVER

Pathophysiology

SARS-CoV-2 has the capacity to infect cells through the angiotensin-converting enzyme 2 (ACE2) receptor that is mainly expressed on the type 2 alveolar cells[8]. The ACE2 receptor is mainly present in the liver, lung, heart, renal and gastrointestinal system[9]. Regarding hepatic involvement, the level of ACE2 expression in cholangiocytes (59.7%) is higher than hepatocytes (2.9%)[10]. SARS-CoV-2 cell entry is mediated by its S protein, which specifically interacts with the host cell ACE2 and transmembrane serine protease 2[11]. The major expression of ACE2 in cholangiocytes reveals that SARS-CoV-2 may cause bile duct dysfunction. Cholangiocytes play critical roles in liver regeneration and immune responses, indicating that viral immunologic injury might be important in liver injury in COVID-19[12].

Even if there is a substantial difference in ACE2 receptor expression between the liver and biliary tract, the liver is not unaffected by the SARS-CoV-2 infection. In fact, current data have shown that in patients with COVID-19 aspartate aminotransferase (AST), alanine aminotransferase (ALT) and lactate dehydrogenase levels are increased, while alkaline phosphatase and gamma glutamyl transferase, representatives of bile duct injury, did not increase significantly[12]. Indeed, in the literature many clinical findings and hepatic alterations are described, and there are several reported potential mechanisms used by the virus to cause liver disease.

Most of the available data demonstrate that hepatic injury during SARS-CoV-2 infection is more likely due to systemic inflammation, and it is less likely mediated by a cytopathic effect directed on liver cells. Viral RNA can be detected in liver tissue of patients with COVID-19, but infection of liver cells has not yet been demonstrated[13]. During a viral infection, the innate and the acquired immune systems recognize pathogen associated molecular patterns and specific viral antigens and release inflammatory molecules, such as cytokines and chemokines, that activate macrophages and T cells to clear the virus and kill infected cells. In patients affected by COVID-19, high levels of inflammatory cytokines have been observed, and this context could cause significant liver damage when SARS-CoV-2 infects host hepatocytes. The most involved inflammatory molecules are tumor necrosis factor, interleukin-2 (IL-2), IL-6, IL-7, IL-18, granulocyte-colony stimulating factor, interferon-γ and ferritin[14]. An early hypercytokinemia could lead to multiorgan injuries, including the liver (Figure 1). The literature demonstrates that worse outcomes of COVID-19 are more common in patients with early cytokine elevation[15].

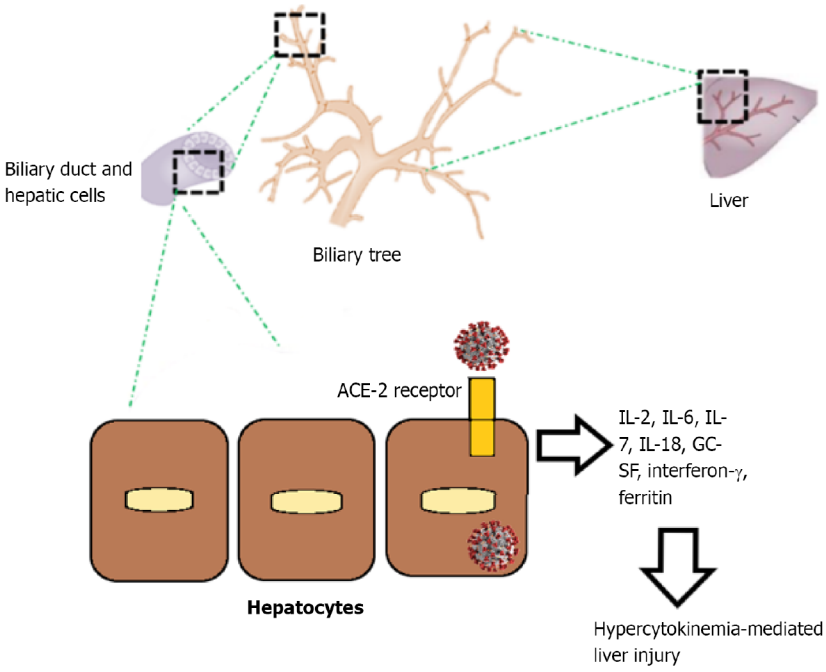
Another potentially harming mechanism is represented by hypoxic injury. The complex vascularization of the liver makes it particularly exposed to circulatory alterations that could be generated in cardiac, circulatory, respiratory failure or in septic shock, causing decreased perfusion of the liver[12]. About 1.1%-20.0% of COVID-19 patients are affected by septic shock, and 23.0% of patients have heart failure[16].

Drugs are also involved with damage mechanisms, particularly causing drug induced liver injury. Antibiotics and non-steroidal anti-inflammatory drugs, which are one of the most common causes of drug induced liver injury in the general population, can contribute to liver damage in COVID-19 patients when used to treat bacterial superinfection, myalgias or fever. Furthermore, drug induced liver injury has been shown in 15.2% of patients receiving remdesivir and in 37.2% of patients in treatment with lopinavir/ritonavir[17]. Finally, dexamethasone, tocilizumab and tofacitinib, used to treat COVID-19, could potentially create hepatic injury *via* pre-existing chronic liver disease reactivation, especially hepatitis B virus[18].

Clinical characteristics

During SARS-CoV-2 infection, patients can be asymptomatic or present clinical symptoms such as fever, dry cough, headache, dyspnea and fatigue and up to acute respiratory distress syndrome, shock and cardiac failure[19,20]. Hepatic involvement can occur through all the pathophysiological pathways previously investigated and still other studies are needed to better characterize them. In current reports, COVID-19 primary liver injury is less common than secondary liver injury[21,22]. SARS-CoV-2 liver involvement is mainly represented by worsening of underlying chronic liver disease, leading to hepatic decompensation and acute-on-chronic liver failure, with higher mortality. Symptoms mainly reported in the literature associated to liver injury and gastrointestinal involvement are diarrhea, nausea, vomiting and loss of appetite. Abnormal liver function was also observed with the increase of AST, ALT, lactate dehydrogenase and bilirubin and the decrease of albumin[21-25].

According to the current data, hepatic dysfunction is significantly higher in critically ill patients and is associated with a poor outcome, underlining its importance in clinical settings. Elevated liver enzymes are observed predominantly in severe and critical cases of COVID-19. For example, increased AST was observed in 62% of patients in the intensive care unit (ICU) compared to 25% in non-ICU patients[23]. Chen *et al*[21] reported that AST, ALT, alkaline phosphatase, gamma glutamyl transferase and bilirubin levels were significantly higher in non-survivors than in survivors. Also hypoalbuminemia was found significantly lower in deceased patients rather than in surviving patients. Furthermore, according to Bangash *et al*[24] the mortality rate in patients with underlying chronic liver disease was 0%-2%. According to the literature data, liver injury is most common in critically ill patients who have diabetes and hypertension[22,25].



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Figure 1 Structure of severe acute respiratory syndrome coronavirus 2 hepatic receptors that induce hepatic injury mediated by cytokine storm. Inflammatory mediators secreted by lymphocytes and macrophages aggravate inflammatory responses causing hepatic damage. IL: Interleukin; GC-SF: Granulocyte colony stimulating factor; ACE-2: Angiotensin-converting enzyme 2.

The fact that liver injury in COVID-19 is mostly hepatocellular rather than cholestatic is demonstrated by the more frequent elevation of ALT, AST and lactate dehydrogenase than of alkaline phosphatase and gamma glutamyl transferase. The latter two did not increase significantly, and jaundice is uncommon[12,26]. Moreover, the current data demonstrate that AST could represent an important hepatocellular injury marker because its elevation is associated with a major mortality risk[27]. According to a meta-analysis, the pooled prevalence of abnormal liver functions (12 studies, 1267 patients) was 19%, and in subgroup analysis patients with severe COVID-19 had higher rates of abdominal pain and abnormal liver function including increased ALT and AST[28].

In a hospital setting, drugs also have to be considered in liver injury; moreover COVID-19 critically ill patients are treated with multiple drugs, such as antibiotics, immunosuppressants and antiviral and antipyretic agents that are associated to abnormal liver function, especially when used in patients with severe COVID-19[23,29]. In the literature, drug induced liver injury has been shown to result in AST and ALT elevation and reactivation of pre-existing chronic liver disease, above all hepatitis B virus infection[17,18]. Indeed, a retrospective study has shown that patients with chronic hepatitis B virus hepatitis had a worse prognosis for COVID-19 and for a higher mortality and a higher incidence of acute-on-chronic liver failure[30]. In a trial by Goldman *et al*[31] comparing remdesivir treatment for either 5 d or 10 d, severe but not life-threatening ALT/AST elevations were reported in 4%-6% of patients and life-threatening AST/ALT elevations in 2%-3% of patients.

COVID-19 AND COAGULATION DISORDERS

Clinical characteristics

SARS-CoV-2 infection can induce multiple vascular district atherothrombosis by simultaneously affecting cerebral, coronary and peripheral vascular beds. Data in the literature highlight how the virus can trigger an exaggerated immune response, which added to the cytopathic effect of the virus can induce endothelial damage and a prothrombotic dysregulation of hemostasis[32-34]. Nowadays, there are several reports and original papers on cases of venous thromboembolism and pulmonary embolism related to COVID-19[35-40].

Incidence of symptomatic and confirmed venous thrombosis in COVID-19 patients hospitalized in the ICU can reach 30%-40%[41]. Data from a study performed in China demonstrated that 25% of COVID-19 patients developed lower extremity deep vein thrombosis without venous thromboembolism prophylaxis[42]. A work by Klok *et al*[36] described pulmonary embolisms in 25 of 184 ICU patients with COVID-19 (13.6%), 72% of which were in central, lobar or segmental pulmonary arteries, despite standard dose pharmacological prophylaxis[36,43]. In Italy, Lodigiani *et al*[35] showed thromboembolic

events (venous and arterial) in 7.7% of patients admitted with COVID-19, corresponding to a cumulative rate of 21.0%.

There are currently fewer data for arterial thrombosis. A study from Wuhan showed that about 12% of patients displayed Hs-troponin I above the threshold of 28 pg/mL[14]. Other data revealed that myocardial injury was diagnosed in 7%-17% of patients hospitalized with SARS-CoV-2 infection and 22%-31% in the ICU setting[16,25]. Dysregulated inflammatory response enhances atherosclerotic plaque disruption[21,44-46]. Previous studies demonstrated that influenza and community-acquired pneumonia are related to an increased risk of myocardial infarction, within the first 7 d of diagnosis and even after hospitalization[47]. Regarding cerebrovascular disease, occurrence of stroke in COVID-19 patients ranges between 2.7% and 3.8%, and these subgroup of patients often displayed comorbidities such as hypertension and were older on average[48,49]. Moreover, there are some reports documenting more serious forms of peripheral arterial disease in patients with SARS-CoV-2 infection[50].

Laboratory findings

Hemostasis dysregulation has been described as an early pathological change in the COVID-19 course. Lower platelet count, mildly increased prothrombin time and increased D-dimer are typical laboratory features of patients with severe SARS-CoV-2 infection, and they are correlated to poor outcomes[51]. Those changes have been described as "COVID-19 associated coagulopathy"[52-54]. Moreover, patients with severe SARS-CoV-2 infection are characterized by high levels of IL-6, thus leading to a subsequent increase in proteins such as fibrinogen and von Willebrand factor (vWF)[55]. High lactate dehydrogenase and ferritin values are other laboratory findings of patients with severe COVID-19 infection, resembling a thrombotic microangiopathy[51,55,56]. Furthermore, complement components C5b-9, C4d and mannose-binding lectin-associated serine protease 2 have been detected in the small vessels of the lung due to complement-associated microvascular injury[57]. High levels of vWF and subsequent ADAMTS13 deficiency seem to be other typical findings of severe COVID-19 infection[52,58,59]. Decreased levels of ADAMTS13 can induce increased platelet-endothelial interaction generating a thrombotic microangiopathy-like state[52,60-63].

Recent data demonstrate that fibrinolysis is impaired in patients with severe COVID-19[64,65]. Critical COVID-19 patients are characterized by low levels of plasminogen, as for a consumptive state [65]. Nougier *et al*[66] reported elevated levels of plasminogen activator inhibitor 1 and low levels of tissue plasminogen activator, along with high thrombin generation, thus demonstrating a significant imbalance between inhibitor and activator factors of fibrinolysis.

Pathogenetic mechanisms

Platelet hyperreactivity, hypercoagulability and hypofibrinolysis induce a pathological state that has been named "immuno-thromboinflammation" during SARS-CoV-2 infection[67]. SARS-CoV-2 binds to the ACE2 receptor on the surface of endothelial and arterial smooth muscle cells, inducing a cytopathic effect and a subsequent endothelial injury[68]. As it is well known, endothelial damage triggers platelet activation, adhesion to the subendothelial matrix and aggregation, thus generating a platelet plug[69]. Moreover, a release of vWF, inefficient cleavage of ultralarge vWF catalyzed by ADAMTS13, direct contact with activating surfaces in the subendothelial matrix, loss of heparan sulfates at the surface of injured blood vessels, disrupted generation of nitric oxide, prostaglandin E2 and prostaglandin I2 and loss of surface expression of ectonucleotidases occur[70,71]. Platelet activation may represent a consequence of the production of a consistent level of thrombin after initiation of coagulation[64].

Furthermore, high IL-6 levels can induce megakaryocytopoiesis generation and platelet formation, which could play a role to generate a hypercoagulability state, in particular within the lung. Intriguing, SARS-CoV-2 can bind directly to platelets since they express both ACE2 and transmembrane serine protease 2 on their surface[72]; this binding can favor platelet activation and the release of clotting factors, inflammatory molecules and leukocyte-platelet aggregates[72].

In patients affected by severe COVID-19 pneumonia, levels of tissue factor (TF) on monocytes are higher than normal, together with P-selectin expression and the amount of platelet-neutrophil and platelet-monocyte aggregates[2]. Therefore, platelet activation due to adenosine diphosphate, thrombin and collagen stimulation is enhanced in these patients. Platelet hyperactivation induced by SARS-CoV-2 infection induce the release of inflammatory molecules such as cytokines, chemokines, growth factors and even procoagulant factors like fibrinogen and vWF[73]. This mechanism seems to generate a vicious cycle since inflammatory molecules worsen endothelial injury by decreasing nitric oxide availability and enhancing oxidative stress and/or favoring leukocyte-endothelial interaction[74,75].

Endothelial injury triggers the release of TF in the blood stream. Moreover, TF can derive from macrophage/monocyte cells and through their microparticles, as a consequence of macrophage activation syndrome shown in patients with severe COVID-19 infection[76]. SARS-CoV-2 can directly induce macrophage activation, which can even occur as a consequence of the inflammatory hyperactivation (cytokine storm), as demonstrated by high levels of interferon-g, C-C motif chemokine ligand 2 and C-X-C motif chemokine 9 and 10 detected in critical COVID-19 patients[77,78].

Neutrophils can also be activated both directly by SARS-CoV-2 and by other inflammatory cells, thus generating neutrophil extracellular traps that may activate Factor XII and the intrinsic pathway of coagulation[79]. Hemostasis dysregulation in SARS-CoV-2 infection is also characterized by a decreased

activity of endogenous anticoagulants like antithrombin, TF pathway inhibitor and anticoagulation proteins C and S[78,80]. Endothelial injury and platelet hyper-activation in severe COVID-19 patients enhance the release of plasminogen activator inhibitor 1 (PAI-1)[81]. High levels of PAI-1 can further inhibit fibrinolysis, thus worsening the thrombotic burden[82,83]. Therefore, as the pulmonary inflammation progresses, there is consumption of plasminogen, along with high levels of PAI-1 and depletion of tPA, thus inducing a state of hypofibrinolysis and allowing perpetuation of prothrombotic state[84].

In patients with SARS-CoV-2 infection, the presence of antiphospholipid antibodies has been demonstrated, which may directly induce endothelial cell activation and enhance TF expression by monocytes, thus contributing to the procoagulant and prothrombotic state[85-87]. Particularly, antiphospholipid antibodies can bind to the platelets and trigger their activation decreasing levels of inhibitors such as activated protein C and antithrombin and increasing clotting factors such as FXa and thrombin [88-90].

SARS-CoV-2 can dysregulate the ACE pathway by its binding to the ACE2 receptor on tissues; high levels of angiotensin (Ang) II favor PAI-1 and TF expression, thus promoting hypercoagulability and impairing fibrinolysis[60]. Furthermore, Ang II receptors on platelets can induce platelet activation and aggregation[91]. Ang 1,7 levels are lower in COVID-19 patients who develop severe disease[92]. Ang 1,7 is a vasoprotective molecule by mediating vasodilation and blocking platelet aggregation through nitric oxide release[93-95]. Therefore, low Ang 1,7 levels can contribute to the procoagulant state in COVID-19 infection. Finally, obesity is a main risk factor for thrombosis due to adipocytokine-mediated mechanisms, increased inflammatory molecules, Ang II/Ang 1,7 imbalance, reactive oxygen species-mediated endothelial dysfunction and lipid and glucose metabolism changes[96-98].

RELATIONSHIP BETWEEN COVID-19, LIVER AND COAGULATION

Liver dysfunction and coagulopathy are often observed in patients with COVID-19. Japanese researchers found that patients with high ALT had higher levels of D-dimer and fibrin/fibrinogen degradation products. In particular elevation of ALT and D-dimer were identified simultaneously[99]. Moreover D-dimer was independently associated to ALT elevation[99].

This study suggests the hypothesis that liver dysfunction could be mediated by microvascular thrombosis, and intrahepatic microvascular thrombosis could theoretically play a role in this physiopathological context. This hypothesis is supported by post-mortem findings by Sonzogni *et al* [100] who found marked derangement of intrahepatic blood vessels with aspects of intravascular thrombosis, suggesting a possible liver damage linked to thrombotic processes. Other evidence has shown a correlation between coagulation and liver damage in COVID-19. In fact, a Chinese study on COVID-19 patients demonstrated the association between AST and ALT values with coagulopathy, identified through laboratory markers such as prothrombin time, international normalized ratio, fibrinogen, D-dimer and platelet count[101]. Pathological findings are consistent with a vascular-related damage caused by impaired blood flow, with lesions similar to histological characteristics of hepatopulmonary syndrome and in obliterative portal venopathy[100]. Indeed it has been shown a diffuse network of sinusoids decorated by CD34 suggesting an abnormal hepatic circulation of blood[100].

A likely explanation could be related to increased hepatic blood flow: this aspect may be linked to heart distress or to thrombotic phenomena in portal and sinusoidal vessels[100]. The abnormal high levels of transaminases in some patients whose liver sample were analyzed post-mortem could be explained by extensive vascular portal and sinusoidal thrombosis, leading to confluent parenchymal necrosis and hepatic cells accelerating apoptosis[100]. Attention must be paid to the evidence of massive pericyte activation in liver samples obtained post-mortem[100]. Pericytes are involved in the recruitment of inflammatory cells in liver injury, and their transformation in myofibroblast-like cells leads to the production of abundant amounts of extracellular matrix proteins and to the consequent vessel wall fibrosis[100].

CONCLUSION

Liver abnormalities and coagulopathy are physiopathological characteristics of COVID-19 that represent the most relevant global health problem. Hypercoagulability, hypofibrinolysis and platelet alterations during SARS-CoV-2 infection induce a sort of "immuno-thromboinflammation," while mildly increased prothrombin time and increased D-dimer are typical laboratory features of patients with severe SARS-CoV-2 infection, described as "COVID-19 associated coagulopathy." These phenomena are clinically relevant with manifestations of thrombosis in a large variety of anatomic districts. Moreover, hepatic alterations are mainly represented by worsening of underlying chronic liver disease leading to hepatic decompensation and liver failure with higher mortality, and they appear to be mediated more by systemic inflammation, direct cytopathic effect on liver cells, hypoxic injury and drugs. Liver dysfunction and coagulopathy are also observed at the same time in patients with COVID-19, probably mediated by microvascular thrombosis, immunological mechanisms and pericyte activation. More data

are needed to better investigate the relevant relationship between coagulation, liver dysfunction and COVID-19 with the aim to understand more deeply the physiopathological mechanisms of SARS-CoV-2 and to evaluate new therapeutic strategies to prevent mortality.

FOOTNOTES

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Country/Territory of origin: Italy

ORCID number: Damiano D'Ardes 0000-0001-5573-4536; Andrea Boccatonda 0000-0001-9303-7790; Giulio Cocco 0000-0003-3458-5468; Stefano Fabiani 0000-0001-6821-103X; Ilaria Rossi 0000-0001-5163-9929; Marco Bucci 0000-0002-5213-7768; Maria Teresa Guagnano 0000-0001-7225-0891; Cosima Schiavone 0000-0002-8453-4936; Francesco Cipollone 0000-0002-5993-9341.

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MINIREVIEWS

Advanced imaging and artificial intelligence for Barrett's esophagus: What we should and soon will do

Marco Spadaccini, Edoardo Vespa, Viveksandeep Thoguluva Chandrasekar, Madhav Desai, Harsh K Patel, Roberta Maselli, Alessandro Fugazza, Silvia Carrara, Andrea Anderloni, Gianluca Franchellucci, Alessandro De Marco, Cesare Hassan, Pradeep Bhandari, Prateek Sharma, Alessandro Repici

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Marco Spadaccini, Edoardo Vespa, Roberta Maselli, Alessandro Fugazza, Silvia Carrara, Andrea Anderloni, Gianluca Franchellucci, Alessandro De Marco, Alessandro Repici, Department of Endoscopy, Humanitas Research Hospital, IRCCS, Rozzano 20089, Italy

Marco Spadaccini, Edoardo Vespa, Roberta Maselli, Gianluca Franchellucci, Alessandro De Marco, Alessandro Repici, Department of Biomedical Sciences, Humanitas University, Rozzano 20089, Italy

Viveksandeep Thoguluva Chandrasekar, Department of Gastroenterology and Hepatology, Mayo Clinic, Phoenix, AZ 85054, United States

Madhav Desai, Prateek Sharma, Department of Gastroenterology and Hepatology, Kansas City VA Medical Center, Kansas City, MO 66045, United States

Harsh K Patel, Department of Internal Medicine, Ochsner Clinic Foundation, New Orleans, LA 70124, United States

Cesare Hassan, Endoscopy Unit, Nuovo Regina Margherita Hospital, Roma 00153, Italy

Pradeep Bhandari, Department of Gastroenterology, Portsmouth Hospitals University NHS Trust, Portsmouth PO6 3LY, United Kingdom

Pradeep Bhandari, School of Pharmacy and Biomedical Sciences, University of Portsmouth, Portsmouth PO6 3LY, United Kingdom

Corresponding author: Marco Spadaccini, MD, Doctor, Department of Endoscopy, Humanitas Research Hospital, IRCCS, Via Manzoni 56, Rozzano 20089, Italy.
marco.spadaccini@humanitas.it

Abstract

Barrett's esophagus (BE) is a well-established risk factor for esophageal adenocarcinoma. It is recommended that patients have regular endoscopic surveillance, with the ultimate goal of detecting early-stage neoplastic lesions before they can progress to invasive carcinoma. Detection of both dysplasia or early adenocarcinoma permits curative endoscopic treatments, and with this aim, thorough endoscopic assessment is crucial and improves outcomes. The burden of missed neoplasia in BE is still far from being negligible, likely due to inappropriate

endoscopic surveillance. Over the last two decades, advanced imaging techniques, moving from traditional dye-spray chromoendoscopy to more practical virtual chromoendoscopy technologies, have been introduced with the aim to enhance neoplasia detection in BE. As witnessed in other fields, artificial intelligence (AI) has revolutionized the field of diagnostic endoscopy and is set to cover a pivotal role in BE as well. The aim of this commentary is to comprehensively summarize present evidence, recent research advances, and future perspectives regarding advanced imaging technology and AI in BE; the combination of computer-aided diagnosis to a widespread adoption of advanced imaging technologies is eagerly awaited. It will also provide a useful step-by-step approach for performing high-quality endoscopy in BE, in order to increase the diagnostic yield of endoscopy in clinical practice.

Key Words: Barrett's esophagus; Endoscopy; Artificial intelligence; Surveillance; Advanced imaging; Neoplasia

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Core Tip: It is recommended that patients with Barrett's esophagus (BE) have regular endoscopic surveillance, with the ultimate goal of detecting early-stage neoplastic lesions, before they could progress to invasive carcinoma. Over the last two decades advanced imaging techniques, from traditional dye-spray chromoendoscopy to more practical virtual chromoendoscopy technologies, have been introduced with the aim to enhance neoplasia detection in BE. As witnessed in other fields, artificial intelligence (AI) has revolutionized the field of diagnostic endoscopy and is set to cover a pivotal role in BE as well. In this commentary, we summarize recent research advances and provide future perspectives regarding advanced imaging technology and AI in BE. It also provides a useful step-by-step approach for performing high-quality endoscopy in BE, in order to increase the diagnostic yield of endoscopy in clinical practice.

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INTRODUCTION

The incidence of esophageal adenocarcinoma (EAC) has been rapidly increasing in the last few decades and is expected to rise further in upcoming years[1,2]. Barrett's esophagus (BE) is a well-established risk factor for EAC[3], progressing from non-dysplastic BE (NDBE) to low-grade dysplasia, high-grade dysplasia (HGD), and EAC. Thus, it is recommended that patients with BE have regular endoscopic surveillance[4,5]. The ultimate goal of endoscopy in BE patients is to detect subtle early-stage neoplastic lesions, before they can progress to invasive adenocarcinoma. Detection of both dysplasia or early EAC typically permits curative endoscopic therapies[6-9], and an accurate endoscopic assessment is crucial, considering the average 5-year survival rate of only 18% associated with invasive EAC[10].

Unfortunately, despite the widespread use of upper gastrointestinal (GI) endoscopy, standard quality indicators are lacking for BE surveillance. Elsewhere in endoscopy, quality benchmarks, such as adenoma detection rate (ADR) for colonoscopy, have already been shown to improve patient outcomes [11]. As ADR improvement reduces interval colorectal cancer[12], the rates of dysplasia and EAC at index upper GI endoscopy, namely the neoplasia detection rate, might have important clinical implications serving as a quality measure in BE[13]. A recent meta-analysis by Visrodia *et al*[14] showed that about 20% of HGD/EAC on BE were detected within 1 year from initial diagnosis, likely being missed at that time, highlighting the paramount importance of performing a high-quality endoscopy. Thus, timely endoscopic detection is key to prevent invasive EAC and has been shown to improve outcomes [15].

Over the last two decades, advanced imaging techniques, moving from traditional dye-spray chromoendoscopy to more practical virtual chromoendoscopy (VCE) technologies, have been introduced with the aim of enhancing neoplasia detection in BE. As witnessed in other fields of advanced endoscopy imaging[16-18], thanks to its ability to automatically recognize specific visual patterns, artificial intelligence (AI) has revolutionized the field of diagnostic endoscopy. The aim of this commentary is to summarize both present evidence and future perspectives in order to provide a useful

step-by-step approach for performing high-quality endoscopy in BE to increase the diagnostic yield of endoscopy in BE.

HOW TO OPTIMIZE THE ENDOSCOPIC EXAMINATION OF BE?

International guidelines recommend high-definition/high-resolution white light endoscopy for examination[4,5]. The use of magnification may also permit closer evaluation of the microstructures of the mucosa, the glandular pattern and its vasculature[19]; thus, use of a scope with optical zoom capabilities is suggested. Furthermore, use of a distal attachment (*i.e.* transparent or black cap) could facilitate visualization. In Figure 1 we provide a step-by-step approach for the high-quality endoscopic evaluation of BE.

During upper GI endoscopy for BE, the distal esophagus should be carefully cleaned with the water jet to remove any debris or mucus. Esophageal landmarks should be documented, including diaphragmatic hiatus, gastro-esophageal junction, and squamo-columnar junction, using the Prague classification system[20]. Then a detailed examination of BE mucosa is recommended to identify (and biopsy) any visible mucosal irregularity (Figure 2). The endoscopist should spend about 1 min *per* cm of BE segment carefully inspecting the metaplastic mucosa[21]. The Paris classification[22] should be used to describe any visible lesion. Once the mucosal irregularities (*i.e.* nodule, erythema, erosion) have been biopsied, four-quadrant random biopsies for every 1-2 cm of the BE segment length should be performed, following the Seattle protocol[4,5], with sensitivity and specificity ranging from 28% to 85% and 56% to 100%, respectively[23].

However, this protocol is far from perfection, as it is prone to several drawbacks. (1) Less than 5% of the BE epithelium is sampled[24], and sampling error is indeed common, increasing by 31% for every cm of BE[25]; (2) Performing such a number of biopsies is time-consuming, which may hamper the widespread adoption of such protocols in clinical practice, with nearly 50% of endoscopists not adhering to it in common practice, significantly decreasing the rate of dysplasia detection[26]; (3) Cost-effectiveness may be also of concern, as highlighted in a recent feasibility study where it was compared to targeted biopsies[27]; and (4) Excessive reliance on random biopsies may undervalue targeted evaluation of subtle lesions, which may therefore be overlooked.

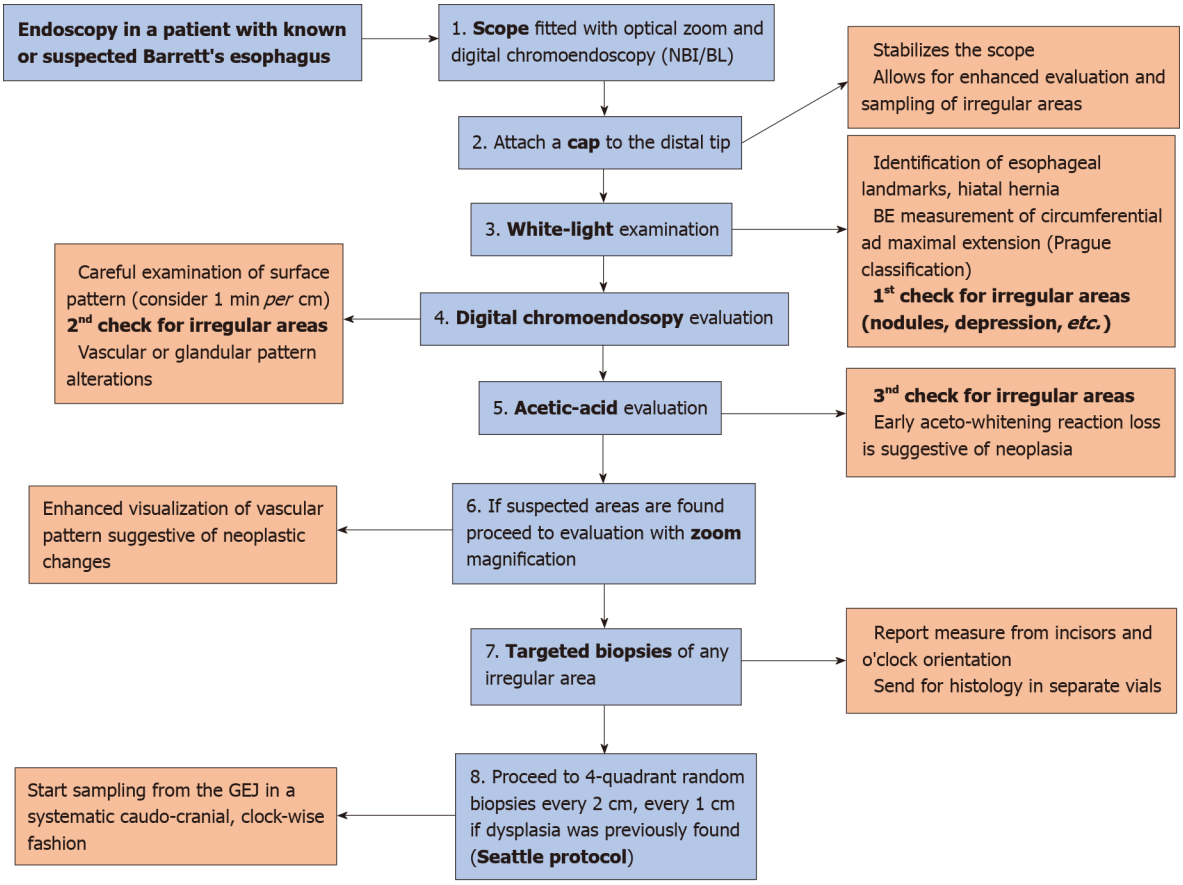
These limitations inherent to the use of white light endoscopy (WLE) with random biopsies may explain why earlier studies underestimated the mortality benefit of endoscopic surveillance. Great effort has been made to implement new technologies that may improve neoplasia detection in BE, and potentially avoid random biopsies; in this regard, the American Society of Gastrointestinal Endoscopy (ASGE) released a Preservation and Incorporation of Valuable Endoscopic Innovations (PIVI) document, delineating the performance thresholds required by imaging technologies using targeted biopsies to eliminate the need for random biopsies during endoscopic surveillance of BE[23]. The performance thresholds are: (1) Imaging technology with targeted biopsies should have a per-patient sensitivity of ≥ 90% and a negative predictive value (NPV) of ≥ 98% for detecting HGD or early EAC, compared with the current standard protocol; and (2) The imaging technology should have a specificity that is sufficiently high (80%) to allow a reduction in the number of biopsies (compared with random biopsies).

Different approaches have been proposed to efficiently target esophageal biopsies, with some of them being difficult to implement in a standard community setting [*i.e.* confocal laser endomicroscopy (CLE), volumetric laser endoscopy (VLE) Autofluorescence imaging (AFI)]. In this commentary we summarize the current knowledge on these strategies, which may be easily integrated in common practice such as both dye-based and electronic chromoendoscopy.

Dye-based chromoendoscopy

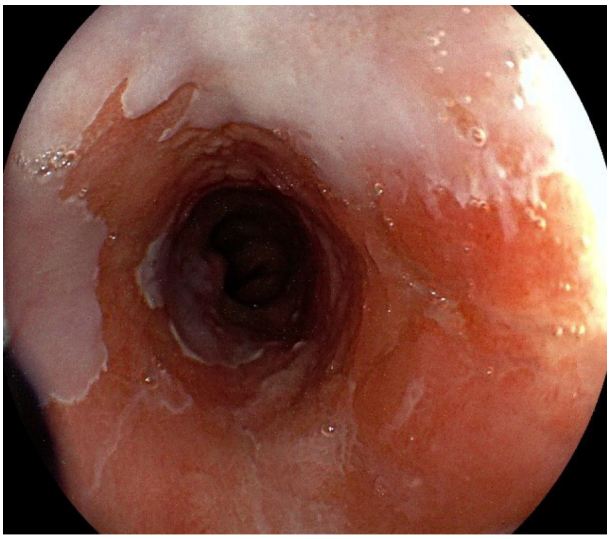
Chromoendoscopy is the most extensively used advanced imaging technique for improving neoplasia detection in BE. The first methodology introduced was dye-chromoendoscopy (DCE). Through a catheter, the dye is sprayed over BE mucosal surface, in an easy but relatively time-consuming process; dedicated equipment and dye must be readily available, as well as expertise in endoscopic mucosal surface evaluation after its application. The most commonly used dyes for BE surveillance are acetic acid (AA), methylene blue, and indigo carmine; however, the only DCE technology that met the PIVI standards in a meta-analysis performed by the ad hoc ASGE committee[23] is AA (sensitivity 96.6%, NPV 98.3%, specificity 84.6%), which performed well enough to be compared to current random biopsies protocols. It works by transiently denaturizing cells in the surface epithelium, leading to a whitening effect. A localized area that loses the whitening reaction earlier than the surrounding mucosa should be considered suspect for neoplasia (Figure 3).

Recent insights regarding AA chromoendoscopy come from a randomized, cross-over, feasibility trial, where AA-guided targeted biopsies (Portsmouth protocol) were compared to the standard Seattle's protocol[27]. Patients under BE surveillance with no history of neoplasia were included, undergoing two endoscopies, one with each protocol, 8 wk apart. A total of 200 patients were randomized across six centers, and 174 underwent both procedures. A total of 2139 biopsies were taken



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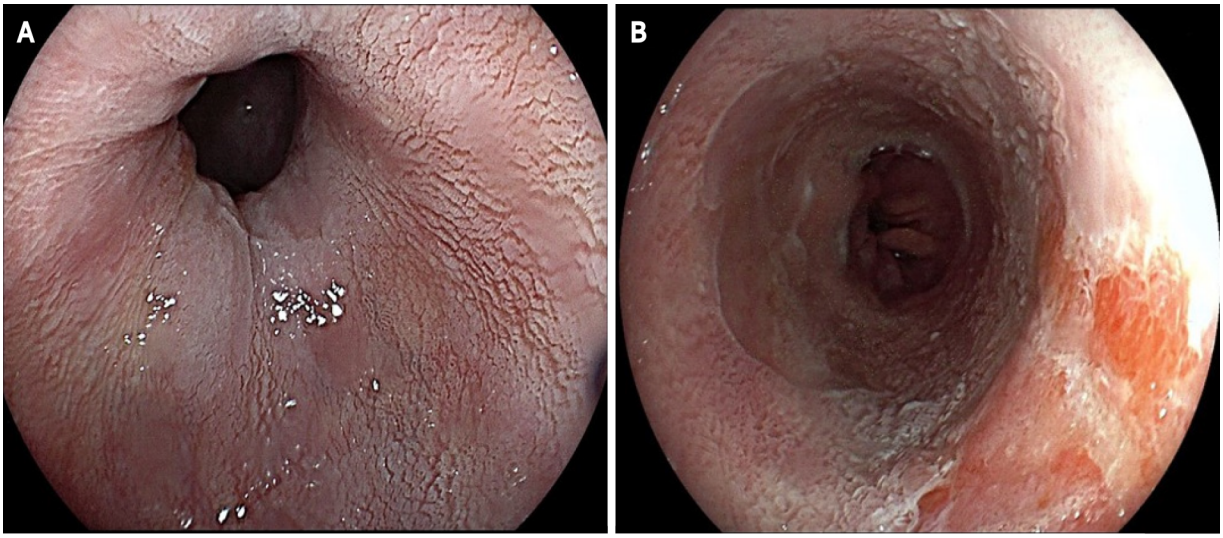
Figure 1 Eight-step flowchart to perform high-quality endoscopic evaluation of Barrett's esophagus.



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Figure 2 White light appearance of non-dysplastic Barrett's mucosa with a slightly elevated lesion (Paris 0-IIa). Histology: Intramucosal adenocarcinoma.

in the nontargeted arm and 226 in the AA arm. HGD and cancer were detected with both protocols (AA and nontargeted biopsies). Five low-grade dysplasia were detected (two with AA and four with nontargeted biopsies; one lesion was detected with both techniques). A paired analysis demonstrated a 6.50-fold decrease in the number of biopsies *per* pathology found by the Portsmouth protocol compared with the Seattle protocol, and a 9.65-fold decrease when restricted to high-risk neoplasia (HGD and cancer), raising an important cost-saving issue. Although the results were promising, the authors



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Figure 3 Acetic acid chromoendoscopy in Barrett's esophagus. A: Appearance of non-dysplastic mucosa (note the white, regular, and homogeneous glandular pattern); B: Early aceto-whitening loss area consistent with an intramucosal adenocarcinoma.

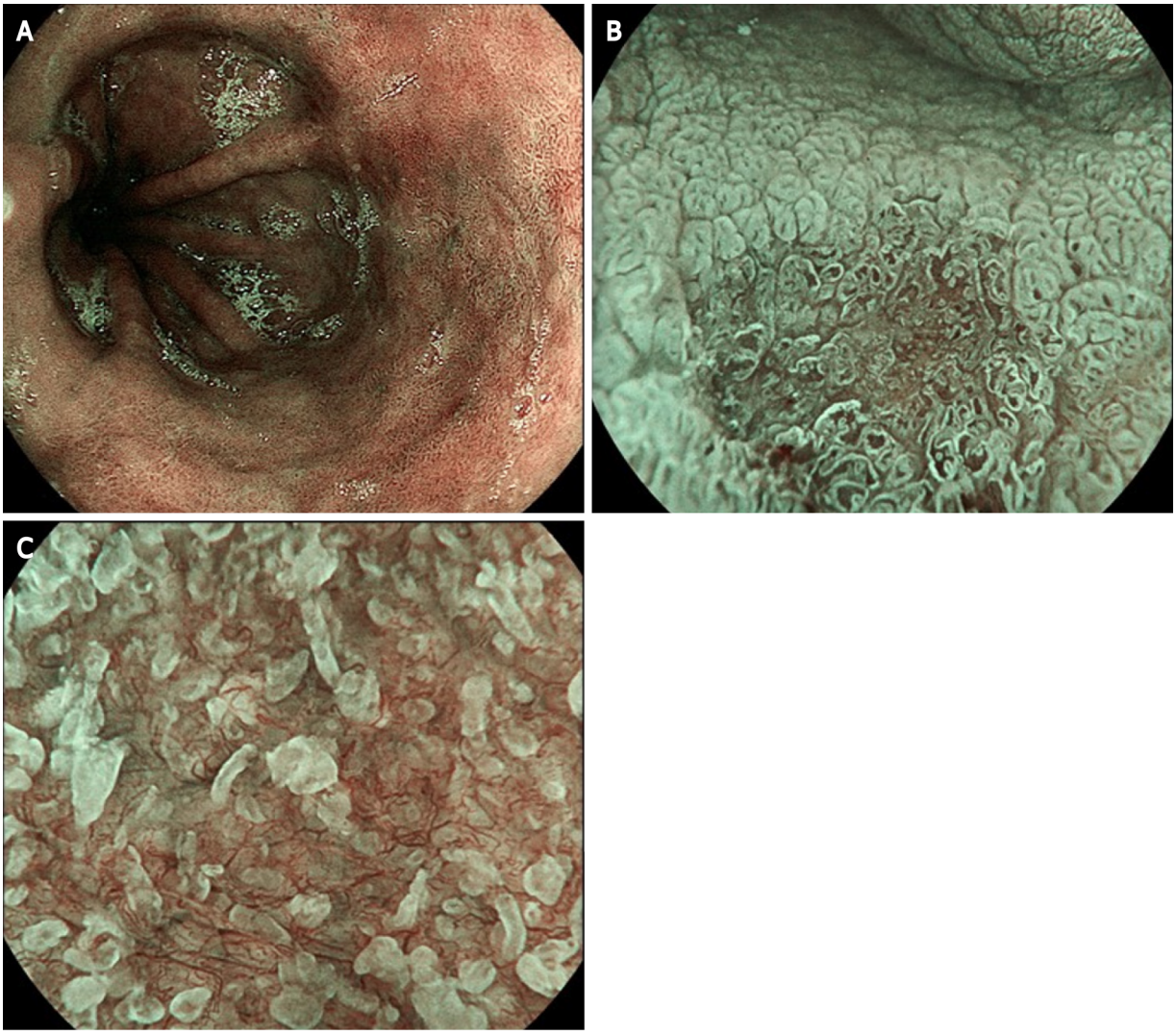
concluded that a noninferiority, tandem, crossover trial would require an estimated 2828 patients in order to prove a significant lack of missed pathology, postponing a conclusive indication.

VCE

VCE has rapidly spread in BE surveillance. Over the last decade, several pre-processing chromoendoscopy technologies have been developed and introduced in clinical practice, including narrow band imaging (NBI; Olympus, Tokyo, Japan), blue light imaging (BLI; Fujifilm, Tokyo, Japan), and i-Scan (PENTAX Medical, Montvale, NJ, United States). Such techniques are practical in first instance (no need to apply any dye, rapid button transition to white light to VCE), allowing immediate enhanced visualization of mucosal and vascular patterns, a critical step to detect early neoplastic lesions. Furthermore, they add no cost, being part of the scope, with no additional risk to the patient.

Several studies have investigated the role of VCE imaging in BE. Most evidence has been accumulated on NBI (Olympus), which is based on the optical phenomenon that the depth of light penetration into tissue depends on its wavelength; NBI applies shorter wavelengths (400-540 nm) to maximally highlight the surface mucosa and vascular pattern. Moreover, the narrow spectrum is matched to the maximum absorption of hemoglobin, in order to make structures rich in hemoglobin look darker (surface capillaries appear brown, submucosal vessels appear cyan) than the surrounding mucosa. Such optical enhancement allows for better visualization and characterization of suspected dysplastic areas. In this regard, a specific NBI-based classification has been developed, based on the so-called BING criteria: Mucosal surface pattern, which can be distinguished in regular (circular, ridged/villous, or tubular) or irregular (absent or irregular); and vascular pattern, distinguished in regular (blood vessels regularly situated along or between mucosal ridges with normal branching patterns) or irregular (focally or diffusely distributed vessels not following the normal architecture of the mucosa). The BING criteria identified patients with dysplasia with 85% overall accuracy, 80% sensitivity, 88% specificity, 81% positive predictive value, and 88% NPV, with a good overall inter-observer agreement (k = 0.681)[28]. Several studies and subsequent meta-analyses have investigated the role of NBI as advanced imaging technology in BE surveillance, showing that NBI-targeted biopsies may improve dysplasia detection, at least when performed by experienced endoscopists in advanced imaging, matching the PIVI thresholds[29-31]. In particular, the most recent meta-analysis performed a methodologically robust comparison between NBI and standard WLE in a per-patient analysis, including six studies that reported the diagnostic utility of NBI for detecting dysplasia of all grades[31]. The pooled sensitivity of NBI to detect all-grades of dysplasia was 0.76 [95% confidence interval (CI): 0.61-0.91], the pooled specificity was 0.99 (95%CI: 0.99-1.00).

BLI (Fujifilm) is a novel technology that enhances the contrast between mucosal and surface vascular patterns, similarly to NBI technology (Figure 4). A classification (BLINC) that helps to characterize BE lesions has been recently developed and validated, similarly as seen with NBI, showing good interobserver reliability and improvement of neoplasia detection[32]. BLI has been recently tested in a tandem retrospective comparison with high definition WLE (HD-WLE) in a recent Dutch analysis of 40 still images of BE lesions. The authors found that experts appreciated BLI significantly better than HD-WLE for visualization of macroscopic appearance in a visual assisted scale (median 8.0 *vs* 7.0; $P < 0.001$), for surface relief (8.0 *vs* 6.0; $P < 0.001$), and for lesion demarcation (8.0 *vs* 6.0; $P < 0.001$ and 8.0 *vs* 5.0; $P <$



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Figure 4 Blue light imaging appearance of Barrett's mucosa. A: Non-dysplastic mucosa; B: Irregular vascular pattern of a slightly depressed (Paris 0-IIc) high-grade dysplastic lesion; C: Detail of irregular mucosal and vascular pattern after zoom magnification.

0.001)[33]. Similar results, in a similar setting, were reported for nonexpert endoscopists after an appropriate training phase[34].

Finally, the I-SCAN (PENTAX) improves neoplasia detection on endoscopic imaging of BE as well as the accuracy of histology prediction. In a recent study, accuracy of neoplasia detection was significantly higher in all trainees (76% *vs* 63%) and in six experts (84% *vs* 77%) who used OE *vs* HD-WLE. OE improved the sensitivity of dysplasia detection compared with HD-WLE in six trainees (81% *vs* 71%) and five experts (77% *vs* 67%). Specificity improved in six trainees who used OE *vs* HD-WLE (70% *vs* 55%) and in five experts (92% *vs* 86%)[19].

In conclusion, it is still debatable if a chromoendoscopy directed targeted-biopsies strategy can replace the four-quadrant random biopsies in the near future. Currently, only AA chromoendoscopy, and VCE with NBI, meet the PIVI thresholds when performed by expert endoscopists[23]. International guidelines continue to support a careful HD-WLE examination of BE, followed by Seattle protocol biopsies[4,5]; however, use of adjunct chromoendoscopy can potentially increase diagnostic yield of the standard guideline supported approach by identifying suspicious areas for targeted biopsies.

HOW TO GET PREPARED FOR AN IMMINENT TOMORROW IN BE EXAMINATION

As already mentioned, different approaches have been proposed to help in dysplasia/neoplasia detection during BE surveillance. Even though it has been years since we started investigating advanced modalities such as CLE, VLE and AFI, what yesterday was considered the "future," is today considered the "present" of BE endoscopy. AI in endoscopy has been booming in the last few years, and BE examination itself will likely be a part of this revolution.

AI

AI is a branch of computer science that takes advantage of the development of autonomous machines that are able to mimic human behavior. Over the last decade, AI has been applied in several fields; in medicine, one of the most ambitious aims is to aid interpretation of subtle differences in medical imaging. Computer-aided diagnosis (CAD) systems have thus been developed and trained to autonomously learn to distinguish features that a human being cannot easily distinguish. For instance, AI may overcome the physician-dependent limitations related to poor intra- and inter-observer agreement, a burden that affects many aspects of medical imaging and endoscopy. If the system were to be trained to distinguish between neoplastic and non-neoplastic BE mucosa with almost-perfection, it seems reasonable to imagine that in the near future the burden of pathological examination may be considerably relieved.

Especially *via* deep learning techniques with artificial neural networks, these CAD systems offer many potential advantages for the endoscopic practice; in the last few years, several CAD systems using deep learning machine techniques have been developed in other areas of endoscopy, such as colonic adenomas, with extremely promising results[35]. The Netherlands-based ARGOS consortium is under way in developing a real-time CAD system for BE neoplasia[36]. The development of this deep learning CAD system required the analysis of a total of 494364 normal white light endoscopy images and 1704 unique BE still images. The aim was automatically to detect early neoplastic BE lesions, indicating areas with the highest chance of containing abnormality with a crosshair, The CAD system was in a complex multistep asset: Starting from WLE images collected from all gastro-intestinal segments (Set 1), refinement of CAD system was obtained with a subsequent analysis of 1247 WLE images of BE (Dataset 2:557 NDBE and 690 early neoplasia images) and other 197 WLE images (Dataset 3:168 NDBE and 129 early neoplasia): To ensure maximal accuracy, NDBE images were further reviewed by BE experts endoscopists for confirming the absence of visible lesions and the neoplastic images were re-delineated by other experts; moreover, all suspected neoplastic lesions were histologically confirmed as HGD or adenocarcinoma. It is worth noting that images were acquired with different endoscopic optical systems (both Fujifilm and Olympus). A double external validation was then carried out with 2 other datasets (4 and 5) of 160 different BE images (80 NDBE, 80 neoplastic) and eventually benchmarked with a large group of international endoscopists, including novices and seniors. As a final result, in dataset 4 the CAD system classified images as containing neoplasms or nondysplastic BE with 89% accuracy, 90% sensitivity, and 88% specificity. In dataset 5 values for the CAD system *vs* those of the general endoscopists were 88% *vs* 73% accuracy, 93% *vs* 72% sensitivity, and 83% *vs* 74% specificity. The CAD system achieved higher accuracy than any of the individual 53 non-expert endoscopists. CAD delineations of the area of neoplasm overlapped with those from the BE experts in all detected neoplasia in data sets 4 and 5. The CAD system also identified the optimal site for biopsy (crosshair) of detected neoplasia in 97% and 92% of cases[37]. The first real-time performance (with video) of this CAD system has been recently reported by the same group in 10 patients[38]: Accuracy, sensitivity, and specificity of the CAD system in per-level analyses were 90%, 91%, and 89%, respectively. In total, 9 out of 10 neoplastic patients were correctly diagnosed. The single lesion not detected by CAD, showed NDBE in the endoscopic resection specimen. In only 1 NDBE patient, the CAD system produced false positive predictions. In 75% of all levels, the CAD system produced 3 concordant predictions. In another study by Ebigbo *et al*[39], a different CAD system was trained using a total of 129 endoscopic images[39]; real-time validation was performed with 62 additional images (36 of early EAC and 26 of normal BE) in a concurrent assessment made by the CAD system and a BE-expert endoscopist. All images and lesions were then validated by pathological examination of resection specimens (EAC), as well as forceps biopsies (normal BE). The AI system had excellent performance scores in the classification task with a sensitivity and specificity of 83.7% and 100.0%, respectively, and an overall accuracy of 89.9%.

The number of available CAD systems may be rapidly increasing, with several other reports appearing in the last few years[40-42]: A meta-analysis has pooled data from the all the studies available so far (9 in total)[43]. Overall, CAD showed pooled sensitivity of 89% (CI 83%-93%), specificity 88% (CI 84%-91%), PPV 88% (CI 84%-91%), NPV 89% (CI 83%-93%), LR+ 7.5 (CI 5.2-10.4), and LR− 0.120 (CI 0.074-0.198). Heterogeneity was present both for sensitivity and specificity (0.60 and 0.29 between study SD in the logit scale, respectively). Comprehensively, at 50% of early-stage neoplasia prevalence, a positive result of AI would increase the disease probability to 88%, whereas a negative result would decrease the disease probability to 11%. In addition, in subgroup analysis, it was shown that the use of advanced imaging techniques was associated with an increase of specificity, at least in BE-related neoplasia detection, suggesting a potential synergic effect between advanced imaging and AI.

The field of AI will continue to evolve in next few years providing models that would enhance easy detection and mucosal detailing for endoscopists. This will undergo rigorous prospective validation and testing before being available. Ideal AI based model should be easily taught, reproducible with accuracy and such that can be easily incorporated in community GI endoscopy centers.

CONCLUSION

The burden of missed neoplasia in BE is far from being negligible, and we hypothesize that such a heavy burden may mostly result from inappropriate endoscopic surveillance. As AI systems are set to go on stage with regards to endoscopic clinical practice, these results are a call to action to reduce the rates of undiagnosed BE-related neoplasia by any means, with the combination of CAD to a widespread adoption of advanced imaging technologies being the most promising and eagerly awaited one.

FOOTNOTES

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Country/Territory of origin: Italy

ORCID number: Marco Spadaccini 0000-0003-3909-9012; Edoardo Vespa 0000-0002-5573-447X; Viveksandeep Thoguluva Chandrasekar 0000-0001-5256-4113; Madhav Desai 0000-0001-8871-3627; Harsh K Patel 0000-0002-9180-2150; Roberta Maselli 0000-0001-7291-9110; Alessandro Fugazza 0000-0003-0485-4903; Silvia Carrara 0000-0003-4206-9463; Andrea Anderloni 0000-0002-1021-0031; Gianluca Franchellucci 0000-0002-3440-4981; Alessandro De Marco 0000-0003-2987-0467; Cesare Hassan 0000-0001-7167-1459; Pradeep Bhandari 0000-0002-1241-2083; Prateek Sharma 0000-0003-4003-7548; Alessandro Repici 0000-0002-1621-6450.

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MINIREVIEWS

Rectal neuroendocrine tumors: Current advances in management, treatment, and surveillance

Camilla Gallo, Roberta Elisa Rossi, Federica Cavalcoli, Federico Barbaro, Ivo Boškoski, Pietro Invernizzi, Sara Massironi

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Camilla Gallo, Pietro Invernizzi, Sara Massironi, Division of Gastroenterology and Center for Autoimmune Liver Diseases, Department of Medicine and Surgery, University of Milano-Bicocca, European Reference Network on Hepatological Diseases, San Gerardo Hospital, Monza 20900, Italy

Roberta Elisa Rossi, Division of HPB Surgery, Hepatology and Liver Transplantation, Department of Pathophysiology and Transplantation, University of Milan, ENETS Center of Excellence, Fondazione IRCCS Istituto Nazionale Tumori (INT, National Cancer Institute), Milan 20133, Italy

Federica Cavalcoli, Diagnostic and Therapeutic Endoscopy Unit, Fondazione IRCCS Istituto Nazionale Tumori, Milan 20133, Italy

Federico Barbaro, Ivo Boškoski, Digestive Endoscopy Unit, Università Cattolica del Sacro Cuore di Roma, Center for Endoscopic Research Therapeutics and Training, Fondazione Policlinico Universitario Agostino Gemelli IRCSS, Rome 00168, Italy

Corresponding author: Sara Massironi, MD, PhD, Doctor, Division of Gastroenterology and Center for Autoimmune Liver Diseases, Department of Medicine and Surgery, University of Milano-Bicocca, European Reference Network on Hepatological Diseases, San Gerardo Hospital, Via Pergolesi, 33, Monza 20900, Italy. s.massironi@asst-monza.it

Abstract

Rectal neuroendocrine neoplasms (r-NENs) are considered among the most frequent digestive NENs, together with small bowel NENs. Their incidence has increased over the past few years, and this is probably due to the widespread use of endoscopic screening for colorectal cancer and the advanced endoscopic procedures available nowadays. According to the current European Neuroendocrine Tumor Society (ENETS) guidelines, well-differentiated r-NENs smaller than 10 mm should be endoscopically removed in view of their low risk of local and distant invasion. R-NENs larger than 20 mm are candidates for surgical resection because of their high risk of distant spreading and the involvement of the muscularis propria. There is an area of uncertainty regarding tumors between 10 and 20 mm, in which the metastatic risk is intermediate and the endoscopic treatment can be challenging. Once removed, the indications for surveillance are scarce and poorly codified by international guidelines, therefore in this paper, a possible algorithm is proposed.

Key Words: Rectal neuroendocrine tumors; Endoscopy; Endoscopic submucosal dissection; Resectable advanced disease; Systemic therapy

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Core Tip: Rectal neuroendocrine neoplasms (r-NENs) represent 1%-2% of all rectal tumors, but an increasing incidence rate has been recently reported. Up to 90% of r-NENs are < 10 mm in size, confined to the submucosa, and well-differentiated, so that the endoscopic resection represents their gold-standard therapy. Advanced metastatic NENs are usually poorly differentiated G3 carcinomas treated with chemotherapy, except for rare G1-G2 neoplasms, which can be targeted with less aggressive systemic options. Locally advanced r-NENs invading submucosal layers, with a ≥ 10 mm diameter, but still without distant metastases, represent the real therapeutic challenge among rectal tumors.

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INTRODUCTION

The gastrointestinal tract is the most frequent site for the onset of neuroendocrine neoplasms (NENs). NENs are a heterogeneous group of epithelial neoplasms, ranging from indolent well-differentiated neuroendocrine tumors (NETs) to very aggressive poorly differentiated neuroendocrine carcinomas (NECs). They can arise virtually from any organ or system in the human body. The rectum is the second localization by frequency after the small intestine[1] and rectal NENs (r-NENs) represent 12%–27% of all gastrointestinal NENs[2,3]. On the other hand, 1%–2% of all rectal tumors are neuroendocrine. An increasing incidence of r-NENs has been reported over the past few years, as illustrated in The Surveillance, Epidemiology, and End Results (SEER) database[1], and the same trend was confirmed in the German registry[4], as well as in the Asian registers[5], even if the highest incidence rate (IR) was reported in the United States, where IR was approximately 1.1 *per* 100000 population and increased tenfold between 1970 and 2000s[6]. In Europe, the IR was lower when compared to the SEER database, with the highest IR reported in Norway (0.25 *per* 100000 population) and the lowest in Austria[6]. This is probably due to an underreporting of the disease because of a lack of national registries.

The increasing incidence is related to the increased participation in screening colonoscopy programs. Even if there are limited data on the r-NENs diagnosed through colorectal cancer (CRC) screening programs, in a Polish screening CRC program cohort of 50148 participants, a prevalence of r-NENs of 0.05%-0.07% was reported[7]. Similarly in another English study, the diagnosis rates of NENs identified in the English bowel cancer screening program were 29 rectal and 18 colonic *per* 100000 colonoscopies, accounting for a prevalence of 0.047%[8].

Despite their increasing incidence, r-NENs are not yet sufficiently recognized by endoscopists in Western countries, since a recent study demonstrated that the neuroendocrine nature was suspected for only 18% of neuroendocrine lesions[9].

Suspicion of rNENs before their resection is clinically very important, given the fact that it significantly drives the decision on how to treat them. In fact, it has been reported that patients whose rNENs were diagnosed or suspected before resection showed a much higher complete resection rate than those whose tumors were resected as polyps and then diagnosed[10].

In the increasingly vast panorama of artificial intelligence, its application to gastrointestinal endoscopy may help in detecting and diagnosing r-NENs, thus leading to a further increase in incidence but in parallel to a more effective therapeutic approach.

CLINICAL PRESENTATION AND ENDOSCOPIC APPEARANCE OF RECTAL NENS

Most of r-NENs are asymptomatic and they are diagnosed incidentally during endoscopic evaluation for CRC screening or unrelated gastrointestinal symptoms. Less frequently, r-NENs may present with anal discomfort, rectal bleeding, and change in bowel habits[11]. Most r-NENs, which arise from the neuroendocrine epithelial cells, appear as small, round polypoid lesions characterized by a smooth, normal-appearing, or yellow-discolored mucosa[12], with round shape pit pattern, type I on Kudo

classification, and invisible vessels, as described by Sano as type I[13] (Figure 1). R-NENs are usually located about 4 to 10 cm above the dentate line, on the frontal or lateral wall of the mid-rectum[14]. Up to 90% of r-NENs, at the time of the diagnosis, are well-differentiated epithelial lesions, less than 10 mm in size[15], usually developing towards the submucosal layer, but without invading the *muscularis propria* layer. However, atypical endoscopic findings (including semi-pedunculated appearance, hyperemia, central depression, erosion, and ulceration) have been reported for r-NEN exceeding 5 mm in diameter[14]. In addition, it has been recently reported that virtual chromoendoscopy with NBI, showing an absence of pit pattern with large amorphous areas (Kudo V), may be of value in detecting invasive r-NENs[13]. Unfortunately, the macroscopic appearance of r-NENs resembles that of hyperplastic or adenomatous polyps, making the differential diagnosis from other polypoid lesions challenging, and often the diagnosis is established at pathological examination after routine snare polypectomy or mucosectomy[16].

Moreover, the expanding use of artificial intelligence in endoscopy with computer-aided softwares could help the detection and characterization of polypoid lesions including r-NENs[17]. In the last years, EUS has extended the role of endoscopic evaluation of rectal NENs and it proved to help defining accurately the tumor size, the depth of invasion, and the presence of pararectal nodal metastases[18]. On EUS, rectal NETs usually appear as well-defined, hypoechoic lesions, located in the second and third wall layer[19] (Figure 2). In addition, EUS evidence of lobulated forms, irregular margins, and echogenic foci, may predict a higher grade of malignancy[20].

RISK FACTORS FOR INCIDENCE/PREVALENCE, METASTASES, PROGRESSION, AND RECURRENCE

The risk factors associated with r-NENs are not fully clear given the overall low incidence rate of these tumors and the consequent scarcity of large epidemiological studies on rare cases. R-NENs include a heterogeneous group of neoplasms that are not completely indolent, as they used to be traditionally considered, but they are characterized by a risk of metastatic disease ranging from 3% to 60%. The identification of specific subgroups that are at increased risk of developing r-NENs is, therefore, of extreme relevance for early detection and removal of these neoplasms. According to a large Asian retrospective study[21], higher levels of cholesterol and ferritin, the presence of metabolic syndrome, and a family history of cancer were associated with an increased prevalence of r-NENs. Ko *et al*[22] observed that higher fasting plasma glucose and hypertriglyceridemia, younger age, and a previous history of malignancies were significantly associated with an increased risk of r-NENs. According to a retrospective cross-sectional study that compared the risk factors for 101 cases of r-NENs found during screening colonoscopies, male gender and low high-density lipoprotein cholesterol levels were significantly associated with these neoplasms[23].

As metabolic syndrome appears to be a recognized risk factor for the development of r-NENs, this might partially explain the increasing incidence of these tumors together with the improvement in screening colonoscopy. The actual mechanism underlying the association between metabolic risk factors and r-NENs is still unclear; however, it might be possible that insulin resistance represents a risk factor in r-NEN pathogenesis, also considering that insulin and insulin-like growth factors have been associated with cell proliferation, differentiation, and apoptosis[24].

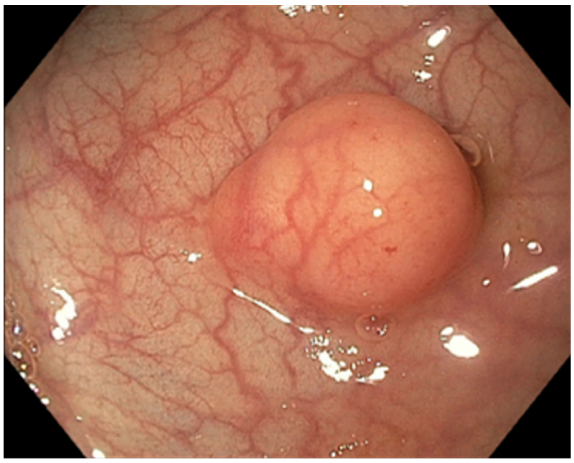
Several predictors of metastases, progression, and/or recurrence of r-NENs have been proposed; however, the relevance of each of these factors has not been clearly established and clear-cut prognostic factors which might help in the management of these neoplasms have not been yet incorporated in current guidelines. Table 1 summarizes available prognostic factors.

The most important factor predicting aggressive disease is the size of the primary tumor, with lesions > 20 mm being metastatic in 60%-80% of the cases[25]. On the other hand, patients with r-NENs measuring 10-19 mm develop synchronous or metachronous metastases in 4%-20% of the cases[25]. According to some large series, the optimal size cut-off to predict the risk of metastases is 15 mm[26-28]; in detail, Concors *et al*[28], in their study assessing a total of 4893 r-NENs patients identified in the National Cancer Database, observed that tumors larger than 15 mm are associated with a higher risk of distant metastasis, even if one should keep in mind that local and distant metastases might occur also in lesions < 15 mm, thus suggesting that the tumor's behavior is not influenced only by the size. As recently highlighted by Capurso *et al*[29], size alone has limited accuracy, as 26% of patients with stage IV and 16% with G3 neoplasm have a primary tumor ≤ 10 mm; instead, both staging and grading could accurately predict r-NEN prognosis. According to this study, the ENETS TNM staging accurately predict prognosis in patients with r-NENs, as stage IV was associated with a worse overall survival (OS) (hazard ratio [HR] = 8.16; $P = 0.002$) and progression-free survival (PFS) (HR = 14.26; $P < 0.0001$).

As concerned tumor grading, r-NENs are also classified according to the WHO 2019 classification of digestive system tumors[30], which divided NEN into NETs and NECs based on their molecular differences. NETs are then graded based on the proliferation index (mitotic count and Ki67-related proliferation index) and divided into three groups (NET G1, NET G2, and NET G3), while NECs are by definition high-grade neoplasms. There is growing evidence that, besides the diameter of the primary

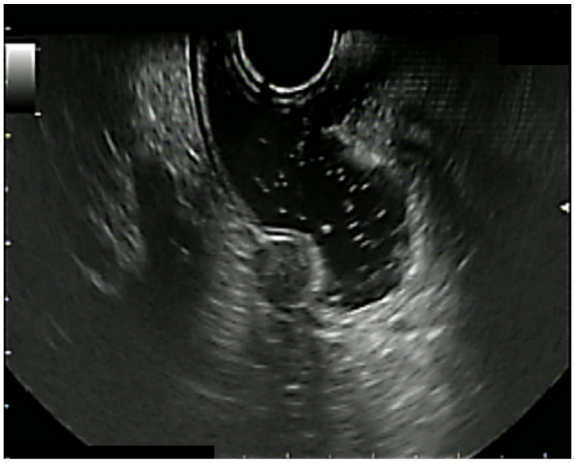
Table 1 Current evidence about available prognostic factors for either metastatic spread or tumor progression/recurrence in rectal neuroendocrine neoplasms

Prognostic factor	Cut-off	Risk of metastases	Evidence
Tumor size	15 mm	14%	Retrospective large study[27]
Grading	G2	50%	Retrospective study[30]
	G3	33%	Retrospective study[30]
Lymph node status	N2 (≥ 5 positive lymph nodes)	Not quantified	Retrospective study[35]
Depth of invasion	Invasion of the *muscolaris propria*	Not quantified	Retrospective study[37]



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Figure 1 Endoscopic aspect of a rectal neuroendocrine neoplasm.



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Figure 2 Endoscopic ultrasound aspect of a rectal neuroendocrine neoplasm.

neoplasm, the tumor grade might play a relevant role in the development of metastases. In a recent retrospective study including 98 patients with r-NENs[31], characterized by a proportion of metastatic disease of 12%, patients with G2 or G3 tumors, regardless of size, were found to be at high risk for the development of metastases; only in G1 tumors, the size (*i.e.*, > 20 mm) was found to be an important predictor of aggressive behavior. Therefore, the authors concluded that grade is a dominant risk factor for metastasis in small and diminutive r-NENs. Again Capurso *et al*[29] confirmed that, besides stage, grade could predict prognosis in r-NENs, with G3 tumors being associated with a worse OS (HR = 15.57; $P = 0.0004$) and PFS (HR = 6.42; $P = 0.0007$).

Other important prognostic factors explored in the literature are: The depth of invasion (with involvement of the muscularis propria), the presence of lymphovascular invasion and/or perineural

invasion, the presence of regional nodal metastasis, atypical histology (including anaplasia, frequent mitotic cells, cellular pleomorphism, and mucin production), and mitotic rate, all of which have been suggested as possible prognostic factors for aggressive behavior[32], although evidence is elusive. To date, in fact, it is not yet clear whether these can be considered as independent risk factors[28,33].

The lymph node status is considered as a relevant prognostic factor and current guidelines have always classified r-NENs as N0 *vs* N1 according to the presence or absence of metastatic lymph nodes, respectively[34,35]. As reported in a recent study[36], which included 687 patients with r-NENs retrieved from the National Cancer Database, there is a significant difference in survival among patients with zero positive lymph nodes (N0), 1 to 4 positive lymph nodes (N1), and ≥ 5 positive lymph nodes (N2), which might suggest a new nodal staging system to provide a more accurate prognosis.

Furthermore, these findings might highlight the importance of an adequate lymphadenectomy in this specific setting, even if further studies are warranted to help quantify the optimal number of lymph nodes that need to be examined.

Finally, it should be underlined that the evaluation of lymphovascular invasion may frequently suffer from inter-observer variations[37].

Fahy *et al*[38], in a study including 70 r-NENs, reported that tumor size, depth of invasion, presence of lymphovascular invasion, and mitotic rate all correlated with poor outcome and generated a carcinoid of the rectum risk stratification (CaRRS) score based on these factors. The score was reliably correlated with recurrence-free survival and disease-specific survival, and it was based on histopathologic variables which might be assessed at biopsy and might guide the treatment strategy.

In summary, according to the ENETS 2016 Consensus Guidelines and the Union for International Cancer Control/American Joint Committee on Cancer (UICC/AJCC) indications, the size of the primary tumor, considering a potential more updated cut-off of 15 mm, and the degree of differentiation with G2 and, indeed, G3 tumors, are considered to be the main independent predictors of a dismal prognosis in r-NENs[39,40]. The number of positive nodes considering ≥ 5 positive lymph nodes as a cut-off and the depth of invasion with the invasion of the *muscolaris propria* are also associated with a poor prognosis [40].

Further studies are needed to determine whether more aggressive surgical approaches, as well as standardized follow-up protocols, might be beneficial in this specific subgroup of neoplasms.

TREATMENT OF RECTAL NENS

The therapeutic approach of R-NENs depends on whether a localized, locally advanced, or advanced metastatic disease is present.

Localized disease

Due to the progressive greater awareness of these neoplasms, the improvement of endoscopic technology, and the increasingly adequate training of endoscopists, 90%-100% of r-NENs are to date detected with a diameter ≤ 10 mm. These lesions are usually intramucosal and nearly every ≤ 10 mm intramucosal r-NEN is diagnosed as a completely resected incidental tumor. Less than 10 mm NENs have a low risk of both lymphatic invasion and distant metastases, nearly 0.7% and less than 2%, respectively. Endoscopic ultrasound (EU) is highly recommended to exclude this remote occurrence, and to choose the best-suited type of endoscopic resection and its feasibility[39]. Even if, to date, not all studies unequivocally affirm that the *en bloc* resection of small r-NENs is associated with a statistically significant increase in OS, complete mini-invasive endoscopic *en bloc* resection still represents the therapeutic aim for these lesions. A wide variety of endoscopic procedures have been traditionally used to resect r-NENs.

Standard polypectomy: Standard polypectomy is performed by using a hot or cold snare. This kind of polypectomy does not guarantee a sufficient complete resection rate of the lesion margins, thus, this technique is no more recommended to treat r-NENs[39].

Endoscopic mucosal resection: Conventional endoscopic mucosal resection (EMR) consists of a snare resection of the lesion, which is usually lifted by the submucosal injection of saline solution, in order to guarantee a lower risk of wall perforation and incomplete margin resection, especially in case of sessile lesions[41]. It is a safe, cost-effective, and technically easy procedure, but it results in a remarkable rate of piece-meal resection, and, thus, a high rate of incomplete removal. According to the most reliable evidence, in fact, conventional EMR only leads to 56%-59% of R0 resections in ≤ 10 mm r-NENs[26,42]. Moreover, a submucosal fluid injection may paradoxically flatten or even depress loose connective tissue lesions, increase the tissue tension, and even displace the lesion to a hardly accessible different localization.

The underwater EMR (U-EMR) technique was inspired by the observation that the mucosa and submucosa float apart from the muscular layer when colon air is removed from the lumen and it is replaced with water. By doing so, the colon lumen gets less outstretched, it gives birth to a pseudopedicle, and a larger mucosal surface can be captured. U-EMR has been shown to be effective in

removing *en bloc* > 20 mm lesions; a multicenter randomized controlled trial comparing conventionally injection-assisted and U-EMR in 219 15-70 mm colorectal laterally spreading tumors demonstrated a significantly higher *en bloc* resection rate for U-EMR (51% *vs* 25%, $P = 0.001$). To date, no comparative studies are available on the *en bloc* resection efficacy between conventional EMR and U-EMR for < 20 mm r-NENs[43].

According to the largest samples available so far, EMR performed with a suction cap, which represents another modified EMR technique (m-EMR), reaches an *en bloc* resection success rate of 100% and a histological complete resection rate of 93% for ≤ 10 mm rNENs[44].

The circumferential incision m-EMR (CI-EMR) technique, first described as an option technique for CRC, performed by marking dots by argon plasma coagulation (APC) around the lesion and lifting only the mucosal layer from the muscularis propria with a mixture of glycerin fructose and methylene blue, similarly guarantees an *en bloc* resection rate and a complete resection rate of 97% and 94%, respectively [45]. If an elastic band is placed around the rectal lesion in addition to its cap-assisted suction and its injection-mediated lifting, an even higher rate of complete resection can be guaranteed: In a prospective Korean study enrolling 77 patients who underwent a ligation-assisted m-EMR (L-EMR) of a ≤ 10 mm r-NEN, 100% of them achieved an *en bloc* histologically complete resection of the lesion[46]. The m-EMR technique performed with a double-channel gastroscope rather proved to be less effective in terms of complete resection rate and it was associated with a higher rate of adverse events[47].

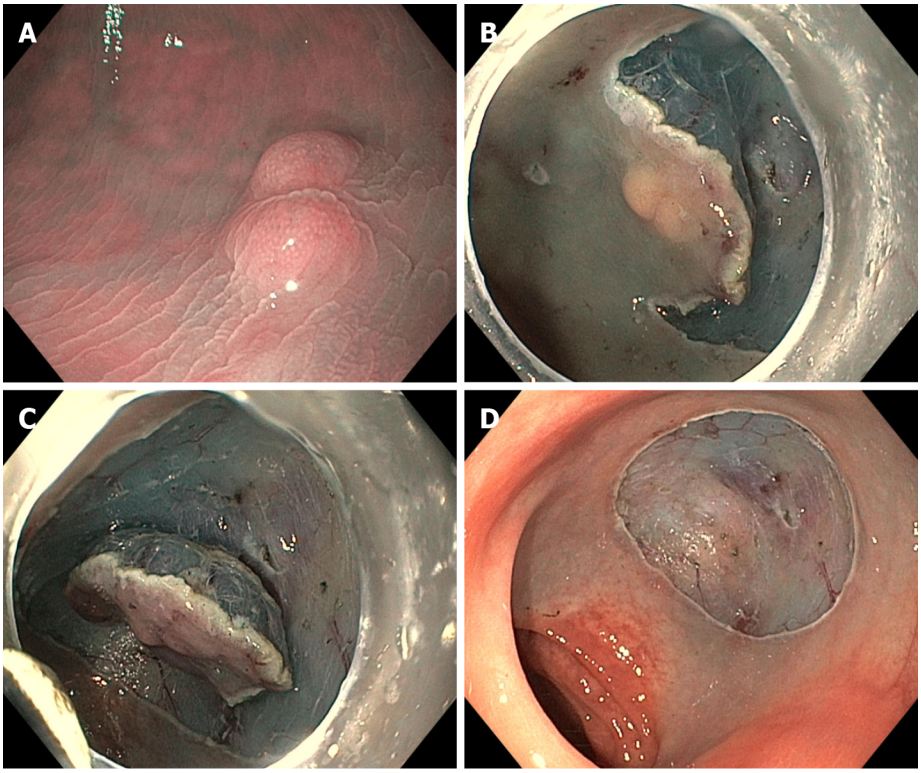
Pooled data deriving from a systematic review and meta-analysis including 11 studies for a total of 811 patients who underwent endoscopic treatment by EMR or any m-EMR of ≤ 10 mm localized r-NENs limited to the mucosal layer, demonstrated a statistically significant higher rate of *en bloc* endoscopic removal (odds ratio [OR] = 0.13, 95% confidence interval [CI]: 0.02-0.74, $P = 0.02$) and complete histological resection (OR = 0.23, 95%CI: 0.10-0.51, $P < 0.01$) for m-EMR when compared to conventional EMR. The safety of the different procedures proved to be equal[48]. However, it is interesting to notice that, according to a multivariate analysis including 277 any size r-NENs treated by conventional EMR (243 of them), dual-channel EMR, and CI-EMR (for a total of 44 m-EMRs), the histological complete resection rate was similar among the techniques, proving that the tumor size influences the histological complete resection regardless of the endoscopic treatment modality[49].

Whatever the endoscopic mucosal resection technique used, the EMR area should be always marked after the rectal lesion resection, in order to facilitate future salvage therapy in case that the histological examination of the tumor margins does not result in complete resection.

Endoscopic submucosal dissection: Endoscopic submucosal dissection (ESD) consists of a delimitation of a circumferential excision zone by using an electrocauterization knife around the lesion, followed by the creation of a cushion beneath the lesion by the injection of saline solution, or better of 0.4% sodium hyaluronate diluted with the same volume of normal saline solution[50] and, thus, by the execution of a dissection underneath the submucosal layer under direct visualization[37] (Figure 3). It represents a technically difficult procedure, requiring endoscopists' specific training and with a long learning curve [51]. It also requires a longer execution time and is associated with a higher peri-procedural adverse event rate (especially bleeding and perforation) compared to EMR, but no significant differences were demonstrated between ESD and any m-EMR technique in terms of operating time for r-NENs[52].

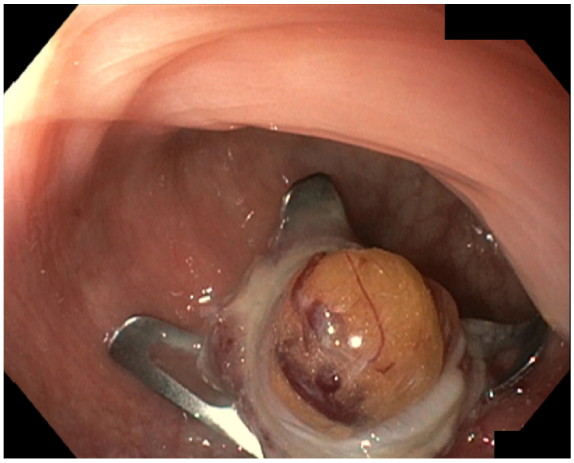
Sometimes even ESD may lead to incomplete resection and thus persistent positive vertical margin, due to deep submucosal invasion of tumor cells. In these situations, other endoscopic resection techniques may overcome the problem: The full thickness resection device (FTRD) Ovesco (Ovesco, Tubingen, Germany) is an over-the-scope clip (OTSC) consisting of a clip and a snare both preloaded on an applicator cap. The FTRD system allows endoscopic full-thickness resection, providing at the same time the closure of the wall defect; it has been shown to be a good option in case of subepithelial lesions (Figure 4).

A broad systematic review and meta-analysis including ten retrospective studies and 650 patients undergoing r-NEN resection, demonstrated a significantly higher complete resection rate in the ESD group compared with the EMR group (relative risk [RR] = 0.89, 95%CI: 0.79-0.99), but a comparable complete resection rate between the ESD group and the m-EMR group (RR = 1.03, 95%CI: 0.95-1.11)[52]. Remarkably, a comparative observational study on 115 patients did not demonstrate statistical significance between ESD and U-EMR in terms of R0 on < 10 mm G1 lesions not invading the *muscularis propriae* (ESD *vs* U-EMR 86.1%R0 *vs* 86.1%R0, $P = 0.996$)[53]. It should be emphasized, however, that EMR often presupposes a piecemeal resection, while ESD guarantees an *en bloc* resection, thus representing a possible bias in the definition of complete histological resection R0. As a matter of fact, U-ESD has been already proposed as a promising modified ESD technique[54]. Furthermore, another systematic review and meta-analysis including 25 studies for 1094 patients, reported L-EMR and ESD to be the most effective endoscopic techniques in guaranteeing histological complete resection of ≤ 10 mm r-NENs (94.8% and 89.6% for L-EMR and ESD R0 *vs* 59.1% and 72.4% for polypectomy/EMR and CI-EMR R0, respectively)[26]. Interestingly, an additional meta-analysis regrouping seven studies for a total of 386 patients, proposed L-EMR to be the most suitable technique for endoscopic resection of ≤ 10 mm r-NENs as far as it proved to achieve a higher R0 rate than ESD (OR = 4.08, 95%CI: 2.42-6.88, $P < 0.00001$), to take significantly less operative time (SMD: -1.59, 95%CI: -2.27 to -0.90, $P < 0.00001$), and to be associated with no significantly higher complications rate (OR = 0.56, 95%CI: 0.28-1.14, $P = 0.11$)[55].



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Figure 3 Endoscopic submucosal dissection of a rectal neuroendocrine neoplasm. A: Rectal neuroendocrine neoplasm aspect before the procedure; B: Initial submucosal dissection beneath the lesion; C: Almost completed submucosal dissection beneath the lesion; D: Final aspect of the endoscopic submucosal dissection eschar.



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Figure 4 The full thickness resection device Ovesco over-the-scope OTSC system is a single-use metallic clip preloaded on an applicator cap that can be attached to any standard endoscope. The endoscopic clip can be placed underneath an epithelial/subepithelial lesion and it guarantees at the same time its full-thickness removal and the wall defect closure. In this picture, the Ovesco clip is placed underneath a rectal neuroendocrine neoplasm and grabs its complete thickness.

Conversely, one study reported that ESD, in comparison to m-EMR, achieved a higher R0 resection rate (100% *vs* 70%) and lower recurrence (0% *vs* 17%) in 55 patients with 10-16 mm r-NENs. Thus, ESD appears to be the most appropriate endoscopic technique for the resection of > 10 mm r-NENs[25]. Both m-EMR and ESD were demonstrated to be feasible also for salvage treatment in incomplete primary endoscopic resection, especially for ≤ 10 mm r-NENs[56].

What has been revealed so far must always consider that comparative studies between endoscopic resection techniques are heterogeneous, and limited in samples and volume, with limited follow-up time. To date, a Chinese open-label double-arm randomized clinical trial comparing cap-assisted EMR to ESD for the treatment of rectal NENs less than 10 mm is about to start the recruiting phase

(NCT03982264).

Resectable advanced disease

Locally advanced r-NENS, which are neoplasms invading submucosal layers, conventionally with a lateral spreading diameter ≥ 10 mm, but still without distant metastatic invasion, represent the real frontier of radical removal in the field of rectal tumors. They are associated with a moderate metastatic frequency rate of 5%−15% and with a moderate risk of lymphatic involvement[57], and thus, a long debate on the most appropriate resection technique is nowadays still ongoing. According to the ENETS and UICC/AJCC guidelines, rectal NENs measuring 10−19 mm at their first endoscopic diagnosis, should be investigated in detail to exclude an invasion of the muscularis mucosae and of the locoregional lymph nodes. Rectal magnetic resonance imaging (MRI) and/or rectal endoscopic ultrasound (r-EUS) represent the most reliable investigating techniques for staging rectal lesions: If the rectal lesion is limited to the mucosa or submucosa, which corresponds to a T1 lesion, regardless of its lateral spreading > 10 mm, the only local resection treatment (may it be endoscopic or transanal) has proved to be effective in guaranteeing a radical resection and a very limited recurrence rate over follow-up[58]. Radical surgical resection for 10-19 mm r-NENS without muscularis or lymphatic invasion does not relate to a higher rate of radical resection and radically reduces the patients' quality of life[59].

Contrariwise, T2 or N+ stage lesions should be accurately studied by total body imaging such as 68-Ga-DOTATATE-PET and CT scanning. Once distant metastases are excluded, a surgical radical attempt by laparoscopic low anterior resection (LAR) with or without total mesorectal excision (TME), or even abdomino-perineal resection should be preferred. It has to be noted that, according to the latest European and American guidelines, rectal tumors above 20 mm are automatically considered as T2 stage lesions, as far as they are related to an up to 80% rate of distant metastases.

Transanal endoscopic microsurgery: Transanal endoscopic microsurgery (TEM) is a minimally invasive surgical technique that allows the local full-thickness excision of rectal lesions localized up to 20 cm from the anal verge, avoiding the segmental surgical resection[60]. TEM is usually performed under general anesthesia, in the lithotomy or clasp knife position; it implies the use of anal retractors to dilate the anal sphincter and to maintain exposure[61]. It requires a multi-channel transanal device, which combines the use of a rigid rectoscope with the magnified tridimensional vision. The device is able to control the endoluminal pressure, in order to precisely follow the electrocautery dots previously performed to delimitate the scheduled operating area[37]. Normal saline solution is injected into the submucosal plane with an injector syringe to create a visible submucosal cushion for the elevation of the lesion; the tumor then is excised with electrocautery or an ultrasonic knife under direct vision, then the wound can be closed with absorbable sutures[61].

R-NENs between 10 and 20 mm, limited to the submucosal layer, and without lymphatic invasion can be either approached with a complete *en bloc* endoscopic resection through ESD or with a complete microsurgical excision through TEM.

Both ESD and TEM demonstrated to be superior to m-EMR for > 10 mm r-NENS, but the two of them have not been appropriately compared in prospective observational studies yet[62]. According to single centers experiences, TEM implies a longer operative time than ESD and a major incidence of anesthesia-related adverse events (such as acute retention of urine); moreover, anal dilation or retraction may cause higher postoperative morbidity[63]. On the other hand, TEM provides deeper vertical resection margins in a full-thickness fashion[63]. Furthermore, in case of local recurrence, the existence of fibrosis in the submucosal layer may represent a limit for the mucosal lifting during ESD and thus may reduce the complete *en bloc* resection success rate[64].

Hence, according to these limited experiences, ESD might be preferred to TEM in case of a first incidental 10-19 mm r-NEN, based on its lower morbidity rate, shorter operative time and, consequently, lower costs; TEM on the contrary might be the first therapeutic choice of scar embedded recurrent 10-19 mm r-NENs located within the first 20 cm of the rectum from the anal verge. Overall, to date, the indication to perform one of the two proposed techniques still depends more on the team expertise rather than on robust superiority evidence[63]. Prospective comparative studies should be launched to better define the treatment algorithm of resectable 10-19 mm r-NENs.

Salvage TEM for incomplete endoscopic resection proved to be effective also in case of > 10 mm lesions[65].

Conventional transanal resection (TAR) represents the exact equivalent to TEM for < 20 mm r-NENs localized within 6 cm from the anal verge.

Low anterior resection or intersphincteric resection, with or without TME: R-NENs extended to the muscularis mucosae or those with lymph node involvement, as previously mentioned, are indicated for surgical resection. Low anterior resection (LAR) and intersphincteric resection, with appropriate lymph-node resection through TME, represent the most commonly performed laparoscopic techniques, which are chosen depending on the localization of the r-NEN. Either laparoscopic LAR or intersphicteric resection often implies a temporary protective ileostomy[66]. Anal preservation, if possible, is mandatory, as far as it is associated with a much higher quality of life. Fortunately, the anal localization of rectal neoplasms is rare, so that anal preservation is statistically guaranteed in most cases.

In the case of anus involvement, abdominoperineal resection with definitive colostomy represents the most appropriate surgical technique.

Systemic therapies for advanced disease

As previously mentioned, only around 10% of r-NENs measure > 10 mm at the diagnosis, so it can be assumed that r-NENs are diagnosed as an advanced metastatic disease in a low number of cases[15]. While most r-NETs are localized, their management should be tailored depending on the presence or absence of metastases-predicting factors, including tumor size, endoscopic aspect, T stage, grade, and lymphovascular invasion, as explained before. Endoscopic ultrasonography is the most relevant technique for locoregional assessment[37]. Moreover, it has been recommended to perform pelvic MRI (otherwise, CT scan) as part of the initial workup to evaluate loco-regional spreading and additional explorations in all cases of high-risk r-NENs (*i.e.*, the ones that show factors predicting aggressive disease, see above): They should include contrast-enhanced thoracic-abdominal-pelvic CT scan, liver MRI – especially diffusion-weighted sequences – which has a better sensitivity than CT scan and somatostatin-receptor isotopic imaging (scintigraphy or positron-emitting tomography)[37]. In the presence of advanced disease with distant metastases, a systemic approach represents the first-line therapeutic strategy. In this context, the goal of systemic therapy is disease control and palliation of symptoms. In fact, none of the up-to-date available systemic treatments yet provided a radical cure for r-NENs, but rather a disease progression-free stabilization with variable duration, depending on the different prognostic factors. Given the fact that r-NENs are usually non-functional, systemic therapies are usually used for their antiproliferative effect rather than for their symptom palliation effect. There are several systemic therapeutic options for advanced r-NENs, not substantially different from the ones for other NENs, although studies evaluating specific response rates in r-NENs are limited. Finally, the potential advantage of palliative surgery, either as primary tumor resection or debulking surgery, is still controversial.

Somatostatin analogs: The use of somatostatin analogs (SSAs), mainly lanreotide and octreotide, is the standard first-line therapy in both functioning and non-functioning NENs, arising from both the gastrointestinal and pancreatic tract[67,68]. However, these studies collected only a few cases of r-NENs and none of these studies were focused specifically on r-NENs; in particular, only in the CLARINET study, 14 patients with hindgut NENs were included[68], demonstrating a significantly prolonged PFS (HR = 0.47; 95%CI: 0.30-0.73) in lanreotide treated patients; the treatment of r-NENs with SSAs is thus based on a very low number of patients.

Octreotide long-lasting release is used at a dose of 30 mg every 4 wk i.m. and lanreotide auto-gel is used at a dose of 120 mg every 4 wk, injected subcutaneously. They are traditionally very well-tolerated drugs[69]. A tumor gross-size reduction with SSAs was achieved in no more than 10% of the cases[69].

A superiority trial comparing octreotide and lanreotide is today active (NCT03289741).

Interferon-alpha: Interferon-alpha (INF-α) has been also approved as a symptomatic therapeutic option, especially in addition to SSAs for refractory syndromes[70].

It can be used in advanced NENs as an anti-proliferative option whenever other traditional anti-proliferative systemic drugs are not feasible[71]. An open-label single-arm interventional pharmaco-immunological study has recruited patients with histologically proved NETs to evaluate the potential immunomodulatory synergy of the association of metronomic cyclophosphamide and IFN-α (NCT02838342); no preliminary results have been posted so far.

Cases of r-NENs included in these studies are sporadic.

Targeted agents (everolimus and sunitinib): Based on the results of two randomized, double-blind, prospective, placebo-controlled studies, the mTOR inhibitor everolimus has been used in advanced pancreatic (RADIANT-3) and extra-pancreatic NENs (RADIANT-4). Specifically, in the RADIANT-4 trial, an everolimus benefit in terms of PFS compared to placebo (HR = 0.56; 95%CI 0.40-1.05) has been demonstrated in the GI or lung NENs subgroups' analysis[72].

The multitargeted tyrosine kinase inhibitor (TKI) sunitinib has demonstrated an improved PFS from 5.5 to 11.4 mo only in metastatic pancreatic NENs (p-NENs)[73]. Therefore, it cannot be recommended in GI-NENs outside clinical trials, which are still ongoing to date (NCT00056693 and NCT02315625).

Other multikinase inhibitors such as cabozantinib and sorafenib have been proposed as alternative options in metastatic disease, but strong evidence should still be spread (NCT05048901, NCT00605566, and NCT00131911).

Furthermore, the association between TKI and chemotherapy has been studied; a single-arm, open-label trial testing the efficacy of the association between everolimus 10 mg daily and temozolomide 150 mg/m^2 for 7 d every 2 wk has been recruiting patients with a histologically proven primary gastro-entero-pancreatic (GEP) neuroendocrine carcinoma with a Ki67 index of 20%-55%; the results are about to be published (NCT02248012).

Radioligand therapy: Radioligand therapy (RLT) represents a therapeutic option in progressive well-differentiated G1/2 NENs with homogenous somatostatin receptor expression[74].

NETTER-1, a recent multicenter prospective phase III trial including 229 patients with metastatic gastro-intestinal well-differentiated G1 or G2 NENs, compared patients' prognosis between those undergoing 177Lu-DOTATATE (Lutathera) in association with octreotide long-lasting release treatment, and those only undergoing high-dose octreotide long-lasting release; 177Lu-DOTATATE proved its superiority in terms of PFS (median PFS > 19.9 mo) and promising results are on the point to be published also regarding its effect on the OS and the patients' quality of life[75]. This study did not explicitly enroll patients affected by r-NENs, rather it focused on midgut neoplasms, with four colonic NENs (none of them were rectal).

The NETTER-2 phase III, multi-center, randomized, open-label trial is today ongoing: It aims to determine if first-line treatment Lutathera in combination with LAR prolongs PFS in G2/3 GEP-NENs patients (NCT03972488). A prospective single-arm, multicenter trial is today enrolling 195 patients with progressed NETs to evaluate the efficacy of 177Lu-DOTATATE in terms of 12-mo PFS (NCT02743741).

Chemotherapy: G3 NENs are represented preferentially by poorly differentiated r-NECs, whereas r-NET G3 are rare. R-NECs represent a distinct category separated from other r-NENs, because of a highly aggressive biological behavior with a poor prognosis and distinctive histological picture (small and large cell carcinomas)[76]. This entity is usually treated with chemotherapy, also according to the latest ESMO guidelines[77], in which the use of chemotherapy is highly recommended for metastatic low-differentiated G3 NECs.

Chemotherapy regimens containing cisplatin or carboplatin in combination with etoposide could be considered as a first-line therapy in patients with advanced GEP-NECs. Irinotecan is a valid alternative to etoposide in the same cases[78]. Regarding the rare cases of advanced G1/2 r-NENs, streptozocin and its association with 5-fluorouracil and doxorubicin are the most used schemes, even if they are associated with a < 25% clinical and radiological response rate[79]. Three-drug regimens (5-FU, dacarbazine, and epirubicin), used historically since the 1990s, are associated with a response rate of up to 30% of cases[80].

Combined radiation therapy and chemotherapy in high-grade r-NECs: Interesting data were published regarding the efficacy of chemotherapy combined with radiotherapy in poorly differentiated and aggressive high-grade G3 rNECs, which by definition have a Ki67 index ≥ 20% and a mitotic index ≥ 20/10 high power fields.

According to a preliminary retrospective univariate and multivariate analysis, radiation therapy (RT) proved to significantly improve OS in patients affected by G3 r-NECs treated by both surgery and RT compared with G3 r-NECs that underwent only surgery (HR = 0.393; 95%CI: 0.206-0.750; P = 0.009)[81]. Furthermore, according to a multicenter American multidisciplinary study, chemotherapy (based on platinum, etoposide, and fluoropyrimidine) with or without RT and surgery, obtained similar outcomes in terms of both PFS (13.0 mo *vs* 13.2 mo, P = 0.75) and OS (49.1 mo *vs* 39.2 mo, P = 0.42)[82]. These findings highlighted that not only does RT improve r-NECs prognosis, but also when combined with chemotherapy, it leads to similar outcomes compared to surgery. Table 2 shows a resume of the main systemic therapies for advanced disease.

SURVEILLANCE

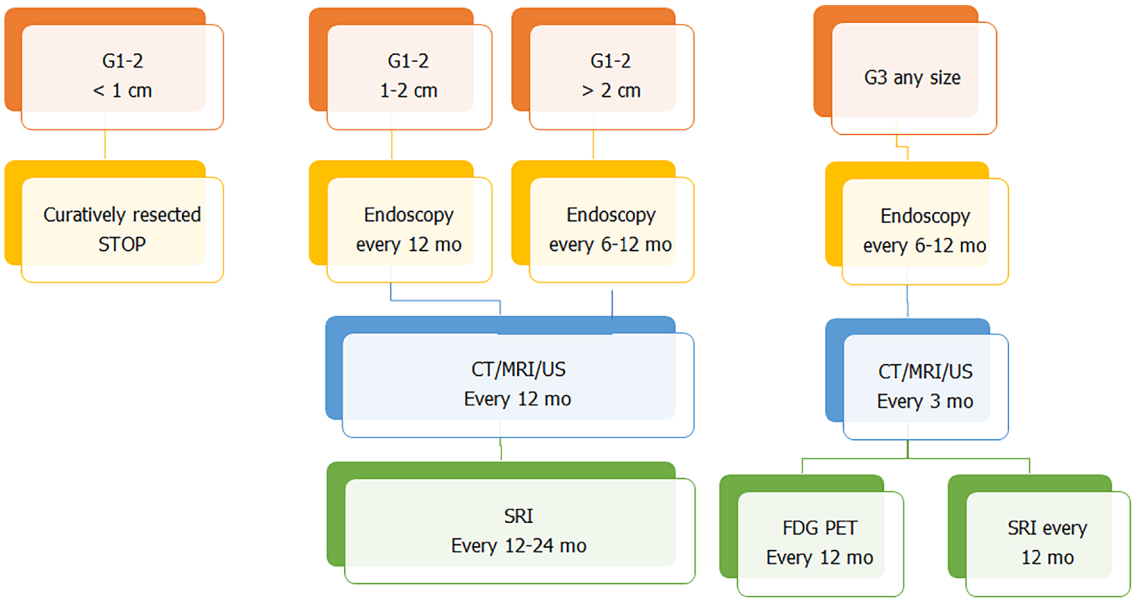
The indications for the follow-up of r-NENs are scarce in the literature. Furthermore, the few indications that we can find in the consensus guidelines are mostly based on expert opinions rather than evidence-based data. Moreover, the main guidelines give follow-up indications mainly based on the size of the r-NEN[83], while it is documented that grade is the most heavily prognostic factor.

According to the ENETS 2017 guidelines, it is advocated that follow-up occurs in specialized NEN centers or at least in hospitals with close collaboration with specialized NEN centers[84]. The suggested type and timing of the follow-up are based on the tumor size, grade, and operative outcome (if curatively resected), respectively. In the case of small (< 10 mm) G1/2 r-NENs, when curatively resected, only one endoscopic check at 12 mo from the endoscopic resection is advisable. No further investigations are needed. For 10-20 mm G1/2 lesions, an annual endoscopic follow-up is recommended. Moreover, classical radiological imaging by using either CT or MRI is recommended. Also, somatostatin receptor imaging (SRI) is advisable every 12-24 mo. For G1/2 lesions > 20 mm, both curatively and non-curatively resected, a closer follow-up (every 3-12 mo) is recommended, by using endoscopy every 6-12 mo, CT or MRI every 3-12 mo, and SRI every 12-24 mo. In rare cases of G3 NEC/NETs, both curatively and non-curatively resected, a 3-mo follow-up is suggested, by performing CT or MRI; an endoscopy is recommended every 6-12 mo; nuclear medicine imaging (both SRI and FDG-PET) is advisable every 12 mo. In any case, an EUS examination may be required if recurrence or progression is suspected (Figure 5).

Table 2 Systemic therapies for advanced disease

Pharmacological class	Therapeutic agent	Superiority *vs* placebo	Main ongoing clinical trials
Somatostatin analogs	Octreotide (30 mg/4 wk i.m.)	Yes[70]	NCT04129255
	Lanreotide (120 mg/4 wk s.c.)	Yes[69]	NCT03289741 (octreotide *vs* lanreotide)
Cytokines	Interferon-α (3 MIU/three times a week s.c.)	Yes[72]	NCT02838342 (association with cyclophosfamide)
Targeted agents	Everolimus (10 mg/d p.o.)	Yes[73]	NCT02248012 (association with temozolomide)
	Sunitinib (50 mg/d p.o.)	Yes[74][1]	NCT00056693NCT02315625
	Cabozantinib (40-60 mg/d p.o.)	NA	NCT05048901
	Sorafenib (800 mg/d p.o.)	NA	NCT00605566, NCT00131911
Radioligand therapy	177Lu-DOTATATE (Lutathera)	Yes[76]	NCT02743741, NCT03972488 (combination with LAR)
Chemotherapy	Cisplatin/carboplatin ± etoposide/irinotecan	Yes[79]	NCT03963193 (etoposide + cisplatin *vs* irinotecan + cisplatin)
	Streptozocin ± 5-FU ± doxorubicin	Yes[80]	NCT00602082
	5-FU ± dacarbazine ± epirubicin	Yes [81]	NA

[1]Only for p-NENs.
LAR: Long-acting release octreotide; NA: Not available; 5-FU: 5-fluorouracil.



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Figure 5 Surveillance flow-chart. CT: Computed tomography; MRI: Magnetic resonance imaging; US: Ultrasound; SRI: Somatostatin receptor imaging; FDG-PET: Positron emission tomography with 18F-fluorodeoxyglucose.

CONCLUSION

R-NENs are among the most frequent digestive NENs, together with small bowel NENs and their incidence has hugely increased over the last few years, likely due to the widespread use of endoscopic screening for CRC and the improvement in endoscopic techniques. As metabolic syndrome appears as a recognized risk factor for the development of r-NENs, this might have partially contributed to the increasing incidence of these neoplasms.

To date, however, there is still an underestimation of the incidence and prevalence of these neoplasms, as they are often not recognized by the endoscopist. In the increasingly vast panorama of artificial intelligence, its application to gastrointestinal endoscopy may help in detecting and thus treating r-NENs. Several predictors of metastases, progression, and/or recurrence of r-NENs have been

proposed, and among them, the diameter of the primary neoplasm and the tumor grade are the most important, although the relevance of each of these factors has not been clearly established. Traditionally, well-differentiated r-NENs smaller than 10 mm are endoscopically removed given their low metastatic risk, whereas tumors larger than 20 mm are suggested to be surgically resected given their high risk of distant spreading. However, a potential more updated cut-off size of 15 mm and the degree of differentiation with G2 and, indeed, G3 tumors, are considered to be the main independent predictors of metastatic spread and dismal prognosis. Moreover, the number of positive nodes (≥ 5 as a cut-off) and the depth of invasion with the invasion of the *muscolaris propria* are also associated with a poor prognosis.

Locally advanced r-NENs with a lateral spreading diameter ≥ 10 mm, but still T1N0 should be addressed at first endoscopically; while surgical techniques should be adopted to address lesions invading the *muscolaris propria* or loco-regional lymph nodes. TEM is the treatment of choice for scar-embedded recurrent resectable lesions.

The indications for surveillance are scarce and mainly based on experts' opinions rather than evidence-based guidelines. According to the ENETS 2017 guidelines, the follow-up is based on the tumor size, grade, and operative outcome.

Large prospective studies should be encouraged to define standardized guidelines for r-NENs and to identify clear-cut prognostic factors and scores which might help in the management of these neoplasms.

FOOTNOTES

Author contributions: Massironi S, Gallo C, and Rossi RE conceived of the contents of the manuscript; Gallo C, Boskoski I, and Barbaro F developed the revision regarding the therapeutic options for r-NENs; Boskoski I and Barbaro F furnished the pictures; Massironi S developed the introduction, the surveillance paragraph, and the conclusive chapter; Rossi RE and Cavalcoli F developed the paragraph regarding the risk factors; Invernizzi P together with all authors, revised entirely the manuscript and contributed to its final aspect.

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Country/Territory of origin: Italy

ORCID number: Camilla Gallo 0000-0002-7598-7220; Roberta Elisa Rossi 0000-0003-4208-4372; Federica Cavalcoli 0000-0002-7745-7573; Federico Barbaro 0000-0002-7928-3757; Ivo Boškoski 0000-0001-8194-2670; Pietro Invernizzi 0000-0003-3262-1998; Sara Massironi 0000-0003-3214-8192.

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ORIGINAL ARTICLE

Basic Study

Quercetin exerts anti-inflammatory effects *via* inhibiting tumor necrosis factor-α-induced matrix metalloproteinase-9 expression in normal human gastric epithelial cells

Hsi-Lung Hsieh, Ming-Chin Yu, Li-Ching Cheng, Mei-Yi Chu, Tzu-Hao Huang, Ta-Sen Yeh, Ming-Ming Tsai

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Hsi-Lung Hsieh, Li-Ching Cheng, Tzu-Hao Huang, Ming-Ming Tsai, Department of Nursing, Division of Basic Medical Sciences, Chang-Gung University of Science and Technology, Taoyuan 333, Taiwan

Hsi-Lung Hsieh, Mei-Yi Chu, Ming-Ming Tsai, Research Center for Chinese Herbal Medicine, College of Human Ecology, Chang Gung University of Science and Technology, Taoyuan 333, Taiwan

Hsi-Lung Hsieh, Department of Neurology, Chang Gung Memorial Hospital, Taoyuan 333, Taiwan

Ming-Chin Yu, Ming-Ming Tsai, Department of General Surgery, New Taipei Municipal TuCheng Hospital, New Taipei 236, Taiwan

Ming-Chin Yu, Ta-Sen Yeh, College of Medicine, Chang Gung University, Taoyuan 333, Taiwan

Ming-Chin Yu, Li-Ching Cheng, Ta-Sen Yeh, Ming-Ming Tsai, Department of General Surgery, Chang Gung Memorial Hospital, Taoyuan 333, Taiwan

Corresponding author: Ming-Ming Tsai, PhD, Associate Professor, Department of Nursing, Division of Basic Medical Sciences, Chang-Gung University of Science and Technology, No. 261 Wenhua 1st Road, Guishan District, Taoyuan 333, Taiwan. mmtsai@mail.cgust.edu.tw

Abstract

BACKGROUND

Gastric injury is the most common digestive system disease worldwide and involves inflammation, which can lead to gastric ulcer or gastric cancer (GC). Matrix metallopeptidase-9 [MMP-9 (gelatinase-B)] plays an important role in inflammation and GC progression. Quercetin and quercetin-rich diets represent potential food supplements and a source of medications for treating gastric injury given their anti-inflammatory activities. However, the effects and mechanisms of action of quercetin on human chronic gastritis and whether quercetin can relieve symptoms remain unclear.

AIM

To assess whether tumor necrosis factor-α (TNF-α)-induced MMP-9 expression

mediates the anti-inflammatory effects of quercetin in normal human gastric mucosal epithelial cells.

METHODS

The normal human gastric mucosa epithelial cell line GES-1 was used to establish a normal human gastric epithelial cell model of TNF-α-induced MMP-9 protein overexpression to evaluate the anti-inflammatory effects of quercetin. The cell counting Kit-8 assay was used to evaluate the effects of varying quercetin doses on cell viability in the normal GES-1 cell line. Cell migration was measured using Transwell assay. The expression of proto-oncogene tyrosine-protein kinase Src (c-Src), phospho (p)-c-Src, extracellular-signal-regulated kinase 2 (ERK2), p-ERK1/2, c-Fos, p-c-Fos, nuclear factor kappa B (NF-κB/p65), and p-p65 and the effects of their inhibitors were examined using Western blot analysis and measurement of luciferase activity. p65 expression was detected by immunofluorescence. MMP-9 mRNA and protein levels were measured by quantitative reverse transcription polymerase chain reaction (qRT–PCR) and gelatin zymography, respectively.

RESULTS

qRT-PCR and gelatin zymography showed that TNF-α induced MMP-9 mRNA and protein expression in a dose- and time-dependent manner. These effects were reduced by the pretreatment of GES-1 cells with quercetin or a TNF-α antagonist (TNFR inhibitor) in a dose- and time-dependent manner. Quercetin and TNF-α antagonists decreased the TNF-α-induced phosphorylation of c-Src, ERK1/2, c-Fos, and p65 in a dose- and time-dependent manner. Quercetin, TNF-α antagonist, PP1, U0126, and tanshinone IIA (TSIIA) reduced TNF-α-induced c-Fos phosphorylation and AP-1–Luciferase (Luc) activity in a dose- and time-dependent manner. Pretreatment with quercetin, TNF-α antagonist, PP1, U0126, or Bay 11-7082 reduced TNF-α-induced p65 phosphorylation and translocation and p65–Luc activity in a dose- and time-dependent manner. TNF-α significantly increased GES-1 cell migration, and these results were reduced by pretreatment with quercetin or a TNF-α antagonist.

CONCLUSION

Quercetin significantly downregulates TNF-α-induced MMP-9 expression in GES-1 cells *via* the TNFR-c-Src–ERK1/2 and c-Fos or NF-κB pathways.

Key Words: Anti-inflammatory; Quercetin; Matrix metallopeptidase-9; Tumor necrosis factor-α; Normal human gastric epithelial cells

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Core Tip: Gastric inflammation is a common digestive system disease. Proinflammatory cytokines, such as tumor necrosis factor-α (TNF-α), and degradation induced by matrix metallopeptidases (MMPs) play a crucial role in gastric injury. We investigated whether quercetin prevents TNF-α-induced gastric inflammation in normal human gastric mucosa epithelial cells. Quercetin significantly blocked MMP-9 activity and cell migration and protected against TNF-α-induced gastric injury. Quercetin appeared to block TNF-α-induced gastric injury by downregulating MMP-9 *via* the TNF-α antagonist-c-Src–extracellular-signal-regulated kinase 1/2 and c-Fos or nuclear factor kappa B pathways. Our data suggest that quercetin, which exhibits high bioavailability, may be effective as an adjuvant therapy for the treatment of gastric inflammation.

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INTRODUCTION

Gastric diseases are among the most common aliments of the digestive system worldwide and include chronic gastritis, duodenal and gastric ulceration, gastric mucosa-associated lymphoid tissue lymphoma, and gastric cancer (GC)[1-3]. Gastric diseases have multifaceted causes that are related to the growing use of nonsteroidal anti-inflammatory drugs (NSAIDs), pathogen-mediated factors [such as

Helicobacter pylori (*H. pylori*) infection], environmental factors (such as alcohol consumption, salt intake, and cigarette smoking), and host genetic and other factors (such as genetic polymorphisms, psychological stress, oxidative stress, and acidity)[4,5]. Gastric diseases accompanied by chronic inflammation involve increased neutrophil and mononuclear cell activities and the release of proinflammatory chemokines and cytokines, such as tumor necrosis factor-α (TNF-α)[6], interleukin (IL)-1[7], IL-6, IL-8, and IL-10[8], cell adhesion molecules [such as circular dichroism 44 and intercellular adhesion molecule-1 (ICAM-1)][9], and metalloproteinases (MMPs)[10]. The induction of MMPs is a major pathological feature and plays a critical role in the progression of gastric diseases[2,11,12]. Among the variants of genetic polymorphisms related to proinflammatory cytokines, those involving ILs and TNF-α are the most extensively studied.

TNF-α is a multifunctional cytokine that was first identified as a serum factor causing necrosis of transplanted tumors. This cytokine participates in the regulation of immune–inflammatory reactions involved in host defense against infectious, autoimmune, and endocrine diseases and cancer, and its actions help to determine the survival or death of various cells[13-16]. The upregulation of TNF-α and its related pathways is associated with some gastric diseases. Excessive TNF-α expression is associated with tumor promotion *via* a strong immune–inflammatory response and angiogenesis, which can modify the risk for gastric, breast, hepatocellular, cervical, or bladder carcinoma[17-20]. Harris *et al*[21] reported that *H. pylori* infection upregulates TNF-α expression and that this upregulation decreases gastric acid secretion[21]. Fan *et al*[22] reported that high expression of TNF-α can stimulate normal gastric epithelial cells to develop into precancerous cells and that these cells may eventually develop into GC. A similar mechanism is also noted in chronic gastritis, intestinal metaplasia, and dysplasia[22].

MMPs are a key group of enzymes that can destroy nearly all elements of the extracellular matrix, including components of the basement membrane[23,24]. MMP-9 expression damages the extracellular matrix, including components of the basement membrane, inducing gastric disease. MMP-9 is often upregulated or dysregulated in severe gastric disease, where it may accelerate severe gastric inflammation and possibly contribute to the development of GC. High MMP-9 expression is also associated with a poor survival rate in patients with GC[25-28]. MMP-9 is induced in a nuclear factor kappa B (NF-κB)-dependent manner[29,30]. Understanding the roles of inflammatory mediators may provide a basis for new therapeutic approaches for treating gastric inflammatory diseases.

Recent research suggests that dietary intake may alter the risk of gastric injury[31,32]. A frequently studied dietary component, quercetin, is a flavonoid polyphenolic compound that is abundant in fruits, red wine, and vegetables. Studies on the bioactivity of quercetin have found that it exerts many favorable effects on human health, such as antioxidant, anti-inflammatory, anticancer, antiallergic, antiviral, free radical-scavenging, and neuroprotective activities[33,34].

Quercetin protects gastric mucosal epithelial cells against injury caused by ischemia–reperfusion[35], ethanol[36], indomethacin[9], hydrogen peroxide[10], or *H. pylori*[37] in various animal models[38]. Quercetin also exerts gastroprotective activity against TNF-α-induced injury to the gastric mucosal epithelium, but the mechanisms responsible for this effect remain unknown. Here, we explored whether quercetin protects against TNF-α-induced MMP-9 damage to gastric mucosal epithelial cells. We examined the responses of the human gastric mucosal epithelial cell line GES-1 to challenge with immune–inflammatory reactions mediated by TNF-α and explored the underlying molecular mechanisms.

MATERIALS AND METHODS

Materials

Roswell Park Memorial Institute (RPMI) 1640 medium, fetal bovine serum (FBS), and TRIzol were purchased from Invitrogen (Carlsbad, CA, United States). Polyvinylidene fluoride (PVDF) membranes, enhanced chemiluminescence (ECL), and Western blot detection systems were obtained from GE Healthcare Biosciences (Chalfont St Giles, Buckinghamshire, United Kingdom). Anti-phospho-c-Src (Cat#2101), anti-phospho-ERK1/2 (Cat#4377), anti-phospho-c-Fos (Cat#5348), anti-phospho-NF-κB (p65) (Cat#3033), c-Src (Cat#2109), ERK2 (Cat#9108), c-Fos (Cat#2250), NF-κB (Cat#8242), and anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Cat#2118) antibodies were purchased from Cell Signaling Technology (Danvers, MA, United States). Anti-mouse (Cat# 7056) and anti-rabbit (Cat# 5054) horseradish peroxidase-conjugated secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, United States). 4′,6-Diamidino-2-phenylindole was purchased from Vector Laboratories (Burlingame, CA, United States). TurboFect Transfection Reagent was purchased from Thermo Fisher Scientific (Waltham, MA, United States). The iScript cDNA Synthesis Kit, SsoFast EvaGreen Supermix, and CFX Connect Real-Time polymerase chain reaction (PCR) detection system were obtained from Bio–Rad Laboratories, Inc. (Hercules, CA, United States). A dual-luciferase reporter system was obtained from Promega (Madison, WI, United States). All inhibitors, including a TNF-α antagonist (SC-356159), PP1 (SC-203212A), U0126 (SC-222395), tanshinone IIA (TSIIA) (SC-200932), and Bay 11-7082 (SC-202490), were purchased from Santa Cruz Biotechnology. Quercetin was obtained from Biotic Chemical Co., Ltd. (New Taipei City, Taiwan). A stock solution of quercetin was resuspended in a

1:9 (v/v) solution of dimethyl sulfoxide (DMSO) and 99% ethanol and diluted to the presented final concentration with culture medium. Human recombinant TNF-α was obtained from R&D Systems (AFL210) (Minneapolis, MN, United States). MMP9 inhibitor (MMP9i) (Cat#15942-500) was purchased from Biomol (Plymouth Meeting, PA, United States). Cell counting Kit-8 (CCK-8) kit reagent was obtained from MedChemExpress Ltd. (Hercules, CA, United States). All other enzymes, reagents, and chemicals were obtained from Sigma–Aldrich (St. Louis, MO, United States).

Cell culture

GES-1 cells were obtained from Xiamen University in China. The cells were cultured in RPMI 1640 medium containing 10% FBS, 100 IU/mL penicillin G, 100 mg/mL streptomycin sulfate, and nonessential amino acids at 37 °C in a humidified atmosphere with 5% CO_2 according to a previously described standard protocol[39]. The cells were either untreated or treated with quercetin or inhibitors of the TNF-α antagonist, MMP-9i, c-Src (PP1), ERK1/2 (U0126), c-Fos (TSIIA), or NF-κB (BAY). These substances were added to the cell culture 1 h before the addition of TNF-α in all experiments.

MMP zymography

Growth-arrested cells were incubated with different treatments for the indicated time intervals. The cells were either untreated or treated with quercetin or inhibitors of TNF-α antagonist, c-Src (PP1), ERK1/2 (U0126), c-Fos (TSIIA), or NF-κB (BAY). After treatment, the conditioned media were collected and mixed with 5 × nonreducing sample buffer and analyzed on a 10% gel containing 0.15% gelatin. After electrophoresis, the gel was washed, incubated, stained, and destained. The gelatinolytic activity appeared as parallel white bands on a blue background[40].

CCK-8 assay

GES-1 cells (5000 cells/well) were seeded into 96-well plates with serum-free RPMI 1640 medium and cultured for 24 h at 37 °C in a humidified atmosphere with 5% CO_2. The culture medium was replaced by quercetin diluted in series to concentrations of 0, 0.01, 0.1, 1, 10, or 100 mg/mL, and the cells were cultured for 24 or 48 h. After incubation with quercetin, 10 mL of the CCK-8 kit reagent was added to each well (100 mL/well), and the incubation was continued for 1 h. The optical density at 450 nm was measured using a multifunction microplate reader (SpectraMax i3; Kelowna International Scientific Inc., New Taipei, Taiwan) using a previously described standard protocol[41]. Cell viability is expressed as the percentage of living cells relative to the control.

Quantitative reverse transcription–PCR

GES-1 cells were seeded into 9-cm dishes with serum-free RPMI 1640 medium, cultured for 24 h at 37 °C, and then incubated with TNF-α for 0, 16, 20, or 24 h. Total RNA was extracted using TRIzol reagent, and the concentration of RNA was measured on a Nano100 Micro-Spectrophotometer (CLUBIO; Taipei, Taiwan). cDNA was then synthesized using an iScript cDNA Synthesis Kit and amplified on a spectro-fluorometric thermal cycler (iCycler; Bio–Rad Laboratories)[42]. To evaluate *MMP9* mRNA expression in GES-1 cells, qRT–PCR was performed according to a previously described standard protocol using *GAPDH* as the internal control[42]. Fluorescence emitted by SsoFast EvaGreen Supermix was measured using the CFX Connect Real-Time PCR detection system. The following primers were used for qRT–PCR: Human *MMP9* (sense, 5′–AGTTTGGTGTCGCGGAGCAC–3′ and antisense, 5′–TACAT GAGCGCTTCCGGCAC–3′) and human *GAPDH* (sense, 5′–ACAGTCAGCCGCATCTTCTT–3′ and antisense, 5′–GACAAGCTTCCCGTTCTCAG–3′). Relative gene expression is expressed using the Delta-Delta-Ct (ddCt) method, where Ct represents the mean threshold cycle.

Western blot analysis

Growth-arrested cells were incubated with different treatments for the indicated time intervals. The cells were either untreated or treated with quercetin or inhibitors of TNF-α antagonist, c-Src (PP1), ERK1/2 (U0126), c-Fos (TSIIA), or NF-κB (BAY). After treatment, the cells were washed with PBS, scraped, lysed in lysis buffer, and collected as a whole-cell extract at 4 °C using a previously described standard protocol[42]. Samples were loaded onto a 10% SDS-PAGE gel, electrophoresis was performed, and the bands were transferred to a PVDF membrane. The PVDF membrane was incubated overnight with specific primary antibodies, including anti-phospho-c-Src, anti-phospho-ERK1/2, anti-phospho-c-Fos, anti-phospho-NF-κB, or anti-GAPDH. The PVDF membranes were washed and then incubated with anti-mouse or anti-rabbit horseradish peroxidase-conjugated secondary antibody (dilution, 1:2000) for 1 h. The relative expression levels of c-Src, ERK1/2, c-Fos, and NF-κB were normalized to that of GAPDH. Finally, the immunoreactive bands were incubated in enhanced chemiluminescence reagent, and images were recorded on a UVP BioSpectrum 500 Imaging System (UVP, Inc.; Upland, CA, United States). Densitometry was quantified using UN-SCAN-IT gel software (Silk Scientific, Inc.; Orem, UT, United States).

Cell migration assay

To evaluate the influence of TNF-α-induced MMP-9 expression on the metastatic activity of GES-1 cells, an *in vitro* Transwell assay (Becton-Dickinson, Franklin Lakes, NJ, United States) was used. Cells at a concentration of 1 × 10^5 cells/100 mL in serum-free RPMI 1640 medium were added to the upper chamber, and the Matrigel-uncoated insert was inserted in the lower chamber, which contained 20% FBS + RPMI 1640 medium. The migration assay was run for 24 h following a previously described standard protocol[39,42].

Immunofluorescence

The influence of TNF-α-induced MMP-9 expression in GES-1 cells and the potential involvement of NF-κB/p65 translocation from the cytoplasm into the nucleus in this process were investigated. GES-1 cells were seeded into culture plates with coverslips. The cells were either untreated or treated with quercetin or inhibitors of c-Src (PP1), ERK1/2 (U0126), or NF-κB (BAY). After treatment, the cells were washed, fixed, permeabilized, blocked, and stained with anti-NF-κB. The coverslips were affixed to slides with mounting medium and stained with 4',6-diamidino-2-phenylindole as previously described[43]. Fluorescence images were photographed using a fluorescence microscope (Leica Microsystems, Wetzlar, Germany).

Luciferase assay

The human AP-1–Luc and NF-κB–Luciferase (Luc) response element reporter plasmids pGL4.44GL4.44 [*luc2P*/AP1 RE/Hygro] (No. E411A; JQ858516) and pGL4.32 [*luc2P*/NF-κB-RE/Hygro] (No. E849A; EU581860), and the dual-luciferase reporter system were obtained from Promega (Madison). Two plasmids were transfected into GES-1 cells using TurboFect Transfection Reagent according to a previously reported protocol[44]. The cells were either untreated or treated with quercetin or inhibitors of TNF-α antagonist, c-Src (PP1), ERK1/2 (U0126), or NF-κB (BAY). After treatment, the cells were collected, lysed, and centrifuged, and aliquots of the supernatants were tested for promoter activity using the dual-luciferase assay system. Firefly luciferase activity was standardized to that of *Renilla* luciferase.

Statistical analysis

All data were obtained from at least three independent experiments and are presented as the mean ± SEM. The data were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's *post-hoc* test to identify significant differences between multiple groups. Statistical analyses were performed using GraphPad Prism 6.0 (GraphPad Software, Inc.; La Jolla, CA, United States). A P value < 0.05 was considered significant. The statistical methods of this study were reviewed by Dr. Jian-Hao Chen from Estat Statistics Consulting Company (Taipei, Taiwan).

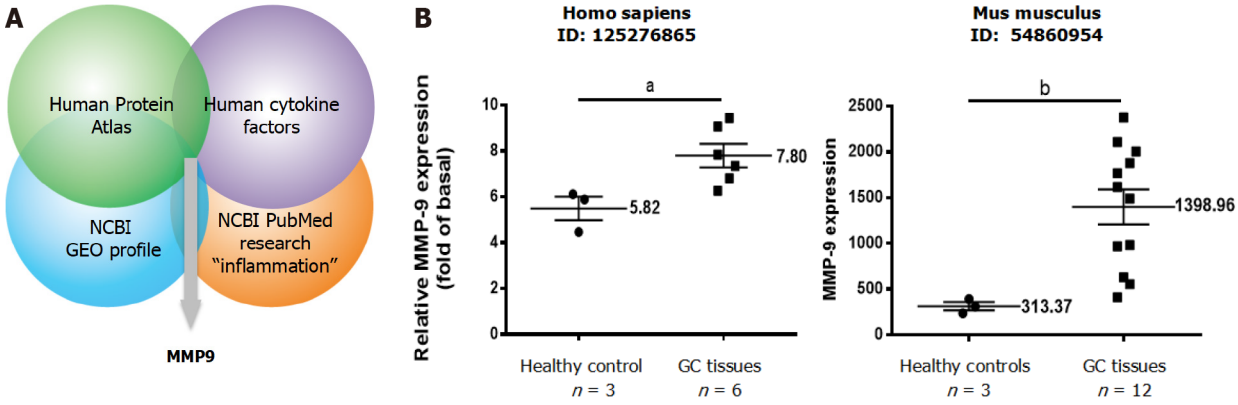
RESULTS

Omics approach to identify inflammation-related proteins involved in GC

To examine the potential roles of inflammation-related proteins in GC, we used an omics approach based on four datasets from the following sources: (1) The public Human Protein Atla (https://www.proteinatlas.org); (2) The public United States National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO) profiles (https://www.ncbi.nlm.nih.gov/geoprofiles/); (3) Human cytokine factors (https://www.abcam.com/human-cytokine-antibody-array-membrane-174-targets-ab193657.html); and (4) NCBI PubMed research "inflammation" papers. The combination of the four datasets revealed significant upregulation of *MMP9* in GC relative to paired normal tissues, as shown in Figure 1A. The NCBI GEO profiles revealed consistent significant *MMP9* upregulation (Human, ID: 125276865; Mouse, ID:54860954) in GC tissues relative to paired normal tissues (Figure 1B). Therefore, we selected *MMP9* for further study.

Role of MMP-9 expression in TNF-a-induced inflammation in GES-1 cells

MMP-9 expression is involved in gastric inflammation, may contribute to the development of GC, and is associated with poor survival rates[25-28]. To explore the role of TNF-α-activated MMP-9 in GC, GES-1 cells were treated with different concentrations of TNF-α (10, 20, or 30 ng/mL) for designated times (0, 16, 20, or 24 h), and MMP9 mRNA and protein levels were measured. TNF-α at a dose of 10 mg/mL for 16 or 20 h did not affect MMP-9 protein expression in GES-1 cells, but exposure for 24 h increased MMP-9 expression compared with 0 h (P < 0.05). TNF-α at a dose of 20 mg/mL for 24 h significantly increased MMP-9 expression compared with 0 h (P < 0.05). TNF-α at a dose of 30 mg/mL for 20 or 24 h resulted in the highest MMP-9 expression of GES-1 cells compared with 0 h (P < 0.05 and P <0.01, respectively). TNF-α induced MMP-9 enzymatic activity in a time- and concentration-dependent manner (Figure 2A). qRT–PCR was performed to measure the *MMP-9* mRNA level in TNF-a-induced GES-1 cells. After TNF-α stimulation, *MMP-9* mRNA was significantly upregulated in a time-dependent manner after 16, 20,



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Figure 1 Identification studies of matrix metallopeptidase-9, a potential inflammation-related protein involved in gastric cancer. A: Identification of potential inflammation-related proteins involved in gastric cancer (GC) based on an omics approach including four integrated datasets. The strategy included proteomic profiling; B: Relative expression levels of matrix metallopeptidase-9 in paired GC and adjacent normal tissues in humans and mice. A *t* test was used for comparisons between the two groups. Data are expressed as the mean ± SEM of three independent experiments. $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* controls. GC: Gastric cancer; GEO: Gene Expression Omnibus; MMP-9: Matrix metallopeptidase-9; NCBI: National Center for Biotechnology Information.

and 24 h of incubation compared with 0 h ($P < 0.05$, $P < 0.05$, and $P < 0.001$, respectively) (Figure 2B). These observations suggest that MMP-9 expression was induced by TNF-α at both the transcriptional and translational levels in GES-1 cells. Therefore, we chose to use 30 ng/mL TNF-α in GES-1 cells for further assessments of TNF-α-induced changes in MMP-9 expression. We explored whether a TNF-α antagonist can repress TNF-α-activated MMP-9 expression in GES-1 cells. The cells were either untreated or treated with a TNF-α antagonist (0.3, 1, and 3 mM) for 1 h before the addition of TNF-α. MMP-9 inhibition significantly repressed TNF-α-activated MMP-9 expression in GES-1 cells in a time-dependent manner after 16 and 24 h of incubation compared with 0 h ($P < 0.01$ and $P < 0.001$, respectively) (Figure 2C).

Effects of quercetin on TNF-α-induced MMP-9 expression in GES-1 cells

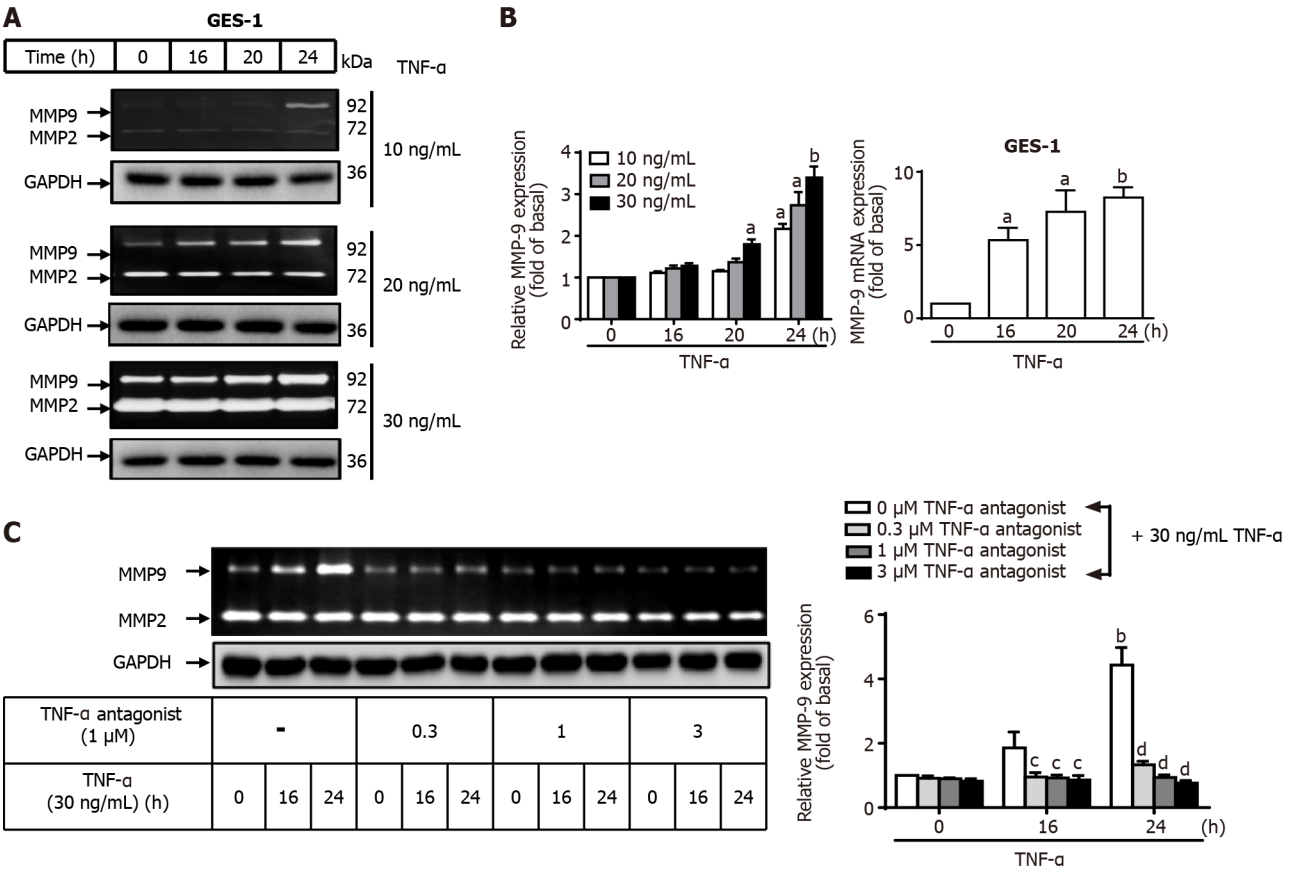
Quercetin has anti-inflammatory effects[33,34]. We first measured the viability of GES-1 cells after quercetin treatment. Quercetin treatment at concentrations of 0, 0.01, 0.1, 1, or 10 mM for 24 to 48 h had almost no effect on GES-1 cell viability (Figure 3A and B). In contrast, 100 mM quercetin significantly reduced GES-1 cell viability after 48 h compared with 0 mM ($P < 0.01$). Therefore, quercetin was used at concentrations up to 1 mM in the following experiments to explore whether quercetin represses TNF-α-activated MMP-9 expression in GES-1 cells and its mechanism of action. The cells were either untreated or treated with quercetin (0, 0.1, 0.3, or 1 mM) for 1 h. Then, TNF-α was added, and the cells were incubated for 0, 16, or 24 h (Figure 3C). Quercetin significantly repressed TNF-α-activated MMP-9 expression in GES-1 cells at doses of 0.1, 0.3, and 1 mM and in a time-dependent manner after 16 and 24 h of incubation.

Involvement of c-Src in TNF-α-induced MMP-9 expression in GES-1 cells

Kim *et al*[45] reported that TNF-α-induced MMP-9 expression is mediated *via* mitogen-activated protein kinase (MAPK) in human GC cells[45]. We next explored the pathway through which quercetin reduces TNF-α-induced MMP-9 expression in GES-1 cells. To determine the role of c-Src in TNF-α-induced MMP-9 expression, two inhibitors of TNF-α antagonist or c-Src (PP1) were used. GES-1 cells were pretreated with the TNF-α antagonist (1 mM) or PP1 (0, 0.3, 1, or 3 mM) for 1 h, and the cells were then incubated with TNF-α for 0, 16, or 24 h (Figure 4A). Pretreatment with PP1 significantly attenuated TNF-α-induced MMP-9 expression in cells incubated for 16 or 24 h in a time-dependent manner. This finding suggests that c-Src was involved in these responses. To confirm this effect, Western blot analysis was used to examine the phosphorylation of c-Src in cells exposed to TNF-α for 0, 5, 15, 30, or 60 min (Figure 4B and C). Pretreatment with quercetin, PP1, or TNF-α antagonist blocked TNF-α-activated phosphorylation of c-Src in a time-dependent manner with the exception of incubation with PP1 + TNF-α and TNF-α antagonist + TNF-α for 15 min (Figure 4C). These findings suggest that TNF-α induced MMP-9 expression *via* TNFR-c-Src in GES-1 cells.

Involvement of ERK1/2 in TNF-a-induced MMP-9 expression in GES-1 cells

To evaluate whether ERK1/2 contributes to TNF-α-induced MMP-9 expression, two inhibitors of TNF-α antagonist or ERK1/2 (U0126) were used. GES-1 cells were pretreated with the TNF-α antagonist (1 mM) or U0126 (0, 0.3, 1, or 3 mM) for 1 h and then incubated with TNF-α for 0, 16, or 24 h (Figure 5A). Pretreatment with U0126 significantly attenuated TNF-α-induced MMP-9 expression after 16 and 24 h of



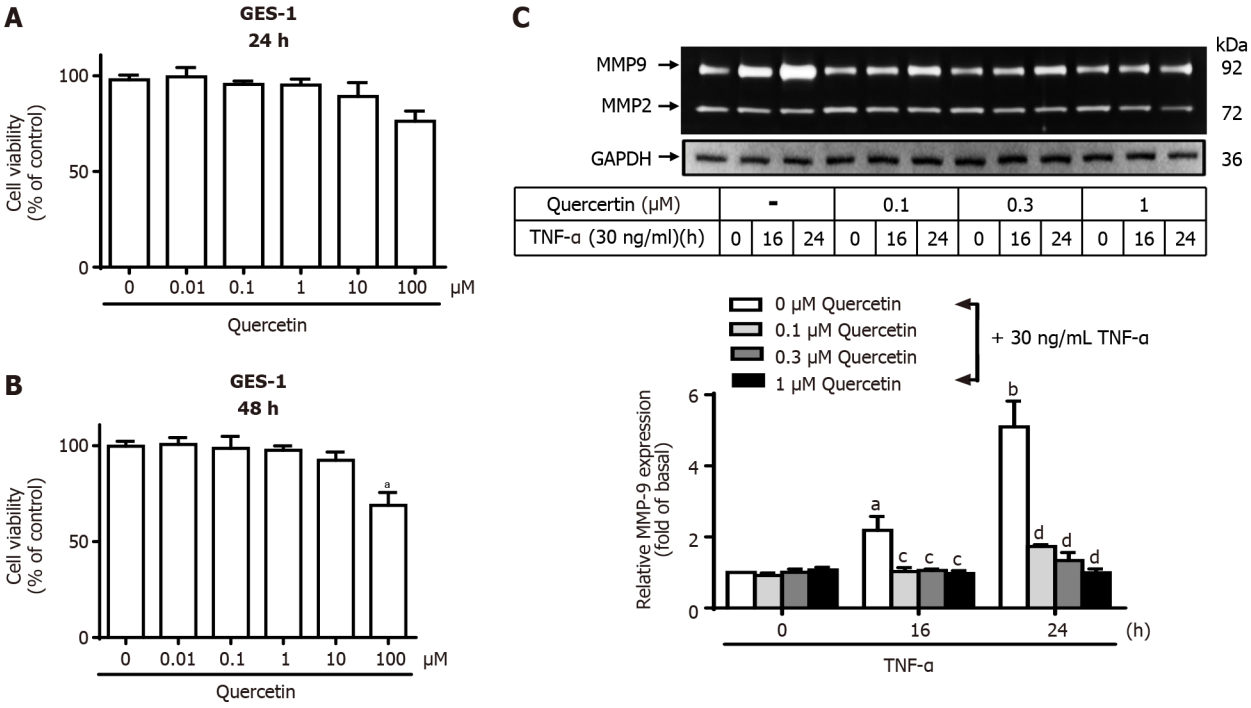
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Figure 2 Tumor necrosis factor-α induces matrix metallopeptidase-9 expression *via* tumor necrosis factor-α receptor in normal human gastric mucosa epithelial cells. A: Tumor necrosis factor-α (TNF-α) was used at concentrations of 10, 20, and 30 ng/mL to stimulate normal human gastric mucosa epithelial cells (GES-1) for 0, 16, 20, or 24 h, and matrix metallopeptidase-9 (MMP-9) enzymatic activity was measured by gelatin zymography as described in the Materials and Methods section; B: *MMP-9* transcripts were analyzed by quantitative reverse transcription-polymerase chain reaction; C: Cells were either untreated or treated with a TNF-α antagonist (TNFR inhibitor) (1 mM) 1 h before the addition of TNF-α (30 ng/mL). The TNF-α antagonist repressed TNF-α-activated MMP-9 expression in GES-1 cells. One-way ANOVA was used for comparisons among different treatment time points ($^{a}P < 0.05$, $^{b}P < 0.01$ *vs* control cells in 0 h). One-way ANOVA was used for comparisons among different treatments ($^{c}P < 0.05$, $^{d}P < 0.01$ *vs* TNF-α-stimulated cells). Data are expressed as the mean ± SEM of three independent experiments. GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; GES-1: Normal human gastric mucosa epithelial cell line; MMP: Matrix metallopeptidase; TNF-α: Tumor necrosis factor-α.

incubation in a time-dependent manner. This finding suggests that ERK1/2 is involved in this response. To further explore the effects of quercetin, U0126, PP1, or TNF-α antagonist on TNF-α-activated ERK1/2 phosphorylation, Western blot analysis was used to measure ERK1/2 phosphorylation after exposure for 0, 5, 15, 30, or 60 min. Pretreatment with quercetin, U0126, PP1, or TNF-α antagonist blocked TNF-α-activated ERK1/2 phosphorylation in a time-dependent manner (Figure 5B and C). These data suggest that TNF-α-induced MMP-9 expression may be mediated through TNFR-c-Src-ERK1/2 in GES-1 cells.

Involvement of c-Fos in TNF-a-induced MMP-9 expression in GES-1 cells

AP-1 and NF-κB play important regulatory roles in the human *MMP-9* promoter region, where they react with cytokines or growth factors[46]. To examine the role of c-Fos in TNF-α-induced MMP-9 expression, two inhibitors of TNF-α antagonist or c-Fos (TSIIA) were used. GES-1 cells were pretreated with TNF-α antagonist (1 mM) or TSIIA (0, 5, 10, or 20 mM) for 1 h and incubated with TNF-α for 0, 16, or 24 h (Figure 6A). Pretreatment with TSIIA significantly attenuated TNF-α-induced MMP-9 expression after 16 and 24 h of incubation in a time-dependent manner. This finding suggests that c-Fos was involved in these responses. To further explore the effects of quercetin, TSIIA, U0126, PP1, and TNF-α antagonist on TNF-α-activated c-Fos phosphorylation, Western blot analysis was used to measure c-Fos phosphorylation in cells incubated for various time intervals (Figure 6B and C). Pretreatment with quercetin or TSIIA, U0126, PP1, or TNF-α antagonist blocked TNF-α-activated phosphorylation of c-Fos in a time-dependent manner. An assay to analyze the activity of human AP-1–Luc response element reporter plasmids was used to explore whether the quercetin-induced reduction in AP-1 promoter reporter activity occurs through the signaling pathways mentioned above. Quercetin attenuated AP-1 promoter reporter activity (Figure 6D), and this effect may occur through the TNFR-c-Src-ERK1/2-c-Fos pathway. Taken together, these results suggest that quercetin reduces TNF-α-induced MMP-9



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Figure 3 Quercetin suppresses tumor necrosis factor-α-induced matrix metallopeptidase-9 expression in normal human gastric mucosa epithelial cells. A and B: Effects of quercetin on the viability of normal human gastric mucosa epithelial cells (GES-1). GES-1 cells were treated with different concentrations of quercetin (0, 0.01, 0.1, 1, 10, or 100 mM) for 24 h (A) or 48 h (B), and cell viability was measured using the cell counting Kit-8 assay; C: Cells were either untreated or treated with quercetin (0, 0.1, 0.3, or 1 mM) for 1 h before the addition of tumor necrosis factor-α (TNF-α) (30 ng/mL) and then incubated for 0, 16, or 24 h as described in the Materials and Methods section. Matrix metallopeptidase-9 zymogen activity was analyzed by gelatin zymography. One-way ANOVA was used for comparisons among different treatment time points ($^aP < 0.05$, $^bP < 0.01$ *vs* control cells in 0 h). One-way ANOVA was used for comparisons among different treatments ($^cP < 0.05$, $^dP < 0.01$ *vs* TNF-α-stimulated cells). Data are expressed as the mean ± SEM of three independent experiments. CCK-8: Cell Counting Kit-8; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; GES-1: Normal human gastric mucosa epithelial cell line; MMP-9: Matrix metallopeptidase-9; TNF-α: Tumor necrosis factor-α.

expression in GES-1 cells *via* the TNFR-c-Src-ERK1/2-c-Fos pathway.

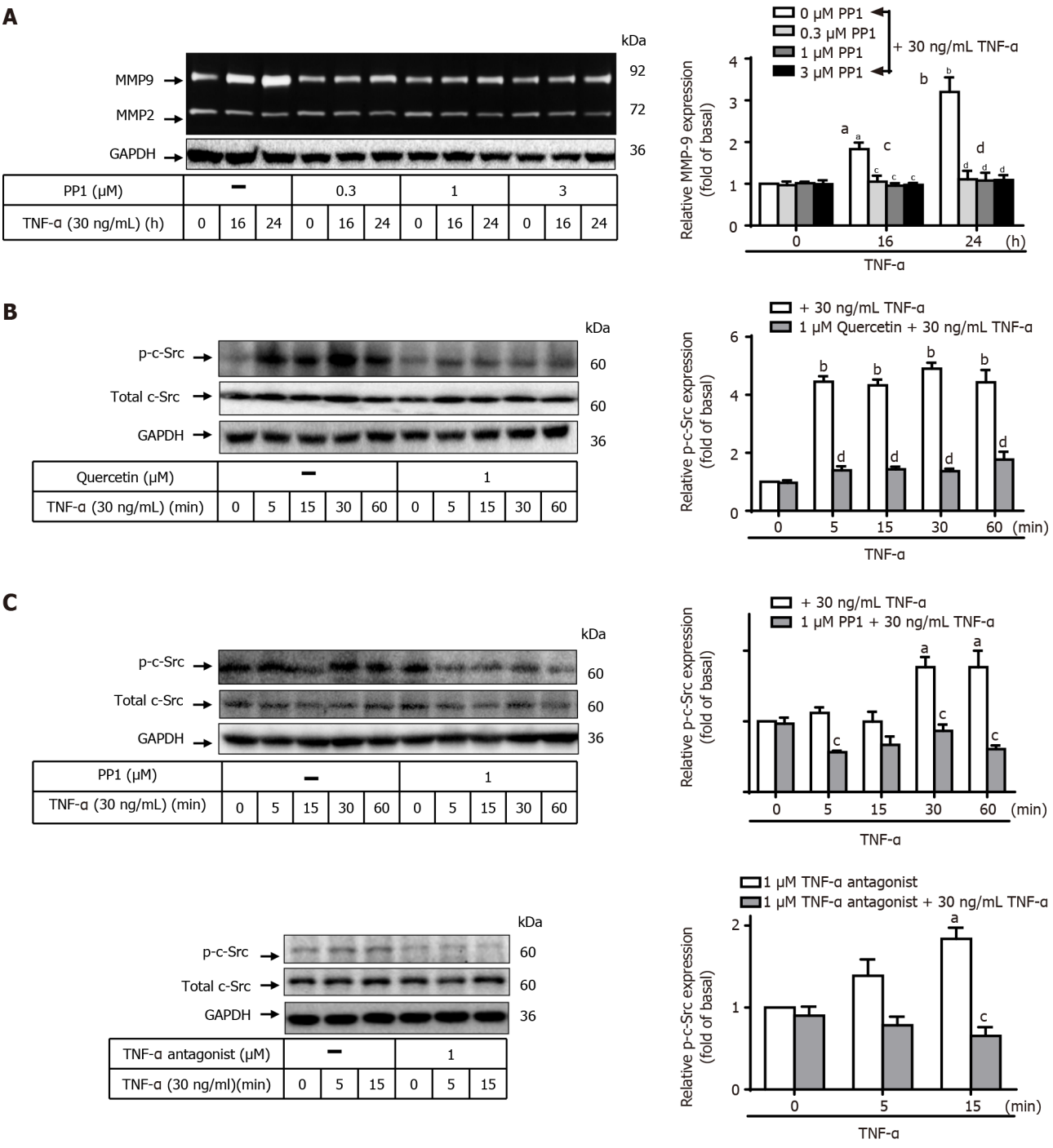
Involvement of NF-κB in TNF-a-induced MMP-9 expression in GES-1 cells

To clarify the role of NF-κB (p65) in TNF-α-induced MMP-9 expression, two inhibitors of TNF-α antagonist or NF-κB (p65) (BAY) were used. GES-1 cells were pretreated with TNF-α antagonist (1 mM) or BAY (0, 5, 10, or 20 mM) for 1 h before the addition of TNF-α and incubated for 0, 16, or 24 h (Figure 7A). Pretreatment with BAY significantly attenuated TNF-α-induced MMP-9 expression after 16 and 24 h of incubation ($P < 0.01$; $P < 0.05$) in a time- and dose-dependent manner. This finding suggests that NF-κB (p65) was involved in these responses. To further examine the effects of quercetin, BAY, U0126, PP1, or TNF-α antagonist on TNF-α-activated NF-κB (p65) phosphorylation, Western blot analysis was used to measure NF-κB (p65) phosphorylation in cells incubated for the indicated time intervals. Pretreatment with quercetin, BAY, U0126, PP1, or TNF-α antagonist blocked TNF-α-activated phosphorylation of NF-κB (p65) in a time-dependent manner (Figure 7B and C).

We used immunofluorescence to assess the effects of quercetin on NF-κB translocation from the cytoplasm into the nucleus. Cells were first stimulated with TNF-α (30 ng/mL) for 0, 5, 10, 15, 30, or 60 min. The data showed that TNF-α induced NF-κB translocation in cells in a time-dependent manner. A maximal response was achieved within 30 min and sustained over 60 min during the period of observation (Figure 7D). Quercetin, PP1, BAY, and U0126 reduced TNF-α-induced NF-κB translocation. PP1 and BAY downregulated MMP9 expression in TNF-α-induced GES-1 cells (Figure 7E). An assay to measure human NF-κB–Luc response element reporter plasmid activity was used to examine whether quercetin could reduce NF-κB promoter reporter activity through the signaling pathways mentioned above. Quercetin attenuated NF-κB promoter reporter activity, and this effect may occur through the TNFR-c-Src-ERK1/2–NF-κB pathway (Figure 7F). Taken together, these results suggest that quercetin reduced TNF-α-induced MMP-9 expression in GES-1 cells *via* the TNFR-c-Src–ERK1/2–c-Fos and NF-κB pathways.

In vitro antimetastatic activities of quercetin

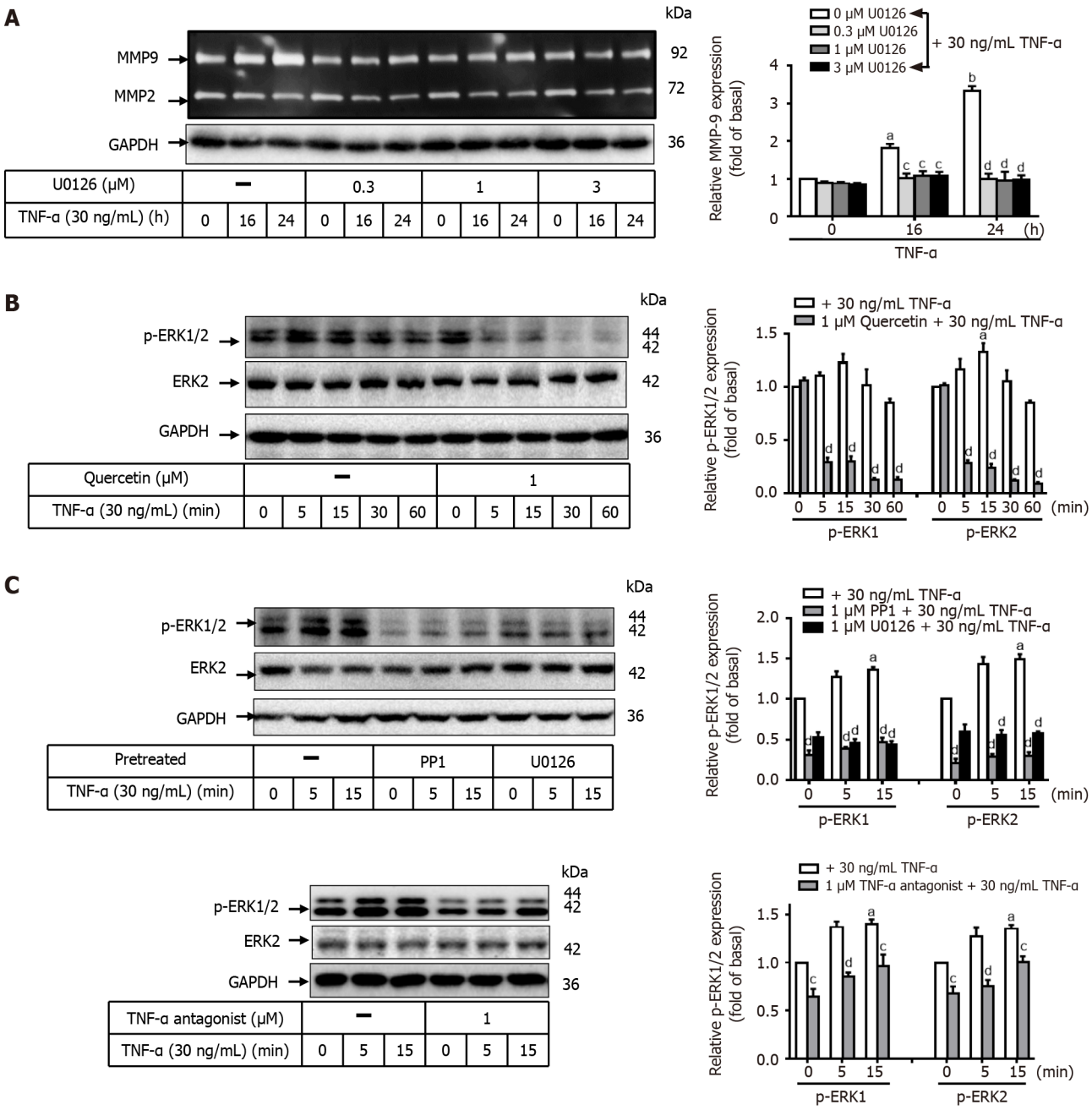
Upregulation of MMP9 is involved in the inflammatory response and cell migration[47-49]. Therefore,



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Figure 4 Quercetin suppresses tumor necrosis factor-α-induced matrix metallopeptidase-9 expression *via* tumor necrosis factor-α antagonist-proto-oncogene tyrosine-protein kinase Src in normal human gastric mucosa epithelial cells. A: Cells were either untreated or treated with PP1 (0, 0.3, 1, or 3 mM) for 1 h before the addition of tumor necrosis factor-α (TNF-α) (30 ng/mL) and then incubated for 0, 16, or 24 h as described in the Materials and Methods section. Matrix metallopeptidase-9 (MMP-9) zymogen activity was analyzed by gelatin zymography; B and C: Cells were either untreated or treated with quercetin (B; 0 or 1 mM) or PP1 (C; 0 or 1 mM) or TNF-α antagonist (1 mM) for 1 h before the addition of TNF-α (30 ng/mL) and incubated for 0, 16, or 24 h. The phosphorylation of c-Src was measured using Western blot analysis after incubation of cells with TNF-α for 0, 5, 15, 30, or 60 min. One-way ANOVA was used for comparisons among different treatment time points ($^aP < 0.05$, $^bP < 0.01$ *vs* control cells in 0 h). One-way ANOVA was used for comparisons among different treatments ($^cP < 0.05$, $^dP < 0.01$ *vs* TNF-α-stimulated cells). Data are expressed as the mean ± SEM of three independent experiments. c-Src: Proto-oncogene tyrosine-protein kinase Src; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; GES-1: Normal human gastric mucosa epithelial cell line; MMP-9: Matrix metallopeptidase-9; TNF-α: Tumor necrosis factor-α.

we further investigated the effects of quercetin on TNF-α-induced MMP9-mediated functional changes in GES-1 cells. First, images of GES-1 cell migration were observed 24 h after TNF-α treatment. Pretreatment with quercetin (1 mM or 10 mM), a TNF-α antagonist (1 mM), or MMP9i (5 mM) significantly blocked TNF-α-induced cell migration (Figure 8). This finding suggests that quercetin repressed TNF-α-induced cell migration by reducing MMP-9 expression in GES-1 cells.

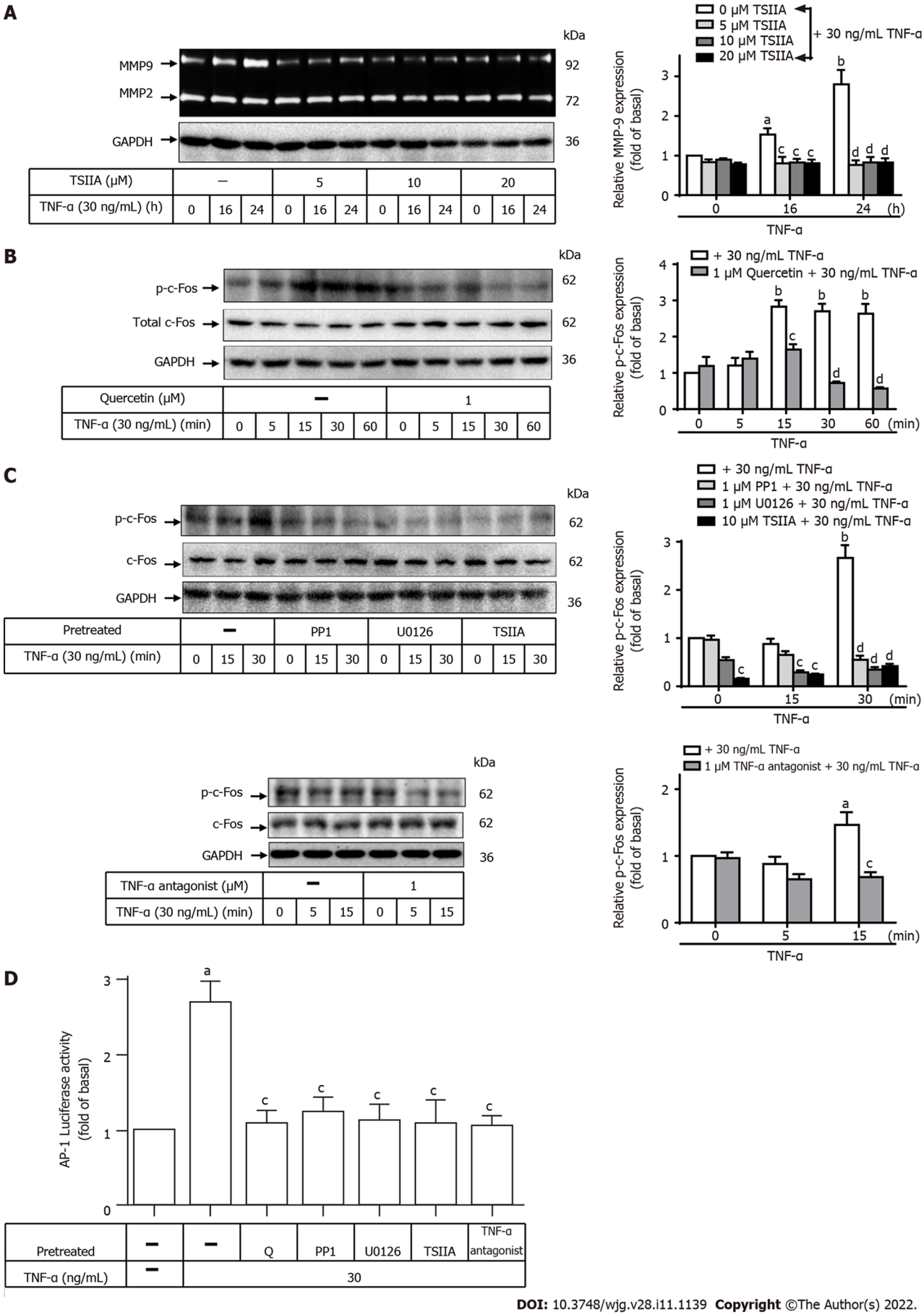


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Figure 5 Quercetin suppresses tumor necrosis factor-α-induced matrix metallopeptidase-9 expression *via* tumor necrosis factor-α antagonist-c-Src-extracellular-signal-regulated kinase 1/2 in normal human gastric mucosa epithelial cells. A: Normal human gastric mucosa epithelial cells (GES-1) were either untreated or treated with U0126 (0, 0.3, 1, or 3 mM) for 1 h before the addition of tumor necrosis factor-α (TNF-α) (30 ng/mL) and incubated for 0, 16, or 24 h as described in the Materials and Methods section. Matrix metallopeptidase-9 zymogen activity was measured using gelatin zymography; B and C: Cells were either untreated or treated with quercetin (B; 0 or 1 mM), PP1 (C; 0 or 1 mM), U0126 (0 or 1 mM), or TNF-α antagonist (1 mM) for 1 h before the addition of TNF-α (30 ng/mL) and incubated for 0, 16, or 24 h. The phosphorylation of extracellular-signal-regulated kinase 1/2 was examined by Western blot analysis in cells incubated for 0, 5, 15, 30, or 60 min. One-way ANOVA was used for comparisons among different treatment time points ($^{a}P < 0.05$, $^{b}P < 0.01$ *vs* control cells in 0 h). One-way ANOVA was used for comparisons among different treatments ($^{c}P < 0.05$, $^{d}P < 0.01$ *vs* TNF-α-stimulated cells). Data are expressed as the mean ± SEM of three independent experiments. ERK: Extracellular-signal-regulated kinase; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; GC: Gastric cancer; GES-1: Normal human gastric mucosa epithelial cell line; MMP-9: Matrix metallopeptidase-9; TNF-α: Tumor necrosis factor-α.

DISCUSSION

Quercetin belongs to a member of the polyphenolic flavonoid family, which has many favorable effects on human health, such as antioxidant, anti-inflammatory, anticancer, antiallergic, antiviral, free radical-scavenging, and neuroprotective activities[33,34]. Quercetin was used in this study due to its anti-inflammatory and gastroprotective effects.



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Figure 6 Quercetin suppresses tumor necrosis factor-α-induced matrix metallopeptidase-9 expression *via* tumor necrosis factor-α

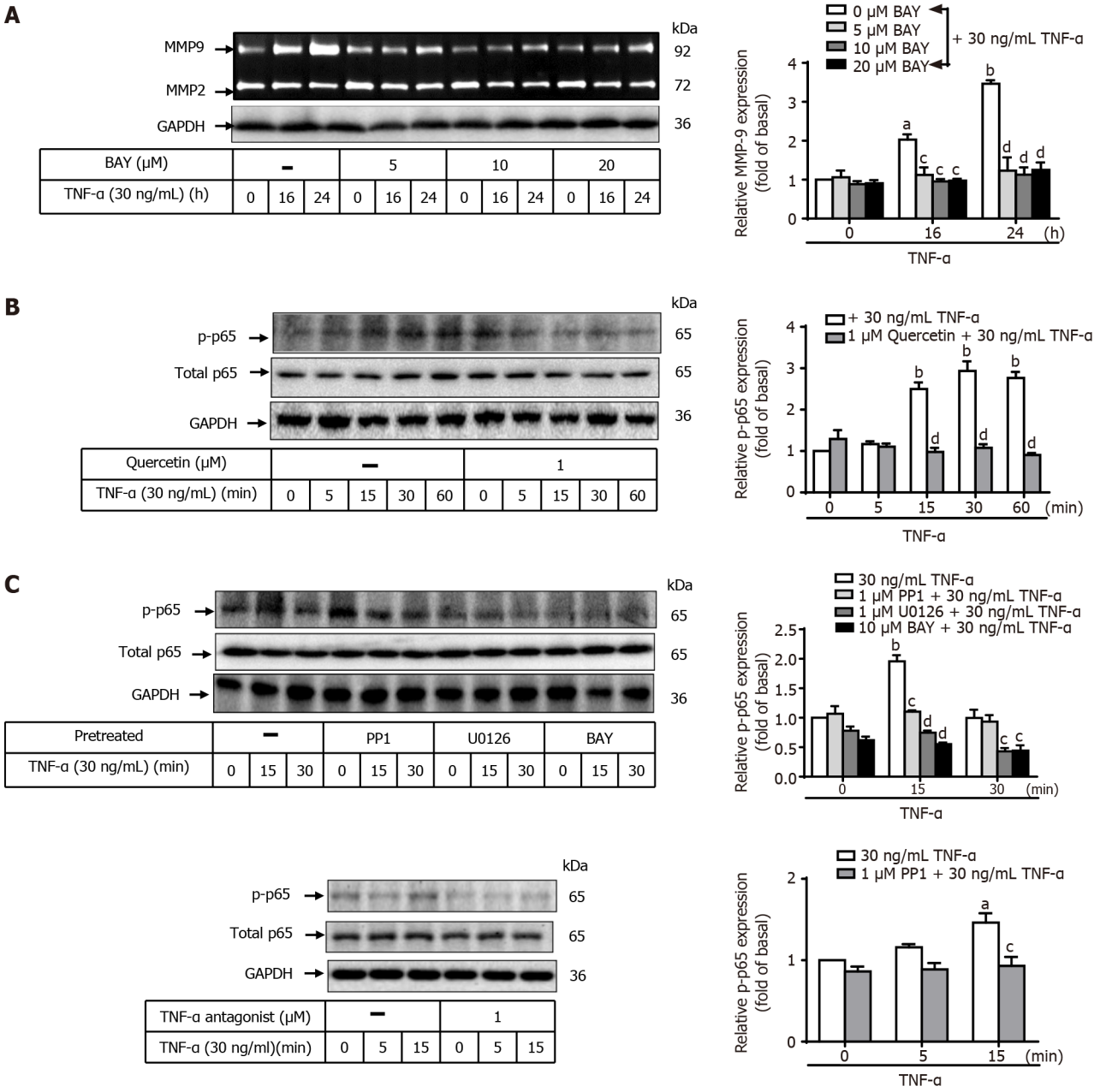
antagonist-c-Src-extracellular-signal-regulated kinase 1/2–c-Fos in normal human gastric mucosa epithelial cells. A: Normal human gastric mucosa epithelial cells (GES-1) were either untreated or treated with Tanshinone IIA (TSIIA) (0, 5, 10, or 20 mM) 1 h before the addition of tumor necrosis factor-α (TNF-α) (30 ng/mL) and incubated for 0, 16, or 24 h as described in the Materials and Methods section. Matrix metallopeptidase-9 zymogen activity was measured using gelatin zymography; B and C: Cells were either untreated or treated with quercetin (B; 0 or 1 mM), PP1 (C; 0 or 1 mM), U0126 (0 or 1 mM), TSIIA (0 or 10 mM), or TNF-α antagonist (1 mM) for 1 h before the addition of TNF-α (30 ng/mL) and incubated for 0, 16, or 24 h. The phosphorylation of c-Fos was measured by Western blot analysis in cells incubated for 0, 5, 15, 30, or 60 min; D: GES-1 cells were transfected with human AP-1–Luc response element reporter plasmids containing the dual-luciferase reporter system. The cells were then pretreated with PP1 (0 or 1 mM), U0126 (0 or 1 mM), TSIIA (0 or 10 mM), TNF-α antagonist (1 mM), or quercetin (1 mM) for 1 h and exposed to TNF-α for 1 h, and luciferase activity was measured. Firefly luciferase activity was standardized to *Renilla* luciferase activity. One-way ANOVA was used for comparisons among different treatment time points ($^{a}P < 0.05$, $^{b}P < 0.01$ *vs* control cells in 0 h). One-way ANOVA was used for comparisons among different treatments ($^{c}P < 0.05$, $^{d}P < 0.01$ *vs* TNF-α-stimulated cells). Data are expressed as the mean ± SEM of three independent experiments. ERK: Extracellular-signal-regulated kinase; AP-1: Activator protein-1; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; GES-1: Normal human gastric mucosa epithelial cell line; Luc: Luciferase; GES-1: Normal human gastric mucosa epithelial cell line; MMP-9: Matrix metallopeptidase-9; Q: quercetin; TNF-α: Tumor necrosis factor-α; TSIIA: Tanshinone IIA.

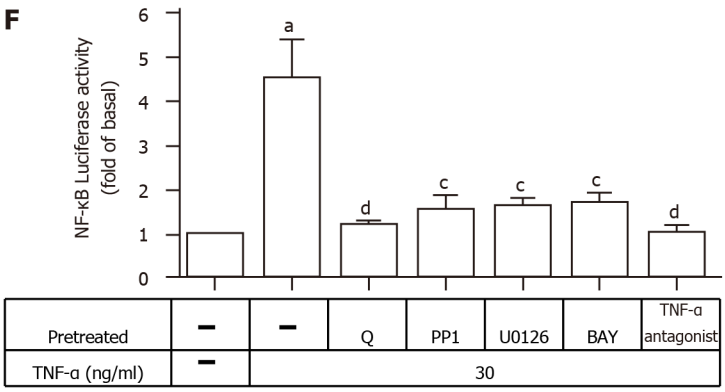
Gastric inflammation is essential to the development of gastric epithelial injury and gastric diseases. Hu *et al*[10] reported that quercetin can protect GES-1 cells from hydrogen peroxide-induced production of reactive oxygen species in a mouse model of acute gastric mucosal injury. Quercetin is thought to be protective against oxidative stress, mitochondrial dysfunction, and apoptosis and to be involved in the initiation of an antioxidant defense[10]. Quercetin has also been observed to modulate the inflammatory response of gastric mucosal epithelial cells against injury caused by ischemia–reperfusion[35], ethanol [36,38], indomethacin[9], and *H. pylori*[37] in animal models. In other cells, quercetin has been reported to inhibit the lipopolysaccharide-induced MMP-9 expression in lung inflammation and the production of proinflammatory cytokines[50]. Ying *et al*[51] reported that quercetin inhibits IL-1b-induced ICAM-1 expression mediated by the MAPK pathway in lung inflammation[51,52], retinal pigment epithelial cells [53], and human endothelial cells[54] *in vivo* and *in vitro*.

The mechanism through which quercetin exerts gastroprotective activity against TNF-α-induced injury to gastric mucosal epithelial cells is unknown. Gastric inflammation and injury induced by proinflammatory cytokines, such as TNF-α produced by activated immune cells, depend on NF-κB and MMP-9 activation through the NF-κB pathway[29,30]. Here, we explored whether quercetin protects against TNF-α-induced MMP-9 damage in GES-1 cells. We challenged these cells by inducing an immune–inflammatory reaction mediated by TNF-α and found that quercetin significantly reduced MMP-9 expression in GES-1 cells. Our findings suggest that quercetin helps to maintain the gastric mucosa and has potential as a treatment for preventing the progression of inflammation.

Investigators have provided evidence that c-Src[55], the MAPK family[56-58], and NF-κB[57,59] participate in pathophysiological processes involved in gastric inflammation and GC. Three major groups of MAPKs have been identified, including ERK1/2, p38, and JNK1/2, which play critical roles in cell proliferation, differentiation, and inflammation[60]. TNF-α-induced MMP-9 expression is regulated through the MAPK pathway in human trophoblastic cells[61], human urinary bladder cancer HT1376 cells[62], lung cancer cells[63], osteoblasts[64], and skeletal muscle cells[65]. This evidence suggests that MMP-9 expression induced by inflammatory mediators, such as TNF-α, is associated with MAPKs in diverse cell types. We observed similar results in our studies. We observed the *in vitro* antimetastatic and anti-inflammatory activities of quercetin in GES-1 cells. Quercetin protected against TNF-α-induced MMP-9 damage in GES-1 cells challenged by induction of the immune–inflammatory reaction mediated by TNF-α. PP1 (c-Src inhibitor), U0126 (ERK1/2 inhibitor), TSAIIA (c-Fos inhibitor), BAY (NF-κB inhibitor), and TNF-α antagonist (TNFR inhibitor) were used to investigate the interactions of c-Src, ERK1/2, c-Fos, NF-κB, and TNFR in the TNF-α-induced response. Using immunofluorescence, we found that TNF-α induced NF-κB (p65) phosphorylation and translocation and that NF-κB/p65–Luc and AP-1–Luc activities were mediated *via* the TNFR-c-Src–ERK1/2–c-Fos and NF-κB pathways. We also found that quercetin reduced TNF-α-induced MMP-9 expression in GES-1 cells *via* the proinflammatory c-Src–ERK1/2–c-Fos and NF-κB pathways. Moreover, quercetin may indeed directly inhibit upstream molecules (such as TNFR or c-Src) and subsequently cause the inhibition of downstream molecules, such as ERK1/2, c-Fos, and NF-κB.

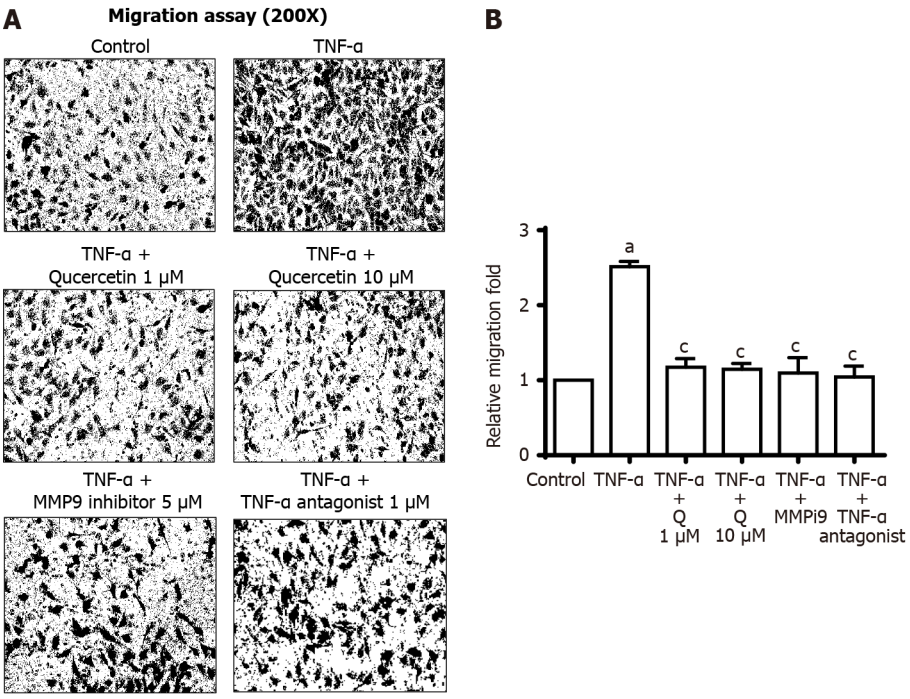
NF-κB plays an important role in the regulation of inflammation. Stimulation of proinflammatory cytokines, such as TNF-α, increases NF-κB activity and involves the phosphorylation and degradation of IκBa, which induces the translocation of active NF-κB to the nucleus and the expression of genes encoding proinflammatory factors, such as cytokines, chemokines, and adhesion molecules[66-69]. In our study, pretreatment with BAY attenuated TNF-α-induced MMP-9 expression in GES-1 cells. MMP-9 is a potential target for new therapies and is being investigated by several research groups[70-72]. A better understanding of the roles of inflammatory mediators will help in the development of new therapeutic approaches for treating gastric inflammatory diseases. Reducing MMP-9 production may help to limit gastric damage in the treatment of gastric diseases. Given the increasing use of NSAIDs worldwide, it is important to identify other agents to control inflammation. Quercetin and a quercetin-rich diet may exhibit potential as food supplements for the prevention of early pathological changes associated with gastric inflammation.


A
MMP9
MMP2
GAPDH
kDa
92
72
36
BAY (μM) | - | 5 | 10 | 20
TNF-α (30 ng/mL) (h) | 0 16 24 | 0 16 24 | 0 16 24 | 0 16 24
0 μM BAY
5 μM BAY
10 μM BAY
20 μM BAY
+ 30 ng/mL TNF-α
Relative MMP-9 expression (fold of basal)
TNF-α
B
p-p65
Total p65
GAPDH
kDa
65
65
36
Quercetin (μM) | - | 1
TNF-α (30 ng/mL) (min) | 0 5 15 30 60 | 0 5 15 30 60
+ 30 ng/mL TNF-α
1 μM Quercetin + 30 ng/mL TNF-α
Relative p-p65 expression (fold of basal)
(min)
TNF-α
C
p-p65
Total p65
GAPDH
kDa
65
65
36
Pretreated | - | PP1 | U0126 | BAY
TNF-α (30 ng/mL) (min) | 0 15 30 | 0 15 30 | 0 15 30 | 0 15 30
30 ng/mL TNF-α
1 μM PP1 + 30 ng/mL TNF-α
1 μM U0126 + 30 ng/mL TNF-α
10 μM BAY + 30 ng/mL TNF-α
Relative p-p65 expression (fold of basal)
(min)
TNF-α
p-p65
Total p65
GAPDH
kDa
65
65
36
TNF-α antagonist (μM) | - | 1
TNF-α (30 ng/ml)(min) | 0 5 15 | 0 5 15
30 ng/mL TNF-α
1 μM PP1 + 30 ng/mL TNF-α
Relative p-p65 expression (fold of basal)
(min)
TNF-α
D
NF-kB p65
Control
TNF-α 5 min
TNF-α 10 min
TNF-α 15 min
TNF-α 30 min
TNF-α 60 min
E
NF-kB p65
Control
TNF-α 30 min
TNF-α+Q 30 min
TNF-α+BAY 30 min
TNF-α+PP1 30 min
TNF-α+U0126 30 min



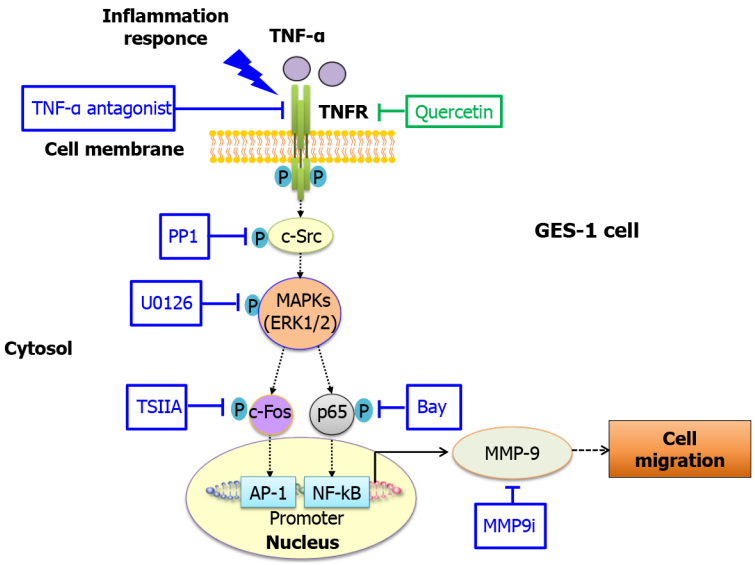
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Figure 7 Quercetin suppresses tumor necrosis factor-α-induced tumor necrosis factor-α antagonist-c-Src–extracellular-signal-regulated kinase 1/2–nuclear factor kappa B (p65) activation in normal human gastric mucosa epithelial cells. A: Normal human gastric mucosa epithelial cells (GES-1) were either untreated or treated with BAY (0, 5, 10, or 20 mM) for 0, 16, or 24 h; compounds were added 1 h before the addition of tumor necrosis factor-α (TNF-α) (30 ng/mL) as described in the Materials and Methods section. Matrix metallopeptidase-9 zymogen activity was measured by gelatin zymography. B and C: Cells were either untreated or treated with quercetin (B; 0 or 1 mM), PP1 (C; 0 or 1 mM), U0126 (0 or 1 mM), BAY (0 or 10 mM), or TNF-α antagonist (1 mM) for 1 h before the addition of TNF-α (30 ng/mL) and incubated for 0, 16, or 24 h. Nuclear factor kappa B (NF-κB) phosphorylation was examined by Western blot analysis; D and E: GES-1 cells were treated with 30 ng/mL TNF-α for 0, 5, 10, 15, 30, or 60 min (D) or quercetin (1 mM), PP1 (1 mM), U0126 (1 mM), or BAY (10 mM) for 1 h (E) before exposure to TNF-α (30 ng/mL) for 30 min; F: GES-1 cells were transfected with the human NF-κB–Luc response element reporter plasmids containing the dual-luciferase reporter system. The cells were then pretreated with PP1 (0 or 1 mM), U0126 (0 or 1 mM), BAY (0 or 10 mM), quercetin (1 mM), or TNF-α antagonist (1 mM) for 1 h and exposed to TNF-α for 1 h. Then, luciferase activity was measured. Firefly luciferase activity was standardized to that of *Renilla* luciferase. One-way ANOVA was used for comparisons among different treatment time points ($^{a}P < 0.05$, $^{b}P < 0.01$ *vs* control cells in 0 h). One-way ANOVA was used for comparisons among different treatments simultaneously ($^{c}P < 0.05$, $^{d}P < 0.01$ *vs* TNF-α-stimulated cells). Data are expressed as the mean ± SEM of three independent experiments. ERK: Extracellular-signal-regulated kinase; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; GES-1: Normal human gastric mucosa epithelial cell line; MMP-9: Matrix metallopeptidase-9; NF-κB: Nuclear factor kappa B; Q: Quercetin; TNF-α: Tumor necrosis factor-α.



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Figure 8 Quercetin exhibit antimetastatic activities *in vitro*. A: Cells were plated in 24-well culture plates, grown to confluence, and starved with serum-free medium for 24 h. Cells were pretreated with a tumor necrosis factor-α (TNF-α) antagonist (1 mM), matrix metallopeptidase-9 inhibitor (5 mM), or quercetin (1 or 10 mM) for 1 h, and monolayer cells were incubated with TNF-α (30 ng/mL) for 24 h as described in the Materials and Methods section; B: One-way ANOVA was used for comparisons between the two groups ($^{a}P < 0.05$, $^{b}P < 0.01$ *vs* control cells). One-way ANOVA was used for comparisons among different treatments ($^{c}P < 0.05$, $^{d}P < 0.01$ *vs* TNF-α-stimulated cells). Data are expressed as the mean ± SEM of three independent experiments. GES-1: Normal human gastric mucosa epithelial cell line; MMP-9: Matrix metallopeptidase-9; MMP9i: MMP-9 inhibitor; TNF-α: Tumor necrosis factor-α.



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Figure 9 Schematic representation of the effects of quercetin on tumor necrosis factor-α-induced matrix metallopeptidase-9 expression and cell migration in normal human gastric mucosa epithelial cells. Schematic diagram of the signaling pathways for quercetin-mediated attenuation of tumor necrosis factor-α (TNF-α)-induced inflammation *via* downregulation of matrix metallopeptidase-9 (MMP-9) expression in normal human gastric mucosa epithelial cells. Quercetin attenuates TNF-α-induced MMP-9 expression in normal human gastric mucosa epithelial cells through the proinflammatory TNFR-c-Src–extracellular-signal-regulated kinase 1/2–c-Fos and nuclear factor kappa B pathways. AP-1: Activator protein-1; c-Src: Proto-oncogene tyrosine-protein kinase Src; ERK: Extracellular-signal-regulated kinase; GES-1: Normal human gastric mucosa epithelial cell line; MAPK: Mitogen-activated protein kinase; MMP-9: Matrix metallopeptidase-9; MMP9i: MMP-9 inhibitor; NF-κB: Nuclear factor kappa B; TNF-α: Tumor necrosis factor-α; TSIIA: Tanshinone IIA.

We evaluated the effects of different concentrations of quercetin on the normal GES-1 cell line using the cell viability assay (Figure 3A and B). We found that cells treated with quercetin at concentrations ≤ 10 mM for 24 to 48 h had almost no effect on GES-1 cell viability. Additionally, many studies have confirmed that the safety and efficacy of quercetin remain inconclusive[73]. In 2010, the American Food and Drug Administration considered that the utilization of high-purity quercetin as a food ingredient in different food products is GRAS ("Generally Recognized As Safe") (https://www.cfsanappsexternal.fda.gov/scripts/fdcc/index.cfm?set=GRASNotices&sort=GRN_No&order=DESC&startrow=1&type=basic&search=341) (GRN No. 341). Based on several published human intervention studies, daily doses of oral quercetin are usually in the range of up to 1000 mg/d (they are the safest at 500 mg/d). Therefore, the daily supplement doses of oral quercetin were noticeably higher than the background daily doses of oral quercetin. Side effects subsequent to supplemental quercetin intake have rarely been described naturally. Moreover, supplemental isolated quercetin at doses clearly exceeding dietary quercetin intake will not aggravate gastric injuries, ulcers, or GC[74-76]. However, based on several published *in vivo* data, quercetin promotes insulin secretion, and insulin can increase tumor growth[77]. Oral quercetin products have a few potential crucial safety behaviors[78], and potential opposite effects in diabetes. In patients who have developed nephropathy or estrogen-dependent cancer, the use of quercetin should be tracked and researched in the future[79-82].

CONCLUSION

We investigated the *in vitro* effects of quercetin on antimetastatic and anti-inflammatory activities in GES-1 cells. Quercetin protected against TNF-α-induced damage caused by MMP-9 in human gastric mucosal epithelial cells (GES-1) challenged by TNF-α-mediated immune–inflammatory reactions. PP1 (c-Src inhibitor), U0126 (ERK1/2 inhibitor), TSAIIA (c-Fos inhibitor), BAY (NF-κB inhibitor), and TNF-α antagonist (TNF-α inhibitor) were used to investigate the roles of c-Src, ERK1/2, c-Fos, and NF-κB in the TNF-α-induced response. Quercetin reduces TNF-α-induced MMP-9 expression in GES-1 cells *via* the proinflammatory TNFR-c-Src–ERK1/2–c-Fos and NF-κB pathways. We propose that quercetin exhibits potential as a novel therapeutic drug for protecting gastric mucosal epithelial cells from damage caused by disease (Figure 9). Quercetin and a quercetin-rich diet may be useful as food supplements for the prevention of early pathological changes associated with gastric inflammation.

ARTICLE HIGHLIGHTS

Research background

Gastric injury involving inflammation is one of the most common diseases of the digestive system worldwide and is associated with gastric ulcers and gastric cancer (GC). Matrix metallopeptidase-9 (MMP-9) plays an important role in the inflammation and progression of GC. Quercetin exhibits anti-inflammatory activities, but its effects and mechanism of action on human chronic gastritis remain unclear.

Research motivation

To assess whether tumor necrosis factor-α (TNF-α)-induced MMP-9 expression is involved in the anti-inflammatory effects of quercetin in normal human gastric mucosa epithelial cell line GES-1.

Research objectives

The objective of this study was to evaluate the anti- inflammatory effects and mechanisms of quercetin in GES-1 cells.

Research methods

A GES-1 cell model was established to evaluate the anti-inflammatory effects of quercetin on TNF-α-induced overexpression of the proinflammatory MMP-9 protein. The cell counting Kit-8 assay was used to examine the effects of quercetin dose on GES-1 cell viability. Cell migration was measured using the Transwell assay. The expression of c-Src, phospho (p)-c-Src, p- extracellular-signal-regulated kinase (ERK) 1/2, ERK2, p-c-Fos, c-Fos, p-p65, and nuclear factor kappa B (NF-κB)/p65 and the effects of their inhibitors were examined using Western blot analysis and the measurement of luciferase activity. p65 expression was detected by immunofluorescence. MMP-9 mRNA and protein expression levels were determined by quantitative reverse transcription polymerase chain reaction (qRT–PCR) and gelatin zymography, respectively.

Research results

qRT–PCR and gelatin zymography showed that TNF-α induced MMP-9 mRNA and protein expression in a dose- and time-dependent manner. These effects were reduced by pretreatment of GES-1 cells with quercetin or TNF-α antagonist in a dose- and time-dependent manner. Quercetin decreased the TNF-α-induced phosphorylation of c-Src, ERK1/2, c-Fos, and p65 in a dose- and time-dependent manner. Quercetin, PP1, U0126, TSIIA, and a TNF-α antagonist reduced TNF-α-induced c-Fos phosphorylation and AP-1–Luc activity in a dose- and time-dependent manner. Pretreatment with quercetin, PP1, U0126, Bay 11-7082, or TNF-α antagonist reduced TNF-α-induced p65 phosphorylation and translocation and p65–Luc activity in a dose- and time-dependent manner. TNF-α significantly increased GES-1 cell migration, and this effect was reduced by pretreatment with quercetin and a TNF-α antagonist.

Research conclusions

Quercetin significantly downregulates TNF-α-induced MMP-9 expression in GES-1 cells *via* the TNFR-c-Src–ERK1/2–c-Fos and NF-κB pathways.

Research perspectives

We propose that quercetin potentially represents a new approach for reducing reliance on nonsteroidal anti-inflammatory drugs and an effective therapeutic agent for protecting gastric mucosal epithelial cells. Quercetin and quercetin-rich diets may exhibit potential as food supplements for the prevention of early pathological changes associated with gastric inflammation.

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FOOTNOTES

Author contributions: Hsieh HL, Yu MC, and Cheng LC contributed equally to this work; all authors contributed to this paper with conception and design of the study, literature review and analysis, manuscript drafting, critical revision, and editing, and approval of the final version.

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Institutional review board statement: No human specimens were involved in this study.

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

Data sharing statement: No additional data are available.

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Country/Territory of origin: Taiwan

ORCID number: Hsi-Lung Hsieh 0000-0001-8302-2472; Ming-Chin Yu 0000-0002-6980-7123; Li-Ching Cheng 0000-0003-3214-7798; Mei-Yi Chu 0000-0003-1264-7824; Tzu-Hao Huang 0000-0002-4960-6789; Ta-Sen Yeh 0000-0002-1830-9466; Ming-Ming Tsai 0000-0002-4495-6616.

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ORIGINAL ARTICLE

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Assessment of pathogens and risk factors associated with bloodstream infection in the year after pediatric liver transplantation

Yeong Eun Kim, Ho Jung Choi, Hye-Jin Lee, Hyun Ju Oh, Mi Kyoung Ahn, Seak Hee Oh, Jung-Man Namgoong, Dae Yeon Kim, Won Kyoung Jhang, Seong Jong Park, Dong-Hwan Jung, Deok Bog Moon, Gi-Won Song, Gil-Chun Park, Tae-Yong Ha, Chul-Soo Ahn, Ki-Hun Kim, Shin Hwang, Sung Gyu Lee, Kyung Mo Kim

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Yeong Eun Kim, Ho Jung Choi, Hye-Jin Lee, Hyun Ju Oh, Mi Kyoung Ahn, Seak Hee Oh, Won Kyoung Jhang, Seong Jong Park, Kyung Mo Kim, Department of Pediatrics, Asan Medical Center Children's Hospital, University of Ulsan College of Medicine, Seoul 05505, South Korea

Jung-Man Namgoong, Dae Yeon Kim, Division of Pediatric Surgery, Department of Surgery, Asan Medical Center Children's Hospital, University of Ulsan College of Medicine, Seoul 05505, South Korea

Dong-Hwan Jung, Deok Bog Moon, Gi-Won Song, Gil-Chun Park, Tae-Yong Ha, Chul-Soo Ahn, Ki-Hun Kim, Shin Hwang, Sung Gyu Lee, Division of Hepatobiliary Surgery and Liver Transplantation, Department of Surgery, Asan Medical Center, University of Ulsan College of Medicine, Seoul 05505, South Korea

Corresponding author: Seak Hee Oh, MD, PhD, Associate Professor, Department of Pediatrics, Asan Medical Center Children's Hospital, University of Ulsan College of Medicine, 88, Olympic-ro 43-gil, Songpa-Gu, Seoul 05505, South Korea. seakhee.oh@amc.seoul.kr

Abstract

BACKGROUND

Bloodstream infection (BSI) is one of the most significantly adverse events that can occur after liver transplantation (LT) in children.

AIM

To analyze the profile of BSI according to the postoperative periods and assess the risk factors after pediatric LT.

METHODS

Clinical data, collected from medical charts of children (n = 378) who underwent primary LT, were retrospectively reviewed. The primary outcome considered was BSI in the first year after LT. Univariate and multivariate analyses were performed to identify risk factors for BSI and respective odds ratios (ORs).

RESULTS

Of the examined patients, 106 (28%) experienced 162 episodes of pathogen-confirmed BSI during the first year after LT. There were 1.53 ± 0.95 episodes *per* children (mean ± SD) among BSI-complicated patients with a median onset of 0.4 mo post-LT. The most common pathogenic organisms identified were *Coagulase-negative staphylococci*, followed by *Enterococcus spp.* and *Streptococcus spp.* About half (53%) of the BSIs were of unknown origin. Multivariate analysis demonstrated that young age (≤ 1.3 year; OR = 2.1, P = 0.011), growth failure (OR = 2.1, P = 0.045), liver support system (OR = 4.2, P = 0.008), and hospital stay of > 44 d (OR = 2.3, P = 0.002) were independently associated with BSI in the year after LT.

CONCLUSION

BSI was frequently observed in patients after pediatric LT, affecting survival outcomes. The profile of BSI may inform clinical treatment and management in high-risk children after LT.

Key Words: Bloodstream infection; Liver transplantation; Children; Pathogens

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Core Tip: Although bloodstream infection (BSI) is the most significant risk event after liver transplantation (LT) in children, few studies have indicated associated clinical profile and risks. In this study, BSI was frequently observed in the year after pediatric LT and common pathogens were analyzed. Young age, growth failure, use of liver support system, and long hospital stay were independent risk factors of BSI.

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INTRODUCTION

Pediatric liver transplantation (LT) is a successful state-of-the-art treatment option for children with end-stage liver disease[1,2]. Due to advances in both surgical and medical management, the survival rate after pediatric LT has increased in high-volume centers over time[3,4]. However, transplant patients are still exposed to risks of mortality and morbidity due to preexisting health conditions, the complex surgical procedures, and intense immunosuppression[5,6]. For example, post-LT bloodstream infection (BSI) is a major cause of death in both adult and pediatric LT patients[7-10]. BSI is infection present in the bloodstream, which is normally a sterile environment[8,11]. BSI is diagnosed when bacteria or fungi are detected in blood cultures, and sepsis is the common inflammatory immune response. Considering the perioperative complexity of LT, children may be exposed to the risk of BSI as a severe complication of localized infection in the abdomen, contamination during surgery, or from catheters or invasive procedures[7-10].

Approximately 20%-40% patients experience BSI after LT[8,12,13]. Risk factors of post-LT BSI in adult patients include age and transplant urgency, surgical options, graft types, and postoperative complications[6,8,13-15]. However, risk analysis of post-LT BSI is poorly studied in children[6,8,13,15]. Young age, operative blood loss, type of procedure, such as Roux-en Y method, biliary complications, and cytomegalovirus infection have been found to be risk factors for BSI. Unlike in adult studies, pediatric LT studies are conducted in highly selected cohorts. Notably, half of the indications for pediatric LT are biliary atresia[1,16], for which the Kasai procedure is conducted during the neonatal period. Hence, it is valuable to understand the unique characteristics of BSI in children after LT and to identify risk factors and preventative measures.

Recently, survival outcomes of pediatric LT have been gradually improving at experienced transplant centers[17-20]. Improved understanding of risks could lead to subtle but significant refinements in medical care for pediatric LT patients. In this context, we hypothesized that the characteristics and outcomes of BSI after LT have changed over time, as BSI was previously a highly recurrent problem in our pediatric LT program[13], likely due to catheter-related infection and biliary complications. In this study, we analyzed the profile of BSI according to the post-LT periods and retrospectively re-assessed the risk factors of BSI within the first year after LT in our center.

MATERIALS AND METHODS

Study subjects

Cases of pediatric LT at Asan Medical Center, Seoul, Republic of Korea from December 1994 to June 2020 were retrospectively reviewed. The pediatric group included patients ≤ 18 years of age on the day of transplant, as *per* the definition from the World Health Organization. We also included pediatric patients aged 17 years who were managed by the adult transplant program. A total of 378 cases were reviewed, including 287 (76%) living donor LTs and 91 (24%) deceased donor LTs. Among them, 27 patients required a second transplant. Medical records of the patients were reviewed for the period up to 36 mo after the transplant, or until death.

Definition of variables

Clinical data collection was conducted by retrospective review of medical charts. The primary outcome considered was BSI in the first year after transplant. BSI was defined based on conventional criteria from the Centers for Disease Control guidelines[11] with minor modifications. The clinical significance of BSI was classified according to the corresponding clinical and laboratory findings; skin contaminants, such as *coagulase-negative staphylococci*, *Micrococcus*, and *Propionibacterium* that were cautiously excluded when no signs of clinical sepsis were noted. Catheter-related BSI was reclassified based on the guidelines of the Infectious Diseases Society of America[21] with minor modifications. When no pathogen was found in the peripheral blood culture, catheter-related BSI was defined only if signs of clinical sepsis were present. Positive blood cultures on serial tests without negative conversion were considered the same infection. If the blood culture test returned negative and then positive again within a few days, it was considered a new infection. Secondary BSI was defined when a blood culture showed the same pathogen as a culture in a location other than the blood, such as abdominal drainage, sputum, or urine.

Perioperative variables including age, sex, pre-transplant anthropometry [weight, height, and body mass index (BMI) z-score; based on age-specific data of the World Health Organization[22]], etiology, Pediatric End-stage Liver Disease (PELD), or Model for End-stage Liver Disease (MELD) scores[23] at the time of transplant, donor type, donor age, sex, blood group and type, and BMI were collected. Clinical data including the following variables were also collected: Transplant number, graft type, post-operative surgical complications, operation time, graft weight, volume of red blood cell (RBC) transfusions during the operation, induction and maintenance immunosuppression, and the use of a ventilator, renal replacement therapy, or liver support system such as plasmapheresis. After LT, microbiology, laboratory tests, length of hospital stays, rejection in the first year after transplant, reoperation, cytomegalovirus infection, Epstein-Barr virus infection, post-transplant lymphoproliferative disorder, and recipient and graft survival information was collected. Growth failure was defined when the z-score of weight or height was less than -2.

Antimicrobial prophylaxis

The standard perioperative prophylaxis consisted of ampicillin plus sulbactam (150 mg/kg *per* day) and cefotaxime (100 mg/kg *per* day) administered intravenously within three hours before the operation, and it continued for about seven days or until there were no signs of clinical infection. For acute liver failure, cefotaxime (100 mg/kg *per* day) plus acyclovir (30 mg/kg *per* day) was administered at the time of diagnosis and then switched to the regular regimen after LT. When any signs of clinical sepsis or intraabdominal infections were noted, the antibiotics were promptly switched to vancomycin (50 mg/kg *per* day) plus meropenem (60 mg/kg *per* day) regardless of the documentation of pathogens. Vancomycin was selected based on the center's own experience with methicillin-resistant pathogens [13], while meropenem, assuming a severe intraabdominal infection, was chosen based on the 2010 Infectious Disease Society of America guideline[24]. Then, specific antibiotics were modulated according to the documented pathogen in the cultures. Sulfamethoxazole-trimethoprim (150 mg trimethoprim/m^2 *per* day) for *Pneumocystis jirovecii* prophylaxis and mycostatin (500000 U/day) for fungal infection prophylaxis were provided for six months or more.

Immunosuppression

For induction of immunosuppression, most recipients received oral tacrolimus (0.075 mg/kg) in addition to intravenous basiliximab (12 mg/m^2) and methylprednisolone (20 mg/kg), while cyclosporine-based induction was used in some patients from 1994 to 2001. For maintenance immunosuppression, oral tacrolimus was tapered to a dosage to maintain trough levels of < 5 ng/mL, according to the responses of the liver graft. The oral prednisolone (0.3 mg/kg) was also tapered and then stopped around 3-6 mo postoperatively.

Statistical analysis

In the univariate analysis, the differences of the variables between the groups were assessed using the Mann–Whitney *U* test for continuous parameters. For categorical variables, the χ^2 test or Fisher's exact test, as appropriate, were used. For the continuous variables, receiver-operating characteristic (ROC) curve analysis was performed to identify the optimal cutoff values based on the area under the ROC

curve (AUC). In multivariate analysis, variables with a P value of < 0.1 in the univariate analysis were included for logistic regression, and the odds ratio (OR) and 95% confidence intervals (CIs) were calculated. The sample size was evaluated for multivariate logistic regression[25], and ten variables were included in final analysis. The performance of a statistical model was evaluated in terms of goodness-of-fit, discriminatory ability, and calibration. The differences of cumulative survival rates according to BSI were compared by the Kaplan–Meier method with the log-rank test. The predictive performance of the model was also internally validated through a 10-fold cross-validation and bootstrap resampling method[26-28]. Based on the TRIPOD statement[29], the mean difference between the 200 bootstrapping re-samples was defined as the optimism. All statistical calculations were performed using IBM SPSS Statistics 27.0 (SPSS Inc., Armonk, NY, United States) and R software version 3.6.1 (R Foundation for Statistical Computing, Vienna, Austria). A $P < 0.05$ was considered statistically significant.

RESULTS

Demographics of pediatric patients

The median recipient age of the 378 children was 1.58 years [interquartile range (IQR): 0.83-5.42 years], and the median weight was 10.9 kg (IQR: 8.5-18.3 kg). Biliary atresia (53%) and acute liver failure (23%) were the most common etiology for pediatric LTs. Other perioperative clinical characteristics are summarized in Table 1. Four patients (1.1%) had surgical complications involving a hepatic artery, 13% involving a portal vein, 10% a hepatic vein, and 5.6% a bile duct. Fifty (13%) children had a reoperation within 2 mo after the primary LT. Two hundred twenty-two (59%) children experienced cytomegalovirus viremia and 259 (69%) experienced Epstein-Barr virus viremia. Among them, fourteen (3.7%) suffered from post-transplant lymphoproliferative disorder. Acute cellular rejections were noted among 186 (49%) children.

Characteristics of BSI after pediatric LT

A total of 106 (28%) patients experienced 162 pathogen-confirmed BSIs during the first year after LT (Table 2). Among them, 67% (n = 71/106) had a single episode of BSI, while 33% (n = 35/106) had more than one. There were 1.53 ± 0.95 episodes (mean ± SD) *per* person observed among BSI-complicated patients. The median onset of the first BSI was 0.4 mo post-LT (IQR: 0.03-1.3 mo). Ninety-eight BSIs (60%) occurred within the first month after LT, 48 (30%) between one to six months, and 16 (10%) between six months and one year. Bacteria (99%) were the main pathogens of BSI in this cohort. The most common organisms identified were *coagulase-negative staphylococci*, followed by *Enterococcus spp.* and *Streptococcus spp.* (Table 2 and Figure 1). BSIs caused by Gram-negative bacteria were more prevalent in cases that occurred more than one month after LT. Half (53%) of the BSIs were of unknown origin, while catheter-related BSIs comprised 37% of cases and infections of intraabdominal origin 6%.

Clinical impact of BSI on post-LT outcome

The cumulative survival rates of grafts and patients with and without BSI were estimated using the Kaplan–Meier method (Figure 2). The survival rates of grafts that experienced BSI at 1 year, 5 years, and 10 years were 86%, 77%, and 76%, respectively, while the corresponding rates with no BSI were 95%, 92%, and 88%, respectively ($P < 0.001$ for the log-rank test). The survival rates of patients that experienced BSI at 1 year, 5 years, and 10 years were estimated to be 88%, 80%, and 80%, respectively. The corresponding rates for patients with no BSI were 97, 96%, and 95%, respectively ($P < 0.001$ for the log-rank test).

Risk factors for BSI in the first year after pediatric LT

To identify the risk factors of BSI during the first year after LT, the perioperative clinical variables were compared between the BSI (n = 106) group and the non-BSI (n = 272) group. In the univariate analyses, age, z-score of height, z-score of weight, the presence of growth failure, etiology biliary atresia, liver support system, total volume of RBC transfusion, post-LT hospital stay, portal vein complication, and reoperation were statistically different between the BSI and non-BSI groups (Supplementary Table 1). In the ROC curve analysis, cut-off values of the continuous variables were calculated as age ≤ 1.3 year (AUC = 0.599, P = 0.002), height z-score ≤ -1.22 (AUC = 0.594, P = 0.005), weight z-score ≤ -0.11 (AUC = 0.604, P = 0.002), volume of RBC transfusion > 21.51 cc/kg (AUC = 0.606, P = 0.002), and post LT hospital day > 44 d (AUC = 0.594, P = 0.004), respectively. These cut-off values were re-analyzed in the univariate logistic regression analysis.

In the multivariate analysis (Table 3), age of ≤ 1.3 years (OR = 2.1, P = 0.011), combined growth failure (OR = 2.1, P = 0.045), experience with a liver support system (OR = 4.2, P = 0.008), and longer hospital stay of > 44 d (OR = 2.3, P = 0.002) were independently associated with BSI in the first year after pediatric LT. This logistic regression model showed a Nagelkerke R^2 value of 0.201, and a goodness-of-fit of χ^2 = 8.262 based on the Hosmer–Lameshow test (P = 0.408). The model performance was evaluated

Table 1 Demographics and details of pediatric liver transplantation

Characteristics	Total *n* = 378, *n* (%) or median (IQR)
Primary LDLT: DDLT	287 (76): 91 (24)
Age, year	1.58 (0.83-5.42)
Male: Female	176 (47): 202 (53)
Height, z-score	-0.79 (-1.81-0.27)
Weight, z-score	-0.23 (-1.13-0.62)
Growth failure	94 (25)
Indications	
Biliary atresia	200 (53)
Acute liver failure	88 (23)
Metabolic liver	45 (12)
Malignancy	25 (6.6)
Other liver disease	20 (5.3)
Urgency of LT	
PELD	15.4 (10.6-23.2)
MELD	27.4 (23-29)
Ventilator	25 (6.6)
Renal replacement	19 (5)
Liver support system	20 (5.3)
Graft type	
LDLT, including dual LT	287 (76)
DDLT split	54 (14)
DDLT whole liver	37 (10)
ABO incompatible	11 (3)
Graft-recipient weight ratio, %	2.5 (1.69-3.22)
Total operation time, hours	6.9 (5.9-8.8)
Volume of RBC transfusion, cc/kg	19.2 (7.7-33.3)
Post-LT hospital stay, days	36 (26-51)
Surgical complication	
Hepatic artery	4 (1.1)
Portal vein	51 (13)
Hepatic vein	39 (10)
Bile duct	21 (5.6)
Re-operation	50 (13)
Cytomegalovirus infection	222 (59)
Epstein–Barr virus infection	259 (69)
Post-transplant lymphoproliferative disorder	14 (3.7)
Acute cellular rejection	186 (49)
Chronic rejection	19 (5)
Patients with ≥ 1 BSI episode	106 (28)
Total BSI cases	162 (1.5 times *per* patient)
Graft loss	58 (15)

Patient loss	34 (9)

BSI: Blood stream infection; DDLT: Deceased donor liver transplantation; IQR: Interquartile range; LDLT: Living donor liver transplantation; MELD: Model for End-Stage Liver Disease; PELD: Pediatric End-Stage Liver Disease.

by the discriminatory ability of AUC = 0.744 (95%CI: 0.689-0.80) and the calibration slope of 1.02 (95%CI: 0.721-1.319) (Supplementary Figure 1). The predictive performance of the model was internally validated through 10-fold cross-validation (Supplementary Table 2), and the average validation-corrected AUC was 0.701 (95%CI: 0.641-0.762). Bootstrap-corrected AUC was 0.71 and bootstrap-corrected calibration slope was decreased to 0.8 (Supplementary Figure 2).

DISCUSSION

This large-scale retrospective study aimed to assess prevalence and risk factors of pediatric post-LT BSI based on extensive statistical analysis. We observed that 28% of pediatric LT recipients had BSI episodes in the first year after LT. In previous studies, BSI was noted in 20%-40% of post-LT adults[9,12] and in 20%-45% of post-LT children (Supplementary Table 3)[8,13,15,30,31]. A prior study by Duncan *et al*[15] showed that 28% of pediatric transplant procedures were complicated by BSI in the first year[15], which is consistent with our data. Most of the first episodes of BSI in our cohort occurred in the early phase after LT; about 60% of BSI episodes within a year after LT developed in the first month after surgery (Supplementary Figure 3). A pediatric study in Denmark reported the overall incidence ratio of first BSI in the first year after LT was 1.91 *per* 100 recipients per month, and the incidence was highest in the first month after LT, with an incidence of 6.47 *per* 100 recipients per month[30]. In a study of adult solid organ transplantation, the incidence of BSI was also highest in the early stage after transplantation, and then it gradually decreased[32]. This indicates that more attention should be paid to prevention of BSI in first months after pediatric LT.

Post-LT BSI can be a fatal complication in children and was associated with both graft loss and mortality in this cohort (Figure 2). BSI is associated with 10%-52% of mortality in adults[14,33] and 10%-70% in children[6-8,20]. Our data showed a similar unfavorable impact of BSI on the outcome of LT survival. Shoji *et al*[8] also reported that the one-year mortality rate after LT was higher in patients with BSI compared with those without BSI (28.3% *vs* 3.2%)[8]. Therefore, it is important to prevent BSI after pediatric LT.

Coagulase-negative staphylococcus was the most common pathogen causing BSIs in our study. Gram-positive bacteria predominated as pathogens in the first month, but after that, the proportion of Gram-negative bacteria increased (Supplementary Figure 3). In general, Gram-negative bacteria dominate in pediatric infections after LT (Supplementary Table 4)[8,15,31]. In the study by Duncan, in which *coagulase-negative staphylococci* were defined as skin contaminants and excluded, Gram-negative bacteria were identified in 76% of BSI cases, and *Enterococcus faecium* was the only Gram-positive pathogen isolated[15]. In other studies, *coagulase-negative staphylococcus* was also considered a pathogen if the patient had clinical manifestations of infection, as in our study. These differences might be derived from variations in regional pathogen epidemiology, prophylactic antibiotics protocols, definitions of BSI, surgical techniques, use of intravenous catheters, and immunosuppressive regimens among centers[30,34]. We believe that based on the diversity of the LT program, each hospital should have its own pathogen profile and modified strategy.

The origin of BSI is generally unknown in half of pediatric post-LT cases (Supplementary Table 4)[8,13,30], as also noted in our study (53%). In adult LT, the proportion of BSI cases with an unknown cause has been shown to be lower than that among children[35]. The rationale to explain these differences is not clear; however, the unique preoperative and operative factors of pediatric LT are probably reflected in the onset of BSI after transplant. For example, unlike in adults, pediatric LT patients represent a highly selected cohort; biliary atresia is behind 35%-60% of cases in pediatric LT programs[1,16]. Most pediatric patients undergo a Kasai procedure during the neonatal period, and Roux-en-Y biliary anastomosis is also commonly performed, predisposing the bile duct to enteral bacterial infection. In addition, pediatric groups, especially infants, are not cooperative in maintaining the sanitation of catheter lines. Indeed, their rates of central line-associated BSI are higher than those of adults[36].

Young age, growth failure, liver support, and longer hospital stays were independent risk factors for BSI in multivariate analysis (Table 3). Age under 1 year was a significant risk factor of early BSI and, was explained by the difficulty of keeping peripheral intravenous lines in place and maintaining the central line aseptic[13]. In addition, younger patients have an immature innate immune system, probably resulting in increased risks of general infection, including BSI. Immune cells involved in innate immunity, such as natural killer cells and phagocytes, increase gradually in number after birth[37]. The adaptive immune system also matures as the child grows. The fact that older children were vaccinated prior to LT and thus had immunity to some pathogens may also have had an impact[38].

Table 2 Characteristics of blood stream infection in the first year after liver transplantation

Characteristics	*n* (%)
Number of patients complicated by BSI	106/378 (28)
Number of BSI episodes	162
Number of BSI episodes *per* patient	
0	272 (72)
1	71 (19)
2	24 (6)
3	4 (1.1)
≥ 4	7 (1.9)
Organisms (% of all organisms)	176 (100)
Gram-positive bacteria	133 (76)
Coagulase-negative staphylococci	67 (38)
Enterococcus spp.	21 (12)
Streptococci	17 (10)
Staphylococcus aureus	5 (2.8)
Other gram-positive pathogens	23 (13)
Gram-negative bacteria	41 (23)
Klebsiella spp.	11 (6.3)
Enterobacter spp.	9 (5.1)
Escherichia coli	6 (3.4)
Pseudomonas aeruginosa	3 (1.7)
Other gram-negative pathogens	12 (6.8)
Fungus	2 (1.1)
Candida albicans	2 (1.1)
Time of onset after LT (% of all BSI)	
0-30 d	98 (60)
31 d-6 mo	48 (30)
6 mo-1 yr	16 (10)
Origin (focus) of BSI (% of all organisms)	
Unknown	94 (53)
Catheter-related infection	65 (37)
Intraabdominal infection	11 (6.2)
Urinary tract infection	3 (1.7)
Respiratory infection	3 (1.7)

BSI: Blood stream infection; LT: Liver transplantation.

This is the first study to reveal growth failure as an independent risk factor of BSI, suggesting the clinical importance of nutritional care before LT. Growth failure may reflect a poor general condition or malnutrition, which may render the patient vulnerable to infection. In malnourished children, immunological alterations have been observed, including impaired gut-barrier function, decreased level of complements, and atrophied lymphatic tissue[39]. Adipose tissue has the ability to store cytokines and hormones involved in immune activity, but is decreased in malnourished children, affecting the immune response[40]. Vitamin A and D deficiency have also been reported to be associated with infectious diseases[40]. In the PELD score system, the index growth failure is used as one of the poor prognostic factors for 3-mo mortality in the waiting list for a LT[23].

Table 3 Multivariate analyses of risk factors for blood stream infection after liver transplantation

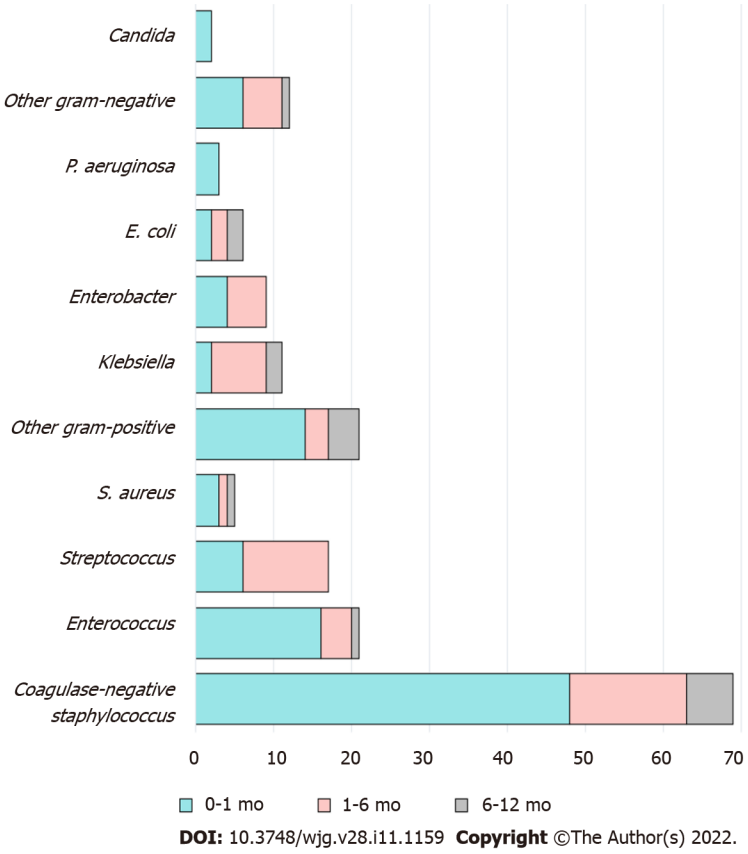
Variables	Non-BSI, *n* = 272		BSI, *n* = 106			Multivariate analysis		
	Median or *n*	(IQR) or (%)	Median or *n*	(IQR) or (%)	*P* value[1]	ORs	95%CI	*P* value
Age	1.83	(0.86-6.0)	1.17	(0.75-3.08)	0.005	2.1	(1.18-3.77)	0.011
Sex, male	124	(45.6)	52	(49)	0.544			
Height, z-score	-0.66	(-1.60-0.34)	-1.23	(-2.17-0.0)	0.005	1.1	(0.54-2.37)	0.722
Weight, z-score	-0.07	(-1.04-0.71)	-0.53	(-1.7-0.4)	0.002	1.2	(0.70-2.25)	0.438
Growth failure	52	(19)	42	(40)	< 0.001	2.1	(1.01-4.47)	0.045
Diagnosis: Biliary atresia	134	(49)	66	(62)	0.023	1.2	(0.66-2.15)	0.546
Ventilator	14	(5.1)	11	(10)	0.066			
Renal replacement	11	(4.0)	8	(7.5)	0.161			
Liver support system	10	(3.7)	10	(9.4)	0.025	4.2	(1.45-12.09)	0.008
PELD	16.2	(6.40-23.7)	15.5	(10.6-24.3)	0.209			
MELD	27.1	(11.3-32.3)	29	(27.8-29.7)	0.746			
LT, DDLT	71	(26)	20	(19)	0.139			
ABO mismatch	8	(2.9)	3	(2.8)	0.954			
Operation time, min	417	(357-536)	411	(350-507)	0.408			
RBC transfusion, cc/kg	17.4	(7.6-29.5)	25.7	(9.8-42.1)	0.003	1.5	(0.87-2.50)	0.146
Post-LT hospital stay	36	(26-50)	41	(28-67)	0.002	2.3	(1.35-3.91)	0.002
Donor, male	128	(47)	44	(42)	0.33			
Donor, body mass index	22.3	(20.3-24.4)	22.7	(21.1-24.5)	0.267			
Reoperation	29	(11)	21	(20)	0.018	1.30	(0.64-2.61)	0.470
Hepatic artery complication	2	(0.7)	2	(2.0)	0.326			
Hepatic vein complication	24	(8.8)	15	(14)	0.126			
Portal vein complication	27	(9.9)	24	(23)	0.001	1.85	(0.95-3.59)	0.070
Bile duct complication	16	(5.9)	5	(4.7)	0.657			
Cytomegalovirus infection	161	(59)	61	(58)	0.771			
Epstein–Barr virus infection	187	(69)	72	(68)	0.839			
Acute cellular rejection	127	(47)	59	(56)	0.117			

[1]Mann–Whitney *U* test, χ^2 test, or Fisher's exact test.

BSI: Blood stream infection; DDLT: Deceased donor liver transplantation; IQR: Interquartile range; MELD: Model for End-Stage Liver Disease; OR: Odds ration; PELD: Pediatric End-Stage Liver Disease; LT: Liver transplantation.

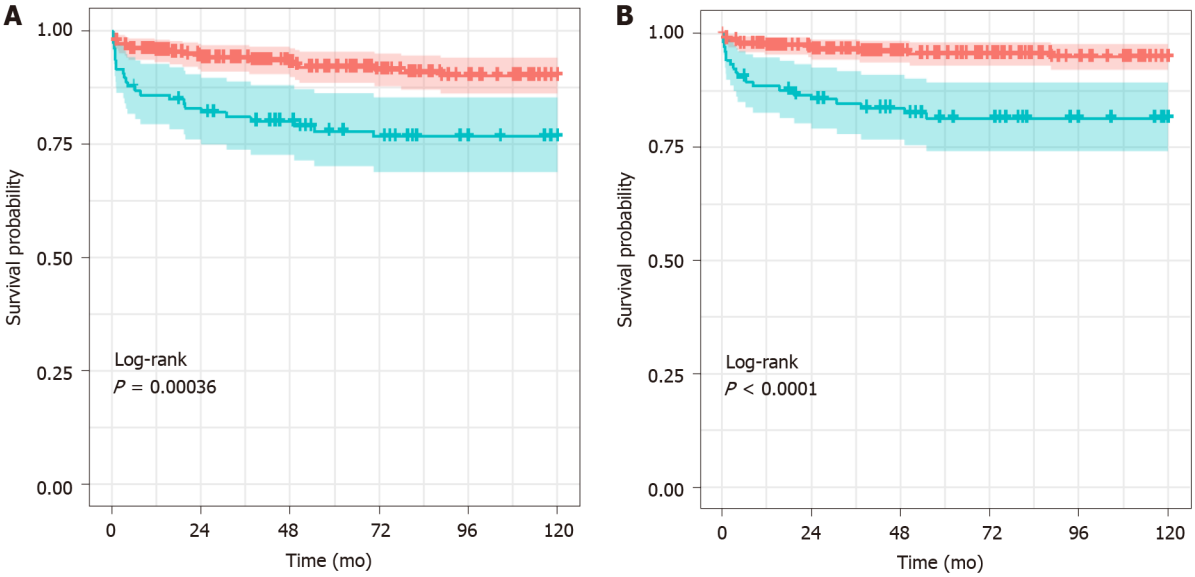
Longer hospital stay was also associated with an increase in BSI, which was confirmed by previous studies[31,34]. However, this should be interpreted with caution, because a longer hospital stay can also be a consequence of BSI. Liver support systems, such as plasma exchange, have been recently adopted as bridging therapies for patients with severe acute liver failure to remove circulating toxic substances [41]. Such treatment may pose a potential risk of infection in that immunoglobulins and complements can be also removed, resulting in immunodeficiency[42]. However, a clear plausible mechanism is not available currently; invasive procedures such as a liver support system or catheterization are risks of BSI from a commonsense standpoint. Blood loss during LT and subsequent massive transfusion are also known risk factors of BSI[8,43,44]. In our study, the total amount of RBC transfusion had a significant correlation with BSI only in the univariate analysis.

A recent meta-analysis suggested that the risk factors of post-LT BSI could be noted among the pre-existing conditions (sex, ascites, and urgency scores for waiting), LT options (ABO incompatibility, operation time, and operative blood loss), and post-LT issues (biliary complication, rejection, hemodialysis, and re-transplantation due to graft failure), for which the OR ranged from 1.28 to 3.37



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Figure 1 Profile of blood stream infection pathogens according to time after liver transplantation.



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Figure 2 Survival outcomes of grafts and patients with primary liver transplantation according to blood stream infection. A: Survival rates of grafts with blood stream infection (BSI) (green line) was lower than those without BSI (red line) ($P < 0.05$ in log-rank test); B: Survival rates of patients with BSI were lower than those without BSI ($P < 0.05$ in log-rank test).

[14]. In children, young age, high operative blood loss, type of procedure (such as the Roux-en Y method), biliary complications, and cytomegalovirus infection were risk factors of BSI (Supplementary Table 3)[8,13,15,30,34]. Presumably, the risks of BSI in children are also different from those in adults with regards to several unique issues such as immunity and LT indication[45,46]. Notably, cytomegalovirus infection, surgical complications of the bile duct, surgical techniques, and blood transfusion were not risk factors for BSI in this analysis. In general, biliary complications are related to

an increased risk of BSI after LT[13,15,31,43]. In our previous report of early BSI, age of < 1 year and bile duct complication were risk factors for BSI[13]. The rationale for the change in the risk factors over time is not clear, and the authors speculate that the refinement and intervention of post-LT care could probably alter the natural history of BSI in transplant children with high risks. For example, there was a remarkable difference in the prevalence of BSI among patients with bile duct complications over the last 10 years (2011-2020, 0%) and before that time (1994-2010, 45.5%) in our study. This improvement may be reflected in the change of the BSI rate and its risk factors according to the transplant era. In the SPLIT data, the rate of BSI significantly decreased with time[6]; our cohort demonstrated similar results.

The present study is limited by several factors. First, this study is a retrospective analysis. In addition, because it is a single-center study, all known risk factors of clinical importance were not included in the analysis to satisfy the minimum events *per* variable for reliable logistic regression analysis[25]. Additionally, the performance of this prediction model was characterized by acceptable goodness-of-fit and fair discriminatory ability. However, prognostic models usually only have an R^2 around 0.2-0.3[47] because substantial future uncertainty after LT is variable at the individual level in reality. In addition, internal validation showed optimism about the apparent performance. To develop a solid prediction model, multi-center studies are needed.

CONCLUSION

Our retrospective study of pediatric patients of LT revealed that BSI was frequently observed and affected the survival outcomes. The profile of the pathogens, onset, and origin site of BSI may be informative to establish individual policy in each surgery center against BSI after the transplant. As clinical practices in pediatric LTs continue to advance, further investigation is necessary to identify how risk factors have been altered by the dynamic nature of early post-LT care and to seek actionable changes in LT care.

ARTICLE HIGHLIGHTS

Research background

Bloodstream infection (BSI) is one of the most significantly adverse events that can occur after liver transplantation (LT) in children. However, risk analysis of post-LT BSI is poorly studied in children.

Research motivation

Our findings are an important step in improving hospital policies for pediatric LT patients and reducing incidence of BSI.

Research objectives

To analyze the profile of post-LT BSIs in children and their risk factor.

Research methods

Clinical data, collected from medical charts of children (n = 378) who underwent primary LT, were retrospectively reviewed.

Research results

BSI was observed in 28% of patients after pediatric LT and affecting survival outcomes. The most common pathogenic organisms identified were *Coagulase-negative staphylococci*. About half of the BSIs were of unknown origin. Young age (≤ 1.3 year), growth failure, liver support system, and hospital stay of > 44 d were independently associated with BSI in the year after LT.

Research conclusions

Our retrospective study of pediatric patients of LT revealed that BSI was frequently observed and affected the survival outcomes. The profile of the pathogens, onset, and origin site of BSI may be informative to establish individual policy in each surgery center against BSI after the transplant.

Research perspectives

The profile of BSI may inform clinical treatment and management in high-risk children after LT.

FOOTNOTES

Author contributions: Oh SH, Namgoong JM, and Kim KM designed the study; Kim YE and Oh SH wrote the manuscript and interpreted the data; Kim YE, Choi HJ, Lee HJ, Oh HJ, and Ahn MK collected patient data; Jhang WK and Park SJ were responsible for patient care and data collection during the intensive care unit stay; Namgoong JM, Kim DY, Jung DH, Moon DB, Song GW, Park GC, Ha TY, Ahn CS, Kim KH, Hwang S, and Lee SG participated in surgery and contributed to the treatment of complications related to surgery and the development of surgical technology; all authors have read and approve the final manuscript.

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

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Country/Territory of origin: South Korea

ORCID number: Yeong Eun Kim 0000-0002-5826-2885; Ho Jung Choi 0000-0003-0701-8038; Hye-Jin Lee 0000-0002-7005-988X; Hyun Ju Oh 0000-0002-0211-3215; Mi Kyoung Ahn 0000-0002-9167-5630; Seak Hee Oh 0000-0002-9672-8877; Jung-Man Namgoong 0000-0002-9237-7440; Dae Yeon Kim 0000-0001-8852-6389; Won Kyoung Jhang 0000-0003-2309-0494; Seong Jong Park 0000-0003-0250-2381; Dong-Hwan Jung 0000-0001-5984-023X; Deok Bog Moon 0000-0002-8209-3540; Gi-Won Song 0000-0002-4235-0434; Gil-Chun Park 0000-0003-1631-3258; Tae-Yong Ha 0000-0001-9932-0212; Chul-Soo Ahn 0000-0002-3844-3646; Ki-Hun Kim 0000-0002-4016-0995; Shin Hwang 0000-0002-9045-2531; Sung Gyu Lee 0000-0001-9161-3491; Kyung Mo Kim 0000-0001-7896-6751.

Corresponding Author's Membership in Professional Societies: Korean Society of Pediatric Gastroenterology, Hepatology and Nutrition.

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ORIGINAL ARTICLE

Observational Study

Real-world effectiveness of direct-acting antivirals in people living with human immunodeficiency virus and hepatitis C virus genotype 6 infections

Hsin-Yun Sun, Chien-Yu Cheng, Chi-Ying Lin, Chia-Jui Yang, Nan-Yao Lee, Bo-Huang Liou, Hung-Jen Tang, Yuang-Meng Liu, Chun-Yuan Lee, Tun-Chieh Chen, Yi-Chia Huang, Yuan-Ti Lee, Ming-Jui Tsai, Po-Liang Lu, Hung-Chin Tsai, Ning-Chi Wang, Tung-Che Hung, Shu-Hsing Cheng, Chien-Ching Hung

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Hsin-Yun Sun, Chien-Ching Hung, Department of Internal Medicine, National Taiwan University Hospital and National Taiwan University College of Medicine, Taipei 100225, Taiwan

Chien-Yu Cheng, Shu-Hsing Cheng, Department of Infectious Diseases, Taoyuan General Hospital, Ministry of Health and Welfare, Taoyuan 330215, Taiwan

Chien-Yu Cheng, School of Public Health, National Yang Ming Chiao Tung University, Taipei 112304, Taiwan

Chi-Ying Lin, Ming-Jui Tsai, Department of Internal Medicine, National Taiwan University Hospital Yun-Lin Branch, Yun-Lin County 640203, Taiwan

Chia-Jui Yang, Department of Internal Medicine, Far Eastern Memorial Hospital, New Taipei City 220216, Taiwan

Chia-Jui Yang, Hung-Chin Tsai, School of Medicine, National Yang Ming Chiao Tung University, Taipei 112304, Taiwan

Nan-Yao Lee, Department of Medicine, National Cheng Kung University Hospital, College of Medicine, National Cheng Kung University, Tainan 704302, Taiwan

Bo-Huang Liou, Department of Internal Medicine, Hsinchu MacKay Memorial Hospital, Hsin-Chu 300044, Taiwan

Hung-Jen Tang, Department of Internal Medicine, Chi Mei Medical Center, Tainan 710402, Taiwan

Hung-Jen Tang, Department of Health and Nutrition, Chia Nan University of Pharmacy and Sciences, Tainan 717301, Taiwan

Yuang-Meng Liu, Department of Internal Medicine, Changhua Christian Hospital, Changhua 500209, Taiwan

Chun-Yuan Lee, Tun-Chieh Chen, Department of Internal Medicine, Kaohsiung Medical University Hospital and College of Medicine, Kaohsiung Medical University, Kaohsiung 807378, Taiwan

Tun-Chieh Chen, Department of Internal Medicine, Kaohsiung Municipal Ta-Tung Hospital, Kaohsiung 801735, Taiwan

Yi-Chia Huang, Department of Internal Medicine, National Taiwan University Hospital Biomedical Park Branch, Hsin-Chu 302058, Taiwan

Yuan-Ti Lee, Department of Internal Medicine, Chung Shan Medical University Hospital, Taichung 402306, Taiwan

Yuan-Ti Lee, School of Medicine, Chung Shan Medical University, Taichung 402306, Taiwan

Po-Liang Lu, Department of Internal Medicine, Kaohsiung Medical University Hospital and College of Medicine, Kaohsiung Medical University, Kaohsiung 807377, Taiwan

Hung-Chin Tsai, Department of Internal Medicine, Kaohsiung Veterans General Hospital, Kaohsiung 813414, Taiwan

Ning-Chi Wang, Department of Internal Medicine, Tri-Service General Hospital, National Defense Medical Center, Taipei 114202, Taiwan

Tung-Che Hung, Department of Internal Medicine, Ditmanson Medical Foundation Chia-Yi Christian Hospital, Chia-Yi 600566, Taiwan

Shu-Hsing Cheng, School of Public Health, Taipei Medical University, Taipei 110301, Taiwan

Chien-Ching Hung, Department of Tropical Medicine and Parasitology, National Taiwan University College of Medicine, Taipei 100233, Taiwan

Chien-Ching Hung, Department of Medical Research, China Medical University Hospital, Taichung, 404332, Taiwan

Chien-Ching Hung, China Medical University, Taichung 406040, Taiwan

Corresponding author: Chien-Ching Hung, MD, PhD, Doctor, Full Professor, Staff Physician, Department of Internal Medicine, National Taiwan University Hospital and National Taiwan University College of Medicine, No. 7 Chung-Shan South Road, Taipei 110, Taiwan. hcc0401@ntu.edu.tw

Abstract

BACKGROUND

Hepatitis C virus (HCV) genotype 6 (HCV-6) infection is prevalent predominantly in Southeast Asia, and the data on the virologic response of HCV-6 to direct-acting antivirals (DAAs) are sparse in people living with human immunodeficiency virus (HIV) (PLWH).

AIM

To assess the virologic response of HCV-6 to DAAs in PLWH.

METHODS

From September 2016 to July 2019, PLWH coinfected with HCV-6 initiating DAAs were included. Laboratory investigations were performed at baseline, the end of treatment, and 12 wk off-therapy.

RESULTS

Of the 349 PLWH included (mean age 48.9 years, 82.5% men), 80.5% comprised people who inject drugs, 18.1% men who have sex with men, and 1.4% heterosexuals. Coexistent hepatitis B virus infection was present in 12.3% of the included PLWH, liver cirrhosis 10.9%, hepatocellular carcinoma 0.9%, and previous HCV treatment experience 10.9%. The mean baseline plasma HCV RNA was 6.2 $\log_{10}$IU/mL. Treatment with glecaprevir/pibrentasvir was initiated in 51.9%, sofosbuvir/ledipasvir 41.5%, sofosbuvir/velpatasvir 6.3%, and sofosbuvir/daclatasvir 0.3%. At DAA initiation, antiretroviral therapy containing tenofovir alafenamide was given in 26.4%, tenofovir disoproxil fumarate 34.4%, non-tenofovir alafenamide/tenofovir disoproxil fumarate 39.3%, non-nucleoside reverse-transcriptase inhibitors 30.4%, protease inhibitors 4.0%, and integrase strand transfer inhibitors 66.8%; 94.8% of the included patients had CD4 counts ≥ 200 cells/mm^3 and 96.0% had plasma HIV RNA < 50 copies/mL. Overall, 96.8% achieved undetectable plasma HCV RNA (< 30 IU/mL) at end of treatment; and 92.3% achieved sustained virologic response 12 wk off-therapy in the intention-to-treat analysis (93.5% in patients receiving

sofosbuvir-based DAAs and 91.2% in those receiving glecaprevir/pibrentasvir).

CONCLUSION
Similar to the observation made in HIV-negative patients, sustained virologic response 12 wk off-therapy with DAAs is high in PLWH coinfected with HCV-6.

Key Words: Viral hepatitis; End-of-treatment response; Sustained virologic response; People who inject drugs; Antiretroviral therapy; Tenofovir

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Core Tip: Similar to the observation made in human immunodeficiency virus-negative patients, virologic response 12 wk off-therapy achieved with direct-acting antivirals (DAAs) was high in human immunodeficiency virus-positive patients coinfected with hepatitis C virus genotype 6. Estimated glomerular filtration rate declined with DAA initiation in patients receiving sofosbuvir and tenofovir disoproxil fumarate or tenofovir alafenamide, which recovered after the completion of DAA treatment.

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INTRODUCTION

With the introduction of highly effective direct-acting antivirals (DAAs), the treatment paradigm of acute or chronic hepatitis C virus (HCV) infections has shifted and the majority of HCV-infected patients with access to DAAs can be cured[1,2]. Nevertheless, an estimated 71 million people globally have chronic HCV infection, of which a significant number develop cirrhosis of the liver or liver cancer [3]. Furthermore, 1.34 million deaths are caused by viral hepatitis, constituting a mortality rate comparable to that of tuberculosis and higher than that of human immunodeficiency virus (HIV) infection[3].

Currently, HCV is classified into seven genotypes and 67 subtypes[4]. Each genotype has its own major geographic distribution[5,6]. The prevalence of HCV viremia due to genotype 6 (GT6) is high in Southeast (34.8%-95.6%) and East Asia (27.4%)[6]. People who inject drugs (PWID) and individuals with thalassemia major are noted to have a higher prevalence of HCV GT6 infection[7]. In Taiwan, genotypes 1b (60.1%), 2a (15.5%), and 2b (11.9%) are the main HCV genotypes in the general population, with genotype 6a being rare[8]. After the outbreak of HCV infection among HIV-positive PWID in Taiwan, genotypes 1a (29.2%), 6a (23.5%), and 3a (20.2%) have emerged as the main circulating HCV genotypes in this population[9].

Compared to patients with HCV non-GT6-related cirrhosis, those with HCV GT6-related cirrhosis have a higher risk of developing hepatocellular carcinoma[10]. In clinical trials, HCV/HIV-co-infected patients are no longer considered special populations, with a sustained virologic response (SVR) rate of 90% or higher with the use of pangenotypic DAAs[11]. In the real world, however, 90% of HCV/HIV-coinfected patients are ineligible for participation in clinical trials[12]. A systematic review concludes that the SVR rates with DAAs are high in patients with HCV GT6 infections in the modern era of DAAs except for patients with cirrhosis of the liver and prior treatment[13]. However, few patients with HIV/HCV GT6 coinfections were included in both clinical trials and real-world studies[14-16]. The present multicenter study aimed to assess the real-world SVR rates in HIV/HCV GT6-coinfected patients receiving contemporary DAAs.

MATERIALS AND METHODS

Study population and setting

In Taiwan, DAAs were conditionally included in the National Health Insurance coverage since January 2017[17,18]. In January 2019, the HCV treatment program providing free-of-charge testing and DAAs was expanded to cover all patients with HCV viremia, including those with acute HCV infections.

Hepatologists and HIV-treating physicians were permitted to screen and treat HIV/HCV-coinfected patients who meet the inclusion criteria. Standardized clinical care and data collection, including serum albumin, alanine aminotransferase and aspartate aminotransferase, prothrombin time and partial thromboplastin time, hepatitis B virus (HBV) serological markers, HCV genotype, abdominal sonography, and plasma HCV RNA load at baseline, the end of treatment (EOT), and 12 wk off-therapy, are strictly required by the HCV treatment program.

People living with HIV (PLWH) in Taiwan are provided with free-of-charge combination antiretroviral therapy (ART) and monitoring of plasma HIV RNA load, CD4 lymphocyte count, renal and hepatic function, lipid profile, and serological markers of viral hepatitis and viral loads, if necessary, according to the national HIV treatment guidelines[19]. HIV care is provided by HIV-treating infectious disease specialists in collaboration with case managers at designated hospitals around Taiwan.

Eligible patients included in this multicenter retrospective observational study were PLWH aged 20 years or older who were diagnosed with HCV GT6 coinfection, either HCV treatment-naïve or -experienced, and received oral DAAs. According to Taiwan's National Health Insurance regulation, oral DAAs for HCV GT6 infections include glecaprevir/pibrentasvir (GLE/PIB) for 8 wk (for patients without cirrhosis) or 12 wk (for those with compensated cirrhosis, Child-Pugh A), sofosbuvir/ledipasvir (SOF/LDV) +/- ribavirin for 12 wk, SOF/velpatasvir (SOF/VEL) +/- ribavirin for 12 wk, and SOF/daclatasvir (SOF/DCV) +/- ribavirin for 12 wk. The regimen of oral DAAs for HCV GT6 infections was chosen at the discretion of the treating physicians.

Data collection

A standardized data collection form was used to record information on demographics (year of birth and sex), clinical characteristics (HIV and HCV transmission routes and ART regimens), and laboratory test results (CD4 count, plasma HIV RNA load, hemogram, and biochemistry). The estimated glomerular filtration rate (eGFR) was assessed using the Chronic Kidney Disease Epidemiology Collaboration equation. The Fibrosis-4 index was calculated according to a previous report[20]. Serum samples to determine HCV RNA load were obtained at baseline (*i.e.* at DAAs initiation), EOT, and 12 wk off-therapy. This retrospective study was approved by the Institutional Review Board or Research Ethics Committee of each participating hospital and the requirement for informed consent was waived.

The Roche cobas® HCV GT test with real-time reverse transcription-polymerase chain reaction was used in eight hospitals to identify HCV genotypes 1 to 6 and subtypes 1a and 1b *via* genotype- and subtype-specific primers and fluorescent dye-labeled oligonucleotide probes; the Abbott Realtime HCV Genotype II assay was used in 7 hospitals. If there were mixed types or any indeterminate results, a sequencing assay was used for final confirmation.

End-points

The primary efficacy end-point, analyzed according to the Food and Drug Administration (FDA) Snapshot algorithm, was SVR with undetectable HCV RNA 12 wk off-therapy (SVR12), defined as having HCV RNA < 30 IU/mL 12 wk after DAA treatment completion. The safety end-point was any adverse event leading to failure of completion of the DAA treatment course. The secondary end-point was HIV virologic suppression after completing DAA therapy, which was defined as plasma HIV RNA load < 50 copies/mL.

Biostatistics

All analyses were performed using the Statistical Program for Social Sciences (SPSS Statistics Version 21, IBM Corp., Armonk, New York, United States). Categorical variables were compared using X^2 or Fisher's exact test and non-categorical variables using Student's *t* test or a Mann-Whitney *U* test. The statistical methods of this study were reviewed by the staff of National Taiwan University Hospital - Statistical Consulting Unit.

RESULTS

From September 2016 to August 2019, a total of 349 PLWH with HCV GT6 infections receiving DAAs were included, with a mean age of 48.9 years (Table 1). The study population consisted mainly of PWID (80.5%), followed by men who have sex with men (MSM) (18.1%), and heterosexuals (1.4%). All had received ART at the time of DAA initiation; 94.8% had CD4 counts ≥ 200 cells/mm^3, 96.0% had plasma HIV RNA loads < 50 copies/mL, and 7.7% had adjusted ART regimens for concerns about potential drug-drug interactions with DAAs (7.2%) or simplification (0.5%).

Cirrhosis of the liver and hepatocellular carcinoma were documented in 10.9% and 0.9% of the included PLWH, respectively. Thirty-eight PLWH (10.9%) had received HCV treatment, mainly interferon-based therapy (n = 35, 10.0%), before DAA initiation. Seroconversion of anti-HCV antibody from negativity to positivity within 1 year was documented in 5.2%. Injection drug use (IDU) (79.9%) was the predominant transmission route of HCV infection followed by sexual transmission (12.3%). Mixed infection with other genotypes was noted in 2.3% (n = 8), mainly genotype 2 (3) followed by 1a

Table 1 Demographic and clinical characteristics of 349 people living with human immunodeficiency virus and hepatitis C virus genotype 6 co-infections

Variables	Data
Age at DAA initiation, mean (SD), yr	48.9 (11.7)
Male sex, *n* (%)	288 (82.5)
Transmission route of HIV infection, *n* (%)	
Men who have sex with men	63 (18.1)
Heterosexuals	5 (1.4)
People who inject drugs	281 (80.5)
CD4 counts ≥ 200 cells/mm^3, *n* (%)	331 (94.8)
Plasma HIV viral loads < 50 copies/mL, *n* (%)	335 (96.0)
eGFR at baseline, mean (SD), mL/min/1.73m^2	94.0 (20.5)
Receiving ART, *n* (%)	349 (100.0)
Switch of ART before DAA, *n* (%)	27 (7.7)
Backbone antiretroviral agent, *n* (%)	
TAF-based	92 (26.4)
TDF-based	120 (34.4)
Non-TDF/TAF-based	137 (39.3)
The third antiretroviral agent	
nNRTI	106 (30.4)
PI	14 (4.0)
InSTI, *n*/N (%)	233 (66.8)
RAL	10/233 (4.3)
EVG	92/233 (39.5)
DTG	131/233 (56.2)
Cirrhosis of the liver, *n* (%)	38 (10.9)
Hepatocellular carcinoma, *n* (%)	3 (0.9)
HCV treatment-experienced, *n* (%)	38 (10.9)
DAA, *n*	4
Interferon/ribavirin, *n*	35
HCV seroconversion[1] within 1 yr, *n* (%)	18 (5.2)
Transmission route of HCV infection, *n* (%)	
Sexual transmission	43 (12.3)
Injection drug use	279 (79.9)
Blood transfusion	1 (0.3)
Unknown	26 (7.4)
HCV RNA viral load, mean (SD), $\log_{10}$ IU/mL	6.2 (1.1)
Mixed HCV infection, *n* (%)	8 (2.3)
Positive HBsAg, *n* (%)	43 (12.3)
Undetectable HBV DNA load (< 20 IU/mL) before DAA initiation, *n*/N (%)	10/13 (76.9)
Undetectable HBV DNA load after completion of DAA therapies, *n*/N (%)	6/7 (85.7)
DAA agents, *n* (%)	
GLE/PIB	181 (51.9)

SOF/LDV	145 (41.5)
SOF/VEL	22 (6.3)
SOV/DCV	1 (0.3)
Plasma HIV RNA < 50 copies/mL after DAA, *n*/N (%)	289/306 (94.4)

[1]HCV seroconversion was defined as change of anti-HCV antibody from negativity to positivity. ART: Antiretroviral therapy; DAA: Direct-acting antiviral; DTG: Dolutegravir; EVG: Elvitegravir; GLE/PIB: Glecaprevir/pibrentasvir; HBsAg: Hepatitis B surface antigen; HCV: Hepatitis C virus; HIV: Human immunodeficiency virus; InSTI: Integrase strand transfer inhibitor; *n*: Patient number; N: Number of patients with data available; nNRTI: Non-nucleoside reverse-transcriptase inhibitor; PI: Protease inhibitor; RAL: Raltegravir; SD: Standard deviation; SOF/DCV: Sofosbuvir/daclatasvir; SOF/LDV: Sofosbuvir/ledipasvir; SOF/VEL: Sofosbuvir/velpatasvir; TAF: Tenofovir alafenamide; TDF: Tenofovir disoproxil fumarate.

(2), 1b (1), both 1a and 1b (1), and 1 (1). Hepatitis B surface antigen testing was positive in 43 PLWH (12.3%). Of the 13 HBV-coinfected PLWH with determinations of HBV DNA load before DAA initiation, 10 (76.9%) had undetectable HBV DNA (< 20 IU/mL). After DAA completion, 85.7% (6/7) had undetectable HBV DNA.

At DAA initiation, ART included tenofovir alafenamide (TAF)-based regimens in 26.4%, tenofovir disoproxil fumarate (TDF)-based regimens in 34.4%, and non-TAF/TDF-based regimens in 39.3%. The third agent of the ART regimen varied between non-nucleoside reverse-transcriptase inhibitors in 30.4% of the included PLWH, protease inhibitors (PIs) in 4.0%, and integrase strand transfer inhibitors (InSTIs) in 66.8% (raltegravir 4.3%, elvitegravir 39.5%, and dolutegravir 56.2%).

GLE/PIB (51.9%) was the most frequently prescribed DAA, followed by SOF/LDV (41.5%), SOF/VEL (6.3%), and SOF/DCV (0.3%). Before DAA initiation, the mean plasma HCV RNA load was 6.2 $\log_{10}$ IU/mL, and the overall virologic response at EOT and SVR12 was 96.8% and 92.3%, respectively, in the FDA Snapshot algorithm (Figure 1). At EOT, plasma HCV RNA was detectable in 2 PWID (plasma HCV RNA 238 and 10,743,433 IU/mL after treatment with GLE/PIB and SOF/VEL, respectively) and 1 heterosexual (plasma HCV RNA 963 IU/mL after treatment with GLE/PIB). Additionally, 8 PLWH (2.3%) had no data available for assessment at EOT (Figure 1), including 4 PLWH (3 treated with GLE/PIB and 1 SOF/VEL) who were lost to follow-up, 3 (2 GLE/PIB and 1 SOF/VEL) who missed the blood testing but achieved SVR12 during follow-up, and 1 (SOF/LDV) who died of a morphine overdose.

At 12 wk off-DAA therapy, 14 PLWH (11 PWID and 3 MSM) were classified as having no data (Figure 1), including 13 PLWH (7 treated with GLE/PIB, 4 SOF/LDV, and 2 SOF/VEL) who were lost to follow-up and 1 (treated with SOF/LDV) who died of a morphine overdose. None discontinued DAA due to adverse effects. Of the 13 PLWH (3.7%, including 11 PWID, 1 MSM, and 1 heterosexual) classified as having virologic non-response (9 treated with GLE/PIB and 4 SOF/LDV), their median plasma HCV RNA load was 5.0 $\log_{10}$ IU/mL (interquartile range 3.7-6.7 $\log_{10}$ IU/mL). Reinfection was considered by the treating physicians as the cause of virologic non-response in 10 PLWH and treatment failure in 1 PLWH; and the causes in the remaining 2 PLWH were unclear. Of the 10 PLWH with HCV reinfection, 5 had HCV genotyping after SVR12, and 3 underwent a genotype switch (GT3 in 2 PLWH and 1a in 1).

The virologic response to SOF-based regimens (168 PLWH) and GLE/PIB (181 PLWH) at EOT by FDA Snapshot algorithm was 97.6% and 96.1%, respectively (P = 0.793) (Supplementary Figure 1), and the SVR12 rate was 93.5% and 91.2%, respectively (P = 0.203) (Figure 2). In the per-protocol (PP) analysis, the virologic response rates were 99.4% (164/165) and 98.9% (174/176) at EOT, and 97.5% (157/161) and 94.8% (165/174) at SVR12 for SOF-based regimens and GLE/PIB, respectively (both P > 0.05).

There was no statistically significant difference in virologic response at EOT (97.5% *vs* 94.3%; P = 0.210) and SVR12 (92.1% *vs* 92.9%; P = 0.999) between the 279 PLWH acquiring HCV infection *via* IDU and the 70 PLWH who were MSM or heterosexuals (Supplementary Figure 2, and Figure 3). In the PP analysis, no statistically significant difference was detected in virologic response between the two groups (IDU *vs* sexual transmission) at EOT [99.3% (272/274) *vs* 98.5% (66/67); P = 0.482] and SVR12 [95.9% (257/268) *vs* 97.0% (65/67); P = 0.999]. Of the 11 PWID who had no data available for assessment of virologic response 12 wk off-therapy, loss to follow-up was the main reason (90.9%, 10/11).

Improvement of elevated serum transaminases and Fibrosis-4 index scores was observed as soon as 4 wk after DAA initiation (Supplementary Figure 3A-C). The median absolute and percentage changes of eGFR were compared among the six groups of PLWH according to the agent used (SOF, TDF, and TAF), which included 53 PLWH receiving SOF/TDF, 44 SOF/TAF, 71 SOF/non-TDF/non-TAF, 67 non-SOF/TDF, 48 non-SOF/TAF, and 66 non-SOF/non-TDF/non-TAF during the DAA treatment course and after DAA discontinuation.

The sequential median eGFR at baseline, during the DAA course, at SVR12, and post-SVR12 of the six groups, are shown in Figure 4. The median eGFR of the PLWH receiving SOF-containing regimens declined initially after the initiation of DAA but recovered later, while that of PLWH taking non-SOF-containing regimens increased or remained the same after DAA initiation and declined after DAA discontinuation. The median absolute and percentage changes of eGFR are presented in

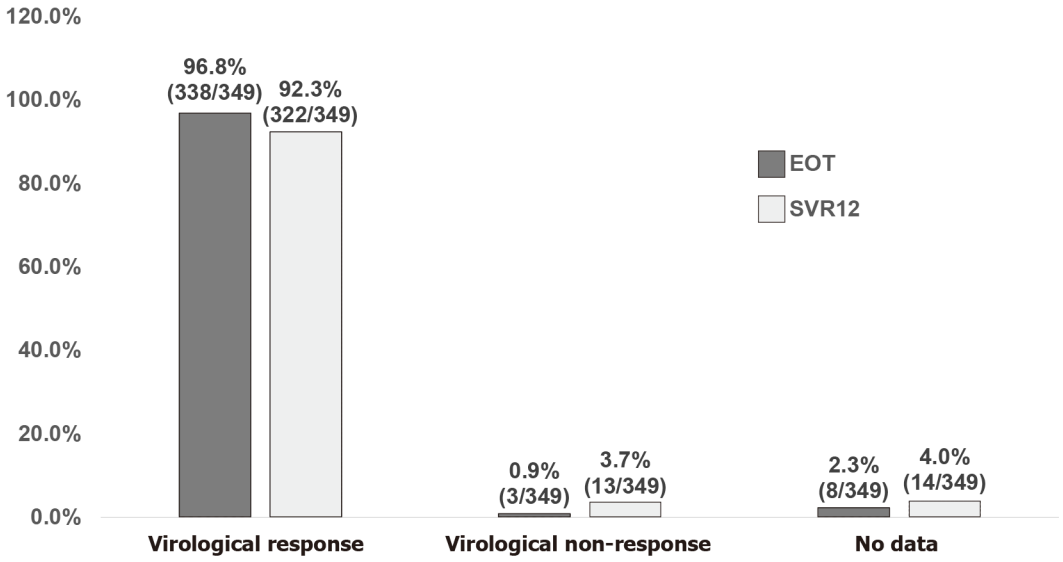


Figure 1 Overall virologic responses at end of treatment and sustained virologic response 12 wk off-therapy. EOT: End of treatment; SVR12: Sustained virologic response 12 wk off-therapy.

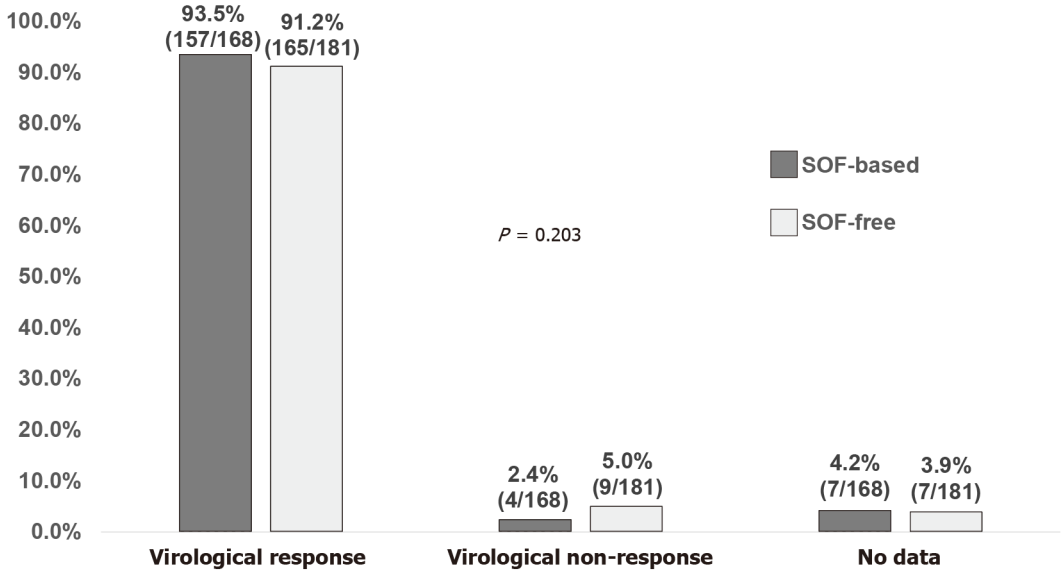


Figure 2 Sustained virologic response 12 wk off-therapy stratified by sofosbuvir-based or sofosbuvir-free regimens. SOF: Sofosbuvir.

Supplementary Figure 4A and B. The eGFR — both absolute and percentage changes — after DAA initiation decreased most significantly in PLWH receiving SOF/TDF compared with other regimens (P = 0.025 and 0.013, respectively) and improved during the DAA course and after DAA discontinuation (Supplementary Figure 3A and B). Plasma HIV RNA loads remained < 50 copies/mL in 94.4% after DAA discontinuation.

DISCUSSION

Our study with a large number (n = 349) of HIV/HCV GT6-coinfected patients receiving DAAs shows that the overall SVR12 rates in the intention-to-treat and the PP analyses were 92.3% and 96.1%, respectively. The majority of the included PLWH acquired HIV (80.5%) and HCV (79.9%) *via* IDU, and loss to follow-up was the main reason (92.9%, 13/14) for the lack of data required in the assessment of virologic response 12 wk off-therapy. Eleven of the 13 PLWH (84.6%) with virologic non-response were PWID, of which 10/13 (76.9%) were likely due to re-infection. Nevertheless, the SVR12 rates were similar when stratified by HCV transmission risk between those with and without IDU (Figure 3), as were the rates between SOF-based regimens and GLE/PIB (Figure 2). The median eGFR in the included PLWH treated with SOF/TDF declined most significantly after DAA initiation (Figure 4,

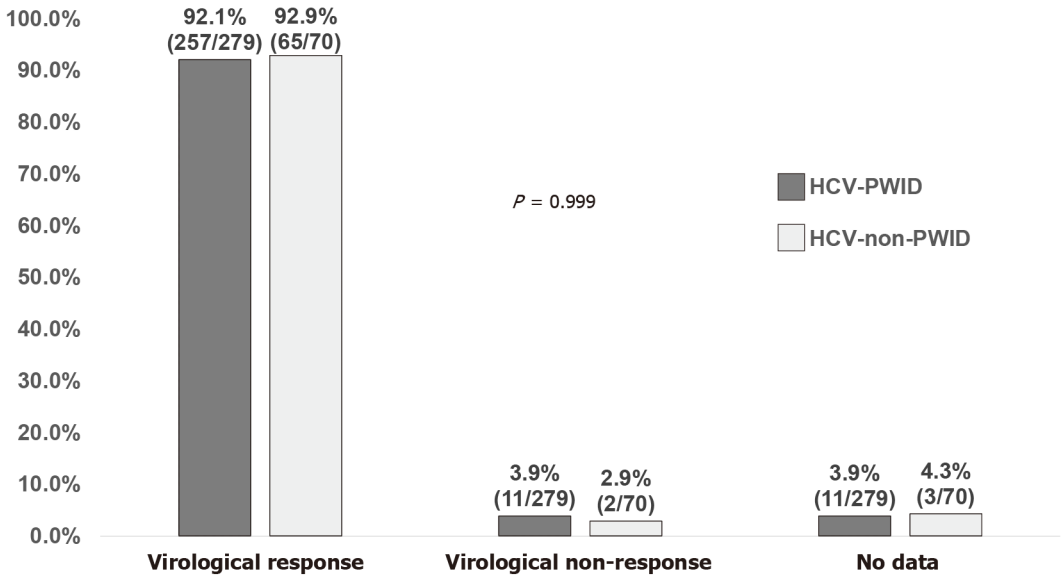


Figure 3 Sustained virologic response at sustained virologic response 12 wk off-therapy stratified by transmission risk of hepatitis C virus infection. HCV: Hepatitis C virus; PWID: People who inject drugs; non-PWID: Non-people who inject drugs.

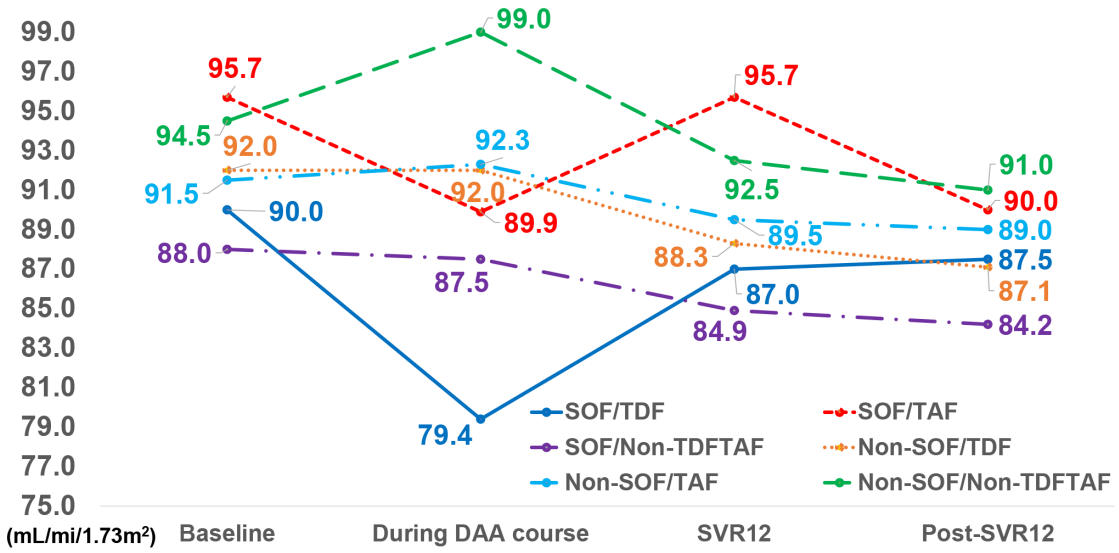


Figure 4 Comparisons of sequential changes of median eGFR from baseline, during DAA course, SVR12, and post-SVR12 stratified by SOF/TDF, SOF/TAF, SOF/non-TDF/non-TAF, non-SOF/TDF, non-SOF/TAF, and non-SOF/non-TDF/non-TAF regimens. eGFR: Estimated glomerular filtration rate; DAA: Direct-acting antivirals; SVR12: Sustained virologic response 12 wk off-therapy; SOF: Sofosbuvir; TDF: Tenofovir disoproxil fumarate; TAF: Tenofovir alafenamide.

Supplementary Figure 4A and B).

DAAs used in the current study were GLE/PIB, SOF/LDV, SOF/VEL +/- ribavirin, and SOF/DCV +/- ribavirin. Their excellent efficacy has been well-documented in a wide variety of patients, including treatment-naïve or -experienced patients and those with and without cirrhosis of the liver, HIV coinfection, chronic renal disease, and solid-organ transplantation[21-23]. For patients with HCV GT6 infections, the reported overall SVR12 was 98% in 108 patients receiving GLE/PIB, 100% in 171 receiving SOF/VEL +/- voxilaprevir, and 64.1-100% in 427 receiving SOF/LDV[13]. A meta-analysis shows that the pooled SVR of DAAs in patients with HCV GT6 infections is 95%, similar to that of patients with HCV GT1 and GT3 infections, respectively[24]. Nevertheless, only 28 HIV/HCV GT6-coinfected patients were included in previous studies[14-16]. Our study provides real-world evidence to fill the current knowledge gap regarding use of DAAs in the HIV/HCV GT6-coinfected population.

Despite excellent effectiveness of DAAs against HCV infections, there are numerous concerns (low treatment completion rates, high rates of loss to follow-up and reinfections) and barriers (psychiatric diseases, poor access to health services, ongoing drug use, inadequate HCV testing, and reluctance of physicians) to including PWID for DAA treatment[25]. In our study consisting mainly of PWID, the

SVR12 rate of 95.9% in PLWH acquiring HCV infection *via* IDU and the rate of loss to follow-up (3.9%, 11/279) seen in the PP analysis are in line with those of previous reports in the literature. In two Spanish cohorts, SVR12 rates were lower for ongoing drug users [with or without opioid agonist therapy (OAT)] than non-drug users (79% *vs* 95%; $P < 0.001$)[26]. Furthermore, ongoing drug users had a high rate of loss to follow-up (17%) and reinfection (3.5%)[26]. Nevertheless, in the PP analysis, SVR12 rates did not differ among never-injectors (97%), PWID without OAT (95%), and those with OAT (95%) ($P = 0.246$). Ongoing drug use was associated with lower SVR12 rates, mainly due to loss to follow-up and not virologic failure. The German Hepatitis C-Registry also reported similar rates of SVR12 (93.7-95.9% in the PP analysis) and loss to follow-up (8.5%-10.2% in PWID with or without OAT)[27].

A meta-analysis performed on post-treatment HCV reinfection rates among people with recent drug use and IDU and those receiving OAT concluded that HCV reinfection rates were higher in the IDU than the OAT group (6.2 *vs* 3.8/100 person-years), and HCV reinfections developed early post-treatment [28]. Thus, the authors advocated that HCV reinfection should not be the reason to withhold DAA from people with ongoing IDU, but harm reduction services should be integrated into DAA treatment programs to avoid reinfection. Moreover, regular HCV testing to detect early reinfection should be performed to initiate retreatment[28]. To eliminate HCV among PWID, concerted efforts should be made to follow the recommendations for action in a health system framework[25].

Acute kidney injury (AKI) has been reported in 1%-15% of patients receiving SOF-based DAAs[29]. Risk factors for AKI following SOF-based DAAs include baseline stage of chronic kidney disease, presence of ascites and diabetes, and concurrent use of nephrotoxic drugs[29]. Concurrent SOF use increases intracellular tenofovir (TFV) diphosphate concentrations in HIV/HCV-coinfected patients receiving TDF-based ART *via* inhibition of TDF hydrolysis by SOF[30,31]. There is a positive correlation between the TFV area-under-the-curve concentration and the levels of urine retinol binding protein-4 and beta-2 microglobulin in a dose-dependent manner in HIV/HCV-coinfected patients receiving SOF/LDV[32]. A study reported significant decreases in eGFR in 273 HIV/HCV-coinfected patients receiving SOF/LDV with concomitant use of ART containing TDF-free, non-boosted TDF, or TDF plus boosted PIs, but the eGFR changes were small and reversible at 12 wk off-therapy[33]. No significant renal dysfunction was observed in HIV/HCV-coinfected patients with TDF-based ART or TDF plus boosted PIs in combination with SOF/LDV[34,35]. Our findings and the literature should provide reassurance that eGFR changes are minimal with the current ART and DAAs among PLWH.

Our study had several limitations. First, our study population consisted mainly of PWID who may have poor treatment adherence and a higher rate of loss to follow-up and reinfection, and only a small proportion of our patients had cirrhosis of the liver or were HCV treatment-experienced. Thus, our findings may not be generalizable to other populations. Second, inherently limited by the observational study design, detailed data regarding the types of adverse effects of DAAs, ongoing injection, enrollment into an OAT program, ongoing substance use during DAA, and adherence of the included PLWH were not available. Nevertheless, it has been known that DAAs are well-tolerated[21-23], and none of our included PLWH discontinued DAAs due to adverse effects. The main reason for DAA discontinuation was loss to follow-up. Third, not all (77.9%-91.1%) of the eGFR data during the DAA course, at SVR12, and post-SVR12 were available, which may have compromised our analysis and interpretation of the pre- and post-treatment eGFR changes. Failure to determine the urinary biomarkers precludes us from getting a better understanding regarding the causality of eGFR changes in PLWH who received TFV-based ART with or without concomitant use of SOF-based DAAs. Finally, HCV strains of PLWH with virologic failure were not available for analysis of emergent resistance mutations to provide more insight into retreatment options.

CONCLUSION

In conclusion, similar to the results in HIV-negative patients with HCV GT6 infections, our PLWH coinfected with HCV GT6 had an SVR12 rate of 96.1% with DAAs in the PP analysis. Small declines of eGFR were observed with DAA initiation in PLWH receiving SOF-based DAAs and TDF- or TAF-based ART, which recovered after the completion of DAA treatment.

ARTICLE HIGHLIGHTS

Research background

Hepatitis C virus (HCV) genotype 6 (GT6) infection is common in Southeast Asia, and its virologic response to direct-acting antivirals (DAAs) in people living with HIV (PLWH) in a large scale is unknown.

Research motivation

The virologic responses of HCV GT6 to DAAs in PLWH will guide national DAA treatment policies for

HCV infection in this population.

Research objectives
The study aimed to assess the virologic responses of HCV GT6 to DAAs in PLWH with HCV GT6 infections.

Research methods
From September 2016 to August 2019, the overall virologic responses at the end of treatment and sustained virologic response 12 wk off-therapy were assessed in the 349 included PLWH with HCV GT6 infections receiving DAAs.

Research results
The overall virologic response at end of treatment and sustained virologic response 12 wk off-therapy. SVR12 was 96.8% and 92.3%, respectively, in PLWH with HCV GT6 infections receiving DAAs.

Research conclusions
PLWH coinfected with HCV GT6 responded well to DAAs.

Research perspectives
National DAA treatment policies of HCV infection in Southeast Asia should take PLWH coinfected with HCV GT6 into consideration.

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FOOTNOTES

Author contributions: Sun HY, Cheng CY, and Hung CC managed and supervised the study; Sun HY, Cheng CY, and Hung CC contributed to the study concept and design; Sun HY, Lin CY, Yang CJ, Liou BH, Tang HJ, Tsai MJ, Huang YC, Liu YM, Lee CY, Lu PL, Chen TC, Lee YT, Tsai HC, Wang NC, Cheng SH, Hung TC, Lee L, and Cheng CY were involved in the collection and assembly of clinical data; Sun HY and Hung CC participated in the data analysis; Sun HY, Cheng CY, and Hung CC undertook interpretation of the data and drafted the report; All authors reviewed and approved the final version of the report.

Institutional review board statement: This retrospective study was approved by the Institutional Review Board or Research Ethics Committee of each participating hospital.

Informed consent statement: The requirement for informed consent was waived.

Conflict-of-interest statement: Hung CC has received research support from Janssen, Merck, Gilead Sciences, and ViiV and speaker honoraria from Abbvie, Bristol-Myers Squibb, Gilead Sciences, and ViiV, and served on advisory boards for Gilead Sciences, Janssen, ViiV, and Abbvie. Sun HY has received research support from Gilead Sciences. Other authors have no competing interest to disclose.

Data sharing statement: No additional data are available.

STROBE statement: The authors have read the STROBE Statement—checklist of items, and the manuscript was prepared and revised according to the STROBE Statement—checklist of items.

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Country/Territory of origin: Taiwan

ORCID number: Hsin-Yun Sun 0000-0003-0074-7721; Chien-Yu Cheng 0000-0002-1886-0370; Chi-Ying Lin 0000-0002-4016-9228; Chia-Jui Yang 0000-0002-5925-2064; Nan-Yao Lee 0000-0002-1206-842X; Bo-Huang Liou 0000-0002-3241-5492; Hung-Jen Tang 0000-0003-2738-6583; Yuang-Meng Liu 0000-0002-9366-5065; Chun-Yuan Lee 0000-0002-0282-8376; Tun-Chieh Chen 0000-0002-9258-0692; Yi-Chia Huang 0000-0002-5658-0265; Yuan-Ti Lee 0000-0003-4577-4379; Ming-Jui Tsai

0000-0002-5646-2170; Po-Liang Lu 0000-0002-7423-6783; Hung-Chin Tsai 0000-0002-5441-4386; Ning-Chi Wang 0000-0002-0190-2116; Tung-Che Hung 0000-0001-7871-4706; Shu-Hsing Cheng 0000-0002-6256-0527; Chien-Ching Hung 0000-0001-7345-0836.

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LETTER TO THE EDITOR

New treatment for gastroesophageal reflux disease: Traditional Chinese medicine Xiaochaihu decoction

Li-Ying Xu, Bin-Yan Yu, Lu-Sha Cen

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Li-Ying Xu, Department of Emergency Medicine, The First Affiliated Hospital of Zhejiang Chinese Medical University, Hangzhou 310006, Zhejiang Province, China

Bin-Yan Yu, Department of Acupuncture and Moxibustion, The First Affiliated Hospital of Zhejiang Chinese Medical University, Hangzhou 310006, Zhejiang Province, China

Lu-Sha Cen, Department of Ophthalmology, The First Affiliated Hospital of Zhejiang Chinese Medical University, Hangzhou 310006, Zhejiang Province, China

Corresponding author: Lu-Sha Cen, PhD, Attending Doctor, Department of Ophthalmology, The First Affiliated Hospital of Zhejiang Chinese Medical University, No. 54 Youdian Road, Hangzhou 310006, Zhejiang Province, China. cenlusa2@sina.com

Abstract

Gastroesophageal reflux disease (GERD) has a high prevalence worldwide. Li *et al* performed a well-designed study on the efficacy of modified Xiaochaihu decoction (MXD) for GERD, which showed that MXD is an optional therapy for GERD beyond proton pump inhibitors (PPIs). The herbal granule administration mode minimized the bias from traditional herbal formula in clinical trials. One limitation of that study was that it lacked records of side effects and rescue medication. As a chronic disease with recurrent symptoms, GERD rehabilitation requires prolonged observation of the clinical course with MXD therapy.

Key Words: Gastroesophageal reflux disease; Xiaochaihu decoction; New treatment; Traditional Chinese medicine; Modified Xiaochaihu decoction; Chronic disease

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Core Tip: Modified Xiaochaihu decoction (MXD) has been applied for gastroesophageal reflux disease (GRED) for thousands of years in Asian countries. Li *et al* proved that MXD is an ideal optional therapy for GERD. The drug selection of herbal granule makes double-blind research achievable. Side effects of MXD should be noted. As a chronic disease, GERD rehabilitation requires prolonged observation of the clinical course with MXD therapy.

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TO THE EDITOR

We read with interest a clinical study by Li *et al*[1]. The researchers performed a prospective, double-blinded, and double-simulation study on the efficacy of modified Xiaochaihu decoction (MXD) for gastroesophageal reflux disease (GERD) and its effect on esophageal motility. The study enrolled 288 participants with GERD, and then divided them into the treatment and control groups, receiving MXD plus omeprazole simulation and omeprazole plus MXD simulation, respectively, for 4 wk. The GERD-Q scale score and esophageal manometry were evaluated. The results showed that MXD had a similar ability to increase the pressure at the lower esophageal sphincter and reduce ineffective swallowing, compared with omeprazole in mild-to-moderate GERD. The recurrence rate was significantly lower than that of the control group within 3 mo at follow-up visits. We greatly appreciate the dedication of the authors towards studying the effect of MXD on GERD as an optional therapy.

As we all know, GERD has a high global prevalence with limited therapeutic options, and its incidence is increasing annually. Patients with GERD present with a variety of symptoms including heartburn, effortless regurgitation, dyspepsia, bloating, and abdominal pain or discomfort, as well as cough and laryngitis, which severely impact their quality of life[2]. Since the 1990s, proton pump inhibitor (PPI) therapy has evolved as the standard treatment of choice for GERD[3]. However, it has become evident that symptoms attributed to GERD remain despite ongoing PPI treatment in up to 40% of patients[4], and PPI therapy increases the risks of infections, osteoporosis, hepatic failure, pancreatitis, jaundice, and persistent gynecomastia[5-7]. Therefore, multivariable treatment beyond PPIs is urgently required.

Traditional Chinese medicine theory believes that the herb Chaihu could regulate qi-flow to harmonize digestive disorders. The formula Xiaochaihu decoction, which has demonstrated therapeutic effects such as for nausea, poor appetite, and epigastric distension, has been used in treating GERD for thousands of years in Asian countries. However, high-level evidence-based traditional Chinese medical research is still lacking and is restricted by many factors. For instance, the herb formula is customized according to patient characteristics including severity of disease, gender, and age. Besides, pharmaceutical effects may be influenced by the way that Chinese herbs are decocted. Thus, it is hard to provide the same drug dose for each patient. In this research, drug selection of herbal granules makes double-blind research achievable. For the concealed allocation, herbal granules were supplied instead of traditional decoction, which greatly reduced the bias from herb treatment.

A limitation of this study was a lack of record keeping of the side effects and rescue medications. With the growing popularity of traditional Chinese medicines, reports of herbal side effects are common. Bupleurum, the main component of MXD, was found to increase the risk of chronic hepatotoxicity[8,9]. Meanwhile, Itoh *et al*[10] reported that long-term oral administration of MXD caused cholestatic liver injury, interstitial pneumonia, and even death[10]. Therefore, medical safety evaluation tests, such as liver and kidney function indices, should be assessed. Rescue medication or treatment for these adverse events should also be considered.

Besides, although the sample size was well calculated according to previous literature reports and formula, the sample size for esophageal manometric indicators, which was from 7–52, was relatively small.

The authors mainly focused on the efficiency of MXD therapy for a short duration. As we know, GERD is generally a chronic disease with recurrent symptoms and requires long-term management[11]. Further studies are needed to evaluate the effects of prolonged MXD therapy on GERD rehabilitation.

FOOTNOTES

Author contributions: Xu LY and Cen LS designed the research; Yu BY analyzed the data; Xu LY and Cen LS performed the research and wrote the letter; Cen LS revised the letter.

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Country/Territory of origin: China

ORCID number: Li-Ying Xu 0000-0001-8238-106X; Bin-Yan Yu 0000-0001-6680-5133; Lu-Sha Cen 0000-0001-7223-340X.

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ABOUT COVER

Editorial Board Member of *World Journal of Gastroenterology*, Saburo Matsubara, MD, PhD, Associate Professor, Department of Gastroenterology and Hepatology, Saitama Medical Center, Saitama Medical University, 1981, Kamoda, Kawagoe-shi, Saitama 350-8550, Japan. saburom@saitama-med.ac.jp

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REVIEW

Epidemiology of stomach cancer

Milena Ilic, Irena Ilic

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Milena Ilic, Department of Epidemiology, Faculty of Medical Sciences, University of Kragujevac, Kragujevac 34000, Serbia

Irena Ilic, Faculty of Medicine, University of Belgrade, Belgrade 11000, Serbia

Corresponding author: Milena Ilic, MD, PhD, Professor, Department of Epidemiology, Faculty of Medical Sciences, University of Kragujevac, S. Markovica 69, Kragujevac 34000, Serbia. drmilenailic@yahoo.com

Abstract

Despite a decline in incidence and mortality during the last decades, stomach cancer is one of the main health challenges worldwide. According to the GLOBOCAN 2020 estimates, stomach cancer caused approximately 800000 deaths (accounting for 7.7% of all cancer deaths), and ranks as the fourth leading cause of cancer deaths in both genders combined. About 1.1 million new cases of stomach cancer were diagnosed in 2020 (accounting for 5.6% of all cancer cases). About 75% of all new cases and all deaths from stomach cancer are reported in Asia. Stomach cancer is one of the most lethal malignant tumors, with a five-year survival rate of around 20%. There are some well-established risk factors for stomach cancer: *Helicobacter pylori* infection, dietary factors, tobacco, obesity, and radiation. To date, the most important way of preventing stomach cancer is reduced exposure to risk factors, as well as screening and early detection. Further research on risk factors can help identify various opportunities for more effective prevention. Screening programs for stomach cancer have been implemented in a few countries, either as a national or opportunistic screening of high-risk individuals only. Generally, due to its high aggressiveness and heterogeneity, stomach cancer still remains a severe global health problem.

Key Words: Stomach cancer; Epidemiology; Incidence; Mortality; Survival; Predictive factors; Prevention

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Core Tip: Despite the decline in incidence and mortality during the last decades, stomach cancer is one of the main health challenges worldwide. According to the GLOBOCAN 2020 estimates, stomach cancer caused approximately 800000 deaths, and ranks as the fourth leading cause of deaths from cancer in both genders combined. Around 1.1 million new stomach cancer cases were diagnosed in 2020. There are some well-established risk factors for stomach cancer: *Helicobacter pylori* infection, dietary factors, tobacco, obesity, and radiation. To date, the most important way of preventing stomach cancer is reduced exposure to risk factors, as well as screening and early detection.

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INTRODUCTION

Stomach cancer was the fifth most common malignant tumor in the world in 2020 with approximately 1.1 million new cases, and is the fourth leading cause of cancer death, with around 800000 deaths[1,2]. Over 85% of stomach cancer cases are registered in countries with high and very high Human Developing Index (590000 and 360000 cases, respectively)[1]. The highest number of cases of stomach cancer (almost 820000 new cases and 580000 deaths) was registered in Asia (mainly in China)[1,2-4]. The estimated five-year survival rate is lower than 20%[2,5-8].

Worldwide, stomach cancer incidence and mortality correlate with increasing age and are relatively rare in persons of both gender younger than 45 years[2,4,7]. The frequency of stomach cancer in men is approximately double that in women[1-3]. In men, stomach cancer was the most commonly diagnosed cancer in 2020 in seven countries (all countries were in Asia: Iran, Afghanistan, Turkmenistan, Uzbekistan, Tajikistan, Kyrgyzstan, and Bhutan) and the leading cause of death from cancer in ten countries (Iran, Afghanistan, Tajikistan, Kyrgyzstan, and Bhutan in Asia, Mali and Cape Verde in Africa, Colombia and Peru in South America, and Costa Rica in Central America)[1,2]. Although stomach cancer was not the most diagnosed cancer in women in any country, stomach cancer was the leading cause of death from cancer among females in three countries (Tajikistan, Bhutan, and Peru)[1,2]. The incidence and mortality rates from stomach cancer were generally low in Northern America and Northern Europe in 2020 and equivalent to rates registered across most of the African regions[1,2].

In the first half of the 20th century, gastric cancer was the leading cause of death from malignant tumors in the United States and Europe[9,10]. Over the past decades, the incidence and mortality due to stomach cancer have substantially declined in many countries[1,2].

Stomach cancer is a multifactorial disease[9-12], including both lifestyle and environmental risk factors *Helicobacter pylori* (*H. pylori*) infection, low socioeconomic status, dietary factors, such as high intake of salty and smoked food and low consumption of fruits and vegetables, fiber intake, in addition to tobacco smoking, alcohol use, low physical activity, obesity, radiation, gastroesophageal reflux disease, positive family history and inherited predisposition. However, the etiology of stomach cancer has not yet been sufficiently elucidated.

Topographically, stomach cancer is classified into two subsites: cardia stomach cancer (arising from the upper stomach) and noncardia stomach cancer (arising from the other parts of the stomach), which differ in epidemiologic patterns and etiology[13]. The majority of all stomach cancers (approximately 90%) are adenocarcinomas, while other types (including lymphoma, sarcoma, neuroendocrine tumors) are rare[12,14]. Two major histologic types of stomach cancer adenocarcinomas are diffuse and intestinal, which differ in epidemiological peculiarities, such as age at diagnosis, gender ratio, *etc.*[15,16].

Despite the strong declining trends in incidence and mortality, stomach cancer remains an important part of the global burden of cancer. Many of the risk factors remain insufficiently understood and need to be the focus of further research in order to achieve more specific, targeted prevention measures.

INCIDENCE

Worldwide, there is a considerable geographic variation in stomach cancer incidence. Stomach cancer incidence rates in 2020 were highest in Eastern Asia (22.4 per 100000 people), followed by Central and Eastern Europe (11.3 per 100000 people), and South America, Polynesia and Western Asia (equally about 8.6 per 100000 people) (Figure 1A)[2]. The lowest rate (3.3 per 100000 people) was registered in Southern Africa.

More than three quarters (75.3%; 819944) of all stomach cancer cases are residents of Asia[2]. Most (86.7%; 944591 cases) stomach cancer cases were residents of more developed regions. The least number of stomach cancer cases was recorded in Micronesia/Polynesia.

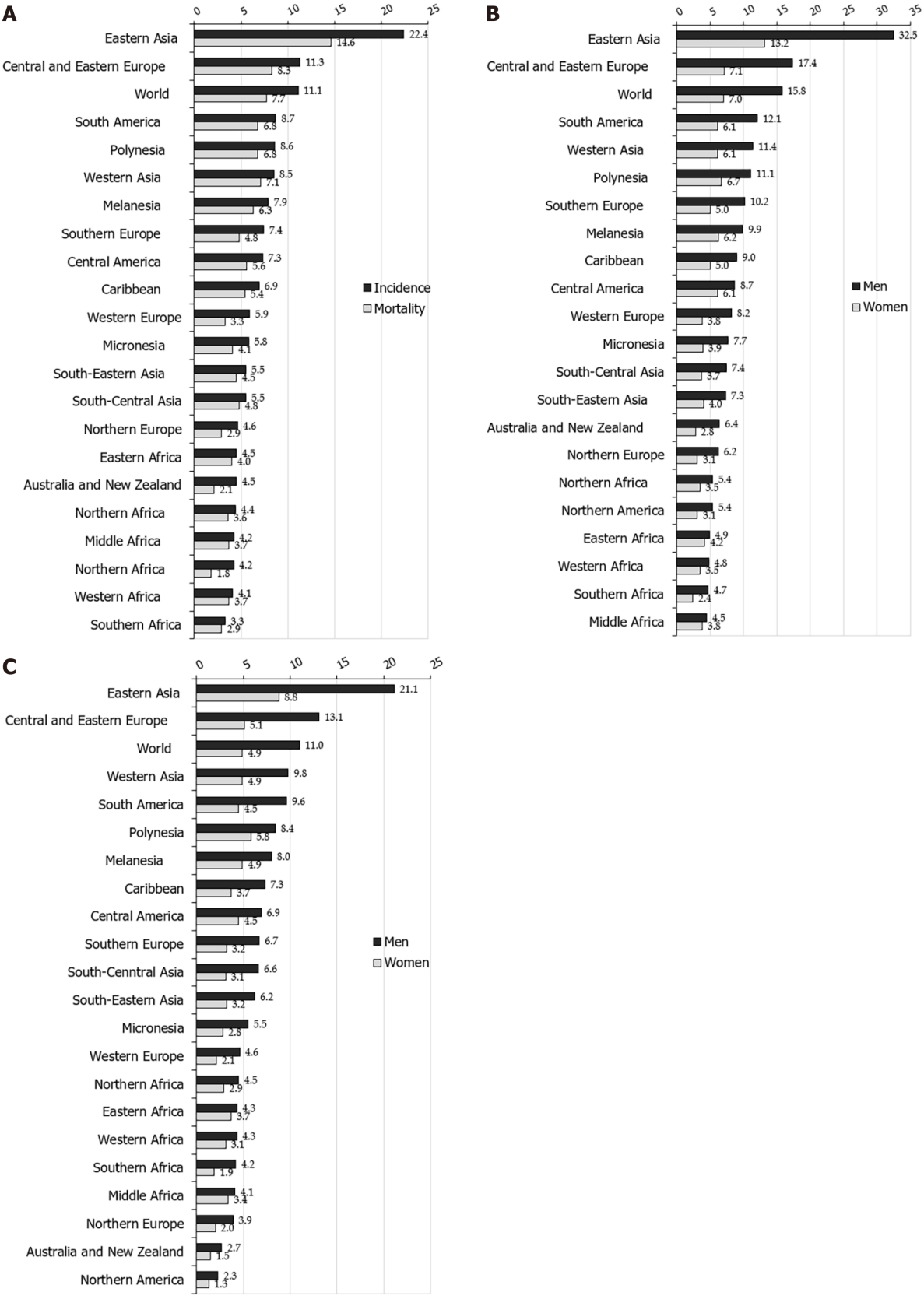


Figure 1 Stomach cancer incidence and mortality, by regions. A: Stomach cancer incidence and mortality; B: Stomach cancer incidence in men and women; C: Stomach cancer mortality in men and women. GLOBOCAN 2020 estimates[2]: Age Standardized Rate (using World standard population, per 100000).

Notable variations in the incidence of stomach cancer in 2020, as well as for mortality, exist around the world (Figures 1-4)[2]. The highest incidence rates were recorded in countries of eastern Asia (Mongolia, Japan, Republic of Korea), while the highest death rates were observed in countries of western Asia (Tajikistan, Kyrgyzstan, Iran). The lowest incidence and mortality rates of stomach cancer

were recorded in Northern America and Northern Europe, Australia/New Zealand and some African countries. Patterns in females were broadly similar to those observed in males, but large differences were observed between sexes and throughout different countries/regions (Figures 1-4)[2]. Globally, the incidence rate of stomach cancer in males was 15.8 per 100000 in 2020, and in females 7.0 per 100000 (Figure 1B)[2]. The gastric cancer incidence rates were about 2 to 3 times higher in males than in females (ranging from 32.5 per 100000 in Eastern Asia to 4.5 per 100000 in Middle Africa for men, and in women ranging from 13.2 in Eastern Asia to 2.4 in Southern Africa) (Figure 1B)[2]. By countries, the differences were fifty-fold: the incidence rates of gastric cancer in men ranged from 48.1 per 100000 in Japan to 1.0 per 100000 in Mozambique in 2020 (Figure 2A). Also, similar differences were observed by regions: the highest incidence rates were reported in Eastern Asia (Japan: 48.1, Mongolia: 47.2, Republic of Korea: 39.7), while the lowest rates were recorded in South Africa (Mozambique: 1.0, Lesotho: 2.1). The incidence rates of stomach cancer in women ranged from 20.7 per 100000 inhabitants in Mongolia (followed by Tajikistan: 18.7, Republic of Korea: 17.6 and Japan: 17.3) to about 0.5 in Indonesia and Mozambique in 2020 (Figure 2B).

However, the distribution of stomach cancer did not have a clear geographical pattern: namely, even though the highest risk populations in the world are in Asian countries (*e.g.* Japan, Mongolia, Republic of Korea), some other countries in Asia register relatively low rates (such as Sri Lanka, Indonesia, Thailand) (Figure 2A and B)[2]. On the other hand, in some low-risk populations, there are some high-risk groups for stomach cancer, such as Koreans and Japanese who live in the United States[17,18].

Also, rates varied across races. Stomach cancer incidence in men in the United States was highest in blacks, followed by Asians/Pacific Islanders, Hispanics, and American Indian/Alaska natives[7]. In women, the highest rates were registered in Hispanics, followed by blacks and Asian/Pacific Islanders, and American Indian/Alaska natives. In the United States, for both sexes, the lowest rates were recorded in whites.

Also, incidence and mortality of gastric cancer in all indigenous groups exceeded the frequency among their non-indigenous counterparts: the highest gastric cancer rates were registered in Indigenous Siberians, Mapuche in Chile and among Alaskan Inuit[19]. Additionally, increasing incidence trends were observed in some indigenous groups, especially in Inuit residing in the circumpolar region and in Maori in New Zealand.

Although differences in stomach cancer incidence in different parts of the world are still not fully clear, most of the variation in stomach cancer incidence worldwide is due to variations in exposure to environmental or lifestyle related risk factors[20-22]. Additionally, migrant studies[23] and secular trends of gastric cancer rates also indicate that environmental factors have an important role in the etiology of gastric cancer[24]. The most important established risk factor for gastric cancer is infection with *H. pylori*[25]. Internationally, variations in *H. pylori* infection prevalence show similarities with variations in stomach cancer prevalence; in developing countries, *H. pylori* infection prevalence in adults is 76% *vs* 58% in developed countries[26]. The prevalence was estimated to be 77.6% in South Africa, 55.8% in China, 52.2% in Mexico, 24.6% in Australia and 22.1% in Denmark[27]. In the United States of America, the prevalence in non-Hispanic blacks was 53%, in Mexican Americans was 62%, but was 26% among non-Hispanic whites[28]. In part, the geographical variation of *H. pylori* infection rates correlate with the frequency of stomach cancer across populations. On the other hand, certain highly infected populations (*e.g.* in Africa and South Asia), unlike the East Asian countries, do not have a high incidence of stomach cancer, which can be explained, at least in part, by the differences in prevalence of genotypes of *H. pylori* (in East Asian the vacA m1 genotype is predominant, whereas the m^2 genotype predominated in Africa, South Asia, and Europe)[29].

Additionally, several other environmental factors are also considered as contributors to gastric cancer occurrence[21,22,30]. Differences between sexes and international variations could likely be due to tobacco smoking[31]. Based on the Global Burden of Disease study[22], the drop in burden of stomach cancer was associated with improved Socio-demographic Index, then to high-sodium diet in both genders combined, as well as to smoking in males, in particular in east Asian populations.

In addition, some research points to the role of aging[24] and hereditary and genetic factors[6] in stomach cancer burden. Incidence differences by sex have never been fully explained, but some theories have suggested a protective role of female sex-specific hormones[15,32]. A higher stomach cancer incidence in males than in females may be due to differences in the incidence of different subtypes of adenocarcinoma according to histology (intestinal or diffuse) and location (proximal or distal)[12,13,33]. Diffuse adenocarcinoma is more common in younger and female patients, whereas intestinal adenocarcinoma is more common in males and the elderly[15]. Intestinal adenocarcinoma dominates high-risk areas and is considered responsible for much of the international variation in incidence. The observed differences in stomach cancer incidence worldwide could be due to diagnostic capacity and changes in the quality of registries, where coverage, completeness and accuracy vary by country[34].

MORTALITY

Nearly three quarters of stomach cancer deaths (74.8%; 575206 deaths) were registered in Asia[2]. Most

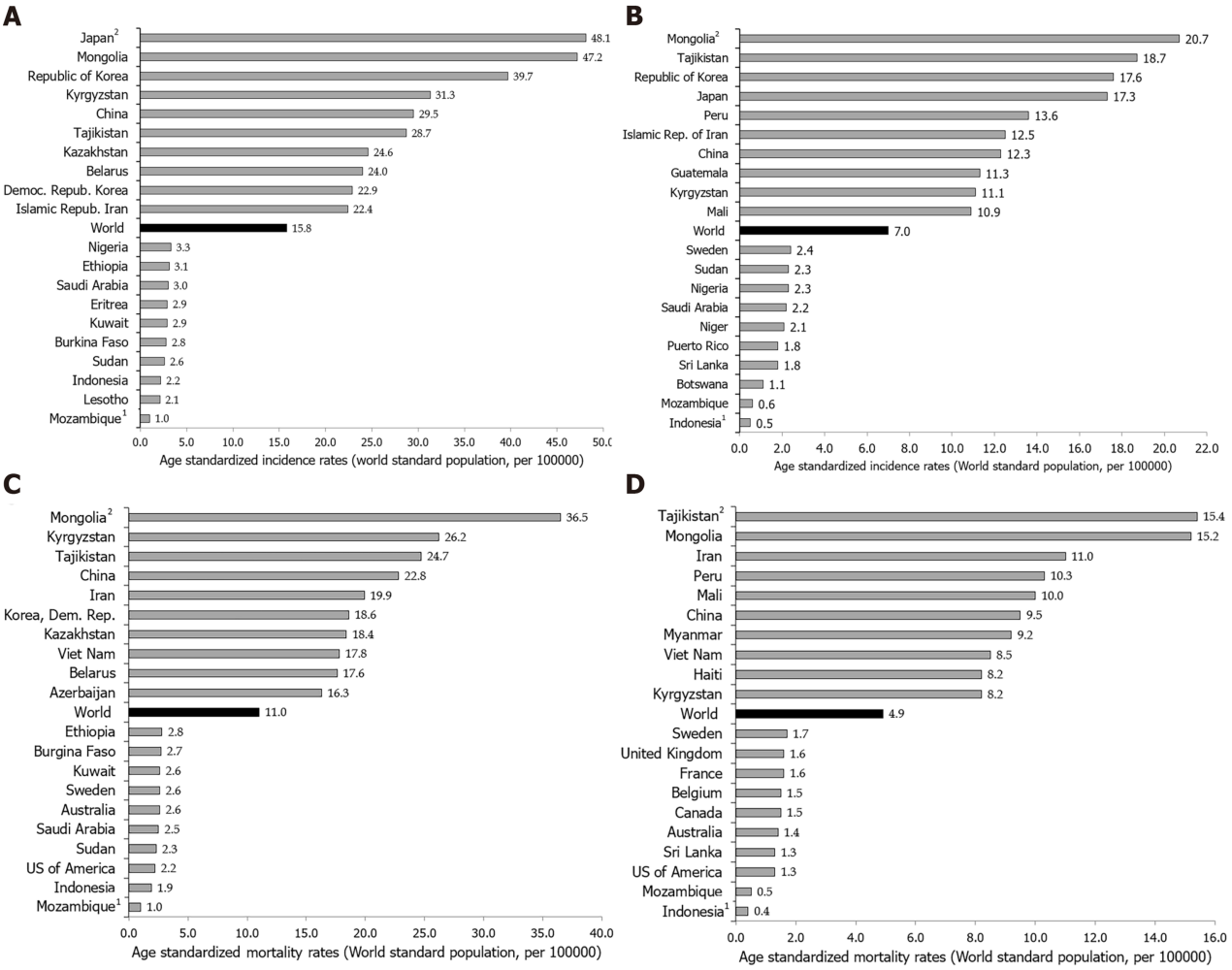


Figure 2 Stomach cancer incidence and mortality, by countries. A: Stomach cancer incidence in men; B: Stomach cancer incidence in women; C: Stomach cancer mortality in men; D: Stomach cancer mortality in women. GLOBOCAN 2020 estimates[2]. [1]Country with the lowest rates; [2]Country with the highest rates.

(83.7%, 643609 deaths) of those who died due to stomach cancer were residents of more developed regions. The least number of deaths were recorded in Micronesia/Polynesia.

Stomach cancer mortality varies greatly across populations and regions. Mortality rates for stomach cancer in 2020 in both genders were highest in the Eastern Asia region (14.6 per 100000 people), followed by South America, Polynesia, Western Asia and Central and Eastern Europe (equally about 8.5 per 100000 people) (Figure 1A)[2]. The lowest mortality rates (about 2.0 per 100000 people) were registered in Northern Africa and Australia. The differences in mortality rates were thirty-fold between the population with the highest rate (Mongolia - 24.6), and the one with the lowest rate (Mozambique - 0.7).

Stomach cancer mortality by gender shows significant geographic variations[1,2,6-8]. Globally, the mortality rate of stomach cancer in males in 2020 was 11.0 per 100000, and in females 4.9 per 100000 (Figure 1C)[2]. The region with the highest mortality rates due to stomach cancer in 2020 in both genders was Eastern Asia (21.3 and 8.8 per 100000, respectively) (Figure 1C)[2]. The lowest rates of stomach cancer mortality in both sexes were in North America (2.3 and 1.3 per 100000, respectively). In men, the risk of dying from stomach cancer was highest in Mongolia (36.5), followed by Kyrgyzstan, Tajikistan and China (approximately 25.0 per 100000) (Figure 2C). By contrast, the risk of death from stomach cancer was lowest in men in Mozambique (1.0) and Indonesia (1.9). Women living in Tajikistan and Mongolia had the greatest risk (approximately 15.0 per 100000) of death from stomach cancer, while the risk for women in Indonesia and Mozambique was lowest (less than 1.0 per 100000) (Figure 2D).

Gastric cancer mortality rates begin to rise in middle-aged persons, with the highest rates observed in the elderly (aged 75 years and older) age group for both males and females (Figure 4B).

Stomach cancer mortality showed apparent geographical variability. Generally, the large differences in mortality rates are between developing and developed countries. Considering developed countries, this mortality pattern could be explained by increased hygiene standards, dissemination of food refrigeration, better preservation of food, high intake of fresh fruits and vegetables and eradication of *H. pylori*[11,22,35]. In the second decade of the 21st century in Japan, mortality due to stomach cancer

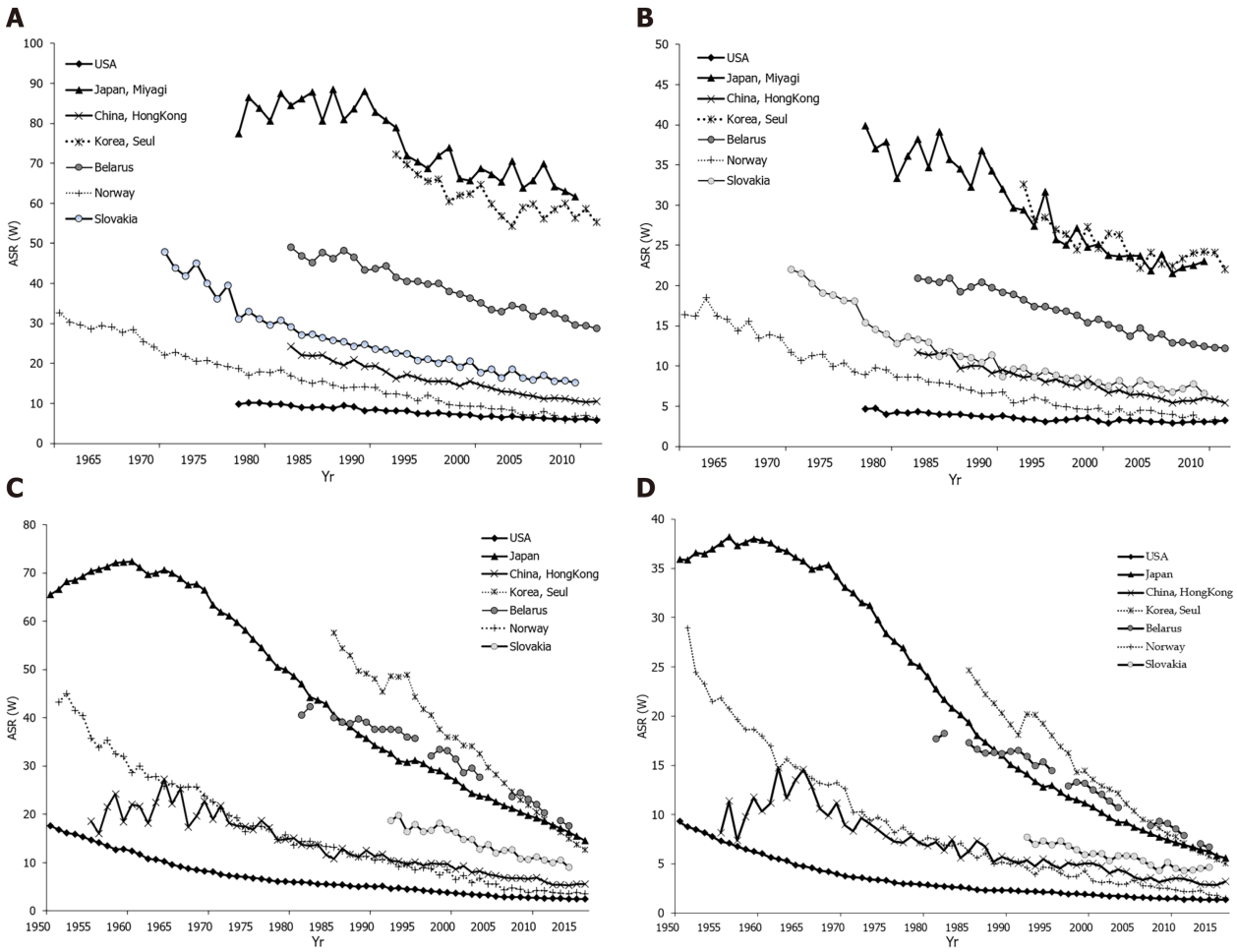


Figure 3 Stomach cancer incidence and mortality trends. A: Stomach cancer incidence trends among men in selected countries; B: Stomach cancer incidence trends among women in selected countries; C: Stomach cancer mortality trends among men in selected countries; D: Stomach cancer mortality trends among women in selected countries. GLOBOCAN 2020 estimates[2]. Age Standardized Rate (using World standard population, per 100000).

reached the levels of Western countries (Figure 4B), which could be attributed to the introduction of gastric cancer screening and to changes in lifestyle, such as the reduction in salt use and an increase in the consumption of fresh fruits and vegetables, improvement in food storage, smoking reduction and prevention of infection with *H. pylori*[22,36,37]. However, reasons for the significant international variations in stomach cancer mortality rates are not fully clear. Diffuse adenocarcinoma is more common in females, while intestinal adenocarcinoma is dominant in males, this subtype being responsible for most of the international variations[15].

There is a wide variation in the relative contribution of cardia and noncardia cancers to the overall number of stomach cancer cases, with a higher proportion of cardia cancers in countries with lower stomach cancer incidence and mortality rates (such as the United States, Canada and Denmark)[38]. In males in Europe, the proportion of cardia and noncardia stomach cancers ranged between 11.6% (Belarus) and 72.0% (Finland), with higher proportions observed in Northern Europe and lower proportions in Eastern Europe. Among other countries worldwide, the proportion of cardia stomach cancers ranged between 5.8% (Republic of Korea) and 64.8% (Iran). In females, a similar geographic pattern was observed, although rates were lower: in Europe, the proportion of cardia and noncardia stomach cancers ranged between 10.6% (Italy) and 44.5% (United Kingdom), while worldwide it ranged from 4.3% (Republic of Korea) to 31.5% (Australia).

Low incidence rates of stomach cancer, which notably became a rare diagnosis among the white United States population, are attributed to the "unplanned triumph" of prevention, which involves a decreased *H. pylori* prevalence and improved food storage and preservation[9,39].

Cancer mortality data are influenced by data on incidence, as well as the success of treatment. Although the World Health Organization estimates present detailed and high-quality information on the incidence and mortality of stomach cancer recorded by cancer registries (regional or national) around the world, these estimates should be interpreted with considerable caution, due to the limited quality and coverage of cancer data worldwide, especially in low- and middle-income countries, due to issues of local data quality, registry coverage, and analytical capacity[2,40,41]. The effect of the coronavirus disease 2019 pandemic on cancer burden is not yet clear, particularly taking into consid-

eration the geographical variations and evolution of the pandemic across countries (because of the lockdown, possible delays in cancer diagnoses, *etc.*)[2].

The differences in availability of improvements in stomach cancer diagnosis and treatment may have had some role in the observed variations in mortality rates worldwide, but this contribution remains open to further quantification[42]. Screening programs and early detection of stomach cancer which have been implemented in Japan[43] and in Korea[44] can partly explain the differences in mortality rates. Also, in Japan, advancements were made in the surgical treatment of early disease, resulting in a better survival rate compared to other countries[45]. However, stomach cancer survival remains unacceptably low in most areas of the world[46,47].

The high prevalence of *H. pylori* infection is widely recognized as the key contributor to high rates of stomach cancer mortality[48]. There is abundant evidence that exposure to other risk factors (tobacco, diet, alcohol use, *etc.*) may have contributed to the apparent international differences in mortality rates of gastric cancer[49-51]. Also, disparities in socio-economic status could have an influence on stomach cancer mortality rates, mediated by varying exposures to infection, environmental factors, as well as barriers in accessing medical care[22,52].

TEMPORAL TRENDS

Declining gastric cancer incidence rates are the dominant epidemiological pattern globally[1,2]. Figures 3A and B show data, for males and females, on stomach cancer incidence secular trends for selected populations. In both sexes, the underlying pattern was a rapid decline in incidence rates over the whole considered time period, regardless of the background stomach cancer risk. There were two exceptions to this pattern. The first exception was seen in the Japanese population (Miyagi prefecture) where, particularly in males, very high rates were observed until the 1990s, and then declined but remained high. The second exception was for the United States population where over the entire time period the rates were constantly very low. The exact reason for the decrease in the incidence of gastric cancer in the last few decades is not completely known, but it most likely includes improvements in diet, food storage and declining prevalence of infection with *H. pylori* due to a general improvement in sanitation and increased use of antibiotics[53]. Eradication of *H. pylori* can be achieved with antibiotic therapy; but, the treatment of asymptomatic carriers is not practical because many countries have a very high infection burden (*e.g.*, over 75% of adult persons living in sub-Saharan Africa have *H. pylori* infection) and reinfection is relatively easy[54,55]. Figure 3C and D represent secular trends for stomach cancer mortality, for males and females, in selected countries over the period 1961 to 2016[2]. Also, downward trends for stomach cancer mortality rates show a very similar pattern as well as incidence trends. In men, the steep decreasing trends for stomach cancer mortality were observed in all selected countries continuously over the observed period. Two exceptions to the mortality pattern were seen. The first exception was for Slovakia where, particularly in women, the rates showed a slower downward trend up to the 2000s, with a flattening of the mortality trend from the 2010s onwards. The second exception was for the United States population where mortality rates remained constantly very low over the entire time period. Stomach cancer mortality in both women and men has shown a significant declining trend in most developed countries over the past 50 years[1,2]. A similar trend, although starting later, has been seen in some countries in Asia, such as Japan and China[37,50]. Factors that led to a decline in mortality involve increased availability of fresh fruits and vegetables, reduced use of salt, reduced incidence of *H. pylori* infection due to improved hygiene and use of antibiotics, and the implementation of screening programs[56,57].

By age, incidence and mortality patterns for stomach cancer in women were broadly similar to those in men, regardless of the background stomach cancer risk being high or low (Figure 4). In selected countries, stomach cancer was predominantly a disease of the elderly, and almost 90% of all cases were diagnosed after the age of 55 years (Figure 4A). For both sexes, stomach cancer mortality continuously increases with age, and is two times higher in those older than 70 years (Figure 4B).

The favorable trend of stomach cancer incidence in developed countries could largely be attributed to a decrease in *H. pylori* prevalence: this is reflected by the "birth cohort effect" where in some countries (including Korea, Japan, the United States) rates of *H. pylori* have been declining in younger generations [24,34,38]. Intestinal adenocarcinoma dominates high-risk areas and is considered responsible for much of the variation in incidence. Recent studies indicate an increase in gastric cancer incidence (cardia and noncardia stomach cancers combined) in persons under the age of 50 in both low- and high-risk countries (such as the United Kingdom, the United States, Canada, Belarus, Chile)[58]. The increasing prevalence of autoimmune gastritis and dysbiosis of the stomach microbiome could have contributed to the increase in stomach cancer incidence among younger generations[59].

In both men and women, trends in the prevalence of cigarette smoking are related to trends in incidence and mortality of stomach cancer with a lag of roughly several decades[22,60]. Besides, stomach cancer mortality trends were minimally influenced by changes in the coding of this disease in the second half of the twentieth century[61].

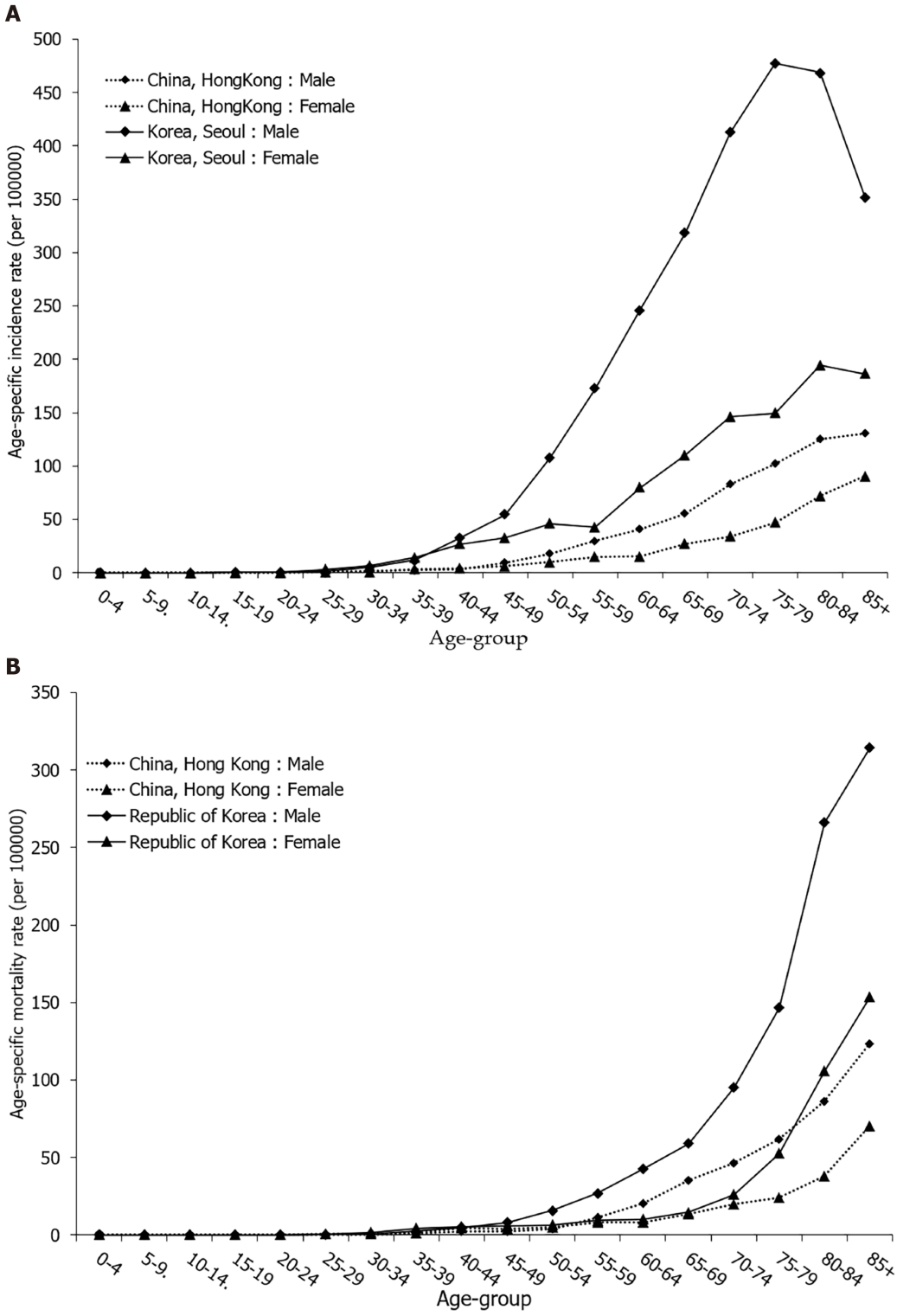


Figure 4 Stomach cancer incidence and mortality trends by age and sex. A: Stomach cancer incidence trends by age and sex in selected countries in 2012; B: Stomach cancer mortality trends by age and sex in selected countries in 2016. GLOBOCAN 2020 estimates[2].

SURVIVAL

In general, survival for patients with stomach cancer is poor[5,62]. In addition, there is global variation in stomach cancer survival[34,47]. Worldwide, with the exception of Japan and Korea, most areas have an overall 5-year relative survival of stomach cancer of about 20%-30%[63]. A 5-year relative stomach cancer survival rate of about 20% is observed in Western developed countries and in developing countries according to an international comparison of data from population based cancer registries[64-66]. In contrast to North America and Europe[66], stomach cancer survival is higher in East Asia: *e.g.*, 5-year survival rate is 67% in Korea and 69% in Japan[67,68], followed by Jordan (56%) and Costa Rica

(46%) in 2010-2014[69]. Also, a notable increase in stomach cancer survival was seen in China (from 30.2% to 35.9%) in recent years[69]. These differences are in part explained by the early stomach cancer detection due to the screening programs implemented in East Asia[63]. The relatively high overall survival for stomach cancer in Japan is the result of a high proportion of patients being diagnosed in the early stage of the disease: in 1995-2000, 53% of stomach cancers were diagnosed at an early stage in Japan[70] in contrast to about 27% in the United States[71]. Differences in tumor biology and stomach cancer subtype (with East/Central Asia and Eastern Europe having a larger proportion of noncardia stomach cancers than North America and Western Europe) may also contribute to survival differences [8,46,72,73]. Worldwide, the proportion of cardia stomach cancers, indicating worse prognosis, ranged from 6% in South Korea to 72% in Finland for men, and for women it ranged from 4% in South Korea to 52% in Serbia[38]. Similarly, cases with the intestinal subtype had a higher survival rate than patients with diffuse tumors[70]. Many factors influence the survival of stomach cancer, including the type of cancer, stage at diagnosis, age, sex, race, overall health, and lifestyle[74-76]. Generally, due to high aggressiveness and heterogeneity, stomach cancer still remains a severe global health problem[47,77].

ETIOLOGY AND RISK FACTORS

Stomach cancer is a multifactorial disease. The notable international variation, time trends, and the migratory effect on stomach cancer frequency suggest that environmental and lifestyle factors are very important in the development of this disease.

In 1994, the International Agency for Research on Cancer has classified *H. pylori* infection as a carcinogen in humans due to the evidence which links *H. pylori* infection and risk of gastric cancer[48]. *H. pylori* as a carcinogen most likely acts indirectly, by causing gastritis, which is a precursor to stomach atrophy, metaplasia, and dysplasia. While the risk of stomach cancer correlated with the duration of *H. pylori* infection, no association was found for the histologic subtype of stomach cancer (intestinal or diffuse), or sex. Based on a meta-analysis of cohort studies, the risk of stomach cancer in people with *H. pylori* infection was 2.36[78]. Chronic or recurrent *H. pylori* infection is a major cause of stomach cancer; the relative risk is estimated to be 2.7-3.8 for cancer of cardia, and 1.1-11.1 for noncardia stomach cancer [20]. *H. pylori* infection is attributed to 592000 (63.4%) of all cases of stomach cancer globally[26]. *H. pylori* is a stomach colonizing bacterium; how *H. pylori* is transmitted has not been elucidated definitely, but the person-to-person pathway is most likely a contact pathway[48]. *H. pylori* infection is acquired in childhood, and population prevalence is associated with socioeconomic status[79]. High prevalence of *H. pylori* infection, and little international variation, suggest that other factors are important in the etiology of gastric cancer[53]. The main risk factors for noncardia stomach cancer are *H. pylori* infection, tobacco smoking and dietary factors, while gastroesophageal reflux disease, obesity and possibly tobacco smoking play an important role in the development of cardia gastric cancer[6,9-13,22].

The main cofactors responsible for the development of stomach cancer are smoking and diet[30,31,80-82]. After adjusting for alcohol intake or the presence of chronic *H. pylori* infection in the stomach, an independent association with smoking was confirmed[83,84]. Over 45 case-control studies and 27 cohort studies confirmed the association of tobacco with stomach cancer, with the average relative risk being RR = 1.5-2.0[85]. One recent meta-analysis of prospective observational studies suggests that the summary relative risk was higher in men (1.63) than in women (1.30)[31]. The risk of stomach cancer increases significantly with cigarette smoking (40% for smokers and 82% for heavy smokers) and alcohol consumption[86]. It is estimated that in developing countries the gastric cancer risk attributable to smoking is 11% in men and 4% in women, while in developed countries the risk is 17% in men and 11% in women[85,87].

While some authors believe that diet has no role in the etiology of gastric cancer, the American Cancer Society states that smoked foods, salted fish and meat, and pickled vegetables represent risk factors for gastric cancer[88]. Some bacteria, like *H. pylori*, can convert nitrates and nitrites (commonly found in meat products) to substances which are shown to cause gastric cancer in animals[48,89]. It is also known that adherence to the Mediterranean diet is significantly inversely correlated with gastric cancer[89,90].

The correlation between salt intake (high in salt, smoked foods, salted fish and meat) and stomach cancer risk has been indicated in several epidemiological studies[91-94]. Sodium chloride is known to increase gastrocarcinogenesis using N-methyl-N-nitro-N-nitrosoguanidine in a rat experiment[95], as well as in a human study[96]. The mucin layer which covers and protects the stomach epithelium is damaged by high doses of salt, which also cause high osmotic pressure that further damages epithelial cells. Prolonged damage to the mucous membrane leads to chronic atrophic gastritis and intestinal metaplasia, which are precursors for stomach cancer.

Higher consumption of fruits and vegetables has been associated with a lower risk of malignant tumors in a number of epidemiological studies (over 200 case-control and cohort studies)[97,98], while results are particularly numerous and consistent for stomach cancer[99]. The intake of fresh fruits and vegetables, which contain antioxidant vitamins (*e.g.* vitamins A and C), reduces gastric cancer risk. In a cohort of 900000 adults (404576 men and 495477 women) who were not diagnosed with malignancy at

the time of enrollment, 57145 people died after 16 years, with the highest weight subjects having a higher mortality rate from malignant tumors in general: men had a 52% higher rate, and women a 62% higher rate, compared to people with normal body weight[100]. Higher mortality was found for esophageal, colon, liver, gallbladder, pancreatic and kidney cancer, but also for non-Hodgkin's lymphoma and multiple myeloma.

Patients with gastroesophageal reflux disease (GERD), especially with the long-standing forms, had a significantly increased risk for cardia stomach cancer and the majority of studies noted a 2-4-fold increase in risk[10,101,102], although not all[103]. One of the explanations for the association between GERD and cardia gastric cancer is that GERD may cause metaplasia with potential progression to adenocarcinoma[104]. On the other hand, a lack of association between GERD and noncardia stomach cancer might be explained, at least in part, by the association with atrophic gastritis which might be associated with a decrease in gastric acid secretion and lower risk of GERD[105].

Of the demographic factors, socio-economic status, older age and male gender play an important role [9-11]. Socio-economic deprivation, within any population, is consistently linked with increased gastric cancer risk[106,107]. The risk of developing stomach cancer increases with age[7,34]. Stomach cancer rarely develops before the age of 40, more than 80% of stomach cancers occur between 60 and 80 years of age. Stomach cancer affects men more than women[1-3]. The consistency of risk difference by sex has never been adequately explained although possible explanations included differences in environmental exposures and lifestyle factors, as well as the theory regarding the potentially protective role of female sex-specific hormones[108]. According to the results of studies conducted in the United States, stomach cancer occurs more often in African Americans compared to the white population[7]. Possible reasons for this increased risk include socioeconomic factors, prevalence of *H. pylori* infection, cigarette smoking, and obesity[7,22,109,110].

It is estimated that around 10% of stomach cancer cases aggregate in families, and only 1%-3% are hereditary[10,13,111]. A positive family history of stomach cancer in a first-degree relative is a risk factor for stomach cancer, but the magnitude of risk varies with different ethnic groups and geographic regions, ranging from 2 to 10[112,113]. Although familial aggregation could be a risk factor because of shared genetic factors, the influence of shared environment cannot be ruled out, *e.g.*, passage of *H. pylori* infection from parents to children, the same dietary factors, *etc.* Although migrant studies indicate a significant reduction in the risk of gastric cancer in Japanese immigrants, the results of many studies point out that exposure to environmental factors in childhood is important for determining gastric cancer risk[48,53,79]. Namely, migrant studies show that exposure in childhood is important in stomach cancer etiology: *e.g.* infection with *H. pylori* often occurs before the age of 10, *i.e.* often before the migration, also children born in the immigrant country are likely to acquire the infection from family members who have migrated from their native country[114].

Gastric cancer risk is increased in many genetic disorders, such as hereditary diffuse gastric cancer, Peutz-Jeghers syndrome, and familial adenomatous polyposis[10,112]. Persons who have mutations or deletion in genes such as *p53*, *BRCA2*, *MSH2*, and *MLH1*, have an increased risk of stomach cancer[10, 111].

Some studies found a link between stomach cancer and antioxidant use, non-steroidal anti-inflammatory drugs, statins, physical activity, and radiation[6,9-13]. Some researchers have suggested a correlation between the excess or deficit of iodine, goiter, and stomach cancer, as well as a decrease in stomach cancer mortality after performing effective iodine prophylaxis[115].

Other potential risk factors in relation to stomach cancer include poor oral hygiene and tooth loss [116], hookah and opium use[117], Epstein-Barr virus infection[118], and consumption of pickled vegetables[119], but the results are not convincing, at least not yet. In addition, in many persons with stomach cancer there is no one specific stomach cancer risk factor.

PREVENTION

During the past century, Western developed countries experienced a major reduction in stomach cancer incidence and mortality, without the introduction of specific primary and secondary prevention measures. Generally, favorable trends in the frequency of stomach cancer are thought to be an important part a consequence of changes such as the reduction in the use of salt and an increase in the consumption of fruit and fresh vegetables due to improvements in food storage (refrigerators, freezers). This phenomenon has been dubbed the "unplanned triumph" of prevention[9,39].

Primary and secondary prevention strategies are the focus of stomach cancer prevention.

Primary prevention measures involve improvements in environment and lifestyle habits such as tobacco control/smoking cessation, reducing salt intake, increasing fruit and vegetable intake, developing other healthy behaviors (such as Mediterranean diet, higher intake of fiber, physical activity), *H. pylori* eradication, other medications (intake of non-steroidal anti-inflammatory drugs, statins), refraining from high alcoholic beverages, sanitation and hygiene improvements. The WHO has set a global goal of reducing the intake of salt to less than 5 g (2000 mg of sodium) per person per day by the year 2025[120]. A meta-analysis of randomized trials (all trials were performed in areas with a high

incidence of stomach cancer, mostly in Asia), in a total of 6695 participants followed from 4 to 10 years showed that the risk of stomach cancer can be reduced by 35% with the treatment of *H. pylori*[121]. In addition to endoscopic and histological surveillance, the American and European guidelines recommend eradication of *H. pylori* in all persons who have atrophy and/or intestinal metaplasia and all persons who are first-degree relatives of stomach cancer patients[122,123]. According to the Asian Pacific Gastric Cancer Consensus, population-based screening and treatment of *H. pylori* infection is recommended in regions which have an annual stomach cancer incidence of more than 20/100000[124]. Eradication of *H. pylori* can be achieved with antibiotic therapy; but, the treatment of asymptomatic carriers is not practical as many countries have a very high infection burden (*e.g.*, over 75% of adult persons living in sub-Saharan Africa have *H. pylori* infection) and reinfection is relatively easy[54].

Japan has had a national endoscopic surveillance program since the early 1970s because of the high stomach cancer risk[125]. It is recommended that all people older than 40 years undergo screening with a double-contrast barium X-ray radiography and endoscopy every year[126]. A study in China demonstrated that a preventive intervention which included eradication of *H. pylori*, nutritional supplements, and screening (with double-contrast radiography and endoscopy) resulted in a 49% reduction in relative risk for overall mortality in a high-risk group of individuals[127].

Upper gastrointestinal endoscopy is the gold standard for stomach cancer diagnosis and due to its high detection rate it is used for stomach cancer screening in high-risk areas (such as Japan, Korea, Venezuela and other areas), but the available evidence shows that endoscopic surveillance of premalignant gastric lesions showed conflicting results[128]. Besides, the procedure is expensive, unpleasant for the patient and carries a risk of hemorrhage and perforation[129,130].

Stomach cancer screening might be possible *via* the detection of potential markers of gastric atrophy (a stomach cancer precursor lesion)[125,131,132], including serum pepsinogens, serum ghrelin, *H. pylori* serum antibodies, gastrin-17, or antigastric parietal cell antibodies, but the results are not convincing, at least not yet.

CONCLUSION

Worldwide, stomach cancer incidence and mortality have declined significantly during the past five decades. However, stomach cancer remains a global health problem as the fifth leading cancer and fourth most common cause of cancer-related deaths in the world. Further illumination of risk factors can help identify various opportunities for prevention. Primary and secondary prevention strategies with more effectiveness are needed in order to reduce stomach cancer incidence and mortality, particularly in populations with a high burden of stomach cancer.

FOOTNOTES

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Country/Territory of origin: Serbia

ORCID number: Milena Ilic 0000-0003-3229-4990; Irena Ilic 0000-0001-5347-3264.

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REVIEW

Antibiotics, gut microbiota, and irritable bowel syndrome: What are the relations?

Zarina Mamieva, Elena Poluektova, Valery Svistushkin, Vasily Sobolev, Oleg Shifrin, Francisco Guarner, Vladimir Ivashkin

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Zarina Mamieva, Elena Poluektova, Oleg Shifrin, Vladimir Ivashkin, Department of Internal Disease Propaedeutics, N.V. Sklifosovsky Institute of Clinical Medicine, I.M. Sechenov First Moscow State Medical University (Sechenov University), Moscow 119991, Russia

Valery Svistushkin, Vasily Sobolev, Department of Ear, Throat and Nose Diseases, N.V. Sklifosovsky Institute of Clinical Medicine, I.M. Sechenov First Moscow State Medical University (Sechenov University), Moscow 119991, Russia

Francisco Guarner, Digestive System Research Unit, Vall d'Hebron Research Institute, Barcelona 08035, Spain

Corresponding author: Zarina Mamieva, MD, Research Assistant, Department of Internal Disease Propaedeutics, N.V. Sklifosovsky Institute of Clinical Medicine, I.M. Sechenov First Moscow State Medical University (Sechenov University), No. 8 bld. 2 Trubetskaya st., Moscow 119991, Russia. mamievazarina@mail.ru

Abstract

Irritable bowel syndrome (IBS) is a functional gastrointestinal disorder in which recurrent abdominal pain is associated with defecation or a change in bowel habits (constipation, diarrhea, or both), and it is often accompanied by symptoms of abdominal bloating and distension. IBS is an important health care issue because it negatively affects the quality of life of patients and places a considerable financial burden on health care systems. Despite extensive research, the etiology and underlying pathophysiology of IBS remain incompletely understood. Proposed mechanisms involved in its pathogenesis include increased intestinal permeability, changes in the immune system, visceral hypersensitivity, impaired gut motility, and emotional disorders. Recently, accumulating evidence has highlighted the important role of the gut microbiota in the development of IBS. Microbial dysbiosis within the gut is thought to contribute to all aspects of its multifactorial pathogenesis. The last few decades have also seen an increasing interest in the impact of antibiotics on the gut microbiota. Moreover, antibiotics have been suggested to play a role in the development of IBS. Extensive research has established that antibacterial therapy induces remarkable shifts in the bacterial community composition that are quite similar to those observed in IBS. This suggestion is further supported by data from cohort and case-control studies, indicating that antibiotic treatment is associated with an increased risk of IBS. This paper summarizes the main findings on this issue and contributes to a deeper

understanding of the link between antibiotic use and the development of IBS.

Key Words: Gut microbiota; Irritable bowel syndrome; Antibiotics; Intestinal barrier; Gut motility; Gut sensitivity

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Core Tip: Irritable bowel syndrome (IBS) is among the most common gastrointestinal disorders; however, its etiology and underlying pathophysiology have yet to be fully elucidated. The present review focuses on the existing evidence on the pathogenic role of the gut microbiota in the development of IBS. Moreover, it provides a comprehensive review on the magnitude of changes in the gut microbiota in response to antibiotics. The paper contributes to a deeper understanding of the link between antibiotic use and the development of IBS.

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INTRODUCTION

Recent advances in culture-independent techniques have greatly expanded our understanding of the human gut microbiota and its functionalities. It is becoming increasingly recognized that gut bacteria play a pivotal role in host homeostasis and are involved in the progression and development of numerous human diseases.

The gut microbiota is established early in life, remains relatively stable thereafter, and is subject to shaping by environmental and host factors (*e.g.*, age, diet, lifestyle, and medications)[1,2]. With regard to the environment, antibiotics have been reported to play a particularly important role in the modulation of the gut microbial community. However, most studies in this area were undertaken 30 to 40 years ago and relied on culture-based techniques. Global antibiotic use has grown 66% since 2000 and continues to grow at a high rate[3,4]. This fact, along with rapid technological advancements for culture-independent analysis, has reinforced the need to take a fresh look at antibiotic-induced changes in the human gut microbiota and clinical consequences of antibiotic intervention. Several studies have reported that antibiotic treatment is associated with an increased risk of irritable bowel syndrome (IBS)[5-8].

IBS is a common gastrointestinal disorder affecting 10%–15% of the population in Europe and North America[9]. This condition negatively affects the quality of life of patients and imposes a significant socioeconomic burden[10]. Over the past few decades, the gut microbiota has emerged as a potential factor that contributes to the pathophysiology of IBS[11,12]. Microbial dysbiosis within the gut has been implicated in intestinal barrier dysfunction, visceral hypersensitivity, impaired gastrointestinal motility, and altered immune response[13-17]. Moreover, various studies have consistently shown the efficacy of microbiota-directed therapies, including prebiotics, probiotics, nonabsorbable antibiotics, dietary changes, and fecal microbial transplantation, in alleviating IBS symptoms[18].

In this paper, we provide a brief overview of the human gut microbiota and its impact on host homeostasis. We highlight what is currently known regarding the role of gut bacteria in the pathophysiology of IBS. Furthermore, we provide an overview of the most up-to-date literature about the impact of antibiotics on gut microbiota composition and discuss a possible link between antibiotic use and the development of IBS. Finally, we identify knowledge gaps and uncertainties that must be filled to orient future research in this area.

GUT MICROBIOTA AND ITS ROLE IN HOST HOMEOSTASIS

The human gut microbiota is a community of microorganisms that inhabit the gastrointestinal tract and is composed of approximately 10^{14} bacterial cells[19,20]. In healthy adults, more than 90% of gut bacteria belong to four dominant phyla, namely, *Firmicutes*, *Bacteroidetes*, *Actinobacteria*, and *Proteobacteria*, whereas other phyla are far less abundant[21,22].

Currently, the gut microbiota is considered an indispensable "organ" within the body with distinct metabolic and immune functions (Table 1). Most of its effects are mediated through metabolites.

Table 1 Gut microbiota functions

Bacterial phylum	Key representatives	Functions
Firmicutes	Members of the genera *Enterococcus, Ruminococcus, Clostridium, Lactobacillus, Faecalibacterium, Roseburia,* and *Eubacterium*	Metabolism of amino acids[23,24], carbohydrates[25], bile acids, and their salts[22]. Lipid metabolism and cholesterol synthesis[25]. Synthesis of vitamins K2, B1, B2, B6, B7, B9, and B12[26]. Maintenance of a proper immune response[28,29] and intestinal epithelial barrier integrity[31,32]. Protection against enteric pathogens[33]
Bacteroidetes	Members of the genera *Bacteroides* and *Prevotella*	Metabolism of amino acids[24], carbohydrates[25,141], bile acids, and their salts[22,142]. Synthesis of vitamin K2[27]. Regulation of appetite[143]. Maintenance of a proper immune response[28-29] and intestinal epithelial barrier integrity[31]. Protection against enteric pathogens[33]
Actinobacteria	Members of the genera *Bifidobacterium* and *Corynebacterium*	Metabolism of bile acids and their salts[22]. Synthesis of vitamins K2, B1, B2, B6, B7, B9, and B12[26]. Protection against enteric pathogens[33]
Proteobacteria	Members of the genera *Desulfovibrio, Escherichia,* and *Shigella*	Metabolism of amino acids[144]

Thus, some of the most important roles of the gut microbiota include metabolism of dietary compounds[23-25], synthesis of vitamins[26,27], regulation of the immune response[28-30], maintenance of intestinal epithelial barrier integrity[25,31,32], and protection against enteric pathogens[33].

MODERN CONCEPT OF IBS: EVOLVING ROLE OF GUT MICROBIOME

Despite extensive research, the etiology and underlying pathophysiology of IBS remain incompletely understood. Proposed mechanisms involved in its pathogenesis include visceral hypersensitivity, impaired gut motility[13,34], increased intestinal permeability[34-36], emotional disorders[11,37], and changes in the immune system[34,37,38].

Over the past decade, there has been an increasing amount of literature on the role of the gut microbiota in the pathogenesis of IBS. The concept of the "microbiota-gut-brain" axis has been proposed [14-17], supporting the crucial role of microbial dysbiosis in the development of IBS symptoms. It is thought that, in genetically predisposed individuals, environmental factors alter the composition of the gut microbiota, leading to disruption of intestinal epithelial barrier integrity[13]. Once the intestinal barrier is breached, bacteria interact with the immune system of the host, provoke a series of immune reactions, and lead to low-grade mucosal inflammation in the gut wall. Collectively, these changes result in sensitivity and motility abnormalities, emotional disorders, and the development of IBS symptoms (abdominal pain, bloating, and alterations in bowel habits)[35]. Interestingly, the gut microbiota not only initiates such a pathological cascade in IBS but also contributes to all aspects of its multifactorial pathogenesis through the release of metabolites[11,12]. These provisions will be discussed below.

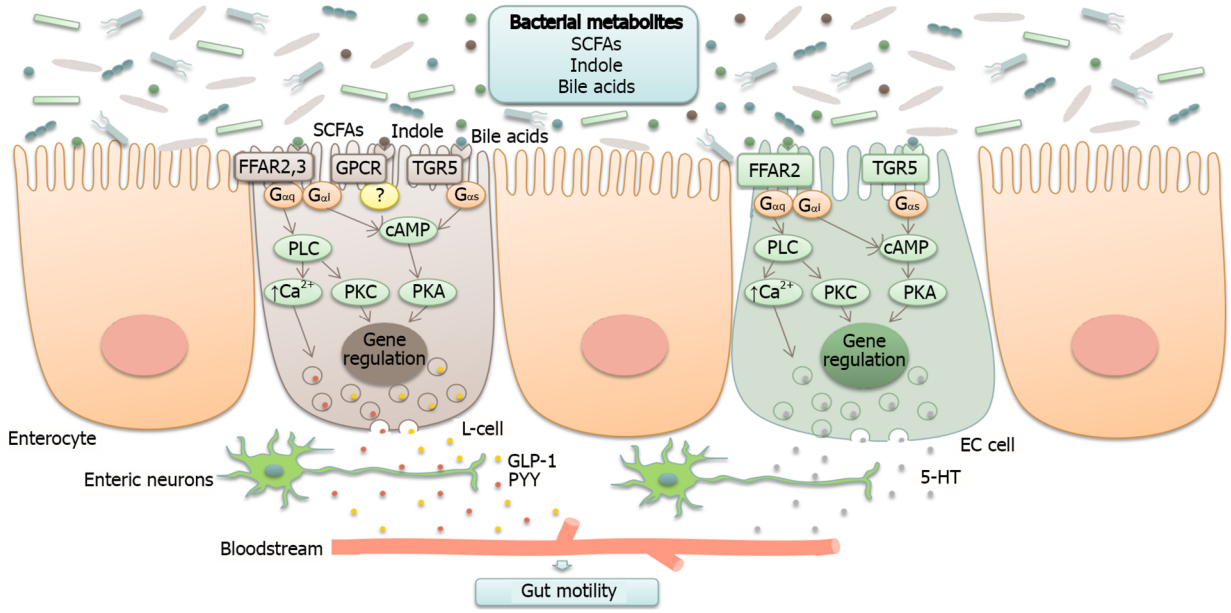
Microbiota and motility/sensitivity abnormalities

The enteroendocrine system modulates gut motor and sensory functions through the secretion of neuropeptides and neurotransmitters[39].

Bacterial metabolites are able to stimulate the production of several neuropeptides, including neuropeptide Y, peptide YY, glucagon-like peptide-1 (GLP-1)[40], cholecystokinin, and substance P (Figure 1)[15,41].

For instance, short-chain fatty acids (SCFAs), secondary bile acids, and indole, which are produced by members of the genera *Clostridium, Bacteroides,* and *Ruminococcus*[23,25], stimulate intestinal L-cells to secrete GLP-1[42]. GLP-1 reduces postprandial motility in the upper gastrointestinal tract (antrum, duodenum, and jejunum) and increases colonic transit[43,44]. A study conducted by Li *et al*[45] reported decreased serum GLP-1 levels and reduced mucosal expression of GLP-1 receptors in patients with constipation-predominant IBS (IBS-C). The authors suggested that lower GLP-1 levels lead to the loss of its prokinetic effects in the colon, resulting in constipation and abdominal pain. In a rat model of bowel dysfunction, administration of the GLP-1 receptor agonist exendin-4 alleviated stress-induced defecation and visceral pain sensitivity[46,47]. Clinical interventions in patients with IBS demonstrated that the synthetic GLP-1 analog ROSE-010 reduced abdominal pain and increased colonic transit[45,48]. The underlying molecular mechanisms responsible for the amelioration of symptoms remain unknown. The authors suggest that modulation of enteric neuronal function and tight junction expression, as well as the activation of serotonergic pathways in the colon, may play a role.

Secondary bile acids and SCFAs, which are mainly produced by *Eubacterium, Bacteroides*, and *Clostridium* (clusters IV, XI, XIII, and XIVa)[22], promote serotonin synthesis from colonic enterochromaffin cells[49]. Serotonin is an important neurotransmitter that, among its other functions, regulates gastrointestinal motility[50]. Serum serotonin levels were found to be increased in those with diarrhea-



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Figure 1 Neurotransmitter modulation by gut microbiota (schematic illustration). Bacterial metabolites, such as short-chain fatty acids (SCFAs), secondary bile acids, and indole, are able to stimulate the production of neurotransmitters, including glucagon-like peptide-1 (GLP-1), peptide YY (PYY), and serotonin (5-HT). They act through G-protein coupled receptors (GPCRs) FFAR2, FFAR3, and TGR5 that are coupled to different types of G proteins ($G_{\alpha s}$, $G_{\alpha q}$, and $G_{\alpha i}$) and activate different pathways known to regulate gene expression and promote exocytosis by raising intracellular Ca^{2+} levels. SCFAs are recognized by FFAR2 and FFAR3. Enteroendocrine L-cells express both of these proteins, whereas enterochromaffin (EC) cells have been reported to express FFAR2. Bile acids are recognized by TGR5 receptors expressed in L-cells and EC cells. The sensing of indole remains elusive, although it is thought to act through GPCR. $G_{\alpha s}$ stimulates adenylate cyclase and elevates cyclic adenosine monophosphate (cAMP), which activates protein kinase A (PKA). $G_{\alpha i}$ inhibits the cAMP pathway. $G_{\alpha q}$ stimulates phospholipase C (PLC), resulting in the generation of diacylglycerol (DAG) and inositol triphosphate (IP_3), which activate protein kinase C (PKC) and induce intracellular Ca^{2+} release[23,138-140]. SCFA: Short-chain fatty acids; GLP-1: Glucagon-like peptide-1; PYY: Peptide YY.

predominant IBS (IBS-D) and reduced in those with IBS-C[34].

The serotonin system represents a potential therapeutic target in IBS. The effects of serotonin are mediated through 5-HT receptors located on the surface of distinct cell types. Fourteen different serotonin receptor subtypes have been identified and classified into seven groups (5-HT_{1-7}), with 5-HT_3 and 5-HT_4 being the most investigated receptors in the intestine. Both receptor subtypes are expressed on neurons within the myenteric and submucosal plexuses of the enteric nervous system, intrinsic and extrinsic sensory neurons, interstitial cells of Cajal, enterocytes, and enterochromaffin cells[51]. 5-HT_3 receptors are involved in the contraction of intestinal smooth muscle and in gut-brain communication through vagal afferent fibers[52]. Activation of 5-HT_4 receptors induces neuronal release of acetylcholine and accelerates the peristaltic reflex[53]. 5-HT_3 receptor antagonists have been shown to improve abdominal pain and global IBS symptoms in patients with nonconstipated and IBS-D[54,55]. 5-HT_4 agonists have been shown to relieve overall and individual symptoms (abdominal pain/discomfort, stool frequency, stool consistency, and straining during defecation) in patients with IBS-C[56-58]. However, cardiovascular side effects were seen with these drugs, and they were either withdrawn from the market (cisapride) or approved for a limited population (tegaserod). Therefore, new safe and well-tolerated 5-HT_4 agonists are under development[59,60].

A number of animal studies have shown the prominent role of the gut microbiota in visceral hypersensitivity[41]. For example, colonization of germ-free rats with the gut microbiota from patients with IBS reduced the pain threshold to colorectal distension[42]. Furthermore, the beneficial effects of probiotic strains (*e.g.*, *Lactobacillus reuteri*, *Lactobacillus plantarum*, *Lactobacillus helveticus*, and *Bifidobacterium longum*) in alleviating visceral sensitivity have been documented[61-63].

Thus, the microbiota influences the main pathogenetic factors of IBS (*i.e.*, motility and sensitivity) both directly and through microbial metabolites.

Microbiota as a regulator of stress and emotional responses

The physiological response to stress is mediated through the hypothalamic-pituitary-adrenal (HPA) axis [64]. Activation of this axis results in the release of corticotropin releasing hormone (CRH) from the paraventricular nucleus of the hypothalamus. CRH acts on the anterior pituitary and induces the production of adrenocorticotropic hormone (ACTH), which in turn stimulates the adrenal cortex to secrete cortisol.

Different types of stressors are known to contribute to the development, maintenance, and exacerbation of IBS symptoms[11]. The results of multiple studies suggest that there is HPA axis dysregulation in IBS. For instance, patients with IBS were found to have excess levels of ACTH in the plasma and cortisol in the serum in response to CRH infusion[65].

Growing evidence indicates that the gut microbiota is involved in the regulation of HPA axis activity. It has been shown that colonization with beneficial microorganisms in early life is of great importance for the normal development of this axis[66]. Moreover, alterations in the gut microbiota may influence the release of ghrelin and galanin, which are endocrine peptides contributing to the stress response through modulation of CRH, ACTH, and glucocorticoid secretion[40,67].

Dysfunction of the HPA axis, along with alterations in neurotransmitter metabolism, appear to be crucial factors in the development of psychiatric disorders, such as anxiety and depression[68,69]. A recent meta-analysis of 27 studies have reported elevated levels of anxiety and depression in patients with IBS as compared to those in healthy controls[70]. Comorbid emotional disorders lead to persistence of symptoms, drive patients to seek medical care, and contribute to poor outcomes[11].

A growing body of literature supports the association between microbial dysbiosis and the development of anxiety and depression. For instance, certain species within the *Lactobacillaceae* and *Bifidobacteriaceae* families are known to produce gamma-aminobutyric acid (GABA). GABA is the main inhibitory neurotransmitter of the central nervous system, playing an important role in the pathogenesis of mood disorders[49,71]. Interestingly, a specific type of GABA receptor (GABA-b) is localized on submucosal and myenteric neurons of the enteric nervous system[72] and is thought to be involved in the modulation of gut motility and sensitivity[37]. Furthermore, members of the genera *Bacillus* and *Escherichia* have been found to produce other neurotransmitters affecting mood and behavior, such as dopamine, serotonin, and norepinephrine[15,73]. In recent studies, germ-free mice have been widely used as a tool for assessing the role of intestinal microbes in brain function and behavior. Studies on germ-free and specific pathogen-free mice indicate that intestinal microbes can cause imbalances of the HPA axis, resulting in an anxiety-like behavioral phenotype[74]. Fecal microbiota transplantation studies have indicated the rodent-to-rodent and human-to-rodent transfer of anxiety-like behaviors[75,76]. Moreover, animal studies have shown that transplantation of the microbiota from depressed patients to rodents is able to induce depression-like behavior. The authors linked microbiota-induced depression in mice to alterations in the cAMP-response element binding protein (CREB) signaling pathway in the olfactory bulb[77] and alterations in carbohydrate and amino acid metabolism[78].

However, despite the data obtained, further research is needed to investigate the difference in emotional disorder levels in patients with postinfectious and other forms of IBS.

Microbiota and host immunity

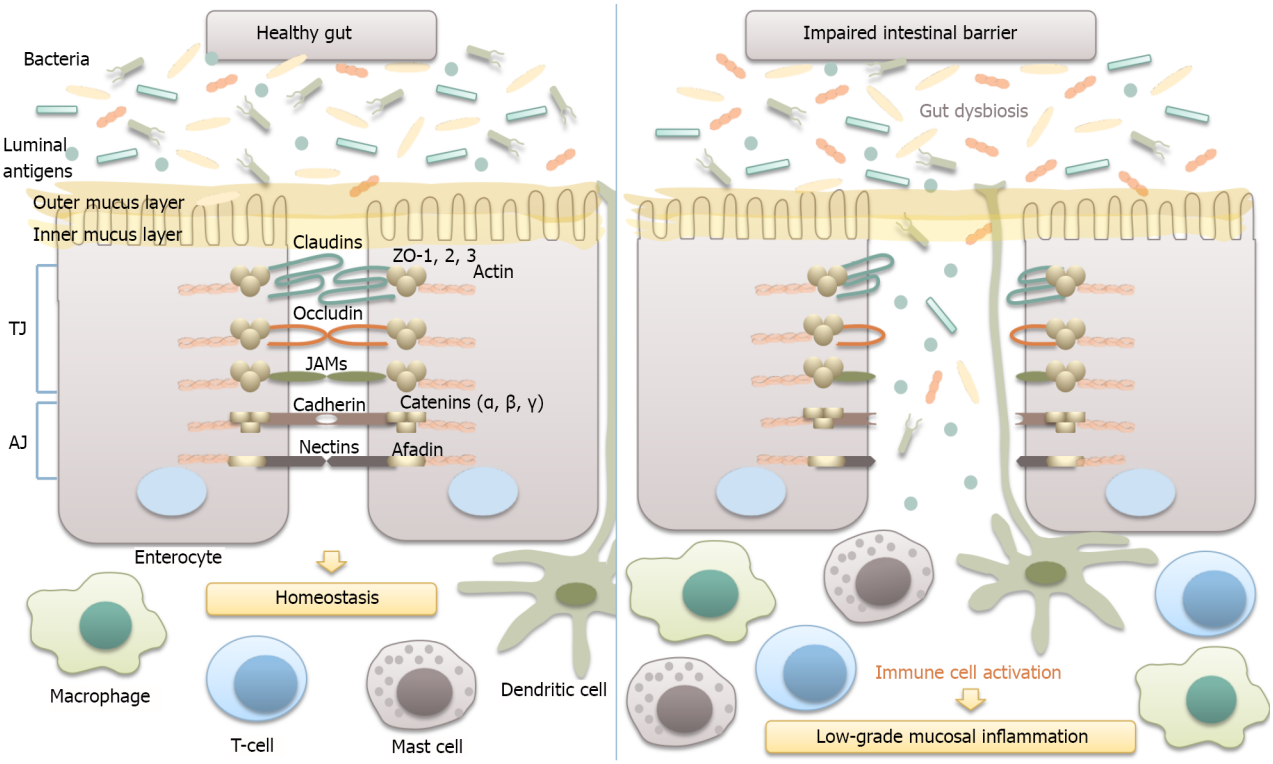
Recently, considerable literature has grown around the theme of immune system activation in IBS. For instance, an increased number of mast cells located in close proximity to enteric nerve fibers have been found in colonic biopsies from patients with IBS and have been associated with the severity of symptoms[11,38,79]. Mast cells are thought to be key players in intestinal mucosal inflammation[79]. Their degranulation causes the release of inflammatory mediators (histamine, serotonin, and proteases), resulting in lymphocyte activation and cytokine imbalance[80]. Patients with IBS were found to have higher levels of proinflammatory interleukin (IL)-6, IL-8, IL-1β, and tumor necrosis factor-α (TNF-α) and lower levels of anti-inflammatory IL-10 in both serum and the intestinal mucosa[81,82]. These changes result in altered pain thresholds and visceral hypersensitivity[38,83]. In addition, mast cell degranulation has been shown to reduce the expression of tight junction proteins, probably through tryptase release[13]. Apart from mast cells, increased numbers of eosinophils and intraepithelial lymphocytes have been observed in colonic biopsies from patients with IBS[11,79].

Gut bacteria play an important role in the modulation of the immune response. For example, butyrate produced by members of the phylum *Firmicutes*[25] induces the differentiation of regulatory T cells[29,84], thereby preventing an excessive immune response and autoimmunity[22,85]. Furthermore, *Lactobacilli* spp. metabolize dietary tryptophan into indole-3-aldehyde, which acts as an aryl hydrocarbon receptor (AHR) ligand[85]. AHR is a ligand-activated transcription factor that is expressed by immune cells and regulates the number of intraepithelial lymphocytes and IL-22 production[86]. Probiotic strains, such as *Lactobacillus rhamnosus*, *Lactobacillus casei,* and *Bifidobacterium breve,* were shown to induce IL-4 and IL-10 production, whereas *L. reuteri* and *L. plantarum* were found to downregulate the expression of TNF-α[87,88].

The importance of the interaction between the gut microbiota and host immune system in IBS is highlighted by a number of studies in patients with postinfectious IBS, indicating activation of the gastrointestinal immune system after acute gastroenteritis[89,90]. Moreover, animal studies have shown that stress-induced changes in the gut microbiota are associated with altered immune response and increased susceptibility to enteric pathogens[91,92].

Microbiota and intestinal barrier integrity

Intestinal epithelial barrier integrity is of great importance for gut homeostasis, as it prevents the translocation of luminal antigens to the mucosa, thus averting the development of low-grade mucosal inflammation in the gut wall (Figure 2).



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Figure 2 Microbiota and intestinal barrier integrity. The intestinal barrier plays an essential role in maintaining host homeostasis. It is mainly composed of the mucus layer, the epithelial layer, and the underlying lamina propria. Intestinal epithelial cells are tightly attached to each other by junctional complexes. Tight junctions (TJs) are composed of several proteins, including occludin, claudins, zonula occludens (ZOs), and junctional adhesion molecules (JAMs), which interact with each other, as well as with the cytoskeleton. The adherence junction is composed of the nectin-afadin system and the E-cadherin-catenin system. Intestinal epithelial barrier integrity prevents the translocation of bacteria and luminal antigens to the mucosa, thus averting their interaction with the host immune system and the development of low-grade mucosal inflammation in the gut wall. TJ: Tight junctions; AJ: Adherence junction; JAM: Junctional adhesion molecules.

An increased density of epithelial gaps has been shown by electron microscopy in gut biopsies of patients with IBS[93]. Furthermore, histological examination of colonic biopsies revealed decreased expression of tight junction proteins, such as occludin; claudins 1, 3, and 5; and zonula occludens-1[13,36,82,93]. Increased serum levels of anti-flagellin antibodies in patients with IBS further support the substantial role of intestinal permeability in the pathogenesis of IBS[94].

The gut microbiota is an important determinant of intestinal epithelial barrier integrity. In particular, certain gut bacteria, such as *Bacteroides thetaiotaomicron, Faecalibacterium prausnitzii,* and *Ruminococcus* spp., were shown to affect the mucus layer thickness and composition[1,22,31]. Moreover, SCFAs, which are produced predominantly by members of the genera *Eubacterium, Clostridium, Ruminococcus,* and *Faecalibacterium,* have been demonstrated to augment the expression of claudins 3 and 4 and occludin. Polyamines (putrescine, spermidine, and spermine), which are produced by certain species within the *Clostridium, Enterococcus, Streptococcus,* and *Lactobacillus* genera, have been shown to stimulate the production of E-cadherin and zonula occludens-1[95]. There is also evidence that probiotic strains of *Bifidobacterium* and *Lactobacillus* promote intestinal barrier function and prevent bacterial translocation [32,96].

Most likely, the preservation of the optimal composition of the microbiota (*e.g.*, a sufficient number of SCFA producers) may serve as a factor preventing the development of IBS.

GUT MICROBIAL COMPOSITION IN PATIENTS WITH IBS

A considerable amount of literature has been published on the compositional changes of the gut microbiota in patients with IBS. Although data from these studies are inconsistent and even conflicting, some common features can be found (Table 2). The discrepancy in findings is possibly due to differences in the population studied (*e.g.*, age, lifestyle, initial microbiota composition, prior antibiotic and/or probiotic use, and diagnostic criteria for IBS) and methodological issues, such as study design and methods for microbiota assessment and data analysis.

The majority of authors report decreased microbial diversity in patients with IBS[97-101]. Furthermore, a substantial number of studies have shown a lower abundance of butyrate-producing

Table 2 Compositional changes in gut microbiota in patients with irritable bowel syndrome (common threads)

Ref.	Subjects	Method	Specimen	Diversity	*Faecalibacterium*	*Enterobacteriaceae*	*Bifidobacterium*	*Lactobacillus*
Dior *et al* [145], 2016	IBS-D (*n* = 16), IBS-C (*n* =15), Controls (*n* = 15)	Real-time PCR	Stool	No data	–	↑ in IBS-D (*Escherichia*)	↑ in IBS-C	–
Ringel-Kulka *et al* [108], 2016	IBS (*n* = 56), Controls (*n* = 20)	16S rRNA	Stool	No data	–	–	–	↑
Maharshak *et al*[102], 2018	IBS-D (*n* = 23), Controls (*n* = 24)	16S rRNA	Stool	↓[1]	↓	↑ (*unclassified genus*)	–	–
			Colonic biopsy	–[1]	–	–	–	↑
Gobert *et al* [146], 2016	IBS-C (*n* = 33), Controls (*n* = 58)	16S rRNA	Stool	No data	–	↑	↓	–
Shukla *et al* [105], 2015	IBS (*n* = 47), Controls (*n* = 30)	16S rRNA; real-time PCR	Stool	No data	–	–	↓	–
Su *et al* [107], 2018	IBS-D (*n* = 40), Controls (*n* = 20)	16S rRNA; real-time PCR	Stool	No data	–	–	↓	↓
Zhuang *et al* [109], 2018	IBS-D (*n* = 30), Controls (*n* = 13)	16S rRNA	Stool	–[2]	–	–	–	↓
Zhong *et al* [147], 2019	IBS-D (*n* = 20), Controls (*n* = 16)	FISH	Colonic biopsy	No data	–	↑ (*E. coli*)	↓	–
Jeffery *et al* [100], 2020	IBS (*n* = 80), Controls (*n* = 65)	16S rRNA, shotgun sequencing	Stool	↓[2]	–	–	–	–
Rangel *et al* [148], 2015	IBS (*n* = 33), Controls (*n* = 16)	Microarray analysis	Stool	↓[2]	↓ (*F. prausnitzii*)	–	–	–
			Colonic biopsy	–[2]	–	–	–	–

[1]Rarefaction analysis.

[2]Shannon diversity index.

↓: Decreased abundance; ↑: Increased abundance; –: No significant differences found; IBS: Irritable bowel syndrome; IBS-D: Diarrhea-predominant irritable bowel syndrome; IBS-C: Constipation-predominant irritable bowel syndrome; FISH: Fluorescence *in situ* hybridization; *E. coli*: *Escherichia coli*; *F. prausnitzii*: *Faecalibacterium prausnitzii*.

bacteria from the genus *Faecalibacterium*, mainly *F. prausnitzii*,[97,98,102,103] as well as an increase in the abundance of the *Enterobacteriaceae* family, including pathogens such as *Escherichia coli* and *Enterobacter* spp.[98,104-106]. Moreover, patients with IBS were found to have a reduced prevalence of *Bifidobacterium*, providing a range of beneficial properties to the host[98,103,104,106,107]. Significant differences in *Lactobacillus* numbers were also observed between patients with IBS and healthy controls, but the findings of different studies were not consistent. Some authors reported an increased amount of *Lactobacillus*[98,99,102,108], while others documented a decrease in the abundance of this commensal[103,104,106,107,109].

Overall, there seems to be some evidence to indicate that patients with IBS have decreased numbers of bacteria contributing to the maintenance of host homeostasis and proper immune response, as well as increased numbers of microbes with proinflammatory properties.

ANTIBIOTICS, GUT MICROBIOTA, AND IBS

Effects of antibiotics on gut microbiota composition

The discovery of antibiotics in the early 20th century was a great milestone in the history of medicine, as

it changed the natural course of most infectious diseases and saved countless lives[110,111]. However, a growing number of studies have shown that inappropriate use of antibiotics promotes the development of antibiotic resistance[112,113]. Furthermore, accumulating evidence indicates that antibiotic exposure in early life increases the risk of obesity and autoimmune and allergic diseases[114-117].

During the past four decades, there has been an increasing interest in the impact of antibiotics on the composition of the gut microbiota. A substantial number of studies in this area were conducted in the 1980s and 1990s and relied on culture-based techniques. However, researchers indicate that up to 80% of gut bacteria are nonculturable[118]. Therefore, the focus has shifted to culture-independent approaches mainly based on 16S rRNA gene sequence analysis.

Extensive research has established that antibiotic treatment induces a dramatic loss of diversity and remarkable shifts in community composition (Table 3), with the time of recovery varying substantially [119-123].

The inconsistency in the results of various studies can be attributed to substantial heterogeneity in sample characteristics (age, ethnicity, diet, *etc.*) and study methodology. Furthermore, antibiotic characteristics, such as their class, pharmacokinetics (absorption and excretion), range of action, and dosing regimen, have been shown to shape the response of the gut microbiota to antibiotic perturbation[124]. For instance, vancomycin is poorly absorbed when administered orally, resulting in high fecal concentrations. Therefore, it significantly alters the composition of the gut microbiota by increasing pathogenic *Proteobacteria*, such as *Klebsiella*, *Escherichia*, and *Shigella*, and decreasing members of the *Bacteroidetes* phylum[122]. Lipophilic antibiotics (*e.g.*, lincosamides and macrolides) are eliminated mainly by biliary excretion and therefore cause profound changes in the intestinal microbiota[125]. For example, treatment with clindamycin resulted in a reduction in microbial diversity and a decrease in *Roseburia, Lachospira, Coprococcus, Dorea,* and *Ruminococcus*. Changes in microbial composition were observed throughout 12 mo after clindamycin exposure[121]. In a recent study conducted by Haak *et al*[123], it was shown that treatment with broad-spectrum antibiotics (ciprofloxacin, vancomycin, and metronidazole) promotes the growth of *Streptococcus* and *Lactobacillus*. Furthermore, the authors found reduced numbers of anaerobes producing SCFAs, such as *Bacteroides*, *Subdoligranulum*, and *Faecalibacterium*. Interestingly, a return toward baseline was observed between 8 and 31 mo, but the composition of the microbiota often remained changed from its initial state.

There is some evidence that antibiotics can indirectly affect the composition of the gut microbiota. This is due to interdependence among different microbial taxa, as they have a variety of shared metabolic pathways[124,126]. Thus, the loss or reduction of certain taxa affects the growth of other members of the community. As an example, vancomycin treatment reduces the number of Gram-negative commensals, although this drug selectively targets Gram-positive bacteria[127].

In a recent systematic review, Zimmerman *et al*[128] summarized data from 129 studies on the effect of antibiotics on the composition of the gut microbiota. The authors concluded that the majority of antibiotics (amoxicillin, amoxicillin/clavulanate, cephalosporins, lipopolyglycopeptides, macrolides, ketolides, clindamycin, tigecycline, quinolones, and fosfomycin) increase the abundance of *Enterobacteriaceae,* mainly *Citrobacter* spp., *Enterobacter* spp., and *Klebsiella* spp. These bacteria contain molecules that directly enhance the inflammatory response of the host and may play a significant role in the alteration of bile acid metabolism[129]. Moreover, expansion of bacteria belonging to the *Enterobacteriaceae* family was associated with inflammatory bowel diseases, both in animal models and in humans [130,131]. Zimmerman *et al*[128] reported that amoxicillin, piperacillin, ticarcillin, cephalosporins (except fifth generation cephalosporins), carbapenems, and lipoglycopeptides facilitate the overgrowth of *Enterococcus,* while treatment with macrolides and doxycycline results in decreased numbers of these bacteria. It has conclusively been shown that piperacillin, ticarcillin, carbapenems, macrolides, clindamycin, and quinolones markedly reduce the abundance of anaerobic bacteria. Finally, the authors documented that the most long-lasting changes in the community structure are caused by ciprofloxacin (1 year), clindamycin (2 years), and clarithromycin plus metronidazole (4 years).

Another negative effect of antibiotic treatment is the loss of colonization resistance. Depletion of beneficial gut commensals, such as *Lachnospiraceae, Ruminococcaceae,* and *Clostridium scindens*, as well as changes in their metabolic activity promote overgrowth of *Clostridium difficile, Enterococcus*, and other pathogens[33,124].

Antibiotics as a risk factor for IBS

Data from large cohort and case-control studies indicate that antibiotics are a risk factor for functional gastrointestinal disorders and IBS in particular. A retrospective study on more than 26000 patients showed that exposure to macrolides and tetracyclines may be associated with the development of IBS [5]. Similarly, a prospective case-control study found that antibiotic treatment of nongastrointestinal infections was associated with the development of IBS [odds ratio (OR) = 2.30; 95% confidence interval (CI): 1.22-4.33; P = 0.01] and other functional gastrointestinal disorders (OR = 1.90; 95%CI: 1.21-2.98; P = 0.005)[6]. A longitudinal study by Krogsgaard *et al*[7] also identified that the use of antibiotics was a predictor for IBS (OR = 1.8; 95%CI: 1.0-3.2). Additionally, a recent meta-analysis showed that the use of antibiotics for infectious enteritis was associated with an increased risk of IBS (OR = 1.69; 95%CI: 1.20-2.37)[8].

Table 3 Effects of antibiotics on gut microbiota composition (based on culture-independent approaches)

Ref.	Method	Antibiotic	Dosing regimen	Diversity	Compositional changes
Pallav *et al* [136], 2014	Pyrosequencing	Amoxicillin	250 mg 3 times daily for 7 d	–[1,2]	↑ *Escherichia, Shigella*
Kabbani *et al* [137], 2017	16S rRNA	Amoxicillin-Clavulanate	875/125 mg twice daily for 7 d	↓[1,3]	↑ *Escherichia, Parabacteroides, Enterobacter* ↓ *Roseburia*
Burdet *et al* [120], 2019	16S rRNA	Ceftriaxone	1 g once daily for 3 d	↓[1,4]	↓ *Firmicutes, Actinobacteria, Bacteroidetes*
Raymond *et al* [135], 2016	Shotgun sequencing	Cefprozil	500 mg twice daily for 7 d	↓[5]	↑*Flavonifractor, Lachnoclostridium, Parabacteroides*, ↓*Bifidobacteriaceae, Coriobacteriaceae, Eubacteriaceae, Oxalobacteraceae, Pasteurellaceae, Veillonellaceae*
Rashid *et al* [121], 2015	Pyrosequencing	Ciprofloxacin	500 mg twice daily for 10 d	↓[1]	↑ *Bacteroides*↓ *Faecalibacterium, Alistipes,* unculturable *Ruminococcaceae*
		Clindamycin	150 mg 4 times daily for 10 d	↓[1]	↓ *Roseburia, Lachospira, Coprococcus, Dorea, Ruminococcus*
Isaac *et al*[122], 2017	16S rRNA	Vancomycin	250 mg *per os* 4 times daily for 2 wk	↓[1,4]	↑ *Escherichia, Shigella, Klebsiella,* ↓ *Bacteroidetes, Faecalibacterium, Ruminococcus*

[1]OTU analysis.
[2]Rarefaction analysis.
[3]Chao1 index.
[4]Shannon index.
[5]Simpson index.

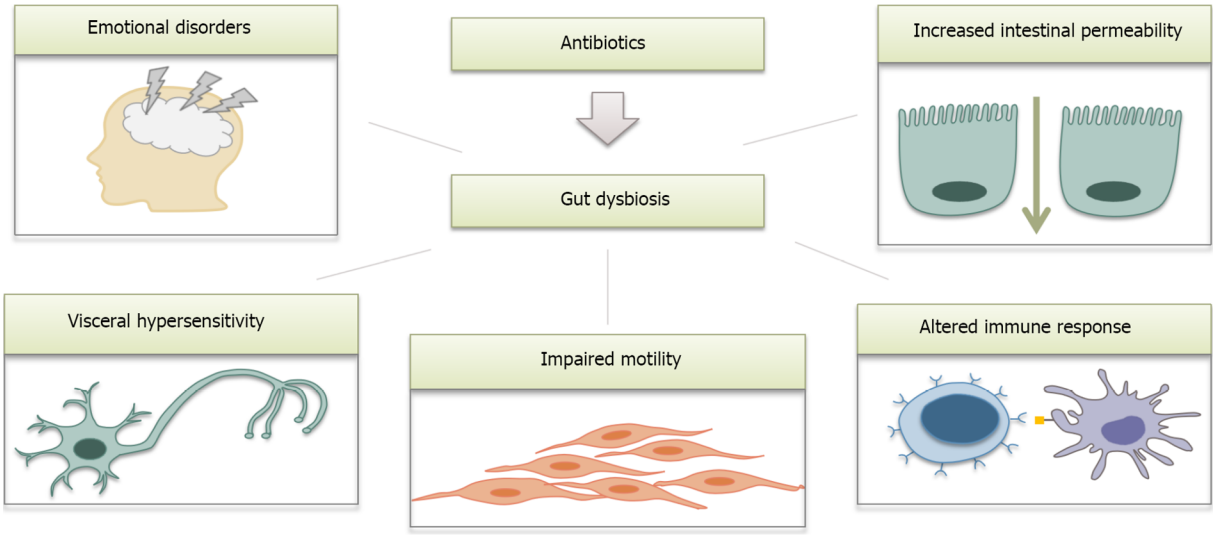
However, nonabsorbable antibiotics can be used to treat IBS. In a double-blind, randomized, placebo-controlled study, treatment with neomycin resulted in a 35% improvement in composite scores of IBS symptoms, compared with only 11% for placebo ($P < 0.05$)[132]. Nonetheless, the use of this antibiotic is limited by the risk for *C.difficile* infection and systemic adverse events. A recent meta-analysis of four studies and 1803 patients showed that rifaximin was more effective than placebo in the overall improvement of IBS symptoms (OR = 1.19; 95%CI: 1.08-1.32 and OR = 1.36; 95%CI: 1.18–1.58, respectively, $P < 0.05$ for both). There was no difference in adverse events between rifaximin and placebo[133]. Due to its safety, rifaximin was approved by the Food and Drug Administration for the treatment of IBS-D.

Similarities in gut microbiota between patients with IBS and those after antibiotic exposure

Analysis of data on changes in the gut microbiota in patients with IBS and those after antibiotic exposure uncovers some common features and trends. For instance, decreased microbial diversity[97-99,121,128] and a reduction in the abundance of *Faecalibacterium,* particularly *F. prausnitzii*[97,98,102,121,122], have been observed in both cases. *F. prausnitzii* is one of the most abundant bacterial species in the gut, exhibiting anti-inflammatory effects through inhibition of IL-8 production, promotion of IL-10 secretion, and upregulation of regulatory T cells[134]. Moreover, patients with IBS were shown to have reduced numbers of *Bifidobacterium*[98,103,104,106,107]. Likewise, several studies have reported a decreased abundance of these commensals after antibiotic exposure[121,128,135]. Most members of the genus *Bifidobacterium* are known to exert beneficial effects on host health, including competitive exclusion of enteric pathogens, metabolism of dietary compounds, and regulation of the immune response[22,26,33]. Furthermore, both IBS and antibiotic exposure are characterized by overgrowth of *Enterobacteriaceae*[98,104,106,136,137]. The *Enterobacteriaceae* family includes pathogenic bacteria (*e.g., Escherichia, Shigella, Klebsiella,* and *Enterobacter)* with proinflammatory properties that may contribute to low-grade inflammation in the gut wall[98].

CONCLUSION

There is clear and consistent evidence from a variety of studies that patients with IBS have altered composition of the gut microbiota and that these alterations are related to the generation of gastrointestinal symptoms. However, studies comparing fecal microbiota in patients with IBS and healthy controls produced variable findings. To date, there is still no consensus on distinct microbiome signatures in IBS. Although some common threads reviewed here were found, prospective large-scale studies need to be carried out to shed light on this issue. Independent analysis of the gut microbiota and its metabolites will help to develop novel microbiota-based treatment strategies that target the



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Figure 3 Possible link between antibiotic use and the development of irritable bowel syndrome (schematic illustration). Antibiotics cause profound changes in the gut microbiota and therefore contribute to all mechanisms involved in the pathogenesis of irritable bowel syndrome.

underlying pathophysiology of IBS rather than focusing on symptom alleviation.

A number of recent studies have addressed the effects of antibiotics on gut microbiota composition, and these effects were found to be quite similar to those observed in IBS. We suggest that the Rome V criteria could provide a new definition of postantibiotic IBS. As major disruptors of the gut microbiota, antibiotics seem to contribute to all aspects of IBS pathogenesis (Figure 3). However, further research in this area is definitely warranted.

FOOTNOTES

Author contributions: Mamieva Z and Poluektova E took the lead in writing the manuscript; Svistushkin V and Sobolev V contributed to interpreting the relevant literature, provided critical feedback, and helped shape the manuscript; Shifrin O, Guarner F, and Ivashkin V substantially contributed to the conception and design of the article and revised the paper; all authors read and approved the final manuscript.

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Country/Territory of origin: Russia

ORCID number: Zarina Mamieva 0000-0002-5673-7920; Elena Poluektova 0000-0002-9038-3732; Valery Svistushkin 0000-0001-7414-1293; Vasily Sobolev 0000-0002-7372-3299; Oleg Shifrin 0000-0001-8148-2862; Francisco Guarner 0000-0002-4051-0836; Vladimir Ivashkin 0000-0002-6815-6015.

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MINIREVIEWS

Emerging role of colorectal mucus in gastroenterology diagnostics

Hesam Ahmadi Nooredinvand, Andrew Poullis

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Hesam Ahmadi Nooredinvand, Andrew Poullis, Department of Gastroenterology, St George's Hospital, London SW17 0QT, United Kingdom

Corresponding author: Andrew Poullis, BSc, FRCP, MBBS, MD, Doctor, Department of Gastroenterology, St George's Hospital, Blackshaw Road, Tooting, London SW17 0QT, United Kingdom. andrew.poullis@stgeorges.nhs.uk

Abstract

Colonoscopy is currently the gold standard for diagnosis of inflammatory bowel disease (IBD) and colorectal cancer (CRC). This has the obvious drawback of being invasive as well as carrying a small risk. The most widely used non-invasive approaches include the use of faecal calprotectin in the case of IBD and fecal immunochemical test in the case of CRC. However, the necessity of stool collection limits their acceptability for some patients. Over the recent years, there has been emerging data looking at the role of non-invasively obtained colorectal mucus as a screening and diagnostic tool in IBD and CRC. It has been shown that the mucus rich material obtained by self-sampling of anal surface following defecation, can be used to measure various biomarkers that can aid in diagnosis of these conditions.

Key Words: Colorectal mucus; Inflammatory bowel disease; Crohn's disease; Ulcerative colitis; Colorectal cancer; Faecal calprotectin

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Core Tip: We now know that non-invasively collected colorectal mucus contains diagnostically informative cells that can be analysed to look for various biomarkers. The presence of some of these biomarkers have the potential role in diagnosis of inflammatory bowel disease and colorectal cancer. This is an exciting field that we believe is worth exploring further.

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INTRODUCTION

The current gold standard for the diagnosis of inflammatory bowel disease (IBD) and colorectal cancer (CRC) is ileocolonoscopy. This however is time consuming, expensive and carries a small risk. Unnecessary colonoscopies could be avoided if reliable non-invasive tests were available to diagnose these conditions.

Non-invasive approaches to measurement of inflammation include serum biomarkers such as C-reactive protein (CRP) although this is not bowel specific. Stool calprotectin is bowel specific and is the best studied test[1] however necessity of collecting stool makes this unpopular with some patients[2-4].

In adults, a faecal calprotectin of > 50 μg/g has a sensitivity and specificity of 93% and 94% respectively for distinguishing between irritable bowel syndrome (IBS) and IBD[1]. While faecal calprotectin has an established position in helping to guide the need for ileocolonoscopy in those with bowel symptoms and the follow up of established IBD, it is not in itself a specific diagnostic test.

For CRC, fecal immunochemical test (FIT) is the most widely used non-invasive screening tool with a sensitivity and specificity of 79% and 94% respectively[5]. Similar to faecal calprotectin, an abnormal FIT prompts further investigations and assessment but is not a diagnostic test.

EMERGING ROLE OF COLORECTAL MUCUS

Over the recent years there has been emerging data looking at the use of non-invasive colorectal mucus sampling as a screening and diagnostic tool for IBD as well as CRC.

Colorectal mucus acts as an interface between colonic mucosa and gut content and is a recipient of cells released from the mucosal surface[6]. Some of these cells embedded in the colorectal mucus are excreted with faeces. This cell containing colorectal mucus can therefore be used for specific cell and biomarker detection[7-9]. There is increasing understanding of the complex roles of the colorectal mucus. The mucus itself is a dilute, aqueous and viscoelastic secretion with specific proteins, the mucins, being the major component[10]. The role, composition, physiology and pathophysiology are outside the scope of this review and have recently been reviewed elsewhere[11]. This review will focus on the emerging role of colorectal mucus in diagnostics.

Colorectal mucus can be obtained during proctoscopy[7,8,12] however this has the obvious drawback of being invasive and therefore unfavorable as a screening tool. Up until recently there was no reliable non-invasive method for colorectal mucus sampling. Over the past few years, a novel technique has been developed based on self-sampling of mucus-rich material from the anal surface immediately following defecation.

The use of colorectal mucus as a source of biomarkers has been evaluated in several studies. In the setting of IBD diagnostics and monitoring and CRC diagnostics colorectal mucus has been shown to be a novel and useful medium.

The first study assessing non-invasively collected colorectal mucus compared 141 patients (58 patients with IBD, 50 patients with IBS and 33 healthy volunteers). The study participants were instructed to swab the external anal area immediately following defecation and samples were collected for cytological and Mucin 2 (MUC2) analysis. This was the first study ever to describe non-invasively collected cytology demonstrating large numbers of preserved inflammatory cells in IBD. Significant differences in MUC2 levels were identified in IBD *vs* non-IBD groups raising the possibility that colorectal mucus was a useful diagnostic medium[9].

In a follow on study the performance of several biomarkers including calprotectin, eosinophil-derived neurotoxin (EDN) and protein S100A12 was evaluated in active IBD[13]. EDN is a major secretory protein of eosinophils and elevated levels of EDN have previously been detected in faeces of IBD patients[8]. S100A12 is another granulocyte protein and it has previously been proposed that measurement of the level of S100A12 in the faeces can help differentiate IBD from IBS[14,15].

The authors found that the medium concentration of all of these biomarkers were significantly higher in IBD patients compared to inflammation free individuals with calprotectin and EDN being more sensitive than S100A12 in detecting patients with IBD. Using a combined test taking into account the result of calprotectin and EDN (designated as CALEDN) resulted in sensitivity and specificity of 91% and 89% respectively in detecting patients with IBD (Figure 1). The concentration of all of these biomarkers was significantly higher in ulcerative colitis (UC) patients than those with Crohn's disease (CD) with the most pronounced difference seen for EDN. Over a follow up period of 30 d following treatment there was a steady decrease of all biomarker levels among patients who demonstrated clinical improvement however correlation was easier to detect in UC patients[13].

Further to the description of inflammatory cells in the colorectal mucus of IBD patients an additional follow on cytology study demonstrated that true diagnostic cytology could be carried out on non-invasively collected colorectal mucus. Using cytology and immunocytochemistry a number of different cell types were identified (neutrophils, plasma cells and erythrophagocytes). Blinded cytological analysis enabled an accurate diagnosis to be made in 61.8% of cases[16].

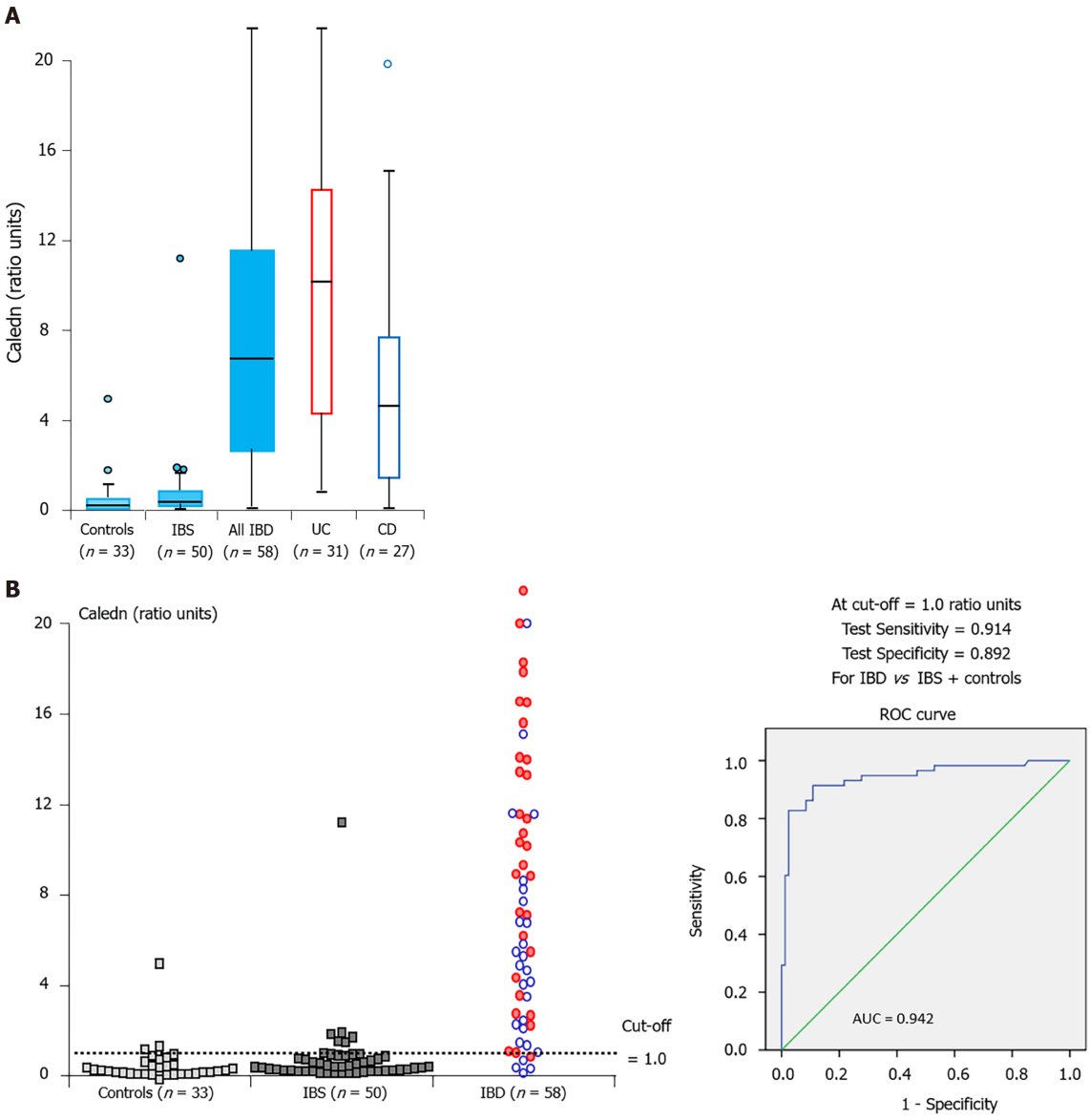


Figure 1 Box and whisker plot and individual result distributions and receiver operating characteristic curve for the combined CALEDN test at stage 1 of the study. In the panel within the inflammatory bowel disease group, blue and red circles correspond to Crohn's disease and ulcerative colitis cases, respectively. AUC: Area under the curve; IBS: Irritable bowel syndrome; CD: Crohn's disease; UC: Ulcerative colitis. Citation: Loktionov A, Chhaya V, Bandaletova T, Poullis A. Inflammatory bowel disease detection and monitoring by measuring biomarkers in non-invasively collected colorectal mucus. *J Gastroenterol Hepatol* 2017; 32: 992-1002. Copyright© The Authors 2017. Published by John Wiley and Sons. A: Box and whisker plot; B: Receiver operating characteristic curve.

Given the success of this new medium in the diagnosis and monitoring of IBD attention has turned to CRC diagnostics. A pilot study was performed looking at whether various biomarkers detected in the colorectal mucus can be reliably used to aid detection of CRC. The diagnostic performance of 24 biomarkers were evaluated. 17 CRC and 35 healthy controls were used to assess these biomarkers. Quantification of haemoglobin, tissue inhibitor of metalloproteinase 1 (TIMP1), M2-pyruvate kinase (M2-PK), peptidyl arginine deiminase-4 (PADI4), CRP, matrix metalloproteinase 9 (MMP9), epidermal growth factor receptor (EGFR), EDN and calprotectin all showed good diagnostic potential[17].

A larger follow on study with 35 healthy volunteers, 62 CRC-free symptomatic patients and 40 CRC patients was conducted to assess the utility of these biomarkers collected in colorectal mucus[18].

The sensitivity and specificity of each of these biomarkers was analyzed in two different scenarios. For assessment of these markers in bowel cancer screening (BCS) comparison was made between the CRC group and healthy controls "screening" arm. A "triage" arm to the study was set up to reflect non-BCS clinical practice and a comparison between CRC cases and symptomatic CRC free patients. Hemoglobin was the best performer with overall sensitivity of 80% and specificity of 94% and 85.5% in the 'screening' and the 'triage' group respectively. These values are comparable to those reported for CRC screening by FIT test[19]. All other biomarkers had a lower sensitivity and specificity especially in the triage group. Of note proximal CRC was associated with higher numbers of false-negative results.

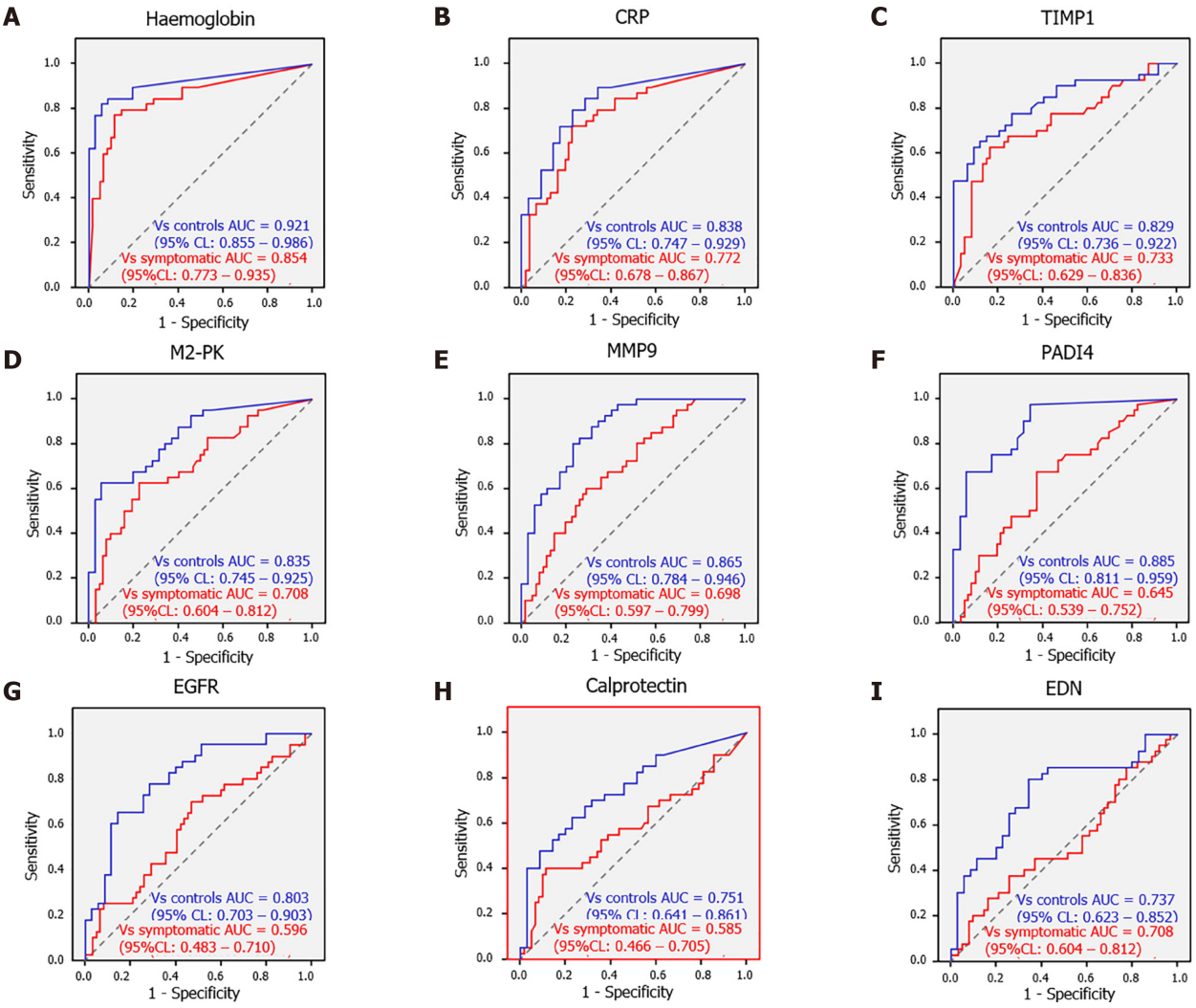


Figure 2 Receiver operating characteristic curves for 'screening' (blue) and 'triage' (red) settings for haemoglobin, C-reactive protein, tissue inhibitor of metalloproteinase 1, M2-pyruvate kinase, matrix metalloproteinase 9, peptidyl arginine deiminase-4, epidermal growth factor receptor, calprotectin and eosinophil-derived neurotoxin. A: Haemoglobin; B: CRP; C: TIMP1; D: M2-PK; E: MMP9; F: PADI4; G: EGFR; H: calprotectin; I: EDN. Citation: Loktionov A, Soubieres A, Bandaletova T, Francis N, Allison J, Sturt J, Mathur J, Poullis A. Biomarker measurement in non-invasively sampled colorectal mucus as a novel approach to colorectal cancer detection: screening and triage implications. *Br J Cancer* 2020; 123: 252-260. Copyright© The Authors 2020. Published by John Wiley and Sons.

EGFR, calprotectin and EDN were worst performing and could not be recommended as reliable diagnostic markers (Table 1 and Figure 2).

Through the use of a questionnaire we found that the method of colorectal mucus sampling was generally very well accepted by the patients arguing that non-invasive testing of colorectal samples for haemoglobin can present an attractive alternative to FIT. The average time taken for carrying out the sampling was about six minutes. Given the immunochemical colorectal mucus sample testing for haemoglobin differs very little from faecal sample testing and the cost being similar to that of FIT, this alternative may boost participation rates.

CONCLUSION

These studies demonstrate that diagnostically informative cells can be obtained from colorectal mucus which can be non-invasively obtained by simply swabbing the external anal area immediately following defecation. The proposed method of colorectal mucus sample collection eliminates the necessity of stool collection and appeared to be very well accepted by the study participants with the vast majority of samples being suitable for biomarker and cytological analysis.

Future work however needs to focus on direct head-to-head comparison between stool calprotectin and colorectal mucus calprotectin in the case of IBD and colorectal mucus haemoglobin concentration and FIT test for colonic cancer screening.

Table 1 Comparison of tested colorectal mucus biomarker performance for colorectal cancer detection versus groups of asymptomatic control subject and patients with abdominal symptoms (based upon receiver operating characteristic curve analysis)

Biomarker	Optimal cut-off level	Sensitivity (%)	AUC *vs* Sympt. Pat-s (95%CI)	Specificity *vs* Sympt. Pat-s (%)	AUC *vs* Control (95%CI)	Specificity *vs* Control (%)	Median biomarker level (CRC)	Median biomarker level (Sympt. Pat-s)	Median biomarker level (Control)
Haemoglobin	109.27 ng/mL	80.00	0.85 (0.77-0.93)	88.55	0.92 (0.85-0.99)	94.29	1708.74 ng/mL[a,b]	0.00 ng/mL[a,c]	0.00 ng/mL[b,c]
CRP	8.90 ng/mL	72.50	0.77 (0.68-0.87)	75.81	0.84 (0.75-0.93)	80.00	22.09 ng/mL[d,e]	1.41 ng/mL[d]	0.00 ng/mL[e]
TIMP1	3.25 ng/mL	67.50	0.73 (0.63-0.84)	75.81	0.83 (0.74-0.92)	85.71	8.26 ng/mL[f,g]	1.42 ng/mL[f,h]	0.71 ng/mL[g,h]
M2-PK	9.00 U/mL	62.50	0.71 (0.60-0.81)	77.42	0.83 (0.74-0.92)	91.43	11.98 U/mL[i,j]	2.97 U/mL[i,k]	0.57 U/mL[j,k]
MMP9	10.38 ng/mL	65.00	0.70 (0.60-0.80)	64.52	0.86 (0.78-0.95)	82.86	20.93 ng/mL[l,m]	6.37 ng/mL[l,n]	0.44 ng/mL[m,n]
PADI4	1.16 ng/mL	67.50	0.64 (0.54-0.75)	62.90	0.88 (0.81-0.96)	94.29	1.51 ng/mL[o,p]	0.88 ng/mL[o,q]	0.00 ng/mL[p,q]
EGFR	305.52 pg/mL	60.00	0.60 (0.48-0.71)	58.06	0.80 (0.70-0.90)	88.57	342.72 pg/mL[r]	187.01 pg/mL[s]	67.72 pg/mL[r,s]
Calprotectin	3.38 μg/mL	57.50	0.58 (0.47-0.70)	56.45	0.75 (0.64-0.86)	80.00	4.01 μg/mL[t]	2.94 μg/mL[u]	0.59 μg/mL[t,u]
EDN	12.83 ng/mL	45.00	0.52 (0.40-0.64)	62.90	0.74 (0.62-0.85)	88.57	8.04 ng/mL[v]	10.10 ng/mL[w]	2.85 ng/mL[v,w]

P value for CM biomarker level comparisons between study groups.
[a,b,d,e,g,j,m,p,q,r]$P < 0.00001$.
[c]$P = 0.01878$.
[f]$P = 0.00008$.
[h]$P = 0.0264$.
[i]$P = 0.00040$.
[k]$P = 0.01352$.
[l]$P = 0.00078$.
[n]$P = 0.00288$.
[o]$P = 0.01428$.
[s,t]$P = 0.00020$.
[u]$P = 0.00042$.
[v]$P = 0.00044$.
[w]$P = 0.00064$.
CRP: C-reactive protein; TIMP1: Tissue inhibitor of metalloproteinase 1; M2-PK: M2-pyruvate kinase; MMP9: Matrix metalloproteinase 9; PADI4: Peptidyl arginine deiminase-4; EGFR: Epidermal growth factor receptor; EDN: Eosinophil-derived neurotoxin; AUC: Area under the curve.

The non-invasive collection of colorectal mucus is a novel and exciting area of gastrointestinal diagnostics and may dramatically change our approach to the investigation and diagnosis of colorectal diseases.

FOOTNOTES

Author contributions: Nooredinvand HA drafted the manuscript; Poullis A proofread and revised the manuscript.

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Country/Territory of origin: United Kingdom

ORCID number: Hesam Ahmadi Nooredinvand 0000-0003-3645-2431; Andrew Poullis 0000-0003-0703-0328.

Corresponding Author's Membership in Professional Societies: British Society of Gastroenterology.

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MINIREVIEWS

Similarities, differences, and possible interactions between hepatitis E and hepatitis C viruses: Relevance for research and clinical practice

Nadia Marascio, Salvatore Rotundo, Angela Quirino, Giovanni Matera, Maria Carla Liberto, Chiara Costa, Alessandro Russo, Enrico Maria Trecarichi, Carlo Torti

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Nadia Marascio, Angela Quirino, Giovanni Matera, Maria Carla Liberto, Department of Health Sciences, Unit of Microbiology, University "Magna Graecia" of Catanzaro, Catanzaro 88100, Italy

Salvatore Rotundo, Chiara Costa, Alessandro Russo, Enrico Maria Trecarichi, Carlo Torti, Department of Medical and Surgical Sciences, Unit of Infectious and Tropical Diseases, "Magna Graecia" University of Catanzaro, Catanzaro 88100, Italy

Corresponding author: Carlo Torti, FRSM, Professor, Department of Medical and Surgical Sciences, Unit of Infectious and Tropical Diseases, "Magna Graecia" University of Catanzaro, Loc. Germaneto, Viale Europa, Catanzaro 88100, Italy. torti@unicz.it

Abstract

Hepatitis E virus (HEV) and hepatitis C virus (HCV) are both RNA viruses with a tropism for liver parenchyma but are also capable of extrahepatic manifestations. Hepatitis E is usually a viral acute fecal-oral transmitted and self-limiting disease presenting with malaise, jaundice, nausea and vomiting. Rarely, HEV causes a chronic infection in immunocompromised persons and severe fulminant hepatitis in pregnant women. Parenteral HCV infection is typically asymptomatic for decades until chronic complications, such as cirrhosis and cancer, occur. Despite being two very different viruses in terms of phylogenetic and clinical presentations, HEV and HCV show many similarities regarding possible transmission through organ transplantation and blood transfusion, pathogenesis (production of antinuclear antibodies and cryoglobulins) and response to treatment with some direct-acting antiviral drugs. Although both HEV and HCV are well studied individually, there is a lack of knowledge about coinfection and its consequences. The aim of this review is to analyze current literature by evaluating original articles and case reports and to hypothesize some interactions that can be useful for research and clinical practice.

Key Words: Hepatitis C virus; Hepatitis E virus; Co-infection; Genomic variability; Extrahepatic diseases; Vaccine

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Core Tip: Hepatitis E virus (HEV) and Hepatitis C virus (HCV) are both RNA viruses characterized by greater variability than DNA viruses and mainly infect the liver. Despite these similarities, the two viruses have different species barriers and disease progression. Coinfection with particular HCV and HEV types could aggravate hepatic and/or extrahepatic diseases, taking into account virus–host interactions between the two viruses during viral replication.

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INTRODUCTION

Viral hepatitis is a global public health problem, affecting more than 325 million people globally. In countries with poor health care standards, coinfection among hepatotropic viruses is possible due to multiple risk factors. This condition increases morbidity and mortality rates in infected patients[1]. Hepatitis E virus (HEV) could influence hepatic or extrahepatic symptoms in patients with chronic hepatitis C virus (HCV) infection[2,3]. Both the prevalence and spreading of HEV and HCV infections worldwide reflect different routes of transmission and high genomic variability[4,5], however coinfections or superinfections with the two viruses in the same individuals may occur, though a paucity of data exist in this respect.

A summary of virological and pathogenic characteristics of both viruses discussed through the text of this review are reported in the Table 1.

EPIDEMIOLOGY

HEV affects around 20 million people worldwide, and the infection is distributed in both developing and industrialized countries[1]. This enteric non-enveloped virus, belonging to the *Hepeviridae* family, *Orthohepevirus* genus, is classified into eight genotypes and 24 subtypes. HEV1 and HEV2 infect only humans in resource limited settings, such as Asia, Mexico, and sub-Saharan and Central Africa[6]. HEV3, emerging in Europe as a sporadic infection, and HEV4 infect both humans and animals. HEV4 shows a high prevalence in Asia[7]. In 2014, HEV5 and HEV6 were isolated from wild boars, while HEV7 (originally infecting dromedaries) was isolated from a human case for the first time[8,9]. Lastly, HEV8 was detected in Bactrian camels[10]. Of note, the nomenclature system of this virus is constantly changing due to frequent identification of novel strains in various animal species[11]. The main routes of transmission are fecal-oral and zoonotic (*i.e.*, undercooked meat or close contact with animals). In industrialized countries, transmission is related to travelers returning from endemic areas and to blood transfusion or organ transplantation[12,13]. Human-to-human transmission was also described in men having sex with men[14], as well as HEV can infect newborns by vertical transmission[15]. Sero-prevalence studies identified specific risk categories, such as veterinarians, forestry workers, butchers and hunters, occurring as sporadic cases of infection[16].

HCV is also very widely disseminated throughout the world. Indeed, approximately 71 million people worldwide are infected by HCV, an enveloped virus belonging to the *Flaviviridae* family and *Hepacivirus* genus. In 2018, Borgia and colleagues identified the eighth genotype in patients from India [17]. The distributions of the genotypes and 86 subtypes are related to risk factors and geography across the world. In developing countries, HCV1 and HCV2 with high subtype diversity are prevalent. HCV3 is predominant in Europe, North America and Southeast Asia. In the Middle East and Central Africa, HCV4 is endemic, while HCV5 was found exclusively in South Africa[18]. HCV6 is present essentially in Japan and nearby areas. HCV7 is responsible for less than 1% of cases of HCV hepatitis. In industrialized countries, the most prevalent subtypes are HCV1a, 1b, 2c, 3a, and 4a[19,20]. HCV1b and 2c are mainly transmitted by blood transfusion and infect older population groups, whereas HCV1a, 3a and 4a are prevalent in intravenous drug users[21,22]. Low standards for healthcare procedures have allowed HCV spreading among patients in hemodialysis units[23]. After 1992, blood screening controlled the spread of this infection. Sexual and mother-to-infant (6%) transmissions increased in subjects coinfected with human immunodeficiency virus (HIV), while breastfeeding does not significantly increase the risk of transmission from mother to baby[24].

Table 1 Similarities, differences and potential interactions across the major points of hepatitis E virus and hepatitis C virus infections

Categories	Similarity	Difference	Interaction
Epidemiology	High prevalence in developing countries[1]	HEV infects humans and animals[6,7]	Co-infections or superinfections[2,3]
Genetic variability	RNA viruses (*quasispecies*), genotype classification, recombination events[4,5]	Replication rate, HEV has non-enveloped or quasi-enveloped virions [12,26]	None
Pathogenesis	Disease progression in immunocompromised patients[32,54]	Microbiota alteration, hepatic severity [23,33,83,84]	HEV could influence hepatic or extrahepatic symptoms in patients with chronic HCV infection [2,3]
Treatment	Choice of therapy[35,50]	PEG-IFN-α and RBV are still the therapies of choice for HEV[35]	DAA therapy can be effective against both viruses[50,53]
Prevention	Public health measures[58]	Vaccine availability[60,62]	Improvement of screening policies[35,50]

DAA: Directly acting antivirals; PEG-IFN: Pegylated interferon; RBV: Ribavirin; HEV: Hepatitis E virus; HCV: Hepatitis C virus.

GENETIC VARIABILITY

RNA viruses have high genetic plasticity, and they can rapidly generate a drug-resistant viral population or evade the host system under pressure. The key of this variability is the polymerase without proofreading activity[25]. During viral replication with a mutation rate ranging from 10^{-6} to 10^{-4} substitutions *per* nucleotide, the virus produces hundreds of progeny (*quasispecies*), which differ by one or a few nucleotides in the genomic sequence. The fitness of *quasispecies* reflects Darwinian evolution and natural selection allows the spread of a better adapted viral population[26]. HEV and HCV are both positive-sense single-stranded (ss) RNA viruses, even if the organization and length of the genome are different.

The HEV genome (7.2 kb) contains three open reading frames (ORFs) between the 5′UTR- and 3′-UTR (polyA-tract) regions. ORF1 encodes enzymes, including RNA-dependent RNA polymerase (RdRp) and non-structural proteins. ORF2 and ORF3 encode for capsid protein and a multifunctional phosphoprotein, respectively. ORF4 is directly involved during replication[27]. By contrast, the HCV genome (9.6 kb), containing one ORF between the 5′-3′UTRs, encodes three structural (C, core) proteins, envelope glycoproteins 1 and 2 (E1 and E2), and finally seven non-structural (p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B) proteins. In particular, NS5B encodes the polymerase enzyme[28].

HEV genetic characteristics make it suitable for infecting humans and animals through various transmission routes, since it is maintained in the environment[12]. Virions are present in two different forms, non-enveloped excreted in the feces of humans or animals and quasi-enveloped coming from blood. Quasi-enveloped virions bind cells in a less effective way, showing minor infectivity[12]. The high similarity among HEV3 and HEV4 strains isolated from humans and animals demonstrated that adaptation is not necessary for infection. On the other hand, HEV1 does not have zoonotic reservoirs, as experimentally, intra-species transmission failed to infect the progeny in pig, rat or goat. Species barriers of HEV1 appear to be related to genetic elements carried on the ORF1 non-structural protein[11]. As far as HCV is concerned, the barrier between species may be responsible for the unique targeting of humans by this virus. However, endemic circulation in an area of the world where human, ape and monkey populations overlap and the discovery of viruses closely related to HCV in animals suggested a zoonotic origin[29].

Recombination events increased the genetic variability for both HCV and HEV viruses. Among HEV genotypes, as well as fragment of human genes and HEV strains, recombination is possible. In particular, two insertions of the ORF1 hypervariable domain on the human RPS17 gene (ribosomal protein S17) increased replication in hepatoma cells[27]. Likewise, during HCV superinfection, recombination events (inter-genotype or inter-subtype), using different breakpoints within the viral genome were identified. The first circulating form was HCV2k/1b with a mapped breakpoint in the NS2 gene. At present, seven inter-genotypes (2k/1b, 21/6p, 2b/1b, 2/5, 2b/6w, 3a/1b, and 2a/1a) and three inter-subtypes (1b/1a, 1a/1c, and 4a/4d) recombinant forms (RFs) are known[30].

PATHOGENESIS AND NATURAL HISTORY

The incubation period of HEV infection ranges from 2 to 10 wk[31]. HEV determines acute hepatitis with a very low incidence (1%-4%), varying severities, which resolves in 2–3 mo[32]. One of the most serious outcomes is fulminant hepatitis (FH), which is characterized by hepatic parenchyma necrosis, renal failure or coma[33,34]. The wide spectrum of clinical illness could indeed be related to the infecting genotype[31]. In 2015, Smith and Simmonds reviewed published papers for causal association

between FH and genomic variability[34]. The correlation appears to be related to epidemiological factors, namely, restricted geographical areas and time span of collected isolates[34]. The majority of people acquiring infection do not have severe consequences. However, HEV1 and HEV2 are the principal genotypes related to severe disease and mortality and HEV1 was the principal responsible for outbreaks in some countries of Asia and Africa between 1987 and 2015[15]. Several studies reported FH to be related to specific nucleotide substitutions in the HEV1, HEV3 and HEV4 genomes. For instance, the U3148 and C5907 substitutions in HEV3 and HEV4 strains were significantly associated with FH [27]. However, HEV3 hardly progresses to acute liver failure[35]. Extra-hepatic manifestations such as membranoproliferative glomerulonephritis and cryoglobulinemia are not rare in HEV infected patients [13] and it was suggested that in severely immunocompromised patients HEV could be implicated in development of hepatic cancer[36]. Also, common neurological disorders in the course of HEV infection were found such as nerve root, plexus disorders and meningoencephalitis[37,38].

HEV can also cause chronic infection, lasting a year or more in immunosuppressed individuals[32], which has only been observed for HEV3 and HEV4[39]. Comparison of HEV3 isolates between blood donors and patients with hepatitis showed just one polymorphism difference (leucine to phenylalanine ORF2 substitution) in sequences from the first category. Anyway, there is no evidence of pathogenesis related with substitutions occurring in virus genomes[33]. Interestingly, the fast progression to liver fibrosis has been associated with slow *quasispecies* diversification during one year of chronic infection [40].

In contrast to HEV, HCV frequently (50%-80%) causes chronic hepatitis, which is associated with liver cirrhosis, steatosis and hepatocellular carcinoma (HCC)[40]. The variability of genotypes/subtypes was associated with pathogenetic significance. HCV1b hypervariable region 1 (HVR1) of E2 protein displays significantly higher genetic variability than HCV3. HCV3 establishes hepatic chronic infection in less cases compared to other HCV types, particularly HCV1b. The hypervariable E2 region of HCV1b displays low evolutionary dynamics during the course of infection, generating few viral variants, which could provide a fitness advantage under immune system and therapy pressures[41], while the lower variability of HCV3 results in a lower chance to establish chronic infection[41]. On the other hand, HCV3 core protein expression is able to induce more intracellular lipid accumulation causing steatosis more than other genotypes[40]. Indeed, HCV3 infection is associated with steatosis more frequently than HCV1. Some amino acid substitutions in HCV3 core proteins upregulate the sterol regulatory element binding protein-1 (SREBP-1), inducing intracellular lipid accumulation[41].

TREATMENT

Usually, acute HEV infection does not require antiviral therapy[35]. Ribavirin (RBV) monotherapy may be considered in cases of severe acute hepatitis or chronic infection in solid-organ transplant recipients. PEGylated-interferon-a (PEG-IFN-α) was effectively administered to patients after liver transplant or hemodialysis[35], although IFN can cause several side effects[34]. RBV therapy with or without PEG-IFN-a is contraindicated during pregnancy[15]. Sustained virological response (SVR) is achieved only in 78% of chronic patients treated with ribavirin for a median period of three months, probably because of viral mutants[35]. Deep sequencing detected the Y1320H, K1383N and G1634R polymerase substitutions on HEV3 isolates from patients who relapsed or failed RBV therapy[35,42]. Clearly, RBV increases viral heterogeneity, leading to the emergence of different viral populations[35].

PEG-IFN-α and RBV were the standard of care to treat HCV until 2011. Direct-acting antiviral (DAA) drugs quickly changed the landscape of infection, as patients achieved a high SVR rate (95%-99%). Five pan-drug combinations are available right now to treat HCV: Sofosbuvir (SOF), sofosbuvir/velpatasvir (SOF/VEL), sofosbuvir/velpatasvir/voxilaprevir (SOF/VEL/VOX), glecaprevir/pibrentasvir (GLE/PIB) and grazoprevir/elbasvir (GZR/EBR)[43]. DAA drugs determined direct pressure on the viral genome, producing *quasispecies* with resistance associated substitutions (RASs) on NS3/4A, NS5A and NS5B target regions escaping therapy[44]. Several RASs on all target regions after treatment with first-/second-generation and IFN-free regimens in specific HCV types were reported[44-46]. Additionally, natural polymorphisms carried on specific subtypes can confer resistance to NS5A inhibitors. In the last EASL guidelines, experts recommended to detect resistance on NS5A (from 24 to 93 amino acid positions) for subtypes 1l, 4r, 3b, 3g, 6u, and 6v prior to first-line treatment[43]. Indeed, patients who failed therapy displayed NS5A RAS at baseline in the same rate of virological failure[47]. The HCV RFs have been reported in few cases around the world, thus pathogenesis and therapy efficacy are not well characterized. Two patients infected by RF 2b/1b achieved viral clearance with an interferon-free regimen[48]. In contrast, a patient infected by the same RF failed two different interferon-free regimens[49].

Of note, new DAA therapies for HCV had an indirect effect on HEV in coinfected patients. SOF is approved for the treatment of chronic HCV infection but can also inhibit HEV replication *in vitro* (especially if co-administered with RBV) and could be an interesting treatment option in coinfected individuals[50], but clinical universal efficacy has not yet been demonstrated[35]. A SOF based DAA regimen excludes occult HCV or HEV infection in patients who received a liver or renal transplant[51]

and successful treatment was reported in some cases of HEV/HCV coinfection. Biliotti and colleagues reported viral clearance of HCV3 and HEV3 in one infected patient after therapy with SOF plus RBV [52]. In a subject infected through liver transplantation, the combination of SOF, daclatasvir (DCV) and RBV led both to HCV-RNA undetectability 6 wk after the initiation of therapy and to HEV-RNA undetectability at 12 wk after initiation of therapy[53]. In another immunosuppressed patient affected by both HCV and HEV infections, SOF in combination with DCV reduced HCV-RNA to undetectable levels after 4 wk of treatment but did not have a significant effect on serum HEV-RNA levels[54]. Lastly, one patient treated for 12 wk with SOF/DCV/RBV and tenofovir cleared HCV and HEV without risk of HBV reactivation[55]. In clinical practice, detection of HCV and potential HEV genome substitutions may be useful to predict treatment failure[25,44].

In 2016, a new molecular mechanism against HCV and HEV was proposed by Wang and colleagues [56]. INF-γ and TNF-α play essential roles in infections by intracellular agents and show a synergistic effect in experimentally transfected cells with HCV or HEV by activating NF-kB signaling. Antiviral activity is related to innate immune responses. Cooperation between INF-γ and TNF-α, activating signaling cascades, protects against HCV and HEV infection[56].

PREVENTION

Prevention of infections is possible through public health measures and screening policies. In endemic areas for HEV, it is important to wash hands frequently, drink bottled water and eat fruits and vegetables washed with safe water[57]. In areas with low endemicity and zoonotic transmission, simple hygiene measures and cooking meat well done can be fundamental to reduce transmission[30]. HCV and HEV may share the same route of transmission, and blood transfusion and organ transplantation can be dangerous for recipient patients and their immunosuppressed status[57,58]. Tests to detect anti-HCV antibodies are standardized. Additionally, HCV core antigen and molecular assay are used to identify patients with ongoing viral infection[43]. On the other hand, a HEV diagnosis needs a combination of an antibody test and molecular assay due to the specificity of the assay being suboptimal and anti-HEV IgM not being a really robust marker[35].

However, vaccines are the best protection against viral infections. HEV genotypes represent one single serotype, with a serological cross-reactivity, thus one vaccine should protect against all types, despite genetic heterogeneity[30]. In China, a vaccine based on the ORF2 protein had high efficiency in a large human population and has been licensed, but is not available elsewhere at this moment[59]. However, mutations on the ORF changed the structure of the ORF2 protein, reducing the protective efficacy of the vaccine. For preventive purposes, naturally attenuated viral variants carrying substitutions in the polymerase region could be used in the future[27]. Very recently, Chen and colleagues evaluated the safety and efficacy of immunization with an accelerated HEV239 vaccine (Hecolin®). Protective antibodies, produced within 21 d, can be useful during an ongoing HEV outbreak or for travelers and humanitarian workers moving to endemic areas in a short time[60]. At present, HEV Vaccine Working Group by the WHO's Strategic Advisory Group of Experts (SAGE) considered the use of Hecolin® for the general populations residing in endemic areas during outbreaks as quickly as possible. However, due to the lack of data about immunogenicity and safety, the Working Group did not recommend the routine use of this vaccine for specific risk groups, such as pregnant women, patients with chronic liver disease and immunocompromised persons[1]. In the next future, human and animal vaccinations should be associated, considering the One Health concept, for preventing transmission and improving public health[57].

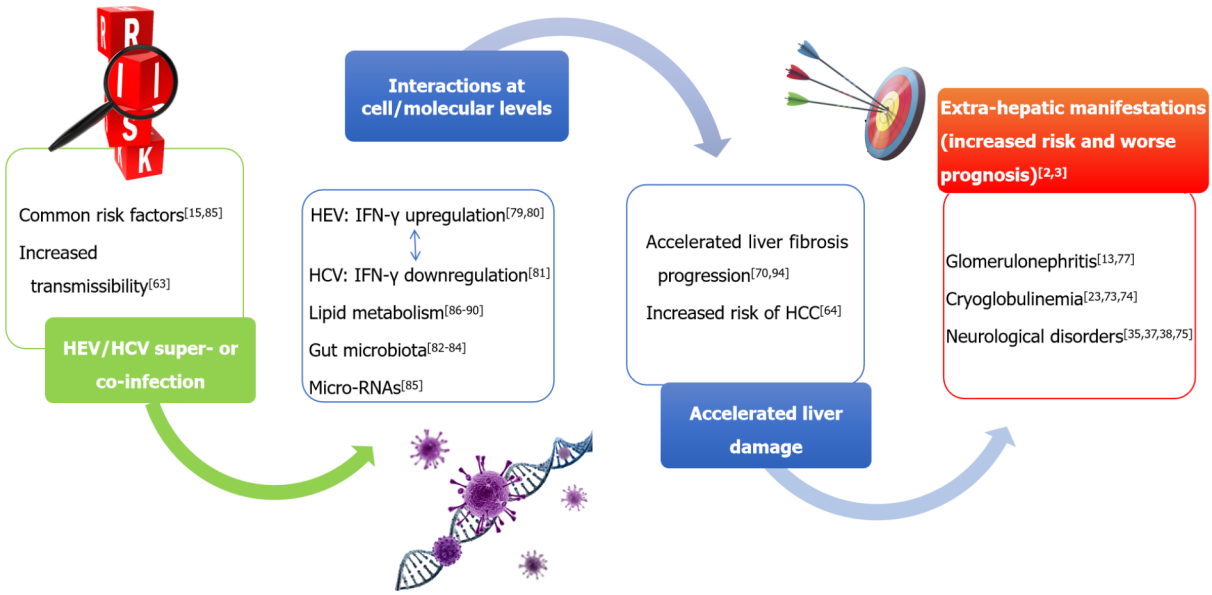
In contrast to HEV vaccine, the HCV vaccine is still under development since there are several limitations, such as easy culture systems not being available, animal models for testing, and viral genetic diversity (genotypes, subtypes and *quasispecies*). The extraordinary variability of HCV determines several opportunities to select, within and between infected individuals, viral variants escaping the immune response[61]. In 2017, University of Oxford in collaboration with other industries developed a candidate vaccine using the entire HCV NS3-5B protein. At present, the vaccine is in phase 1 (EudraCT Number 2016-000983-41) to assess the safety and effectiveness of the immune response against the virus in healthy volunteers. The estimated completion of the study is August 2022[62]. Eradication of HCV by 2030 is the goal of the World Health Organization, and the organization must consider improvements in screening policies and hope for an effective vaccine.

MAJOR CAUSES AND EFFECTS OF COINFECTIONS

A schematic overview of HEV/HCV possible interactions is reported in Figure 1.

Epidemiological considerations

Co-infections or superinfections of HEV with HCV may be due to a common parenteral route of



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Figure 1 Possible interactions at cell-molecular level of hepatitis E virus and hepatitis C virus infecting the same individual. IFN: Interferon; HEV: Hepatitis E virus; HCV: Hepatitis C virus; HCC: Hepatocellular carcinoma.

transmission. Moreover, it was hypothesized that alteration of the intestinal mucosa associated with chronic liver damage due to HCV facilitates HEV translocation from the gut to the liver of patients infected through the oral route[63]. There is a lack of studies investigating the prevalence of possible coinfections with HCV and HEV. At present, it is impossible to provide reliable estimates of the actual prevalence, since information came from few studies and case reports. Future studies, including adequately large sample size, should be planned to estimate the actual prevalence of coinfections. Moreover, the main limitation of the epidemiological surveys conducted so far is that only antibody tests were used[58,64]. Detection of anti-HEV immunoglobulins is related to specificity and sensitivity of commercial kits, among which discordant results were reported in the literature[65]. In 2016, Norder and coworkers[66] evaluated the performance of five commercial assays to determine IgM and IgG levels against HEV. IgM titer was detected by a sensitive HEV IgM/HEV IgG test after the onset of symptoms, providing concordant results in 99% samples from patients with suspected HEV infection. By contrast, recomWell™ HEV IgG/IgM (Mikrogen Diagnostik, Neuried, Germany) and DS-EIA-ANTI-HEV-G/M™ (DSI Srl, Milan, Italy) tests were found to be less specific. In conclusion, investigating the actual rate of coinfections and the effect of both viruses on liver disease progression would require more accurate serological assays and more studies using direct detection of HCV and HEV RNA by molecular tests.

Clinical considerations

Hepatic damage: Infections due to HEV and HCV, even if occurring at different times, can lead to a worse clinical course[58,67]. In fact, serum IgG directed against HEV were associated with a faster evolution towards more severe degrees of fibrosis in patients with chronic HCV infection[58]. Coexistence of the two viruses appeared to be associated with accelerated progression of liver damage as evidenced by the reduced number of platelets, increased transaminases and prolonged prothrombin times observed in patients with chronic HCV hepatitis with HEV exposure during their lifetime (IgG-positive) when compared to HCV mono-infected patients[68]. It is possible that HEV infection in patients infected by HCV with a significant degree of liver fibrosis, accelerates liver damage to such an extent that liver decompensation and death may occur more frequently[69]. These considerations point to the importance of treating HCV and preventing HEV superinfection (either primary prevention or vaccine strategies) in patients affected by chronic HCV infection, a situation which may be particularly frequent or problematic in resource-limited settings. In patients with HCV related HCC, HEV seroprevalence was 11% (compared to 6% in the healthy population), while it reached 42% in patients who underwent liver transplantation for chronic HCV infection[67]. In 2005, Elhendawy and coauthors reported HCV/HEV coinfections in 71.4% of chronic hepatitis patients and in 96.1% of cirrhotic patients with or without HCC, suggesting a possible relationship between the two viruses on progression of liver disease[67]. Recently, the prevalence of HEV infection among adults with chronic liver disease, from 2011 and 2018, was evaluated and anti-HEV IgG positivity was found in 8.6% of HCV chronic positive patients, with a high prevalence in the oldest individuals compared to young age groups[68]. Also, possible effects of HEV infection in increasing the risk of liver cancer over HCV-induced

subclinical liver injury[70] further emphasizes the importance of treatment and preventative strategies for these two viruses to reduce overlap in the same individuals.

Extra-hepatic diseases: Since both viruses may be responsible for extra-hepatic diseases, several studies described these manifestations and correlated them with genetic features[38]. Importantly, HCV does not infect only hepatic cells, and the virus has been found in peripheral blood mononuclear cells, T cells, and monocytes, as well as in B cells and macrophages of colonic tissue. HCV replicates within carotid plaques induce arterial inflammation, probably through the pro-inflammatory cytokine interleukin 1β regardless of viral type[71]. The extrahepatic infection, demonstrated by cell lines producing HCV2a virions, could explain the late relapses observed in clinical trials[72]. Both acute and chronic hepatitis E infections are associated with antinuclear antibodies and cryoglobulinemia in the serum of patients that is similar to untreated HCV infection. The cryoglobulin concentration correlates with the viral load rather than with the degree of inflammation[73]. Serum cryoglobulins in the serum of patients affected by HCV infection are associated with a worse degree of steatosis and fibrosis, and it is not known if the same can happen in HEV infection[74]. Likewise, the risk of evolution to lymphoproliferative diseases associated with HEV cryoglobulinemia with or without HCV cryoglobulinemia is unknown. Furthermore, insulin resistance and metabolic syndrome have already been related to HCV infection, as well as HEV infection recently, which can contribute to the progression of fibrosis in patients with chronic liver disease[3]. As far as HEV is concerned, the neurological disease Guillain-Barre syndrome did not appear to be genotype specific[38], but HEV1 was associated with neurological injury[35], as well as HCV[75]. Moreover, HEV1 and HEV3 were found to be responsible for acute pancreatitis, which has already been described for major hepatitis viruses, in a large number of reports or case control studies[39]. In 2012, a causal link between HEV3 and renal injury was reported[76]. Additionally, mechanisms inducing glomerular disease were found to be similar to those induced by HCV[77]. HCV increased the risk of chronic kidney disease, inducing glomerular injury through the high viral load related to HCV1 or HCV2[23].

Virological and pathogenetic considerations

It is known that HEV inhibits production of type I IFNs[78], while it induces upregulation of IFN-γ by natural killer (NK) or natural killer T lymphocytes[79,80]. The core and some non-structural proteins of HCV (NS3, NS5A and NS5B) were demonstrated to alter the function of dendritic cells (DCs) in vitro, resulting in impaired CD4+ and CD8+ T-cell responses to the virus. Also, patients with chronic HCV infection have reduced interleukin-12 and IFN-γ levels compared to those who cleared the virus[81]. Therefore, at least in principle, HEV could counteract chronicity of HCV through IFN-γ upregulation, but interactions between the two viruses *via* cytokine cross-talk may be complex and not well demonstrated or easy to predict.

Interestingly, liver health is related to the composition of gut microbiota. This is influenced by enteric virome, with whom is in continuous and dynamic equilibrium, and by viruses chronically infecting host tissues[82]. The number of studies on the gut-liver axis and hepatitis infections is presently very low, but microbiota alteration is related to liver disease. HCV-positive people had lower bacterial diversity (*i.e.*, less *Clostridium* and more *Streptococcus* and *Lactobacillus* species) compared with non-infected people[83]. Exacerbation of HEV infection was negatively related to high *Lactobacillaceae* levels[84]. The relationship between gut dysbiosis and viral hepatitis needs to be further investigated, but clearly unfavorable shift in gut microbiota composition driven by the two hepatic viruses may correlate with increase of inflammation and a worse liver stiffness[83,84].

Lastly, at molecular level, microRNAs (miRNAs) play a pivotal role in the progression of liver diseases[82]. The roles of the miRNAs are still under study, but it was already speculated that miR-628-3p, miR-194, miR-151-3p, miR-512-3p, miR-335 and miR-590 are potentially involved in HEV/HCV coinfection[85].

Studies in animal models highlighted the ability of HCV to determine changes in the expression of genes that regulate the lipid metabolism[86]. The role of statins in inhibiting viral replication was subsequently proven[87]. Interestingly, not all statins show an inhibitory effect on HCV replication, suggesting an anti-viral mechanism independent from 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase[88]. However, the capability of fluvastatin in lowering HCV RNA in people with chronic hepatitis C appears to be modest, variable, and often fleeting[89]. In contrast, patients treated with statins who are chronically infected with HEV show significantly higher viral loads than chronically infected patients without statin administration and this underlines the possible impact of lipid metabolism on HEV replication[90], while treatment with proprotein convertase subtilisin/kexin type 9 (PCSK9) inhibitors, such as alirocumab, determines a poor antiviral activity against HEV. These observations led to the hypothesis that the antiviral activity of these molecules is related to their ability to determine an increase in intracellular cholesterol, which is greater for statins than for PCSK9 inhibitors[90]. Possible indirect interactions between the two viruses through their influence on lipid metabolism merit determination.

Table 2 Research perspectives for the next future

No.	Research perspectives for the next future
1	*In vitro* and *in vivo* studies to define pathogenic interactions during HEV/HCV coinfection. Cell lines model could explain interactions between viral proteins and cellular pathway responsible for liver fibrosis progression, liver steatosis and insulin resistance encountered in patients
2	Studies to understand relationships among immune phenomena (autoantibodies, cryoglobulins and autoimmune diseases) in patients infected by both viruses, and their correlated in terms of T- and B-cell responses and human leukocyte antigen type
3	Clinical trials to test safety and effectiveness of DAA in co-infected patients and new therapies. At present, data on DAA treatment is limited to *in vitro* studies or very few case reports
4	*In vitro* studies to evaluate genetic interactions between HEV and HCV during viral replication. Permissive cell lines, infected simultaneously by the two viruses, could show whether there is an interference or synergy between them during viral progeny production

DAA: Directly acting antivirals; HEV: Hepatitis E virus; HCV: Hepatitis C virus.

Special populations

The interactions between the two viruses could be even promoted by immune-suppression induced by HIV, which may facilitate HEV transmission[91]. High prevalence of IgG anti-HEV antibodies (> 15%) was found in people living with HIV (PLWH) affected by HCV chronic infection, in particular if CD4+ T-cell count was below 350 cells/mm^3[92]. In endemic rural areas, HEV/HCV coinfection also occurred frequently among pregnant women, inducing a significant worsening of biochemical liver indices than women with negative HCV serology[2]. HCV pathogenesis during pregnancy is poorly understood, and it was related to preterm delivery, placental abruption, and low birth weight in a large cohort of infected women[93]. HEV replicates in the human placenta, among pregnant women, the fatality rate being around 20% and up to 30% in the third trimester. HEV infection determines fulminant hepatic failure, membrane rupture and spontaneous abortions[27].

CONCLUSION

Since HEV/HCV coinfection is a novel topic, several clinical and research questions remain summarized in Table 2.

As previously discussed, seroprevalence studies demonstrated that the lifetime risk of HEV infection in patients affected by chronic HCV hepatitis is not rare. Although the prevalence of HEV/HCV coinfection is not known, it is reasonable to speculate that in resource limited settings where HEV is a frequent cause of acute hepatitis, superinfections with this virus in patients with chronic HCV infection is quite frequent[94], and the consequences in terms of worsening liver damage and liver decompensation merit to be further investigated. By contrast, since HEV infection is a much rarer cause of chronic liver disease than HCV, chronic co-infections with both viruses are less frequently observed unless in immune-compromised individuals.

Immune phenomena are described for both viruses, and physicians should be aware that patients with autoantibodies and cryoglobulins could be tested for both acute and chronic HEV or HCV infection. However, to the best of our knowledge, no one has described immune alterations in patients affected by HEV/HCV coinfection. We propose, given the relative rarity of the infection, that physicians (who diagnose coinfection) also screen for immune phenomena.

Some DAA drugs, such as SOF, are active against both HEV and HCV *in vitro*, but a regime with SOF and DCV failed to clear HEV RNA in a coinfected patient who did not tolerate ribavirin[54]. Our limited knowledge is based on too few cases being described[52,53,55], and it is not possible to get definitive conclusions on the use of DDA drugs in coinfected patients. It is desirable that researchers focus on *in vitro* studies to better define possible pathogenetic interactions determined by the two viruses. People at risk of HEV or HCV infection (such as transfused or transplanted patients) should be screened regularly to identify coinfected patients. Also, PLWH should be screened for HEV in cases of unexpected elevations of liver enzymes, with or without HCV co-infection.

HEV and HCV are both RNA viruses characterized by greater variability than DNA viruses and mainly infect the liver. Despite these similarities, the two viruses have different species barriers and disease progression. However, coinfection in endemic areas can be a serious public health problem, especially for immunosuppressed individuals or pregnant women. The evolutionary behavior of RNA viruses is responsible for its pathogenesis and antiviral success in infected hosts, as well as vaccine design[26]. Coinfection with particular HCV and HEV types could aggravate hepatic and/or extrahepatic diseases, taking into account viruses–host interaction and the possible genetic interaction between the two viruses during viral replication. At present, the prevention of infections is mainly related to screening policies and public health measures.

FOOTNOTES

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Country/Territory of origin: Italy

ORCID number: Nadia Marascio 0000-0003-0880-8955; Salvatore Rotundo 0000-0001-5441-1727; Angela Quirino 0000-0002-1697-1587; Giovanni Matera 0000-0002-7763-6029; Maria Carla Liberto 0000-0001-8685-4717; Chiara Costa 0000-0003-3389-2650; Alessandro Russo 0000-0003-3846-4620; Enrico Maria Trecarichi 0000-0001-9064-7745; Carlo Torti 0000-0001-7631-5453.

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ORIGINAL ARTICLE

Basic Study

Spinal anesthesia alleviates dextran sodium sulfate-induced colitis by modulating the gut microbiota

Yu Hong, Jie Zhao, Ye-Ru Chen, Zi-Hao Huang, Li-Dan Hou, Bo Shen, Yu Xin

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Yu Hong, Jie Zhao, Bo Shen, Department of General Surgery, Sir Run Run Shaw Hospital of Zhejiang University, Hangzhou 310012, Zhejiang Province, China

Ye-Ru Chen, Department of Anaesthesiology, Sir Run Run Shaw Hospital of Zhejiang University, Hangzhou 310012, Zhejiang Province, China

Zi-Hao Huang, College of Medicine, Zhejiang University, Hangzhou 310012, Zhejiang Province, China

Li-Dan Hou, Biomedical Research Center, Sir Run Run Shaw Hospital of Medical School, Zhejiang University, Hangzhou 310012, Zhejiang Province, China

Yu Xin, Department of Anesthesiology, Sir Run Run Shaw Hospital of Zhejiang University, Hangzhou 310012, Zhejiang Province, China

Corresponding author: Yu Xin, PhD, Attending Doctor, Chief Physician, Department of Anesthesiology, Sir Run Run Shaw Hospital of Zhejiang University, No. 3 Qingchun Road, Hangzhou 310012, Zhejiang Province, China. xinxin_yu@zju.edu.cn

Abstract

BACKGROUND

Inflammatory bowel disease (IBD) is a chronic disease with recurrent intestinal inflammation. Although the exact etiology of IBD remains unknown, the accepted hypothesis of the pathogenesis to date is that abnormal immune responses to the gut microbiota are caused by environmental factors. The role of the gut microbiota, particularly the bidirectional interaction between the brain and gut microbiota, has gradually attracted more attention.

AIM

To investigate the potential effect of spinal anesthesia on dextran sodium sulfate (DSS)-induced colitis mice and to detect whether alterations in the gut microbiota would be crucial for IBD.

METHODS

A DSS-induced colitis mice model was established. Spinal anesthesia was administered on colitis mice in combination with the methods of cohousing and fecal microbiota transplantation (FMT) to explore the role of spinal anesthesia in IBD and identify the potential mechanisms involved.

RESULTS

We demonstrated that spinal anesthesia had protective effects against DSS-induced colitis by alleviating clinical symptoms, including reduced body weight loss, decreased disease activity index score, improved intestinal permeability and colonic morphology, decreased inflammatory response, and enhanced intestinal barrier functions. Moreover, spinal anesthesia significantly increased the abundance of *Bacteroidetes*, which was suppressed in the gut microbiota of colitis mice. Interestingly, cohousing with spinal anesthetic mice and FMT from spinal anesthetic mice can also alleviate DSS-induced colitis by upregulating the abundance of *Bacteroidetes*. We further showed that spinal anesthesia can reduce the increase in noradrenaline levels induced by DSS, which might affect the gut microbiota.

CONCLUSION

These data suggest that microbiota dysbiosis may contribute to IBD and provide evidence supporting the protective effects of spinal anesthesia on IBD by modulating the gut microbiota, which highlights a novel approach for the treatment of IBD.

Key Words: Spinal anesthesia; Inflammatory bowel disease; Gut microbiota; Intestinal barrier; Intestinal inflammation; Intestinal immune

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Core Tip: Inflammatory bowel disease (IBD) is a chronic inflammation with rising trends, but the pathogenesis is still not well understood. The effects of the gut microbiota, particularly the bi-directional interaction between brain and gut microbiota, have gradually attracted increasing attention. In the present study, we found that spinal anesthesia, a regional sympathetic block, alleviated the intestinal inflammation, maintained immunological function, and improved intestinal barrier function by modulating the gut microbiota. And reducing the increase of noradrenaline level in dextran sodium sulfate-treated mice by spinal anesthesia could be one of the mechanisms. The study highlights a novel approach for the treatment of IBD.

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INTRODUCTION

Inflammatory bowel disease (IBD) is a chronic inflammatory disease of the gastrointestinal tract that includes Crohn's disease (CD) and ulcerative colitis (UC). Over the past decades, the incidence of IBD has rapidly increased globally with consistently rising trends, particularly in Asian countries[1,2]. However, the pathogenesis of IBD is still not well understood. Multiple factors, such as environmental factors, the host's genetics, immune responses, and the intestinal microbiome, might participate in the progression of the disease[3]. Currently, the available clinical treatments for IBD include corticosteroids, salicylates, and biologics. However, as a life-long disease, IBD still lacks a radical cure due to its multiple mechanisms.

With the development of genomics, people have gained a better understanding of a "forgotten organ", the gut microbiota. The imbalance of the gut microbiota damages intestinal epithelial barrier function and the immune response, which are related to the occurrence and progression of IBD[4,5]. Intestinal dysbiosis, which means a compositional imbalance of commensal bacteria, is the central characteristic of the gut microbiota in IBD[6]. Probiotics can effectively improve intestinal symptoms and suppress inflammation in IBD[7,8]. More importantly, recent studies have shown that intestinal dysbiosis can be regulated through a central system called the "brain-gut axis"[9]. The brain-gut axis is a bidirectional communication network that links the central nervous system and the gastrointestinal tract. The central nervous system communicates with intestinal targets, such as the muscle layer, gut mucosa, permeability, mucus secretion, immunity, and enteric microbiota, when is activated in response to environmental factors, such as pain and stress[10].

Neuraxial anesthesia includes spinal, epidural, and combined spinal-epidural anesthesia to maintain regional sympathetic blocks. The postganglionic sympathetic neurons, which are located in the celiac, superior mesenteric, and inferior mesenteric ganglia, interact with the intestines at the serosal surface

and innervate the vascular beds as well as the central nervous system. Neuraxial anesthesia has been shown to exert a positive effect on intestinal microvascular perfusion[11,12]. In an animal model of sepsis, thoracic epidural anesthesia was demonstrated to ameliorate perfusion deficits in the muscularis and mucosal layer of the gut[13]. However, whether neuraxial anesthesia affects intestinal inflammation in IBD is still unclear.

In this study, a dextran sodium sulfate (DSS)-induced colitis mouse model was established to explore the role of spinal anesthesia in IBD and identify the potential mechanisms involved. Moreover, cohousing and fecal microbiota transplantation (FMT) were used to help mice from separate lines share microbes across coached individuals[14,15]. This may open up an opportunity to regulate the distribution of the gut microbiota and prevent IBD.

MATERIALS AND METHODS

Animals

Male C57BL/6 WT mice were purchased from the Shanghai Laboratory Animal Center (Chinese Academy of Sciences, Shanghai, China). All animal procedures were conducted in accordance with guidelines for laboratory animal care after approval by the Laboratory Animals Ethics Committee of Zhejiang University. These mice were housed in the standard animal care facility for 1 wk with free access to food and water at the Laboratory Animal Center of Zhejiang University with conventional housing circumstances of a 12 h/12 h light/dark cycle and 22 °C. All animal experiments were completed at the Laboratory Animal Center of Zhejiang University. Efforts were made to minimize any discomfort or pain, and the minimum number of animals was used.

Acute colitis was induced by administration of 2% DSS (MW: 36000-50000 Da, Sigma–Aldrich) in drinking water for 1 wk in male C57BL/6 mice (6-8 wk old). Mice were randomly divided into four groups with 10 mice per group: Untreated normal controls, mice receiving DSS + spinal saline (saline, subarachnoid administration), mice receiving DSS + spinal lidocaine (2% lidocaine, subarachnoid administration), or those receiving DSS + 5-aminosalicylic acid (5-ASA) [200 mg/kg body weight (BW) in saline, *i.g.*]. After cessation of DSS exposure and lidocaine or 5-ASA treatment, the mice were given water *ad libitum* for an additional 1 wk (n = 10). The weight, food intake, and disease activity index (DAI) were recorded every day, and at the end of the experiments, the mice were sacrificed. At autopsy, the colon was rinsed with saline and excised. After the length of the colon was measured, it was cut and fixed in 10% formalin before paraffin embedding as previously described[16]. In addition to protein extracts, the colon was snap-frozen immediately in liquid nitrogen and stored at -70 °C before the preparation of soluble extracts[16].

Hematoxylin and eosin staining

For paraffin-embedded tissue, colon sections cut at 3 μm were stained with hematoxylin and eosin (HE). The inflammatory index was measured by the histological score, which is the sum of scores of four individual inflammatory parameters: Ulceration (0 or 1), inflammation severity (0, 1, 2, or 3), inflammation area involved (0, 1, 2, 3, or 4), and hyperplasia and dysplasia (0, 1, 2, or 3) as detailed previously [16].

Transmission electron microscopy

The tissues from the colon were fixed with 2.5% glutaraldehyde overnight at 4 °C and then postfixed with 1% osmium tetraoxide for 2 h. After three rinses with phosphate buffer solution (PBS), the tissues were then rinsed with distilled water, followed by a graded ethanol dehydration series ending with propylene oxide. The tissues were embedded in resin after infiltration in a mixture of one-half propylene oxide and one-half resin. Sections (120 nm) were cut and stained with 4% uranyl acetate for 20 min and with 0.5% lead citrate for 5 min. Microvilli in the colon were observed by transmission electron microscopy (Philips Tecnai 10, Holland) at the Center of Cryo-Electron Microscopy at Zhejiang University.

Immunohistochemistry

Immunohistochemistry (IHC) of colon sections from mice was performed using formalin-fixed paraffin-embedded tissue as described previously[17]. Sections were stained using primary antibody against CD4 (1:1000; HuaBio, ER1706-80) and appropriate horseradish peroxidase-conjugated secondary antibody (1:1000; MXB, KIT-5006). Images were captured under a light microscope (Olympus BX41, Shanghai, China). Image-Pro Plus 6.0 software (Media Cybernetics, Inc., Rockville, MD, United States) was used to analyze the staining intensity. The semiquantitative results of IHC were based on the average value from three mice per group. Three separate slides from each mouse were analyzed. Five microscopic fields at 100 × magnification were randomly selected, and the integral optical density of the protein of interest was calculated.

Immunoblotting

Colon tissues from each group were homogenized in RIPA buffer (Beyotime, P0013B) with 1 × protease inhibitor cocktail (Beyotime, P1010). The supernatant was collected by centrifugation at 13000 × g for 10 min, and the protein concentration was detected with a bicinchoninic acid protein assay kit (Beyotime, P0012S). An aliquot of 50 μg protein from each sample was separated using SDS-PAGE, transferred to a nitrocellulose membrane, and then blocked with 5% nonfat milk in PBS (pH 7.4). The membranes were incubated with a primary antibody against claudin-1 (1:5000, Proteintech, 130501-1-AP), occludin (1:500, Huabio, ET1701-76), or β-actin (1:5000, ABcam, ab8227) at 4 °C overnight. Blots were incubated in a secondary antibody against rabbit or mouse IgG (1:5000, CST, 7071, and 7072) for 2 h at room temperature and then subjected to chemiluminescent detection using ChemiDoc (BioRad). Digital images were quantified using Image-Pro Plus 6.0 software (Media Cybernetics, Inc., Rockville, MD, United States).

Alcian blue/periodic acid-Schiff staining

The slices of colon tissues were immersed in xylene twice for dewaxing. After the gradient in water, the slices were incubated in Alcian blue reagent for 20 min and distilled water for 3 min. The sample was immersed in 0.5%-1% periodic acid solution and oxidized for 5 min, and it was washed in distilled water at a rapid rate. After applying Schiff reagent staining for 30-60 min (at 37 °C boxes), the slices were washed 2-3 times with sulfate water and distilled water twice. After redying the cell nucleus with hematoxylin solution, the samples were dehydrated with 95% alcohol and 100% alcohol for 1 min and xylene. After sealing with neutral gum, the samples were observed under a microscope.

Real-time quantitative polymerase chain reaction

Total RNA from different groups was extracted using a TRIzol RNA Kit (TransGen Biotech) based on the manufacturer's protocol. Subsequently, cDNA synthesis and antisense RNA amplification were performed using HiScript II Q Select RT SuperMix (Vazyme). Polymerase chain reaction (PCR) was conducted on a StepOne Real-Time PCR System (ABI StepOnePlus, Applied Biosystems; Thermo Fisher Scientific, Inc.) using Hieff UNICON qPCR SYBR Green Master Mix (Shanghai Yeasen Biotechnology Co., Ltd.). The primers were synthesized by BioSun Biotechnology. The following primers were used: Interleukin *(IL)-1β* forward, 5′-AGTTGACGGACCCCAAAAG-3′ and reverse, 5′-TTGAAGCTGGATGCTCTCAT-3′; *IL-6* forward, 5′-AGTCCTTCCTACCCCAATTTCC-3′ and reverse, 5′- GGTCTTGGTCCTTAGCCACT-3′; inducible nitric oxide synthase (*iNOS*) forward, 5′- CTCACCTACTTCCTGGACATTAC-3′ and reverse, 5′- CAATCTCTGCCTATCCGTCTC-3′; *β-actin* forward, 5′- CCACCATGTACCCAGGCATT-3′ and reverse, 5′-AGGGTGTAAAACGCAGCTCA-3′. Each assay was performed in triplicate. Fold changes were calculated after normalizing the change in expression of β-actin using the $2^{-\Delta\Delta cycle}$ threshold method.

Enzyme-linked immunosorbent assay

Serum IL-1β, IL-6, tumor necrosis factor-alpha (TNF-α), and cytotoxic T-lymphocyte-associated protein 4 (CTLA4) concentrations in plasma were measured using an RSGELISA kit (Affymetrix, United States) following the manufacturer's instructions.

Analysis of CD4⁺ T cells and regulatory T cells

Alterations in $CD4^+$ T cell and regulatory T cell (Treg) populations in the intestine were analyzed by staining cells with specific antibodies using flow cytometry performed on a BD FACS S ORP ARIA II (BD Biosciences, Mountain View, CA, United States) according to a method previously described with slight modifications.

Briefly, after DSS and lidocaine treatment, about 1×10^6 cells were collected, and 10 μL of PerCP-Cy5.5-conjugated monoclonal mouse anti-human CD4 antibody and 10 μL of PE-conjugated monoclonal mouse anti-human CD25 antibody (BD Biosciences) were added after washing once with PBS (pH 7.4) (Gibco; Thermo Fisher Scientific, Waltham, MA, United States). To exclude the amount of nonspecific binding, 10 μL of PerCP-Cy5.5-conjugated and 10 μL of PE-conjugated mouse IgG1κ isotype control (BD Biosciences) were used and evaluated as blank controls. After incubation in the dark at 37 °C for 20 min, the cells were washed with PBS and resuspended in diluted Foxp3 buffer A (BD Biosciences), followed by incubation in the dark at room temperature for 10 min. Then, the cells were washed once with PBS, resuspended in 0.15 mL of Foxp3 buffer C composed of 49 parts of Foxp3 buffer A and one part of Foxp3 buffer B (BD Biosciences), and incubated in the dark at room temperature for 30 min. After incubation, the cells were washed once with PBS, and 10 μL of Alexa Fluor 488-conjugated mouse anti-mouse Foxp3 antibody or 10 μL of Alexa Fluor 488-conjugated mouse IgG1 isotype control was added, followed by incubation for 30 min in the dark at 37 °C. The cells were suspended in 0.4 mL staining buffer [0.4% (v/v) formaldehyde neutral buffer solution in PBS (pH 7.4)]. After washing once with PBS, the cells were analyzed by flow cytometry, and the data were further analyzed using FlowJo software (BD Biosciences). $CD4^+$ T cells in the lymphocyte fraction were gated, and the percentages of $CD4^+$ T and Tregs in the $CD4^+$ T cells were calculated.

Intestinal permeability testing

Each group of mice were fasted for 8 h before being sacrificed. After 3 h of administration with FITC-dextran (MW: 4K, R-FD-001, XINQIAO Biotechnology, China, 400 mg/kg in PBS), 300 μL of heart blood was harvested, and plasma was separated. Different concentrations of standard products were prepared with mixed mouse plasma without FITC-dextran gastric filling, PBS, and FITC-dextran solutions. The intracellular fluorescence was measured with a microplate reader at an excitation wavelength of 492 nm and an emission wavelength of 525 nm (DTX880, Beckman Coulter, United States). The FITC-dextran content was calculated according to the standard curve. The fluorescence intensity of FITC-dextran in the blood of mice was positively correlated with the permeability of their intestines.

Gut microbial community sequencing

At the end of the experiment, overnight fasted mice were sacrificed. Fecal samples were collected from the mice, and gut microbiota DNA was extracted. The V3-V4 region of 16S rDNA was amplified with universal primers. The PCR products were then quantified by electrophoresis on a 1.5% agarose gel followed by cDNA purification with the QIAquick Gel Extraction kit (Qiagen). Sequencing and data analysis were subsequently performed on an Illumina HiSeq platform by Novogene (Beijing, China). Briefly, after the raw sequences were identified by their unique barcodes, Ribosomal Database Project Classifier 2.8 was used to perform the assignment of all sequences at 50% confidence. Operational Taxonomic Units (OTUs) present in 50% or more of the colon content samples were identified as core OTUs. PLS-DA of core OTUs was performed using Simca-P version 12 (Umetrics), and to visualize and cluster the bacterial community into different groups, a heatmap was generated using Multi-Experiment Viewer software. Community diversity was measured by the Shannon-Weiner biodiversity index (Shannon index), alpha diversity index (Ace index), and Chao1 richness estimator (Chao 1 index).

Cohousing

For cohousing experiments, siblings (male C57BL/6 mice, 4-12 wk, n = 12) were divided into four groups consisting of a group of WT mice (n = 3), a noncohousing DSS group (n = 3), a cohousing DSS group (n = 3), and a cohousing lidocaine + DSS group (n = 3). The DSS group and lidocaine + DSS group were treated as described above. The cohousing DSS group and the cohousing lidocaine group were transferred to fresh cages to start the cohousing experiments in the same experimental room. The mice remained together for 2 wk with free access to water and chow diet.

FMT

Bacterial strains that were previously isolated from lidocaine mice were mixed and resuspended in PBS. The mice were exposed to DSS for 7 d and then randomly separated into either an FMT group or a DSS group. The FMT and DSS groups were gavaged with fecal microbiota (0.3 mL) or PBS (0.3 mL). After observation for 30 min, all mice were housed separately.

Measurement of serum noradrenaline level

A method for the determination of noradrenaline was established by high-performance liquid chromatography-tandem mass spectrometry. The noradrenaline standard was detected and analyzed by gradient. After blood samples were collected, the serum was separated and frozen at -80 °C. After thawing, methanol was added, and then acetonitrile was added after the internal standard solution was vortex centrifuged again. After stratification, the upper organic phase was placed in another tube, and 10 μL of the organic phase was taken for determination. Chromatographic conditions are as follows: Agilent Zorbax sb-c8 column (2.1 mm × 100 mm, 1.8 μm); mobile phase, water (containing 0.1% formic acid, 2 mmol/L ammonium acetate): Acetonitrile (containing 0.1% formic acid) = 55:45; flow rate, 0.4 mL/min; column temperature, 30 °C; injection volume, 2 μL; autosampler temperature, 10 °C. For mass spectrometry, the ionization mode was electrospray ionization, multireaction ion detection MRM was used in the positive ion mode, the capillary voltage was 3.5 kV, the temperature of the dryer was 350 °C, the flow rate of the dryer was 5 L/min, the atomization gas was 60 psi, the sheath gas temperature was 350 °C, and the sheath gas flow rate was 11 L/min.

Statistical analysis

Experiments were performed in triplicate and repeated at least three times. Data are presented as the mean ± SD or SEM from independent experiments. Statistical analyses involved one-way ANOVA and two-way ANOVA performed with GraphPad Prism 6 (GraphPad Software, Inc., La Jolla, CA, United States) or SPSS 22 (IBM Corp., Armonk, NY, United States). $P < 0.05$ was considered statistically significant.

RESULTS

Spinal anesthesia alleviates DSS-induced colitis

Acute colitis was induced in C57BL/6 mice by the administration of 2% DSS in drinking water for 1 wk. To test whether spinal anesthesia could relieve colitis, colitis mice were given lidocaine (subarachnoid administration), saline (vehicle, subarachnoid administration), and 5-ASA (positive control, *i.g.*) daily until the termination of the experiment after 7 d. The experimental protocol is shown in Figure 1A. The DSS-treated colitis mice exhibited an increasing DAI level with dramatic body weight loss, colon shortening, rectal bleeding, and stool consistency reduction. In contrast, colitis mice treated with lidocaine or 5-ASA showed obvious improvements in body weight (Figure 1B), DAI score (Figure 1C), and colon length (Figure 1D). H&E staining images of colonic tissues of the DSS-treated mice showed obvious intestinal injury, including the loss of crypts and discontinuous brush borders and large lumens. However, these damage signs were noticeably ameliorated by lidocaine treatment, which appeared to protect the colonic mucosal structure by limiting multifocal inflammation (Figures 1E and 1F). Furthermore, SEM showed that the microvilli of colitis mice became shorter and uneven, and the arrangement was disordered. Interestingly, the lidocaine group had more microvilli than the saline group (Figure 1G). Taken together, these results suggested that spinal anesthesia with lidocaine relieved the symptoms of colitis and colonic epithelial injury in a DSS-induced IBD mouse model.

Spinal anesthesia relieves intestinal inflammation activated by DSS

To better understand the alleviation of colitis by spinal anesthesia, the inflammatory response was determined by detecting proinflammatory cytokines and infiltration of immune cells in the colon. As expected, in colitis mice, enzyme-linked immunosorbent assay (ELISA) analysis showed that the levels of IL-1β, IL-6, and TNF-α in plasma were dramatically higher than those in the control mice (Figures 2A, 2B and 2C). The levels of these cytokines were lower in the 5-ASA group than in the saline + DSS group. Interestingly, spinal anesthesia with lidocaine also significantly suppressed the expression of IL-1β, IL-6, and TNF-α in plasma of DSS-induced mice (Figures 2A, 2B and 2C). Meanwhile, the transcriptional levels of cytokines were detected by real-time quantitative PCR (qRT-PCR), and the results revealed that spinal anesthesia also decreased the mRNA levels of *IL-1β*, *IL-6*, and *iNOS* in DSS-induced mice (Figures 2D, 2E and 2F). CD4+/CD25+/FoxP3+ Tregs and CTLA4 cells play an important role in maintaining the healthy intestinal immune state. We then focused on those immune cells. IHC analysis showed that DSS treatment dramatically increased the area of infiltration of CD4+ T cells in the colon, whereas spinal anesthesia or treatment with 5-ASA significantly inhibited the production of CD4+ T cells (Figures 2G and 2H). Importantly, flow cytometry assays showed that spinal anesthesia led to an increase in Tregs (Figure 2I), thereby inducing a change in the proportion of T lymphocytes. Similar results were also obtained when we determined the level of CTLA4 in serum. The lidocaine + DSS group had a higher serum CTLA4 level than the saline + DSS group (Figure 2J). Together, these data suggested that spinal anesthesia may downregulate proinflammatory cytokines and upregulate the levels of CTL4 and CD4+/CD25+/Foxp3+ Tregs to alleviate the inflammatory activity in DSS-induced mice.

Spinal anesthesia protects intestinal barrier function

To examine intestinal barrier function, we first detected intestinal permeability. After spinal anesthesia, the concentration of FITC-dextran in the serum of colitis mice decreased significantly, which indicated the strengthening of intestinal physical barrier function (Figure 3A). Intercellular tight junctions (TJs), whose integrity is a key determinant of paracellular permeability, are important for maintaining epithelial barrier function. Western blot analysis showed that claudin-1 and occludin, two representative TJ proteins, were significantly lower in the DSS groups than in the control group (Figure 3B). However, spinal anesthesia with lidocaine reversed the levels of claudin-1 and occludin in the positive control 5-ASA group. Goblet cells secrete gel-forming mucins to lubricate and protect the intestine. Alcian blue/periodic acid-Schiff staining showed that there was a clear trend of decreasing neutral (Figures 3C and 3D) and acidic (Figures 3E and 3F) mucins after DSS treatment. Spinal anesthesia with lidocaine resulted in a significant increase in mucins. Together, these results showed that spinal anesthesia with lidocaine can protect intestinal barrier function in the DSS-induced IBD mouse model.

Spinal anesthesia changes the intestinal microflora in the IBD mouse model

A wealth of information has been generated regarding the altered microbiota composition, or dysbiosis, at different taxonomic levels that manifests in patients with IBD and animal models of IBD[18,19]. The composition of the fecal microbiota was determined by analysis of -30000 paired reads per mouse of the -250 bp v3-v4 region of the 16S rRNA gene by Illumina MiSeq sequencing (Figures 4A and 4B). The PCoA analysis revealed that specific bacterial alterations occurred during colitis. Ace index varied between the saline + DSS group and the lidocaine + DSS group (Figure 4C). Interestingly, spinal anesthesia significantly rescued the abundance of *Bacteroidetes*, which was suppressed by DSS (Figures 4D and 4E). Other types of bacteria, including *Verrucomicrobia* and *Proteobacteria*, were also affected by spinal anesthesia, although the difference did not reach statistical significance. Together, these data demonstrated that spinal anesthesia with lidocaine can alter the intestinal flora in the IBD mouse model.

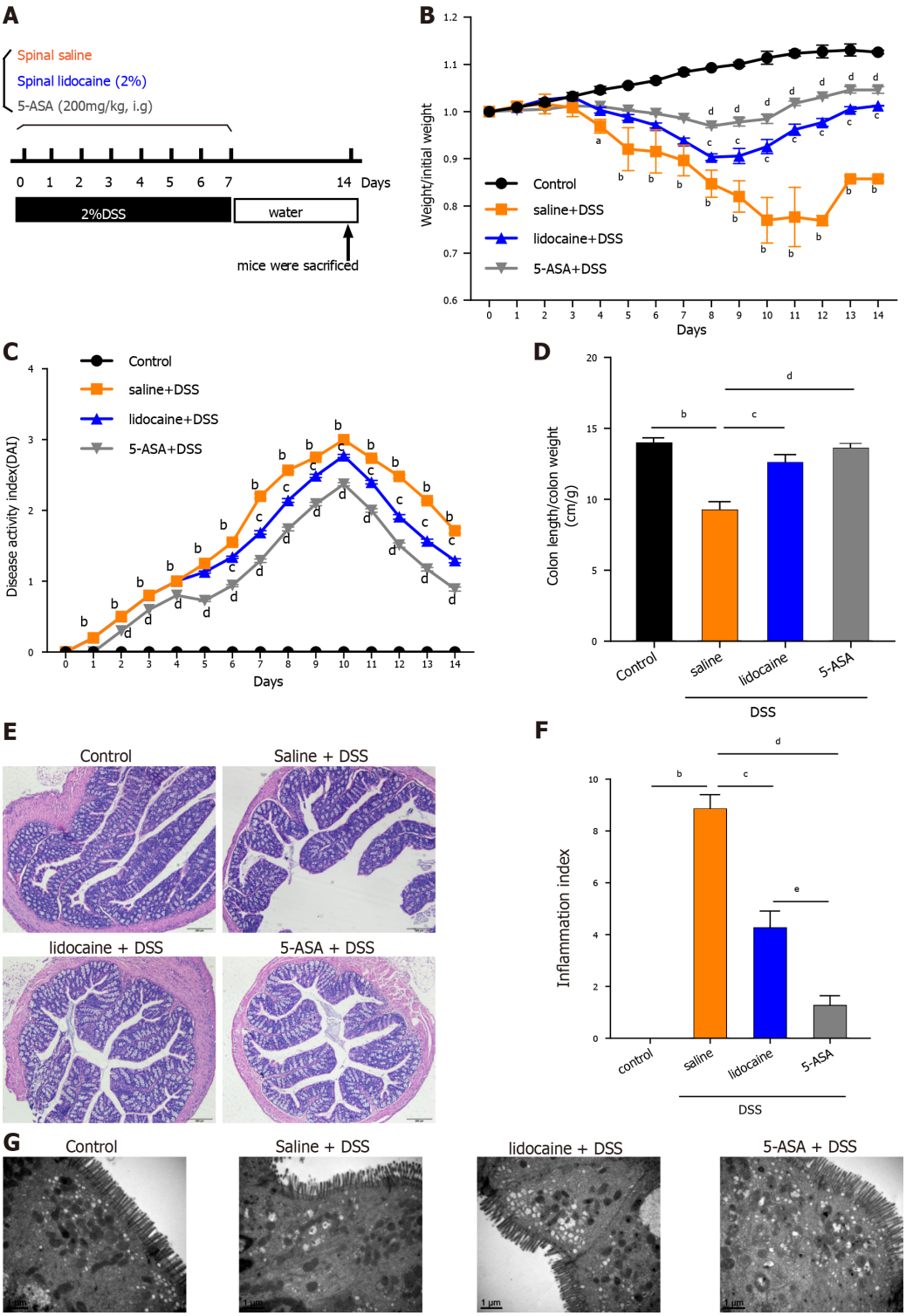


Figure 1 Spinal anesthesia alleviates dextran sodium sulfate-induced colitis. A: Diagram of experimental design; B: Body weight; C: Disease activity index. Each group was evaluated daily (n = 10); D: Colon length of each group measured at the end of the experiment (n = 10); E: Representative images of hematoxylin and eosin staining; F: Inflammation index quantitation; G: Transmission electron microscopy. Data are presented as the mean ± SEM of three independent experiments. P-values were calculated using one-way ANOVA between different groups. $^{a}P < 0.05$, control *vs* saline + DSS; $^{b}P < 0.01$, control *vs* saline + DSS; $^{c}P < 0.01$, saline + DSS *vs* lidocaine + DSS; $^{d}P < 0.01$, saline + DSS *vs* 5-ASA + DSS; $^{e}P < 0.05$, lidocaine + DSS *vs* 5-ASA + DSS. 5-ASA: 5-aminosalicylic acid; DSS: Dextran sodium sulfate.

Cohousing spinal anesthesia and DSS mice have alleviated DSS-induced colitis

To certify whether the gut microbiota contributes to relieving colitis by spinal anesthesia, DSS mice and spinal anesthesia with lidocaine mice were cohoused (Figure 5A). Using microbiota sequencing analysis, we compared the gut microbiota in the cohousing and noncohousing groups. Figure 5B reveals that cohousing altered the relative abundance of intestinal flora. There was no significant difference in various measures, including the Chao1 index, Shannon index, and Ace index (Figure 5C). However,

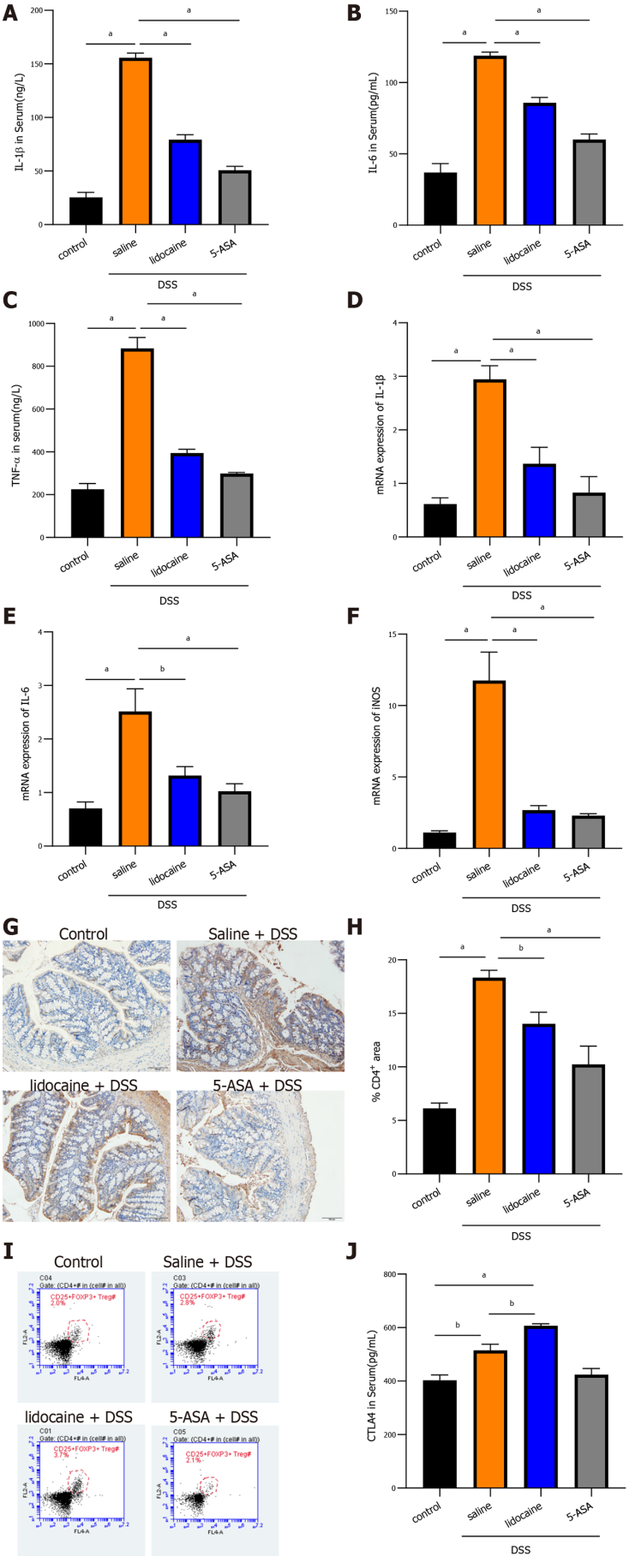


Figure 2 Spinal anesthesia relieves intestinal inflammation activated by dextran sodium sulfate. A-C: Serum levels of interleukin (IL)-1β, IL-6, and

tumor necrosis factor-alpha detected by enzyme-linked immunosorbent assay (ELISA); D-F: The mRNA expression of *IL-1β*, *IL-6*, and inducible nitric oxide synthase detected by real-time quantitative polymerase chain reaction; G: Expression of CD4 detected using immunohistochemistry; H: Quantitation of the immunohistochemistry result; I: Proportion of CD4+/CD25+/Foxp3+ Tregs analyzed by flow cytometry; J: Serum level of cytotoxic T-lymphocyte-associated protein determined by ELISA; Data are presented as the mean ± SEM of three independent experiments. *P*-values were calculated using one-way ANOVA between different groups. [a]*P* < 0.05, [b]*P* < 0.01. 5-ASA: 5-aminosalicylic acid; DSS: Dextran sodium sulfate; IL: Interleukin; TNF-α: Tumor necrosis factor-alpha; iNOS: Inducible nitric oxide synthase.

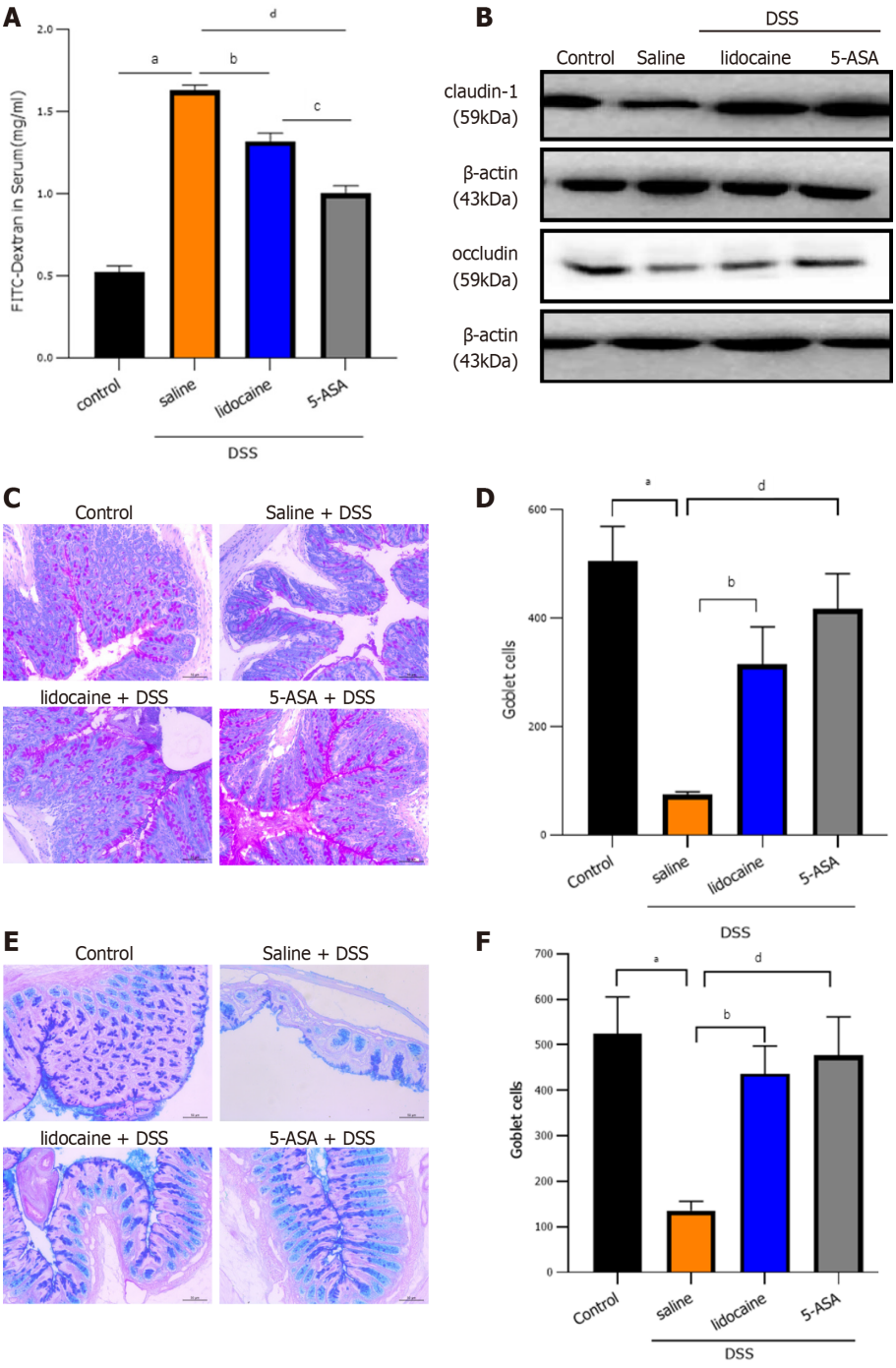
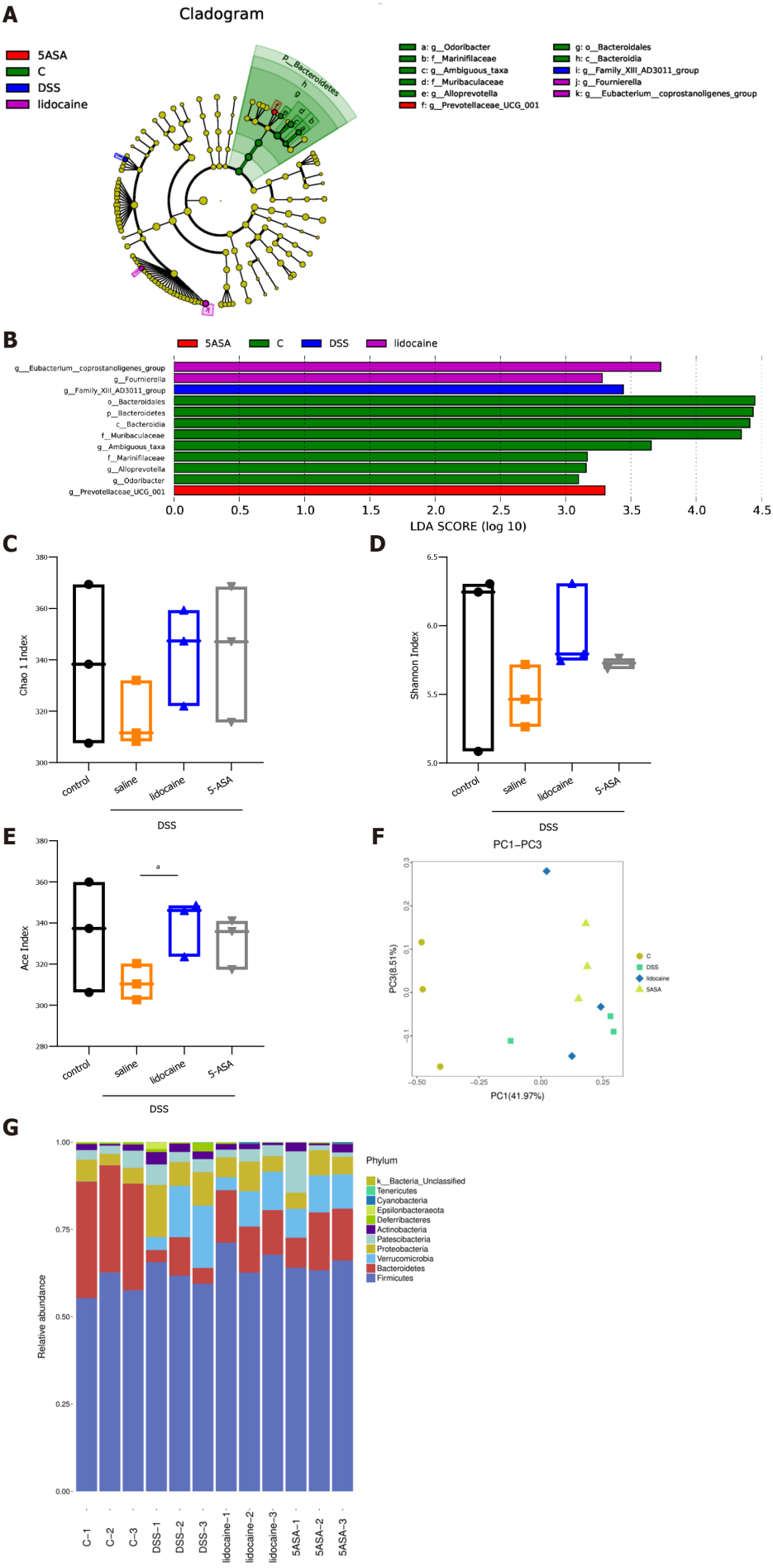


Figure 3 Spinal anesthesia with lidocaine protects the intestinal barrier. A: Intestinal permeability tested by FITC-dextran assay; B: Western blot analysis showed the expression of claudin-1 and occludin as key tight junction proteins in each group; C: Mucins in tissue sections observed by Alcian blue staining; D: Quantitation of the Alcian blue staining result; E: Mucins in tissue sections observed by periodic acid-Schiff staining. F: Quantitation of the periodic acid-Schiff staining result. Data are presented as the mean ± SEM of three independent experiments. *P*-values were calculated using one-way ANOVA between different groups. [a]*P* < 0.01, control *vs* saline + DSS; [b]*P* < 0.01, saline + DSS vs lidocaine + DSS; [c]*P* < 0.01; lidocaine + DSS *vs* 5-ASA + DSS; [d]*P* < 0.01, saline + DSS *vs* 5-ASA + DSS. 5-ASA: 5-aminosalicylic acid; DSS: Dextran sodium sulfate.


A
Cladogram
5ASA
C
DSS
lidocaine
a: g__Odoribacter
b: f__Marinifilaceae
c: g__Ambiguous_taxa
d: f__Muribaculaceae
e: g__Alloprevotella
f: g__Prevotellaceae_UCG_001
g: o__Bacteroidales
h: c__Bacteroidia
i: g__Family_XIII_AD3011_group
j: g__Fournierella
k: g__Eubacterium__coprostanoligenes_group
B
5ASA
C
DSS
lidocaine
g__Eubacterium__coprostanoligenes_group
g__Fournierella
g__Family_XIII_AD3011_group
o__Bacteroidales
p__Bacteroidetes
c__Bacteroidia
f__Muribaculaceae
g__Ambiguous_taxa
f__Marinifilaceae
g__Alloprevotella
g__Odoribacter
g__Prevotellaceae_UCG_001
LDA SCORE (log 10)
C
Chao 1 Index
control
saline
lidocaine
5-ASA
DSS
D
Shannon Index
E
Ace Index
F
PC1–PC3
PC3(8.51%)
PC1(41.97%)
G
Phylum
k__Bacteria_Unclassified
Tenericutes
Cyanobacteria
Epsilonbacteraeota
Deferribacteres
Actinobacteria
Patescibacteria
Proteobacteria
Verrucomicrobia
Bacteroidetes
Firmicutes
Relative abundance
C–1
C–2
C–3
DSS–1
DSS–2
DSS–3
lidocaine–1
lidocaine–2
lidocaine–3
5ASA–1
5ASA–2
5ASA–3

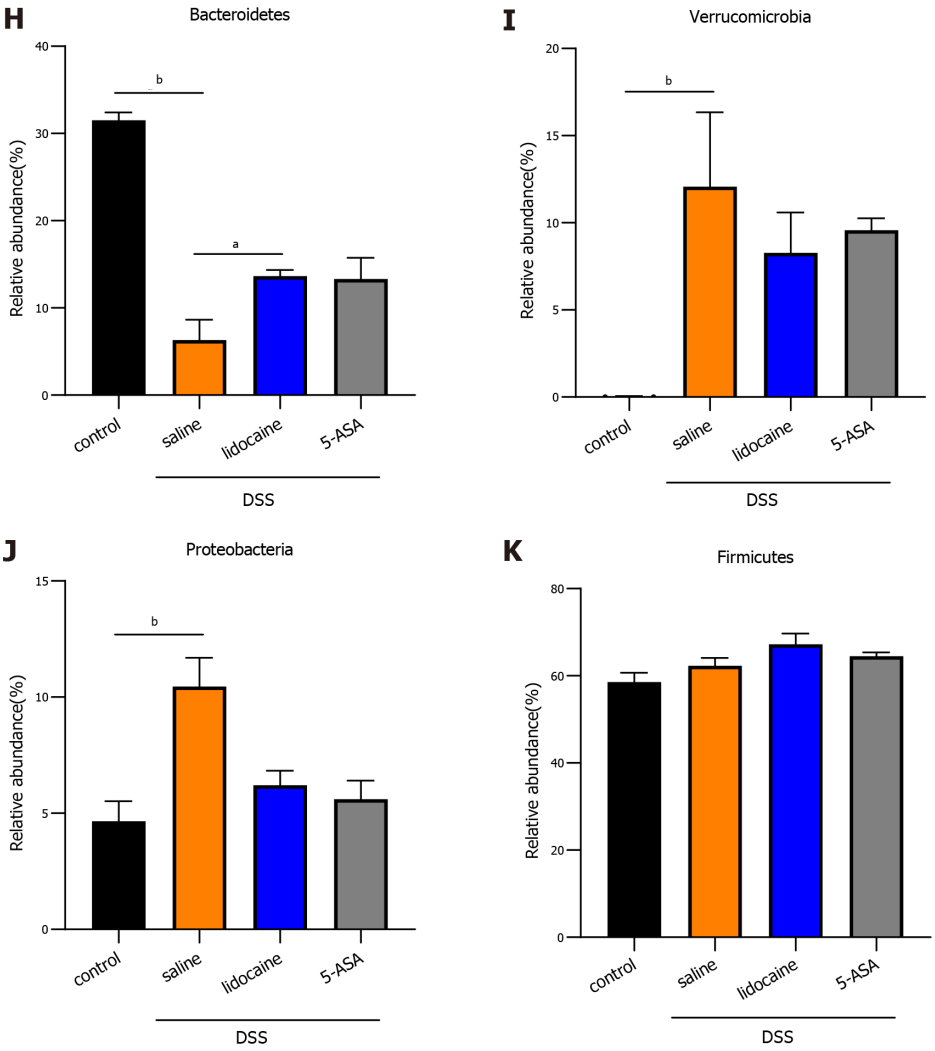


Figure 4 Spinal anesthesia changes the intestinal microflora. A: Cladograms (family level) of the intestinal microflora in different groups; B: Differentiating taxa (family level) of the intestinal microflora in different groups; C: Chao1 index (richness); D: Shannon index (diversity); E: Ace index; F: PCoA plots revealing specific bacterial alterations in different groups; G: Relative abundance plots showing community variation in various groups; H: Abundance of *Bacteroidetes* in different groups; I: Abundance of *Verrucomicrobia* in different groups; J: Abundance of *Proteobacteria* in different groups; K: Abundance of *Firmicutes* in different groups. Statistical significance and variance of Bray-Curtis dissimilarity data were assessed using PERMANOVA; alpha diversity data are represented as the mean ± SEM, and statistical significance was assessed using *t* tests. n = 3 mice per group. *P* values were calculated using one-way ANOVA between different groups. $^{a}P <$ 0.01, saline + DSS *vs* lidocaine + DSS; $^{b}P < 0.01$, control *vs* saline + DSS. 5-ASA: 5-aminosalicylic acid; DSS: Dextran sodium sulfate.

cohousing significantly increased the abundance of *Bacteroidetes* (Figure 5D), which was consistent with the change affected by spinal anesthesia. Meanwhile, cohousing rescued the shortened colon and intestinal permeability in DSS-induced mice (Figures 5E and 5F), and the body weight and DAI of the cohousing spinal anesthesia and DSS mice were better than those of the DSS mice (Figures 5G and 5H).

Transplantation of fecal microbiota from lidocaine-treated mice alleviates DSS-induced colitis

FMT is generally accepted as a promising experimental treatment for patients suffering from gut dysbiosis. We were interested in determining whether transplantation of the intestinal flora from the spinal anesthesia group has an effect on DSS-induced colitis (Figure 6A). FMT also altered the relative abundance of intestinal flora (Figure 6B). Various measures, including the Chao1 index, Shannon index, and Ace index, varied between the cohousing group and the noncohousing group (Figure 6C). FMT significantly increased the abundance of *Bacteroidetes* and decreased the abundance of *Verrucomicrobia*, recovering the change in DSS-induced mice (Figure 6D). Similarly, FMT reduced colon swelling and relieved intestinal permeability (Figures 6E and 6F). The body weight and DAI of the DSS mice treated by FMT were better than those of the DSS mice (Figures 6G and 6H), which was consistent with changes by cohousing treatment. Taken together, these results suggested that the gut microbiota was implicated in relieving colitis by spinal anesthesia due to the key role that it plays in intestinal inflammation.

Spinal anesthesia decreases the noradrenaline level induced by DSS

Several studies[12,13] have indicated that the sympathetic system primarily exerts an inhibitory

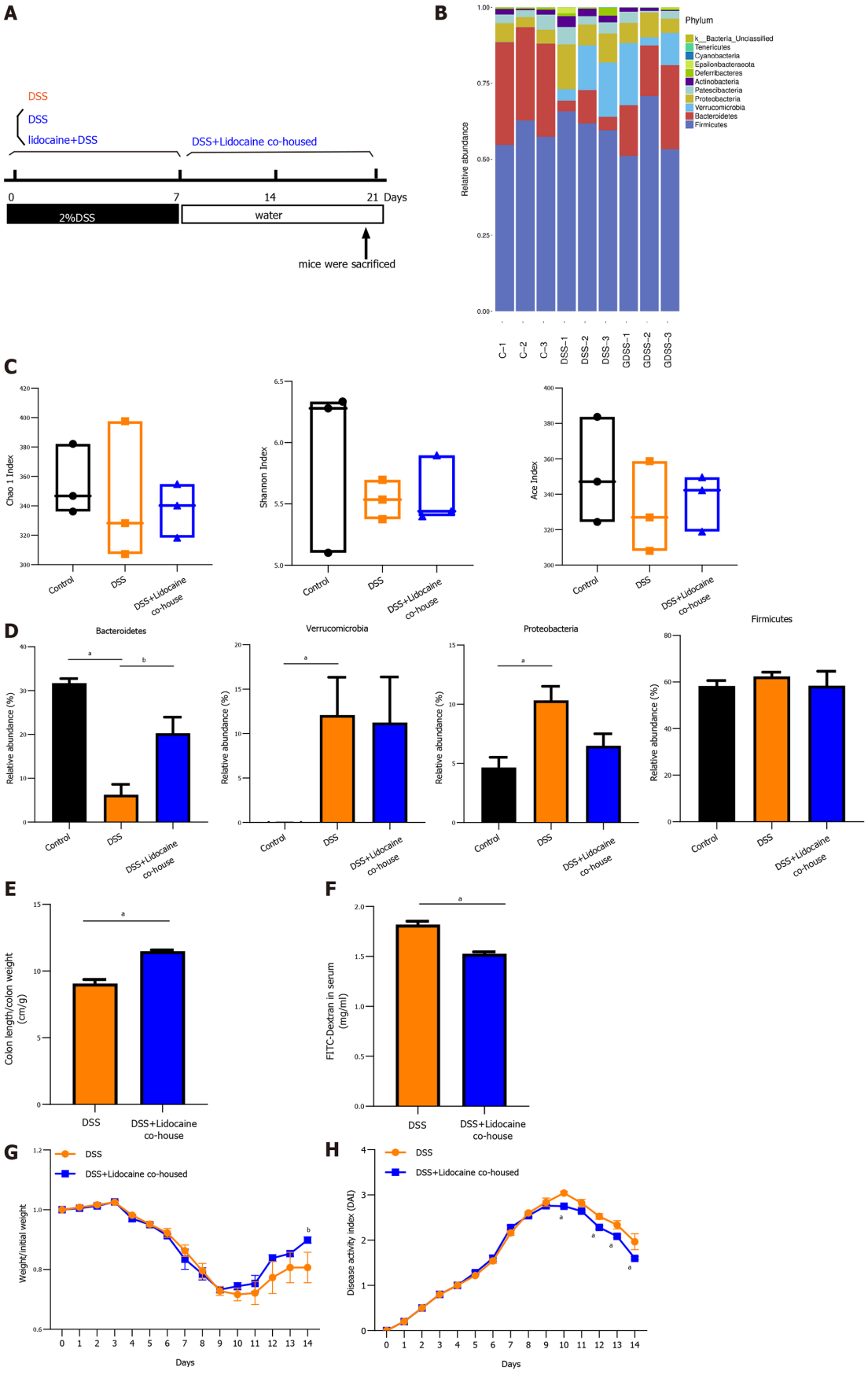


Figure 5 Cohousing alleviates dextran sodium sulfate-induced colitis in mice. C57BL/6 mice were divided into four groups: A normal control group (*n* = 3), a no-cohousing dextran sodium sulfate (DSS) group (*n* = 3), a cohousing DSS group (*n* = 3), and a cohousing lidocaine + DSS group (*n* = 3). The DSS group and lidocaine + DSS group were treated with DSS. The cohousing DSS group and the cohousing lidocaine group were transferred to fresh cages. A: Diagram of experimental design; B: Relative abundance plots showing community variation in various groups; C: Alpha diversity calculated using Chao1 index (richness),

Shannon index (diversity), and Ace index; D: Abundance of *Bacteroidetes*, *Verrucomicrobia*, *Proteobacteria*, and *Firmicutes* in different groups; E: Colon length; F: Intestinal permeability compared between cohousing DSS mice (n = 6) and no-cohousing DSS mice (n = 6); G: Body weight; H: Disease activity index compared between cohousing DSS mice (n = 6) and no-cohousing DSS mice (n = 6). Data are presented as the mean ± SEM of three independent experiments. *P*-values were calculated using one-way ANOVA between different groups. $^{a}P < 0.05$, $^{b}P < 0.01$. Statistical significance and variance of Bray-Curtis dissimilarity data were assessed using PERMANOVA; alpha diversity data are represented as the mean ± SEM, and statistical significance was assessed using *t* tests. n = 3 mice per group. 5-ASA: 5-aminosalicylic acid; DSS: Dextran sodium sulfate.

influence on the gut by the release of noradrenaline. To verify that spinal anesthesia modulates the gut microbiota *via* noradrenaline, we used liquid chromatography to detect the level of noradrenaline in our model (Figure 7A). The results indicated that the level of noradrenaline was increased in the saline + DSS group compared with the control group (Figures 7A and 7B). Spinal anesthesia with lidocaine decreased the noradrenaline level in DSS-induced mice. The altered level of noradrenaline could be one of the mechanisms underlying the effect of spinal anesthesia on the gut microbiota.

DISCUSSION

The pathogenesis of IBD is not fully understood, and changes in intestinal microbes, impairment of the epithelial barrier, and the chronic dysregulated immune response in the gastrointestinal tract are strongly implicated in the development of IBD[20,21]. In particular, increasing evidence from animal and human studies has shown that gut microbes are key factors contributing to IBD by acting on different parts of the gut-brain-microbiota axis[22]. Therefore, our study explored the possibility of improving the clinical outcomes of IBD by acting on this axis. Interestingly, we found that IBD mice treated by lidocaine spinal injection, which is a regional sympathetic block, showed a significant amelioration of symptoms and intestinal permeability. Moreover, this treatment increased the abundance of specific genera, such as *Bacteroidetes*, and regulated gut immunity. Here, we, for the first time, directly highlighted the therapeutic effects of spinal anesthesia on DSS-induced colitis mice.

Neuraxial anesthesia (*i.e.*, spinal, epidural, and combined spinal-epidural techniques) is widely used to induce analgesia for lower extremities and lower abdominal surgery. In addition, the specific beneficial effect of neuraxial anesthesia attributed to sympathetic nerve blockade, which increases gastrointestinal track microvascular perfusion and function, has been observed in animal and clinical studies[23,24]. Recently, several reports have shown that spinal or epidural anesthesia may improve the ischemic and insufficient oxygenation stage of organs[11,12]. In the present study, we found that spinal anesthesia with lidocaine not only relieved intestinal permeability but also increased the diversity of the gut microbiota and changed the composition of microbiota species in colitis mice, which suggests that spinal anesthesia affects part of the gut-brain-microbiota axis to alleviate colitis in DSS-induced mice.

Microbial predominance includes four different phyla, *Bacteroidetes*, *Prodominace*, *Firmicutes,* and *Verrucomycrobia*[25], in healthy people, and an alteration of the intestinal microbiota occurs in IBD patients. Prior studies have noted that polysaccharides, a component of *Bacteroidetes,* can prevent intestinal inflammatory disease, and *Akkermansia* exerts beneficial effects on colitis[26]. In the DSS model, we found that the abundance of *Proteobacteria* and *Verrucomycrobia* increased, but the abundance of *Bacteroidetes* decreased, which might lead to abnormal intestinal immune responses and intestinal imbalance. After spinal anesthesia, we identified that the abundance of *Bacteroidetes* with health-promoting functions significantly increased and the abundance of *Proteobacteria* and *Verrucomycrobia* decreased. Furthermore, our cohousing and FMT experiments revealed that the microbiota of spinal anesthesia mice transferred to DSS colitis mice can effectively relieve disease. These results confirmed that altered gut microbiota caused IBD and that *Bactericides* and *Verrucomycrobia* species are critical in keeping the gut microbiota healthy.

Extensive studies have found that behavioral disorders, such as anxiety, stress, or depression, change the composition of the intestinal flora and influence the recurrence of CD[27,28]. The sympathetic nerve innervated to the gut plays a critical role in affecting the composition of the gut microbiota and maintaining immune homeostasis *via* the hypothalamus-pituitary-adrenocortical axis mainly by secreting catecholamine[9,29,30]. In the stress model, the sympathetic system primarily exerts an inhibitory influence on the gut, decreasing intestinal motor function and secretion *via* the release of neurotransmitters, such as noradrenaline[31]. Noradrenaline, one of the main catecholamines, can influence the microbiota in the gut, leading to the altered release of cytokines and bacterial molecules. Noradrenaline can also increase the growth of several bacteria in nutrient-deficient environments, including *Campylobacter jejuni*, *Escherichia coli*, *Helicobacter pylori*, *Pseudomonas aeruginosa*, and *Salmonella enterica spp*[32]. Catecholamines were found to facilitate the removal of iron from human lactoferrin and transferrin in a dose- and time-dependent manner, which also correlated with bacterial growth[33]. In our study, the level of noradrenaline was increased after DSS treatment, which may influence the microbiota present in the colitis gut. Moreover, spinal anesthesia with lidocaine recovered the level of noradrenaline. This could be one of the mechanisms underlying the effect of spinal anesthesia on the gut

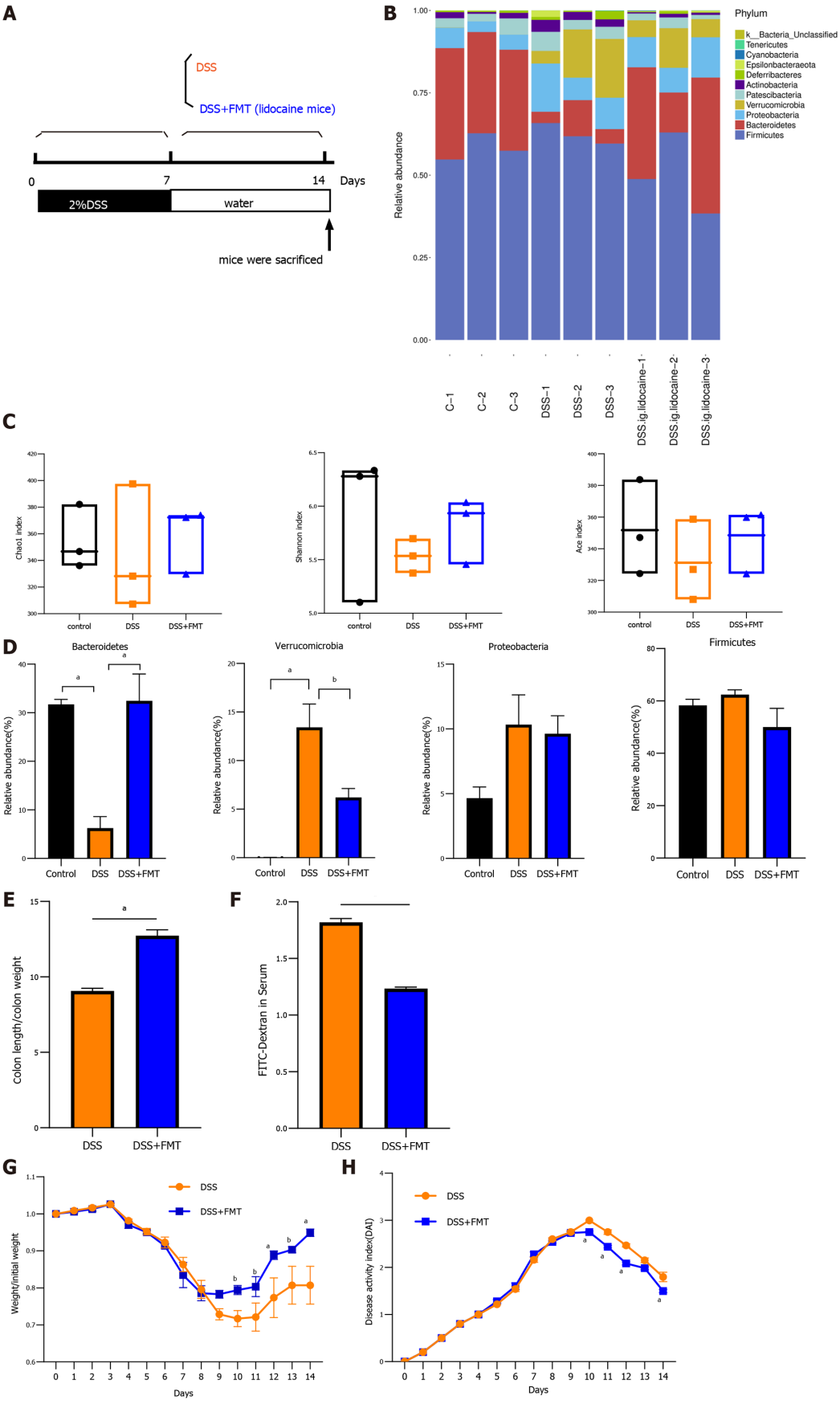


Figure 6 Transplantation of fecal microbiota from lidocaine-treated mice alleviates dextran sodium sulfate-induced colitis. C57BL/6 mice were exposed to dextran sodium sulfate (DSS) for 7 d and then randomly separated into either a fecal microbiota transplantation (FMT) group or a DSS group. The FMT and DSS groups were gavaged with fecal microbiota (from lidocaine mice) and phosphate-buffered saline, respectively. A: Diagram of experimental design; B: Relative abundance plots showing community variation in various groups; C: Alpha diversity calculated using Chao1 index (richness), Shannon index (diversity), and

Ace index ; D: Abundance of *Bacteroidetes*, *Verrucomicrobia*, *Proteobacteria*, and *Firmicutes* in different groups; E: Colon length compared between DSS mice (n = 6) and FMT + DSS mice (n = 6); F: Permeability compared between DSS mice (n = 6) and FMT + DSS mice (n = 6); G: Body weight compared between DSS mice (n = 6) and FMT + DSS mice (n = 6); H: Disease activity index compared between DSS mice (n = 6) and FMT + DSS mice (n = 6). Data are presented as the mean ± SEM of three independent experiments. *P*-values were calculated using one-way ANOVA between different groups. $^{a}P < 0.05$, $^{b}P < 0.01$. Statistical significance and variance of Bray-Curtis dissimilarity data were assessed using PERMANOVA; alpha diversity data are represented as the mean ± SEM, and statistical significance was assessed using *t* tests, n = 3 mice per group. 5-ASA: 5-aminosalicylic acid; DSS: Dextran sodium sulfate.

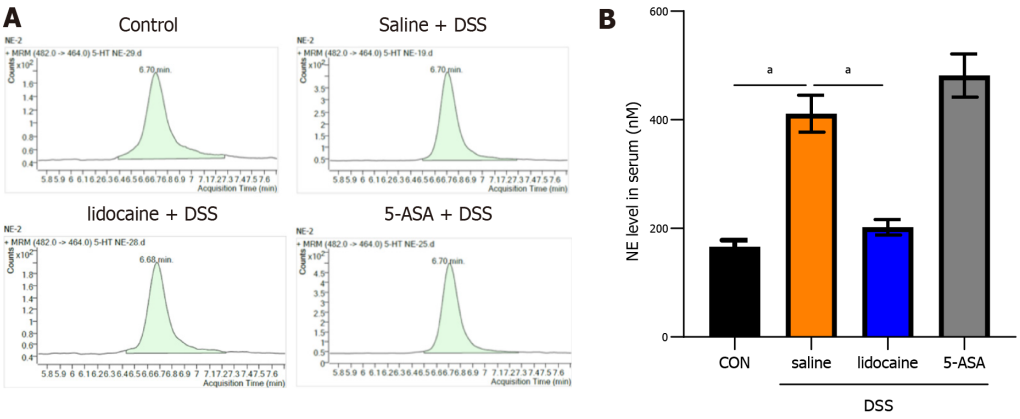


Figure 7 Spinal anesthesia decreases the noradrenaline level induced by dextran sodium sulfate. At the end of the experiment, according to Figure 1A, the serum of mice in each group was collected. A: Noradrenaline level measured by high performance liquid chromatography (HPLC); B: Quantitation of the HPLC result. Data are presented as the mean ± SEM of three independent experiments. *P* values were calculated using one-way ANOVA between different groups. $^{a}P < 0.05$. 5-ASA: 5-aminosalicylic acid; DSS: Dextran sodium sulfate.

microbiota.

It is evident that the sympathetic nervous system has the potential to serve as a therapeutic target for inflammatory disease. Increasing sympathetic tone has a deteriorating effect on IBD, including the development of intestinal inflammation and inhibition of immune defenses[34,35]. This is in line with the finding in the present study that thoracic sympathetic block through spinal anesthesia in colitis mice increased the expression of FoxP3+ Treg cells, which are a subset of CD4+ T lymphocytes and plays an important role in maintaining the immune response[36]. Moreover, the levels of caludin-1 and occludin, the main tight junction proteins of the intestinal epithelial barrier, were changed. All these results indicated that immune function and colon barrier function were impaired, which may be caused by increased noradrenaline levels and altered gut microbiota.

In recent years, diet and short-chain fatty acids have been recommended to replace or increase conventional IBD therapies[37]. Some peptides named food protein-derived bioactive peptides against intestinal inflammation have attracted increasing attention, but their mechanism and effect are under exploration[38]. Experts have said "as we enter a new era of patient-centered health care, treating the 'brain' is as important as the 'gut' for comprehensive, whole-person IBD management"[39]. However, it is difficult to verify the bidirectional relationship of the gut-brain axis, and here, we for the first time used spinal anesthesia to block thoracic nerve in IBD mice, and we luckily found that it was effective for IBD.

CONCLUSION

In conclusion, this study clearly revealed that spinal anesthesia inhibited the development of DSS-induced colitis in mice. We demonstrated that spinal anesthesia alleviated intestinal inflammation, maintained immunological function, and improved intestinal barrier function by modulating the gut microbiota. Reducing the increase in noradrenaline levels in DSS-treated mice by spinal anesthesia could be one of the mechanisms underlying the effect on the gut microbiota. The present study provided evidence supporting the protective effects of spinal anesthesia on IBD by modulating the gut microbiota, which highlights a novel approach for the treatment of IBD.

ARTICLE HIGHLIGHTS

Research background

Neuraxial anesthesia has been shown to exert a positive effect on intestinal microvascular perfusion. In an animal model of sepsis, thoracic epidural anesthesia was demonstrated to ameliorate perfusion deficits in the muscularis and mucosal layers of the gut. However, whether spinal aneathesia as a neuraxial anesthesia affects intestinal inflammation in inflammatory bowel disease (IBD) is still unclear.

Research motivation

The exact etiology of IBD remains unknown, and the imbalance of the gut microbiota is related to the occurrence and progression of IBD. The bidirectional between the brain and gut microbiota has gradually attracted more attention. Finding interventions on the brain-gut axis will be a new vision.

Research objectives

A dextran sodium sulfate (DSS)-induced colitis mouse model was established to explore the role of spinal anesthesia in IBD and to identify the potential mechanisms involved.

Research methods

A DSS-induced colitis mice model was established, and then we used spinal anesthesia on colitis mice to explore the role of spinal anesthesia in IBD and identify the potential mechanisms involved. Moreover, cohousing and fecal microbiota transplantation were used to help mice from separate lines share microbes across cocaged individuals.

Research results

This study clearly revealed that spinal anesthesia inhibited the development of DSS-induced colitis in mice. We demonstrated that spinal anesthesia alleviated intestinal inflammation, maintained immunological function, and improved intestinal barrier function by modulating the gut microbiota. Reducing the increase in noradrenaline levels in DSS-treated mice by spinal anesthesia could be one of the mechanisms underlying the effect on the gut microbiota.

Research conclusions

The study implied a positive effect of spinal anesthesia in relieving intestinal inflammation, protecting intestinal barrier function, and regulating the intestinal microflora in an IBD mouse model. Decreasing the noradrenaline level would be a possible mechanism of spinal anesthesia.

Research perspectives

The present study provided evidence supporting the protective effects of spinal anesthesia on IBD by modulating gut microbiota, which highlights a novel approach for the treatment of IBD.

FOOTNOTES

Author contributions: Hong Y and Zhao J contributed equally to this work; Xin Y, Hong Y, and Chen YR designed the research study; Huang ZH and Chen YR performed the research; Zhao J and Hou LD contributed analytic tools; Shen B and Zhao J analyzed the data and wrote the manuscript; all authors have read and approved the final manuscript.

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Institutional review board statement: The study was reviewed and conducted in accordance with guidelines for laboratory animal care after approval by the Laboratory Animals Ethics Committee of Zhejiang University.

Institutional animal care and use committee statement: All animal procedures were conducted in accordance with guidelines for laboratory animal care after approval by the Laboratory Animals Ethics Committee of Zhejiang University.

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ARRIVE guidelines statement: The authors have read the ARRIVE guidelines, and the manuscript was prepared and

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Country/Territory of origin: China

ORCID number: Yu Hong 0000-0001-5990-3720; Jie Zhao 0000-0002-8795-2770; Ye-Ru Chen 0000-0002-2457-7854; Zi-Hao Huang 0000-0003-0852-9235; Li-Dan Hou 0000-0001-5776-0703; Bo Shen 0000-0003-0727-9748; Yu Xin 0000-0002-6967-8704.

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ORIGINAL ARTICLE

Basic Study

Microbiologic risk factors of recurrent choledocholithiasis post-endoscopic sphincterotomy

Ying Li, Wen-Hui Tan, Jia-Chuan Wu, Zhi-Xin Huang, Yan-Yan Shang, Biao Liang, Jian-Hui Chen, Rui Pang, Xin-Qiang Xie, Ju-Mei Zhang, Yu Ding, Liang Xue, Mou-Tong Chen, Juan Wang, Qing-Ping Wu

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Ying Li, Yan-Yan Shang, Rui Pang, Xin-Qiang Xie, Ju-Mei Zhang, Yu Ding, Liang Xue, Mou-Tong Chen, Juan Wang, Qing-Ping Wu, Guangdong Provincial Key Laboratory of Microbial Safety and Health, Key Laboratory of Agricultural Microbiomics and Precision Application, Ministry of Agriculture and Rural Affairs, State Key Laboratory of Applied Microbiology Southern China, Institute of Microbiology, Guangdong Academy of Sciences, Guangzhou 510070, Guangdong Province, China

Wen-Hui Tan, Jia-Chuan Wu, Biao Liang, Digestive Endoscopy Center, Guangdong Second Provincial General Hospital, Guangzhou 510000, Guangdong Province, China

Zhi-Xin Huang, Jian-Hui Chen, Division of Gastrointestinal Surgery Center, The First Affiliated Hospital of Sun Yat-sen University, Guangzhou 510080, Guangdong Province, China

Corresponding author: Qing-Ping Wu, PhD, Academic Fellow, Chairman, Professor, Senior Researcher, Senior Scientist, Guangdong Provincial Key Laboratory of Microbial Safety and Health, Key Laboratory of Agricultural Microbiomics and Precision Application, Ministry of Agriculture and Rural Affairs, State Key Laboratory of Applied Microbiology Southern China, Institute of Microbiology, Guangdong Academy of Sciences, No. 66 Yard 100 Xieliezhong Road, Guangzhou 510070, Guangdong Province, China. wuqp203@163.com

Abstract

BACKGROUND

Choledocholithiasis is a severe disorder that affects a significant portion of the world's population. Treatment using endoscopic sphincterotomy (EST) has become widespread; however, recurrence post-EST is relatively common. The bile microbiome has a profound influence on the recurrence of choledocholithiasis in patients after EST; however, the key pathogens and their functions in the biliary tract remain unclear.

AIM

To investigate the biliary microbial characteristics of patients with recurrent choledocholithiasis post-EST, using next-generation sequencing.

METHODS

This cohort study included 43 patients, who presented with choledocholithiasis at the Guangdong Second Provincial General Hospital between May and June 2020. The patients had undergone EST or endoscopic papillary balloon dilation and

were followed up for over a year. They were divided into either the stable or recurrent groups. We collected bile samples and extracted microbial DNA for analysis through next-generation sequencing. Resulting sequences were analyzed for core microbiome and statistical differences between the diagnosis groups; they were examined using the Kyoto Encyclopedia of Genes and Genomes pathway hierarchy level using analysis of variance. Correlation between the key genera and metabolic pathways in bile, were analyzed using Pearson's correlation test.

RESULTS

The results revealed distinct clustering of biliary microbiota in recurrent choledocholithiasis. Higher relative abundances (RAs) of *Fusobacterium* and *Neisseria* (56.61% ± 14.81% *vs* 3.47% ± 1.10%, 8.95% ± 3.42% *vs* 0.69% ± 0.32%, respectively) and the absence of *Lactobacillus* were observed in the bile of patients with recurrent disease, compared to that in stable patients. Construction of a microbiological co-occurrence network revealed a mutual relationship among *Fusobacterium*, *Neisseria*, and *Leptotrichia*, and an antagonistic relationship among *Lactobacillales, Fusobacteriales*, and *Clostridiales*. Functional prediction of biliary microbiome revealed that the loss of transcription and metabolic abilities may lead to recurrent choledocholithiasis. Furthermore, the prediction model based on the RA of *Lactobacillales* in the bile was effective in identifying the risk of recurrent choledocholithiasis ($P = 0.03$).

CONCLUSION

We demonstrated differences in the bile microbiome of patients with recurrent choledocholithiasis compared to that in patients with stable disease, thereby adding to the current knowledge on its microbiologic etiology.

Key Words: Choledocholithiasis; Biliary tract; Microbiome; Endoscopic sphincterotomy; Recurrence; *Lactobacillus*

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Core Tip: Treatment of choledocholithiasis by endoscopic sphincterotomy (EST) has become widespread, but recurrence post-EST is relatively common. In this study, we analyzed the bile microbiome of patients with recurrent choledocholithiasis. Increase in Fusobacterium and Neisseria, and the absence of Lactobacillus in bile were the key microbiologic features of recurrent choledocholithiasis. Bile microbiome imbalance might cause poor metabolism of carbohydrates and amino acids and increased glycan biosynthesis in the biliary tract, leading to disease recurrence. The microbiological features in bile could be an effective predictor for choledocholithiasis recurrence post-EST. The findings of our study will help develop new prevention strategies for post-surgery recurrence of choledocholithiasis.

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INTRODUCTION

Cholelithiasis is a common and socially significant health problem worldwide, occurring in approximately 5%-22% of adults, with synchronous common bile duct stone (CBDS) in 20% of these patients[1-4]. In western countries, it is one of the leading gastrointestinal conditions that results in hospitalization [4]. Cholelithiasis with CBDS can lead to biliary obstruction, secondary cholangitis, and obstructive jaundice, endangering lives in some severe cases and often requiring surgical interventions[5]. The introduction of endoscopic treatment started a new era in the treatment of choledocholithiasis[6-9], and management by endoscopic sphincterotomy (EST) or endoscopic papillary balloon dilation (EPBD) has become widespread, replacing open laparoscopic cholecystectomy or open common bile duct exploration with choledochoscopy[10,11].

However, long-term surveys have revealed up to 39% recurrence of choledocholithiasis post-EST, and life-long follow-ups are still needed after surgery[12-14]. Recurrent choledocholithiasis post-EST involves complicated factors, including infections and biliary anatomical abnormalities[15,16]. The elimination of certain pathogens in the bile duct can significantly reduce the recurrence rate[17,18].

Therefore, further investigations into the microbiological etiology and underlying mechanisms of recurrent choledocholithiasis post-EST are crucial for its prediction and prevention in clinical practice.

Complex microbiomes in the biliary system have been observed using next-generation sequencing (NGS)[19]. In these systems, the microbiota metabolize and secrete cholesterol and bile acids; their dysfunction may cause pathophysiological defects and result in stone formation[20,21]. Unlike primary stones, secondary stones in recurrent choledocholithiasis predominately consist of more cholesterol than calcium bilirubinate[22], and their microbiological etiology remains unclear.

In this study, we investigated the microbiological etiology of recurrent choledocholithiasis using NGS to find the key pathogens associated with recurrence post-EST and their metabolic characteristics in disease relapse.

MATERIALS AND METHODS

Study participants and sample collection

Ethical compliance: Consecutive recruitment of eligible patients was carried out in the Department of Endoscopy at Guangdong Second Provincial General Hospital from May to June 2020. All experimental protocols were approved by this hospital's ethics committee (project 2019-QNJJ-14-02). The study design complied with all relevant ethical regulations in accordance with the Declaration of Helsinki and Belgian Privacy Commission. Written consent was obtained from all patients in the study.

Study cohort: In this study, we included 43 choledocholithiasis participants diagnosed using computed tomography (CT) or magnetic resonance cholangiopancreatography (MRCP). All patients were assessed by experienced doctors, without risk of EST-related complications[10]. Patients with a history of malignant diseases, autoimmune diseases, diabetes, structure abnormality of the biliary tract, or any exposure to antibiotics within one month were excluded from the study. All patients accepted laparoscopic cholecystectomy (LC) treatment following an EST or endoscopic papillary balloon dilation (EPBD), during the same hospitalization episode. The components of the stones were recorded according to the method of Dosch[23]. All patients received CT or MRCP examinations one week after the treatment to ensure the complete removal of stone in the biliary tract. All participants underwent at least one-year follow-up with transabdominal ultrasonography every three months, and CT or MRCP was performed once recurrence of choledocholithiasis was indicated through clinical presentations or imaging examinations. Patients were divided into stable and recurrent groups according to their disease evaluation at the end of the follow-up period.

Bile sample collection: Bile samples were collected during endoscopic treatment. The ERCP was performed to confirm the diagnosis of choledocholithiasis, followed by the EST or EPBD treatment, and the bile sample was collected through suction during the treatment. The bile samples were immediately transported to the laboratory and stored at -80 °C until extraction.

Microbiome DNA extraction and 16S rRNA gene amplicon sequencing

Bile sample (3 mL) was centrifuged at 16000 × *g* at 4 °C for 10 min, and the pellet was washed twice with phosphate-buffered saline before DNA extraction. Microbiome DNA was then extracted using the QIAamp PowerFecal DNA kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

The *16S rRNA* gene obtained from each bile sample was amplified by targeting the V3-V4 hypervariable regions using the following primers: 341F 5′-CCTACGGGNGGCWGCAG-3′ and 806R 5′-GGACTACHVGGGTWTCTAAT-3′ using the UCP Multiplex PCR Kit (Qiagen)[24]. The amplicon library was prepared using the QIAseq Ultralow Input Library Kit (Qiagen). An Agilent Bioanalyzer 2100 system (Agilent Technologies, Santa Clara, CA, United States) and Qubit dsDNA HS Assay Kit (Invitrogen Life Technologies, Carlsbad, CA, United States) were used to validate the library pooling. Paired-end sequencing was conducted using the MiSeq platform (Illumina, San Diego, CA, United States) with MiSeq Reagent Kit version V3 (Illumina).

Microbiome sequence curation and analysis

Trimming and quality filtering of the data were performed using the CLC Genomic Workbench version 20.0, with the Microbial Genomics Module (Qiagen). Sequences were matched to the Greengenes database version 13.5.

The amplicon sequencing, and the taxonomic and statistical analyses were performed using Calypso version 8.84[25]. Alpha diversity was determined based on Fisher's alpha index, which was assessed using the analysis of variance test. Microbial diversity was visualized using the canonical correspondence analysis based on the prognosis groups. Key taxonomic discovery analysis related to prognosis was performed using linear discriminant analysis effect size (LEfSe) at the genus level[26]. The relative abundance (RA) measurements of the genera, with biomarker significance were compared using the Wilcoxon rank test.

The core microbiome was identified as described by Ainsworth[27]. Network analysis was performed to identify the co-occurring and exclusive bacteria using Calypso[25]. Genera and orders of the bile microbiome were represented as nodes, taxa RAs as node size, and edges as positive and negative associations. Networks were generated based on the associations between both genera and orders using Pearson's correlation; nodes were colored based on their association with different prognosis groups. Only relationships with statistical significance ($P < 0.05$) were visualized in the network.

Metagenome prediction of the bile microbiome was performed using the amplicon sequencing approach in the Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt)[28]. The statistical differences between the diagnosis groups were examined using the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway hierarchy level using analysis of variance. Correlation analysis was carried out between the key genera and metabolic pathways in bile using Pearson's correlation test. Survival analysis of the identical microbiological risk factors was carried out using Kaplan-Meier analysis.

Statistical analyses

Except for those analyzed using Calypso, data were analyzed using GraphPad Prism v7.00 software (GraphPad, La Jolla, CA, United States). All analyses in the study were statistically significant at $P < 0.05$, and P values were adjusted using false discovery rate, Bonferroni, or area under curve correction.

RESULTS

Clinical features and prognosis of patients with choledocholithiasis one-year post-EST

Forty-three choledocholithiasis patients, who underwent LC following EST were recruited in this study and received a one-year follow-up survey. Thirteen patients had co-occurrence of cholelithiasis; the baseline clinical characteristics of the 43 choledocholithiasis patients were shown in Table 1. The stone components were recorded according to the methods of Dosch[23]; they were classified as brown pigmented stones, black pigmented stones, cholesterol stones, and mixed stones. Four recurrent cases without other complications were observed using routine ultrasonography as well as CT during the follow-up period. No significant differences were found in the clinical features or the stone components between patients with and without recurrent choledocholithiasis.

Bile microbiome characteristics in patients with choledocholithiasis

A total of 702 unique operational taxonomic units were identified in the bile of all patients with choledocholithiasis, indicating the diversity of the microbiome in bile (Figure 1). *Streptococcus* and an unclassified genus of *Enterobacteriaceae* were the most dominant genera; they were detected in the bile of 28 and 29 patients, respectively. The average RAs of *Streptococcus* and *Fusobacterium* in bile were 13.59% and 19.91%, respectively.

Key microorganisms in bile of patients with recurrent choledocholithiasis

The bile microbial structure in patients with recurrent choledocholithiasis was different from that in patients without recurrence, with lower alpha diversity ($P = 0.41$) and distinct beta diversity ($P = 0.03$; Figure 2).

LEfSe biomarker discovery analysis identified *Fusobacteriales* and *Neisseriales* as biomarkers in the recurrent group and *Lactobacillales* in the stable group at the order level (Figure 3A). The RAs of *Fusobacteriales* (56.61% ± 14.81% *vs* 3.47% ± 1.10%) and *Neisseriales* (8.95% ± 3.42% *vs* 0.69% ± 0.32%) were higher in patients with recurrent choledocholithiasis than that in stable patients post-EST ($P < 0.05$), while the RA of *Lactobacillales* was significantly lower in the recurrent group (1.48% ± 1.28%) than that in the stable group (25.04% ± 4.76%; $P < 0.05$; Figure 3B).

Bile microbiological ecosystem analyses in patients with choledocholithiasis with different prognoses post-EST

Core microbiome analyses showed that *Streptococcus, Prevotella, Fusobacterium*, an unclassified genus of *Enterobacteriaceae*, and an unclassified genus of *Clostridiaceae* were the shared core genera in both the stable and the recurrent group. *Veillonella*, *Oribacterium*, *Neisseria*, *Leptotrichia*, and *Campylobacter* were the specific core genera in the recurrent group, while *Enterococcus*, *Clostridium*, and an unclassified genus of *Aeromonadaceae* were the unique genera in the stable group (Table 2). Construction of a microbiological co-occurrence network revealed a mutual relationship among *Fusobacterium*, *Neisseria*, and *Leptotrichia* (Figure 4A).

Additionally, *Lactobacillales*, *Fusobacteriales*, *Enterobacteriales*, *Clostridiales*, and *Bacteroidales* were the shared core orders in both the stable and the recurrent group. *Pasteurellales*, *Neisseriales*, and *Campylobacterales* were the unique core orders in the recurrent group, while *Pseudomonadales*, *Burkholderiales, Bacillales, Aeromonadales*, and *Actinomycetales* were the unique orders in the stable group. Co-occurrence network analyses suggested mutual enhancement among the key recurrence-related pathogens in bile

Table 1 Clinical characteristics of choledocholithiasis patients

	Stable (*n* = 39)	Relapse (*n* = 4)	*P* value
Age (yr) (range)	47 (38-64)	44 (38-46)	0.142
Sex			
Male (cases) (%)	24 (61.54)	3 (75.00)	0.626
Female (cases) (%)	15 (38.46)	1 (25.00)	
History of smoking (cases) (%)	10 (25.64)	1 (25.00)	0.978
Comorbidities			
Type 2 diabetes mellitus	4 (10.26)	1 (25.00)	0.381
Hypertension	6 (15.38)	1 (25.00)	0.620
Hyperlipoidemia	13 (33.33)	2 (50.00)	0.505
Accompanied diagnosis			
Cholelithiasis (cases) (%)	11 (28.21)	2 (50.00)	0.366
Acute cholangitis (cases) (%)	4 (10.26)	1 (25.00)	0.381
Pancreatitis (cases) (%)	1 (2.56)	0 (0.00)	-
Serum biochemical indexes			
ALT (U/L)	161.50 ± 159.11	67.75 ± 75.61	0.210
AST (U/L)	128.51 ± 151.74	34.75 ± 38.20	0.063
Total Bilirubin (μmol/L)	92.19 ± 82.97	24.23 ± 23.50	0.057
Direct Bilirubin (μmol/L)	84.92 ± 91.99	18.73 ± 23.69	0. 060
Amylase (U/L)	118.69 ± 192.30	77.50 ± 30.39	0.544
Follow-up time (d)	369.80 ± 2.67	372.00 ± 4.00	0.101
Recurrent time from EST (d)	-	208.80 ± 87.97	-
Stone components			
Brown pigment (cases) (%)	29 (74.36)	2 (50.00)	0.303
Black pigment (cases) (%)	8 (20.51)	1 (25.00)	
Cholesterol (cases) (%)	0 (0.00)	0 (0.00)	
Mixed component (cases) (%)	2 (5.13)	1 (25.00)	

EST: Endoscopic sphincterotomy.

and antagonistic relationships among *Lactobacillales*, *Fusobacteriales*, and *Clostridiales* in the ecosystem, indicating the role of probiotics in the prevention of recurrence (Figure 4B).

Functional characteristics of bile microbiome in patients with choledocholithiasis with different prognoses post-EST

The metabolites from microorganisms are the key pathogenic factors for the host; therefore, the characteristics of the metabolic pathways in bile were analyzed for deeper insight into the microbiologic etiology of recurrent choledocholithiasis post-EST. Comparative analyses of microbiological functions were carried out at the 2nd hierarchy level of the KEGG pathway. In the stable group, the bile microorganisms were active in the transcription and metabolism related to the nervous system, infectious diseases, biosynthesis of carbohydrates and amino acids; while, in the recurrent group, the microbes were active in translation, replication and repair, metabolism of cofactors and vitamins, glycan biosynthesis and metabolism, genetic information processing, energy metabolism, and biosynthesis of secondary metabolites (Figure 5).

Furthermore, correlations between the key genera in the two groups and the different metabolic pathways were analyzed to identify the influence of certain microbes on the host (Figure 6). In the bile ecosystem of the patients with recurrent disease, *Fusobacterium* and *Campylobacter* had positive correlations with the metabolism of amino acids, replication and repair, and translation ($P < 0.05$), while the unclassified genus of *Enterobacteriaceae* had a negative correlation with all the discrepant metabolic

Table 2 Core microbiome in bile of choledocholithiasis patients with different prognosis

Core microbiome	Type	Group	Recurrent. Occ	Stable. Occ
Clostridium	Unique	Stable	0	0.44
Enterococcus	Unique		0	0.44
Unclassified genus of *Aeromonadaceae*	Unique		0.25	0.49
Fusobacterium	Core	Recurrent&Stable	1	0.41
Prevotella	Core		0.5	0.56
Streptococcus	Core		0.5	0.67
Unclassified genus of *Clostridiaceae*	Core		0.75	0.49
Unclassified genus of *Enterobacteriaceae*	Core		0.5	0.69
Campylobacter	Unique	Recurrent	0.5	0.18
Leptotrichia	Unique		0.75	0.28
Neisseria	Unique		0.75	0.31
Oribacterium	Unique		0.5	0.18
Veillonella	Unique		0.5	0.38

pathways between the two groups ($P < 0.05$). *Leptotrichia* had a positive correlation with all the discrepant metabolic pathways in the bile of the stable group ($P < 0.05$). Correlation analyses indicated that in bile of the recurrent group, increased *Fusobacterium* could alter the metabolism of amino acids, replication and repair, and translation functions, leading to the formation of secondary bile stones.

Microbiologic risk factor analysis of the recurrent group post-EST

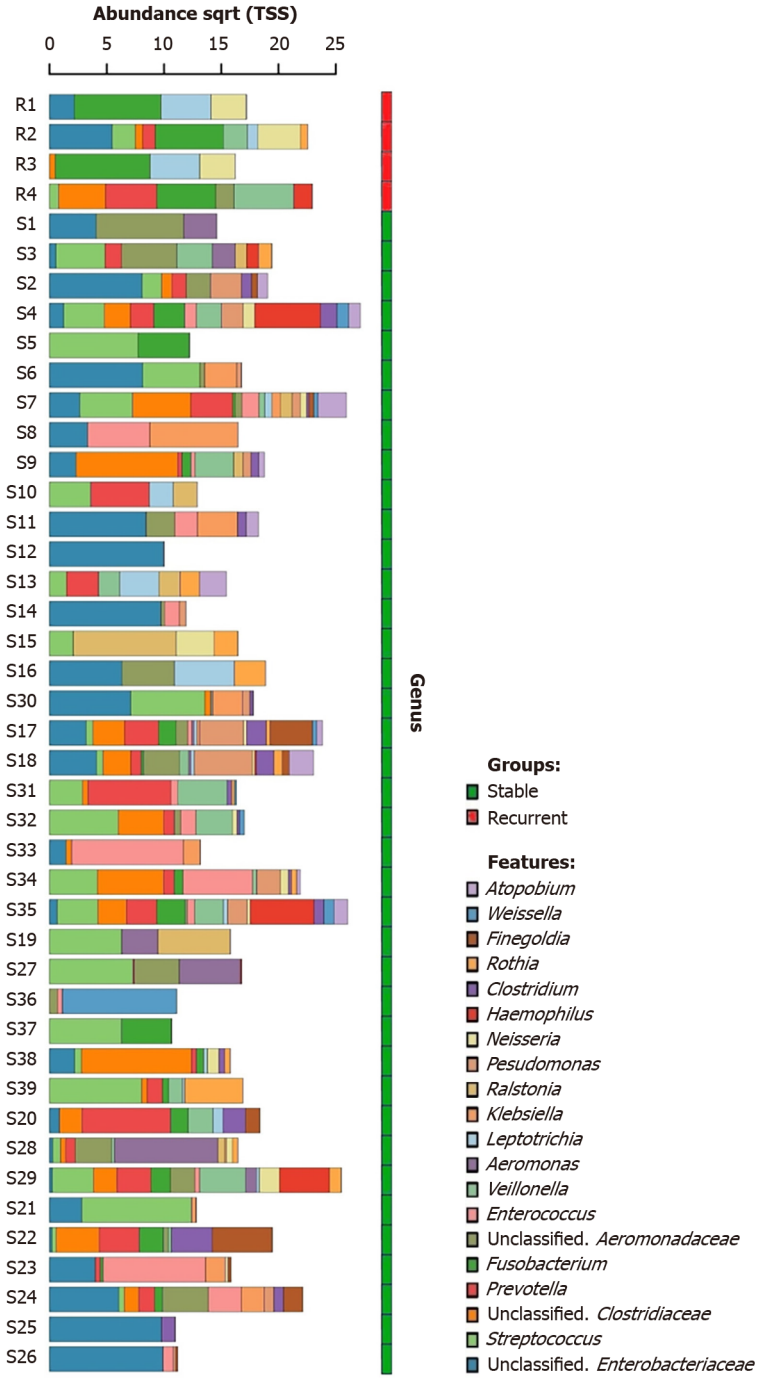
Fusobacteriales and *Neisseriales* were identified as the bile biomarkers in the recurrent group and *Lactobacillales* in the stable group. Kaplan-Meier analysis was carried out to confirm whether these biomarkers can be used as independent predictive factors for recurrence post-EST (Figure 7). The statistical results revealed that patients with *Lactobacillales* in the bile were at a lower risk of recurrence post-EST ($P = 0.03$) than patients, who lacked this order in their bile.

DISCUSSION

It was assumed that the biliary system is sterile in healthy people; however, an increasing amount of NGS-supported evidence shows that bile supports a complex and abundant microbiome in healthy individuals[19,29]. The frequently identified microorganisms using traditional culture techniques are *Enterococcus*, *Klebsiella*, and *Pseudomonas*; these bacteria are active in reducing the bile acid pool and regulating bile acid metabolism[30-33]. However, the contribution of microbes to the biliary system is still unclear. NGS techniques revealed that the most common inhabitants of the biliary tract are *Proteobacteria*, *Firmicutes*, *Bacteroidetes*, *Actinobacteria*, *Fusobacteria*, *Synergistetes*, and candidate phylum *Saccharibacteria* (*TM7*)[34]. Some of these microorganisms regulate the hydrolysis of bile acids to constituent components, cleavage of exogenous aromatic rings, deconjugation of bile acid complexes by hydrolytic enzymes, and the formation of free bile acids[35]. The disturbance of the microbiologic ecosystems in bile may lead to dysfunctional bile acid metabolism, resulting in a series of bile duct diseases[20,21]; however, the most disease-specific pathogens and their unique functions remain unknown.

Similar to the results from previous studies[29,34,36], this study revealed that the biliary tract was composed of a diversity of bacteria, and the majority of microorganisms in the bile were *Streptococcus*, *Prevotella*, *Fusobacterium*, *Enterococcus*, *Veillonella*, and *Clostridium*. *Lactobacillus* and *Lactococcus* were reported as the major genera in bile[36]; however, these two genera could only be detected in 11 patients in this study. These differences could be attributed to the differences in study designs; we included only patients with severe choledocholithiasis, who needed surgical intervention, for the analysis of microbial risk factors for disease recurrence. Another factor could be the difference in bile sampling; we chose the endoscopic route over open surgery for the collection of bile.

Endoscopic treatment such as EST can provide definitive relief to choledocholithiasis; however, the formation of gallstones will not stop unless the etiologic factors are eliminated[12,13,37]. Among all the risk factors for choledocholithiasis recurrence, only biliary infections are correctable; microbiological treatment is the most potential therapy against the recurrence of choledocholithiasis[5,15,16,38,39].

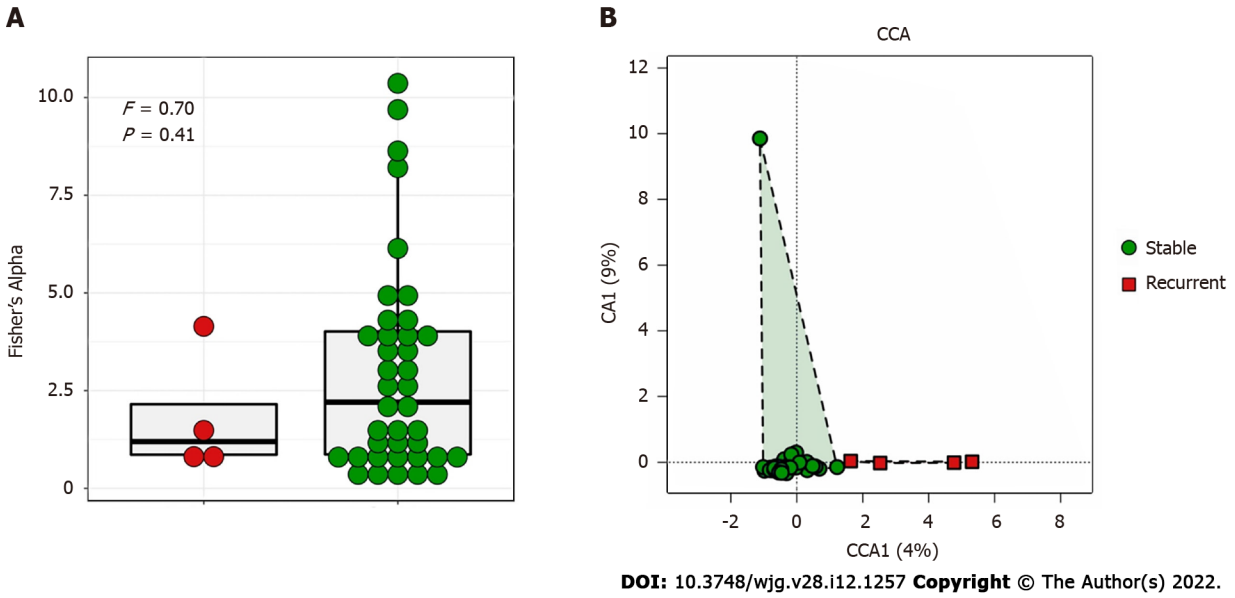


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Figure 1 Dominant bacterial genera in the bile of choledocholithiasis patients. The top 20 dominant bacterial genera in bile are shown in the bar chart, the bile microbiome of recurrent choledocholithiasis patients post-endoscopic sphincterotomy are in the red group and choledocholithiasis patients without recurrence post-EST are in the green group. The genera were listed from the bottom to the top according to their relative abundance in bile samples.

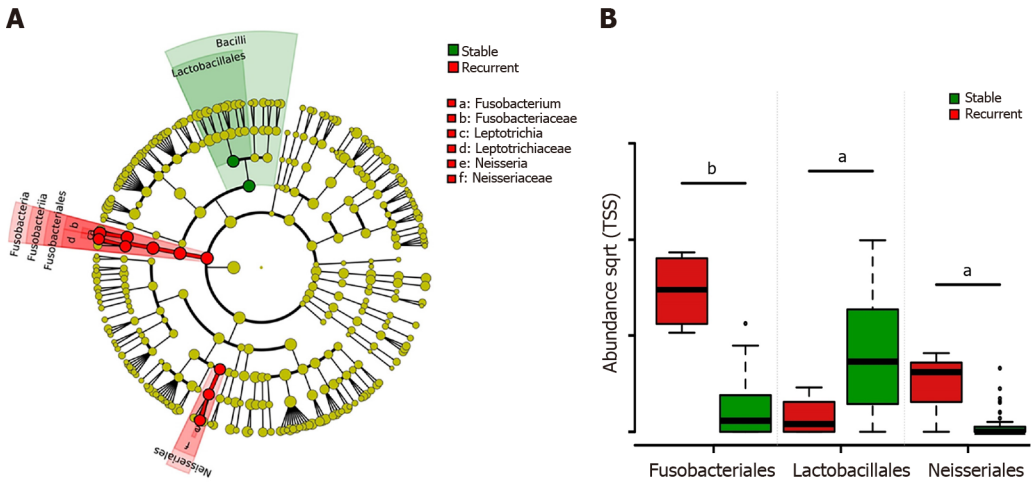
Therefore, investigation into the biliary microbiology characteristics of recurrent choledocholithiasis is crucial to both etiology and prevention studies. To the best of our knowledge, this is the first pilot study to investigate the microbiological risk factors in recurrent choledocholithiasis post-EST. Increased *Fusobacterium* and *Neisseria* were recurrence-related biomarkers in the bile microbiome. Furthermore, we discovered the antagonistic potentials of *Lactobacillus* and an unclassified genus of *Enterobacteriales* against *Fusobacterium* and *Neisseria*, indicating the potential use of probiotics in the prevention of recurrence post-EST.

Bacteria in bile play an active role in gallstone formation[35]. *Escherichia coli* and *Klebsiella* in bile can produce hydrolytic enzymes such as β-glucuronidase, phospholipase A[40], and conjugated bile acid hydrolase; in addition, they can cause deconjugation of bilirubin diglucuronide and precipitation of calcium bilirubinate, which ultimately leads to biliary stone formation[41,42]. We identified *Clostridium* as one of the key microorganisms in the bile microbiome, which, according to Leung *et al*[43], is a more



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Figure 2 Diversity analysis of bile microbiome of choledocholithiasis patients. A: Comparison of alpha diversity of bile microbiome by the Fisher's Alpha Index between stable (green) and recurrent (red) choledocholithiasis patients post-endoscopic sphincterotomy (EST); B: Comparison of beta diversity of bile microbiome using canonical correspondence analysis between stable (green) and recurrent (red) choledocholithiasis patients post-EST.

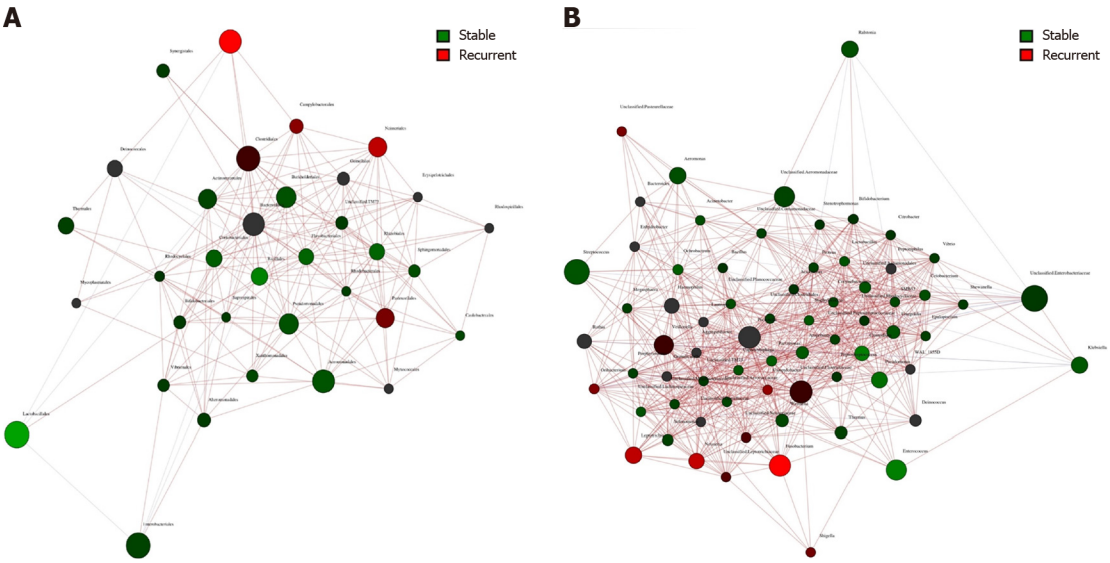


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Figure 3 LEfSe analysis of group-specific microbes in choledocholithiasis patients with different prognosis post endoscopic sphincterotomy. A: Colored cladogram showing microbiota with biomarker significance in choledocholithiasis patients with different prognosis post-endoscopic sphincterotomy (EST) (red for biomarkers in recurrent patients and green for biomarkers in patients without recurrence post EST); B: Relative abundance comparison of microbes with biomarker significance in choledocholithiasis patients with different prognosis post-EST. Statistical significance is expressed as $^{a}P < 0.05$, $^{b}P < 0.001$.

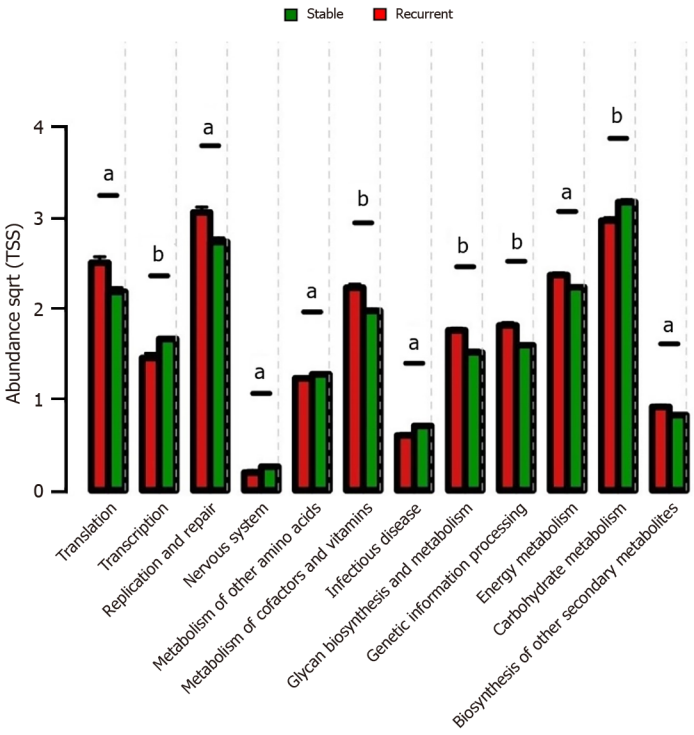
important microorganism in the deconjugation of bilirubin diglucuronide than *E. coli*, because it exhibits a 34-fold higher β-glucuronidase enzyme activity in the biliary tract. A lack of *Lactobacillus* in the bile could be a probable risk factor for choledocholithiasis, because *Lactobacillus* in bile can absorb cholesterol and reduce total serum cholesterol[44,45]. The core microbiome pattern in the bile of patients with choledocholithiasis in this study offers a more comprehensive understanding of the influence of the bile microbiome on biliary stone formation.

Furthermore, functional analysis indicated that the loss of transcription and metabolic abilities, and increased function of translation, replication and repair, metabolism of cofactors and vitamins, glycan biosynthesis and metabolism, genetic information processing, energy metabolism, and biosynthesis of other secondary metabolites could lead to recurrent choledocholithiasis. Most of these microbiologic functions were caused by the increased abundance of *Fusobacterium* and *Leptotrichia* and the loss of an unclassified genus of *Enterobacteriales*. However, little is known about the specific health-related functions of the metabolites of these microbes in the bile, and these important metabolic pathways require further research.



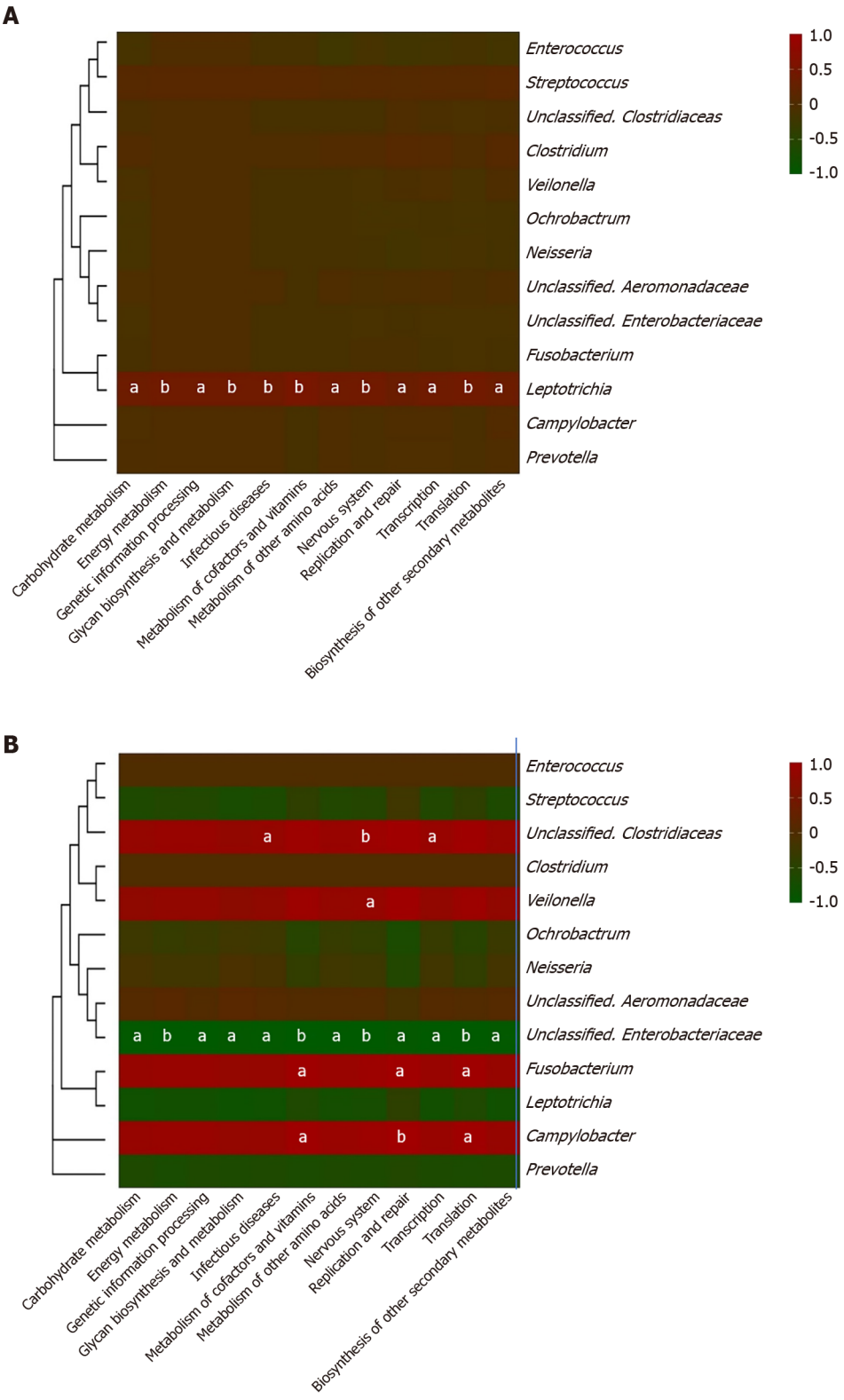
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Figure 4 Co-occurrence network analysis of bile microbiome of choledocholithiasis patients with different prognosis post endoscopic sphincterotomy. A: Co-occurrence and disease-specific bacterial interactions at the order level. Order was presented as nodes (stable group specific order in green and recurrent group specific order in red), order abundance was presented as node size, and edges were represented based on their association tested using Pearson's correlation (positive inter-node correlations in blue, negative inter-node correlations in red); B: Co-occurrence and disease-specific bacterial interactions at the genus level. Genus was presented as nodes (stable group specific genus in green and recurrent group specific genus in red), genus abundance was presented as node size, and edges were represented based on their association tested using Pearson's correlation (positive inter-node correlations in blue, negative inter-node correlations in red).



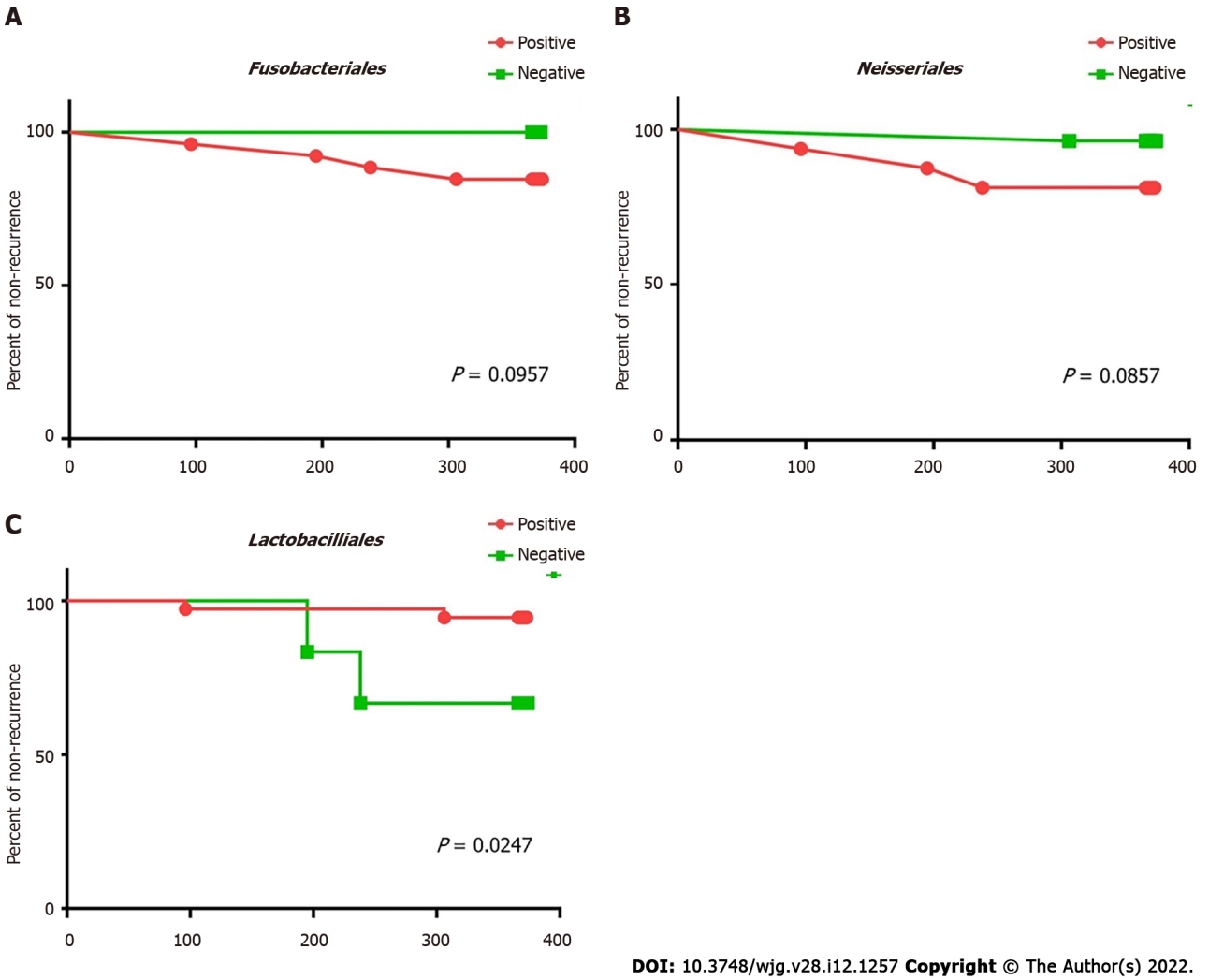
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Figure 5 Comparison of microbial function prediction of bile microbiome of choledocholithiasis patients with different prognosis post endoscopic sphincterotomy. Functional analysis was performed at the 2nd hierarchy level of the Kyoto Encyclopedia of Genes and Genomes pathways in the bile microbiome of choledocholithiasis patients. Wilcoxon test was applied to the comparison of each category of microbial function; those with significant differences are shown in the bar chart. Statistical significance is expressed as $^{a}P < 0.05$, $^{b}P < 0.01$.



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Figure 6 Heatmap of correlation between the core microbiome and key metabolic pathway in choledocholithiasis patients. Thirteen core genera of bile microbiome and their correlations with the twelve discrepant metabolite pathways in different prognosis groups were analyzed using Pearson correlation analysis. The Pearson correlation coefficient between the genus and the metabolic pathway was calculated and shown in colored matrix; red represents a positive correlation, while green represents a negative correlation. A: Matrix heatmap shows the correlations between different genera and metabolite pathways in choledocholithiasis patients without recurrence post-endoscopic sphincterotomy (EST); B: Matrix heatmap shows the correlations between different genera and metabolite pathways in recurrent choledocholithiasis patients post-EST. Statistical significance is expressed as $^{a}P < 0.05$, $^{b}P < 0.01$.



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Figure 7 Kaplan-Meier analysis of recurrent time post endoscopic sphincterotomy with different microbiologic risk factors. A: Kaplan-Meier analysis of recurrent time post-endoscopic sphincterotomy (EST) between choledocholithiasis patients with (red) and without (green) *Fusobacteriales* in bile; B: Kaplan-Meier analysis of recurrent time post-EST between choledocholithiasis patients with (red) and without (green) *Neisseriales* in bile; C: Kaplan-Meier analysis of recurrent time post-EST between choledocholithiasis patients with (red) and without (green) *Lactobacillales* in bile.

Certain microorganisms in bile could predict the time taken before disease recurrence post-EST, and this was evaluated. The existence of *Lactobacillales* is crucial for predicting recurrence time post-EST, because patients with *Lactobacillales* in their bile had a longer progression-free time post-EST than patients without *Lactobacillales*. Therefore, the examination of *Lactobacillales* existence in bile at the time of endoscopic examination could help doctors identify high-risk patients, who are likely to have early choledocholithiasis recurrence post-EST.

The limited number of early recurrent choledocholithiasis patients could have introduced a bias in statistical analysis and could have limited the generalizability of the prediction model in this study. Furthermore, the diagnosis of choledocholithiasis recurrence relied mainly on the imaging examinations; we could have missed some stones which were invisible in the CT, underestimating the rate of choledocholithiasis recurrence. The molecular mechanisms of microorganisms underlying the recurrence post-EST was based on the PICRUSt model. Verification experiments such as analyzing the correlation between the bile microbiome and the stone composition, and animal experiments to ascertain the preventive effects of *Lactobacillus* in choledocholithiasis recurrence are warranted.

CONCLUSION

The microbiological characteristics of bile from patients with recurrent choledocholithiasis post-EST indicated that an increase in *Fusobacterium* and *Neisseria* are potential biomarkers for the identification of high-risk patients in the first EST. It elucidated the role of microbial metabolites in the underlying etiology of choledocholithiasis. A co-occurrent network of the biliary bacterial community was constructed. Potential preventive therapy against recurrent choledocholithiasis through supple-

mentation with *Lactobacillus* and maintenance of the balance of the microbial systems could be promising. These findings could help doctors better understand the etiology of recurrent choledocholithiasis and develop better monitoring and treatment strategies against recurrence post-EST.

ARTICLE HIGHLIGHTS

Research background

Choledocholithiasis is a common and socially significant health problem worldwide, and endoscopic sphincterotomy (EST) has become widespread in treating choledocholithiasis; however, recurrence post-EST is relatively common. The bile microbiome has a profound influence on the recurrence of choledocholithiasis; however, the key pathogens and their functions are not fully elucidated.

Research motivation

To determine the microbiologic risk factors of recurrent choledocholithiasis post EST.

Research objectives

To investigate the biliary microbial characteristics of the recurrent choledocholithiasis post-EST, using next-generation sequencing.

Research methods

This cohort study included 43 choledocholithiasis patients who had undergone EST were followed up for over a year. They were divided into either the stable or recurrent groups and comparison of their bile microbiome was carried out through next-generation sequencing. Resulting sequences were analyzed for core microbiome and statistical differences between the microbiologic compositions and functions. Correlation between the key genera and metabolic pathways in bile, were analyzed using Pearson's correlation test.

Research results

The results revealed distinct clustering of biliary microbiota in recurrent choledocholithiasis, in which higher relative abundances (RAs) of *Fusobacterium* and *Neisseria* and the absence of *Lactobacillus* were observed in the bile of the recurrent patients. Microbiological co-occurrence network revealed a mutual relationship among *Fusobacterium*, *Neisseria*, and *Leptotrichia*, and an antagonistic relationship among *Lactobacillales, Fusobacteriales*, and *Clostridiales*. Functional analysis revealed that the loss of microbiologic transcription and metabolic abilities may lead to the choledocholithiasis recurrence. Furthermore, the prediction model based on the RA of *Lactobacillales* in the bile was effective in identifying the risk of recurrent choledocholithiasis.

Research conclusions

We concluded the microbiologic differences in the bile of recurrent choledocholithiasis patients post EST, thereby adding to the current knowledge on its microbiologic etiology.

Research perspectives

The findings of our study will help develop new prevention strategies for post-surgery recurrence of choledocholithiasis.

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FOOTNOTES

Author contributions: Li Y, Tan WH, Wu JC and Wu QP designed the research; Wu JC, Tan WH and Liang B recruited the clinical cohort, collected samples, and performed the follow-up surveys; Li Y, Huang ZX and Shang YY contributed to the amplicon sequencing; Li Y, Chen JH, Pang R and Xie XQ analyzed the data; Li Y, Wu JC, Huang ZX and Xue L wrote the paper; Zhang JM, Ding Y, Chen MT, Wang J, Tan WH and Wu QP performed critical revisions of the manuscript; Li Y, Tan WH and Wu JC contribute equally to the manuscript; all authors approved the final version of the article.

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Country/Territory of origin: China

ORCID number: Ying Li 0000-0003-4012-6954; Jia-Chuan Wu 0000-0001-8490-2965; Zhi-Xin Huang 0000-0001-9862-1523; Yan-Yan Shang 0000-0002-6079-8195; Biao Liang 0000-0003-1954-8976; Jian-Hui Chen 0000-0002-8881-6949; Rui Pang 0000-0002-3795-9843; Xin-Qiang Xie 0000-0003-3943-7755; Ju-Mei Zhang 0000-0003-2103-6443; Yu Ding 0000-0002-3688-7294; Liang Xue 0000-0003-3606-4615; Mou-Tong Chen 0000-0003-2837-2124; Juan Wang 0000-0002-1545-3593; Wen-Hui Tan 0000-0003-0364-5069; Qing-Ping Wu 0000-0001-6503-359X.

Corresponding Author's Membership in Professional Societies: Chinese Academy of Engineering, Academician of Chinese Academy of Engineering; Chinese Institute of Food Science and Technology, vice president; China Edible Fungus Association, vice chairman.

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ORIGINAL ARTICLE

Retrospective Study

Epidemiological, clinical, and histological presentation of celiac disease in Northwest China

Man Wang, Wen-Jie Kong, Yan Feng, Jia-Jie Lu, Wen-Jia Hui, Wei-Dong Liu, Zi-Qiong Li, Tian Shi, Mei Cui, Zhen-Zhu Sun, Feng Gao

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Man Wang, Wen-Jie Kong, Yan Feng, Jia-Jie Lu, Wen-Jia Hui, Wei-Dong Liu, Zi-Qiong Li, Tian Shi, Feng Gao, Department of Gastroenterology, People's Hospital of Xinjiang Uygur Autonomous Region, Urumqi 830001, Xinjiang Uygur Autonomous Region, China

Mei Cui, Zhen-Zhu Sun, Department of Pathology, People's Hospital of Xinjiang Uygur Autonomous Region, Urumqi 830001, Xinjiang Uygur Autonomous Region, China

Corresponding author: Feng Gao, PhD, Chairman, Chief Doctor, Director, Professor, Department of Gastroenterology, People's Hospital of Xinjiang Uygur Autonomous Region, No. 91 Tianchi Road, Tianshan District, Urumqi 830001, Xinjiang Uygur Autonomous Region, China. drxjgf@sina.com

Abstract

BACKGROUND

Research on celiac disease (CD) in northwest China is still in its infancy. At present, large-sample data on the epidemiological, clinical, and pathological characteristics of CD are limited.

AIM

To investigate the epidemiological, clinical, and pathological characteristics of CD in northwest China.

METHODS

The clinical data of 2884 patients with gastrointestinal (GI) symptoms were retrospectively analyzed. Total immunoglobulin A (IgA) and anti-tissue transglutaminase (tTG) IgA levels were examined in all patients. Gastroscopy and colonoscopy were performed in patients with positive anti-tTG IgA and deficient total IgA levels. Atrophy of the duodenal and ileal villi was examined and histopathological examinations were performed. The modified Marsh–Oberhuber classification system was used to grade villous atrophy in the duodenum or distal ileum. The patients' *Helicobacter pylori* (*H. pylori*) infection status was compared in terms of clinical presentation and Marsh grade. Statistical analyses were performed using the t-test or chi-square test.

RESULTS

Among the 2884 patients, 73 were positive for serum anti-tTG IgA, and 50 were diagnosed with CD. The CD detection rate was significantly higher in Kazakhs

(4.39%) than in Uyghurs (2.19%), Huis (0.71%), and Hans (0.55%). The main symptoms of CD were chronic diarrhea, anorexia, anemia, fatigue, weight loss, sleep disorders, osteopenia, and osteoporosis. The body mass index of patients with CD was significantly lower than that of patients without CD. A total of 69 patients with positive serum anti-tTG IgA and two patients with deficient total IgA levels underwent GI endoscopy. Endoscopy revealed crypt hyperplasia and/or duodenal villous atrophy, mainly manifested as nodular mucosal atrophy, grooves, and fissures. The difference in *H. pylori* infection rates was not statistically significant between CD and non-CD patients but was significantly different among CD patients with different Marsh grades.

CONCLUSION

Among the patients with GI symptoms in northwestern China, the prevalence of CD was more in the Uyghur and Kazakh populations. *H. pylori* infection may be associated with CD severity.

Key Words: Celiac disease; Epidemiology; Gastrointestinal symptoms; Pathology; *Helicobacter pylori* infection

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Core Tip: Celiac disease (CD) is an autoimmune disease caused by the ingestion of gluten in genetically susceptible individuals. The global prevalence of CD is approximately 1.4%. An increase in celiac-specific autoantibody levels can lead to varying degrees of damage to the small intestinal mucosa and consequently to various gastrointestinal and systemic symptoms. This study reports the epidemiological, clinical, and pathological characteristics of CD and its association with *Helicobacter pylori* infection and aims to provide useful information for the clinical diagnosis and treatment of CD.

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INTRODUCTION

Celiac disease (CD) is an autoimmune chronic inflammatory disorder of the small intestine caused by ingestion of gluten in genetically susceptible individuals. Intestinal mucosal gluten-reactive CD4+ T cells are involved in the pathogenesis of CD[1]. The presence of T-cells in the mucosa can cause varying degrees of damage to the small intestinal mucosa, leading to a variety of gastrointestinal (GI) and systemic symptoms[2]. Typical GI manifestations include abdominal pain, abdominal distension, and diarrhea, whereas non-GI manifestations include anemia, osteoporosis, herpetic dermatitis, and neurological symptoms[3,4]. Early epidemiological studies have suggested that CD is common in Caucasian populations, particularly in Europe and North America[5,6]. Several studies in other regions have shown similar CD prevalence rates in the Middle East, Asia, Southeast Asia, and Oceania (0.2%–1%)[7-9]. The global prevalence of CD is approximately 1.4%, which is gradually increasing[9,10]. The prevalence of CD among hospitalized patients has also been investigated. A report from Brazil found that the prevalence of CD was 1.9% among 1030 hospitalized patients[11]. The detection rate of CD was 4.48% in patients with irritable bowel syndrome[12].

There are scarce data on the prevalence of CD in Asia, while no data have been compiled for some countries. The prevalence of CD among asymptomatic adults in Japan is 0.05%, and no study has investigated the prevalence of CD in Japanese children[13]. The prevalence of CD in Indian children is approximately 1%[14]. In Central Asia, the prevalence of HLA-DQ alleles susceptible to CD is similiar to that in Europe; however, epidemiological and clinical studies are lacking[15]. The seropositivity for CD in the Chinese population is mainly concentrated in the northern region. A meta-analysis reported that the CD seroprevalence in the general population in China was 0.27%, whereas the CD seroprevalence in the high-risk population was 8.34%[16]. According to a study screening 118 Chinese children with chronic diarrhea, 14 patients were subsequently diagnosed with CD[17]. Research on CD in China is still in its infancy, with only a few cases reported[17,18]. However, the presence of CD susceptibility genes is not uncommon among the Chinese population, and it is believed that the actual number of CD cases in China may be much higher than the currently reported number of diagnosed cases[19].

Serum endomysium antibodies (EMAs) and antibodies against tissue transglutaminase (tTG) are commonly used serological tests for CD. Studies have shown that the sensitivity and specificity of anti-tTG immunoglobulin A (IgA) were 92.5% and 97.9%, respectively. Though EMA IgA testing is less sensitive, it is more specific than anti-tTG IgA, with sensitivity and specificity of 79.0% and 99.0%, respectively[20]. Anti-tTG IgA is the standard test used to screen for CD, while EMA IgA is widely used to confirm the diagnosis. Human leukocyte antigen (HLA)-DQ2 and HLA-DQ8 genotyping can be used to exclude CD[21,22]; however, these are poor diagnostic tests because not all individuals with these genetic variations develop CD. Duodenal mucosal biopsy remains the gold standard for diagnosing CD, and characteristic changes include villous atrophy, crypt hyperplasia, and intraepithelial lymphocytosis. Therefore, specific serum antibody testing and endoscopic duodenal mucosal biopsy should be performed for patients with suspected CD[23,24].

The clinical presentation of CD is both complex and diverse. However, the diagnosis and treatment of CD is relatively simple. A strict gluten-free diet (GFD) is the most effective dietary intervention for disease control, but it has some limitations. Clinical trials of other non-dietary therapies are currently underway. However, owing to the lack of understanding of the disease, identification of high-risk populations for CD remains a challenge, which leads to high rates of missed diagnoses of early-stage CD, resulting in patients frequently developing serious complications.

Northwest China is a multiethnic region with ethnic groups such as Hans, Uyghurs, Huis, and Kazakhs. People living in this area have similar eating habits, with wheat being the staple food crop. In addition, this region is located in Central Asia and geographically close to Europe, where the incidence of CD is high. Genetic exchanges may have occurred between residents and travelers on the ancient Silk Road in this region. Therefore, many cases of CD may remain undiagnosed in this geographical area owing to insufficient knowledge of the disease. This study explored the prevalence, clinical manifestations, and pathological characteristics of CD in northwest China with the aim of improving clinician awareness of the disease, reducing the rates of missed diagnoses and misdiagnoses, and improving patients' quality of life.

MATERIALS AND METHODS

Patient and public involvement

This retrospective cross-sectional study was conducted in the Department of Gastroenterology of the People's Hospital of Xinjiang Uygur Autonomous Region. The study was approved by the hospital's institutional review board (IRB) (Register number: KY2021052611). All patients who underwent gastroduodenoscopy signed an informed consent form, and the IRB waived the requirement for informed consent for other clinical data. This study was conducted in adherence to STROBE guidelines.

Inclusion and exclusion criteria

The clinical data of 3147 patients, including adults and children, with GI symptoms, such as chronic diarrhea, abdominal pain, abdominal distension, constipation, vomiting, nausea, anorexia, heartburn, acid reflux, and burping, were collected from both inpatient and outpatient services between March 2016 and February 2021. All included patients agreed to undergo tests for CD and all relevant clinical data were kept confidential. To investigate the incidence of ileal villous atrophy and exclude diseases other than CD, anti-tTG IgA-positive patients were further examined using gastroduodenoscopy. Colonoscopy with ileal biopsy was not mandatory for the diagnosis of CD. The exclusion criteria were as follows: Physically healthy patients without GI symptoms; patients with digestive tract tumors or a history of other cancer types; patients with a history of cholecystectomy or gastric, duodenal, colon, or small intestinal surgery; and patients with liver cirrhosis, hepatitis, or acquired immunodeficiency syndrome.

Chronic diarrhea was defined as diarrhea lasting for > 4 wk, or recurrent diarrhea with an intermittent period of 2–4 wk. Anemia was defined as hemoglobin (Hb) levels < 110 g/L in children aged 6 months to 6 years, < 120 g/L in children aged 6–14 years, < 130 g/L in adult men, and < 120 g/L in adult women. Bone mineral density was measured using dual energy X-ray absorptiometry, with T-scores of -2.5 to -1 defined as osteopenia and T-scores of ≤ -2.5 defined as osteoporosis. Weight loss was defined as an unexplained reduction of > 5% in initial body weight within 6 mo. Anxiety and depression were quantified using the Hamilton Anxiety Rating Scale and Hamilton Depression Rating Scale, respectively. General patient information including sex, age, race, body mass index (BMI), GI signs and symptoms, comorbidities, *Helicobacter pylori* (*H. pylori*) infection status, and GI endoscopy and pathology results were collected.

Serological tests

Approximately 3–5 mL of venous blood was drawn from each patient, centrifuged to separate the serum, aliquoted, and frozen at -70 °C until required. Serum total IgA was evaluated using the immunoturbidimetric method, with levels of < 0.82 g/L considered as absence of selective IgA. Anti-tTG IgA levels were measured in patients with normal total IgA levels using enzyme-linked

immunosorbent assays, with anti-tTG IgA levels > 20 CU defined as positive. Testing was conducted in accordance with the kit instructions, and the test kit was sourced from INOVA Diagnostics Inc. (United States). Patients positive for anti-tTG IgA and total IgA deficiency underwent GI endoscopy.

Endoscopic, histological assessments and H. pylori infection

GI endoscopy was performed using an Olympus endoscope (Olympus EVIS LUCERA CV290, Tokyo, Japan). The mucosa of the duodenal bulb, descending duodenum, and terminal ileum were observed using white-light endoscopy. Villous architecture was further observed by near-focus narrow-band imaging, the water immersion method, and indigo carmine staining. Pathological biopsies were performed on the duodenal bulb (two pathological tissue samples), descending duodenum (four pathological tissue samples), and terminal ileum (two pathological tissue samples). Two blinded pathologists made the histopathological diagnoses and graded villous atrophy in the duodenum or distal ileum according to the modified Marsh–Oberhuber classification system[25]. Disagreements in the classification and grading were resolved by consensus. CD was diagnosed when the biopsy result was classified as Marsh grade ≥ 2.

For histological diagnosis of *H. pylori* infection, biopsy specimens were obtained from the antrum, corpus, and angulus of the stomach. Hematoxylin-eosin and Giemsa staining was performed as appropriate. *H. pylori* infection was considered negative if *H. pylori* was absent in all biopsy sites and positive if *H. pylori* was present in at least one biopsy site. If the histological diagnosis of *H. pylori* infection was negative, but the urea breath test showed positive results, the patient was diagnosed with *H. pylori* infection.

Statistical analysis

The SPSS software (version 17.0) was used for all statistical analyses. Normally distributed continuous data were compared using the t-test and are presented as mean ± SD, whereas categorical data were compared using the chi-square or Fisher's exact test and are presented as numbers and percentages. Statistical significance was set at $P < 0.05$.

Data availability

The datasets used and/or analyzed during the study are available from the corresponding author upon reasonable request.

RESULTS

Epidemiological characteristics

Of the 3147 patients with GI symptoms, such as chronic diarrhea, abdominal pain, abdominal distension, and weight loss, 2884 met the inclusion criteria (Figure 1). The participants, with ages ranging from 2 to 96 years, were divided into categories according to age. The majority of subjects fell within the 40-59 years, and ≥ 60 years age groups (34.3% and 30.7%, respectively). There were 1531 men (53.1%) and 1353 women (46.9%). When patients were grouped by ethnicity, 1097 (38.0%) were Hans, 1048 (36.3%) were Uyghurs, 387 (13.5%) were Kazakhs, 283 (9.8%) were Hui, and 69 (2.4%) were of other ethnicities. Table 1 summarizes the incidence of CD based on ethnic group, sex, age group, and BMI, and the correlation analysis results for each variable. Among these factors, there were significant associations based on ethnicity ($P < 0.05$) and BMI ($P < 0.01$). In terms of ethnicity, CD incidence was lowest in Hans (0.55% in Hans, 2.19% in Uyghurs, 4.39% in Kazakhs, and 0.71% in Huis). Among the other ethnicities, one Mongolian and one Uzbek patient were diagnosed with CD; however, this was not analyzed further because of the small sample size. All participants were tested for total serum IgA and anti-tTG IgA levels. Overall, two IgA-deficient patients and 73 anti-tTG IgA-positive patients were identified. The rate of positive serum anti-tTG IgA level was 2.53%. A total of 71 patients underwent GI endoscopy: Two IgA-deficient patients and 69 anti-tTG IgA-positive patients. Pathological classification was performed according to the modified Marsh–Oberhuber classification (Table 2). Two patients with total IgA deficiency had Marsh grades of 0. Among the 69 patients, 10 had Marsh grade 0, 9 had Marsh grade 1, and 50 had Marsh grade ≥ 2. Patients with Marsh grades 0 and 1 were excluded, and 50 patients with Marsh grade ≥ 2 were eventually diagnosed with CD. The overall CD detection rate was 1.73%.

Clinical signs and symptoms

CD was more common in patients with a BMI ≤ 18.49 kg/m^2 (5.50%). No significant differences were noted in CD incidence when patients were evaluated based on age or sex. The incidence rates for abdominal pain in non-CD and CD patients were 50.7% and 54.0%, respectively, and abdominal distension were 49.4% and 58.0%, respectively. The rates of chronic diarrhea, anorexia, anemia, fatigue, weight loss, sleep disorders, osteopenia, and osteoporosis were significantly higher in patients with CD than in those without CD. No significant differences were noted in the incidence of constipation, vomiting and/or nausea, heartburn and/or acid reflux, belching, headache and/or dizziness, anxiety

Table 1 General information of included subjects

	Subjects, *n* (%)		Coeliac disease	Frequency	95%CI	*P* value
Ethnicity						
Han	1097	38.0	6	0.55	0.1-1.0	< 0.001
Uygur	1048	36.3	23	2.19	1.3-3.1	
Kazakh	387	13.5	17	4.39	2.3-6.4	
Hui	283	9.8	2	0.71	0.0-1.7	
Others	69	2.4	2	2.90	0.0-7.0	
Gender						
Male	1531	53.1	22	1.44	0.8-2.0	0.202
Female	1353	46.9	28	2.07	1.3-2.8	
Age						
0-19	283	9.8	3	1.06	0.0-2.3	0.135
20-39	727	25.2	16	2.20	1.1-3.3	
40-59	989	34.3	22	2.22	1.3-3.1	
≥ 60	885	30.7	9	1.02	0.4-1.7	
BMI						
≤ 18.49	159	5.5	14	8.81	4.4-13.3	< 0.001
18.5-23.99	1237	42.9	23	1.86	1.1-2.6	
24-27.99	1064	36.9	7	0.66	0.2-1.1	
≥ 28	424	14.7	6	1.42	0.3-2.5	
Total	2884	100.0	50	1.73	1.3-2.2	

Table 2 The modified Marsh–Oberhuber classification[24]

	Marsh 0	Marsh 1	Marsh 2	Marsh 3			Marsh 4[2]
				3a	3b	3c	
IEL count[1]	< 30/100	> 30/100	> 30/100	> 30/100	> 30/100	> 30/100	< 30/100
Crypt hyperplasia	-	-	+	+	+	+	-
Villous atrophy	-	-	-	Mild	Moderate	Total	Total
	Pre-infiltrative	Infiltrative	Infiltrative-hyperplastic	Flat destructive			Atrophic-hypoplastic

[1]Number of intraepithelial lymphocytes *per* 100 enterocytes.
[2]This category is principally included for historic purposes.
IEL: Intraepithelial lymphocytes.

and/or depression, or *H. pylori* infection between the CD and non-CD patients (Table 3).

Histological presentation

The main endoscopic manifestations of duodenal villous atrophy in patients with CD are nodular mucosal atrophy, grooves, and fissure-like lesions. Overall, 24 patients showed nodular mucosal atrophy, 29 showed grooves and fissure-like lesions, 4 showed mosaic signs, 12 showed scallop-like lesions, 9 showed wrinkle reduction or disappearance, and 15 showed multiple manifestations. Villous atrophy in the terminal ileum was observed in 10 patients with CD, whereas normal terminal ileal mucosa was observed in 40 patients. The histological findings of CD included total villous atrophy, increased intraepithelial lymphocytes, and crypt hyperplasia.

H. pylori infection

The *H. pylori* infection rates in CD and non-CD patients were 48.0% and 57.4%, respectively, and the

Table 3 General clinical symptoms and frequency of coeliac disease

Symptoms[1]	Subjects without coeliac disease (*n* = 2834), *n* (%)		Coeliac disease (*n* = 50), *n* (%)		*P* value	Total (*n* = 2884), *n* (%)		Frequency of coeliac disease among patients with symptoms (95%CI)	*P* value
Chronic diarrhea	258	9.1	21	42.0	< 0.001	279	9.7	7.53 (4.4-10.6)	< 0.001
Abdominal pain	1437	50.7	27	54.0	0.644	1464	50.8	1.84 (1.2-2.5)	
Abdominal distension	1400	49.4	29	58.0	0.228	1429	49.5	2.03 (1.3-2.8)	
Constipation	397	14.0	5	10.0	0.417	402	13.9	1.24 (0.2-2.3)	
Anorexia	640	22.6	20	40.0	0.004	660	22.9	3.03 (1.7-4.3)	
Vomit or/and nausea	802	28.3	18	36.0	0.231	820	28.4	2.20 (1.2-3.2)	
Heartburn or/and acid reflux	740	26.1	12	24.0	0.736	752	26.1	1.60 (0.7-2.5)	
Belch	776	27.4	13	26.0	0.828	789	27.4	1.65 (0.8-2.5)	
Headache or/and dizziness	677	23.9	14	28.0	0.500	691	24.0	2.03 (1.0-3.1)	
Anemia	416	14.7	20	40.0	< 0.001	436	15.1	4.59 (2.6-6.6)	
Fatigue	536	18.9	24	48.0	< 0.001	560	19.4	4.29 (2.6-6.0)	
Weight loss	873	30.8	24	48.0	0.009	897	31.1	2.68 (1.6-3.7)	
Osteopenia or osteoporosis	329	11.6	38	76.0	< 0.001	367	12.7	10.35 (7.2-13.5)	
Sleep disorder	771	27.2	23	46.0	0.003	794	27.5	2.90 (1.7-4.1)	
Anxiety and depression	808	28.5	17	34.0	0.395	825	28.6	2.06 (1.1-3.0)	
H. pylori infection	1627	57.4	24	48.0	0.182	1651	57.2	1.45 (0.9-2.0)	

[1]Note that patients had more than one symptom.

difference was not statistically significant. Abdominal pain was significantly more frequent in patients with CD without *H. pylori* infection than in those with *H. pylori* infection. Of the 50 patients diagnosed with CD, 17 were classified as having Marsh grade 2 and 33 as having Marsh grade 3. The rates of *H. pylori* infection were significantly different among the different Marsh grades (P = 0.032) (Table 4). Further pairwise comparisons showed significant differences in the detection rate of *H. pylori* between CD patients with Marsh grades 2 and 3b (P = 0.025). Patients with *H. pylori* infection were more commonly found to have Marsh grade 2, and more patients without *H. pylori* had Marsh grade 3b.

DISCUSSION

Currently, there is a paucity of clinical and epidemiological data on CD in China. To the best of our knowledge, to date, no large-sized sample data analysis of the pathological characteristics of patients with CD is available in the literature. Additionally, there have been no published reports on the relationship between CD and *H. pylori* infection. The prevalence of CD is high in Europe[9,26]. Northwest China connects Eurasia and lies on the ancient Silk Road. Historically, owing to the possibility of intermarriages between the populations of the two regions, there may have been transfer of CD susceptibility genes present in European populations to this region, leading to an increase in CD incidence. In addition to genetic susceptibility, wheat is the main food crop for this population. These factors may have contributed to the high detection rate of CD in northwest China. Northwest China is a multi-ethnic region in which ethnic groups such as Hans, Uyghurs, Huis, and Kazakhs live together. A previous study by Zhou *et al*[27] found a higher incidence of CD in Xinjiang and a higher detection rate of CD in Kazakhs than in Uyghurs and Hans[27]. Studies have found that HLA-DQ2 and HLA-DQ8 gene carrier rates are high in Kazakhs and Uyghurs[27,28]. Genetic susceptibility may be the reason for the difference in prevalence among different races.

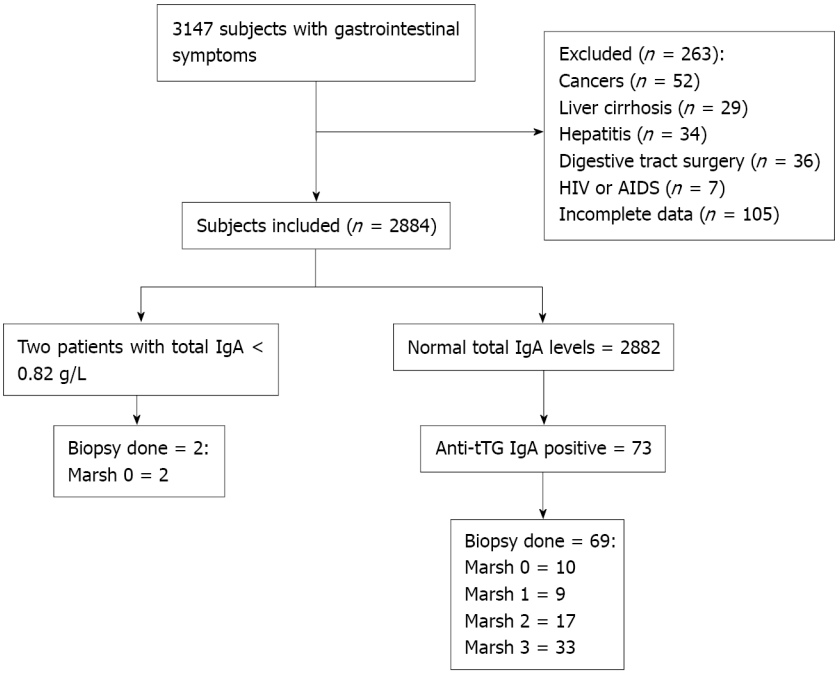
Table 4 Clinical signs and symptoms, celiac grading according to the presence of *Helicobacter pylori* in celiac disease patients

Symptom, sign, associatedcondition or, test	HP (+) (*n* = 24)	HP (-) (*n* = 26)	OR (95%CI)	*P* value
Chronic diarrhea	12 (50.0)	9 (34.6)	1.89 (0.61-5.89)	0.271
Abdominal pain	17 (70.8)	10 (38.5)	3.89 (1.19-12.68)	0.022
Abdominal distension	15 (62.5)	14(53.8)	1.43 (0.46-4.42)	0.536
Constipation	4 (16.7)	1 (3.8)	5.0 (0.52-48.34)	0.182
Anorexia	8 (33.3)	12 (46.2)	0.58 (0.19-1.84)	0.355
Vomit or/and nausea	11 (20.8)	7 (50.0)	2.30 (0.71-7.45)	0.164
Heartburn or/and acid reflux	8 (33.3)	4 (15.4)	2.75 (0.70-10.74)	0.138
Belch	6 (25.0)	7 (26.9)	0.91 (0.26-3.21)	0.877
Headache or/and dizziness	5 (20.8)	9 (34.6)	0.50 (0.14-1.78)	0.278
Anemia	8 (33.3)	12 (46.2)	0.58 (0.19-1.84)	0.355
Fatigue	11 (45.8)	13 (50.0)	0.85 (0.28-2.57)	0.768
Weight loss	10 (41.7)	14 (53.8)	0.61 (0.20-1.88)	0.389
Osteopenia or osteoporosis	20 (83.3)	18 (69.2)	2.22 (0.57-8.65)	0.243
Sleep disorder	11 (45.8)	12 (46.2)	0.99 (0.32-3.01)	0.982
Anxiety or/and depression	6 (25.0)	11 (42.3)	0.46 (0.14-1.52)	0.197
Celiac grading, No. (%)				0.032
Marsh grade 2 (*n* = 17)	12 (50.0)	5 (19.2)	1.0 (ref)	
Marsh grade 3a (*n* = 16)	6 (25.0)	10 (38.5)	0.25 (0.06-1.07)	
Marsh grade 3b (*n* = 13)	3 (12.5)	10 (38.5)	0.13 (0.02-0.66)	
Marsh grade 3c (*n* = 4)	3 (12.5)	1 (3.8)	1.25 (0.10-15.11)	

Patients may have one or more associated symptoms or conditions.

In this study, 2884 patients with GI symptoms were screened for CD according to the global guidelines of the World Gastroenterology Organization[29]. Among them, 73 were positive for anti-tTG IgA and 50 were pathologically diagnosed with CD. CD can occur at any age, and the prevalence rate in women is 2–4 times higher than that in men[30,31]. In line with this, our study found a higher prevalence of CD in female patients than in male patients. The clinical manifestations of CD include delayed growth, malnutrition, chronic diarrhea, abdominal pain, and abdominal distension. Up to 17% of female patients may present with severe clinical manifestations during pregnancy or puerperium [32]. This study found that the main clinical manifestations of patients with CD in Xinjiang included chronic diarrhea, severe malnutrition, osteoporosis, anemia, fatigue, and decreased BMI. BMI is an important index for evaluating and predicting CD, and diarrhea is a typical symptom of CD. The immune response caused by gluten intake in susceptible populations leads to intestinal absorption dysfunction and osmotic diarrhea. In our study, 21 patients with CD-related diarrhea mainly presented with profuse watery and fatty diarrhea. Owing to the lack of knowledge and limited diagnostic criteria for CD, diarrhea often becomes chronic, making the disease more difficult to control. Therefore, most patients exhibit significant weight loss, accompanied by anemia, iron and vitamin D deficiency, and other forms of malnutrition. In Britain, individuals with suspected CD are screened to avoid complications associated with delayed CD diagnosis[24]. Therefore, CD screening should be performed in patients with GI symptoms in China, especially in those with anorexia and significant weight loss. Most patients with CD in Europe initially present with extra-intestinal manifestations and are missed because they are not tested for CD[33]. The European Society for Pediatric Gastroenterology Hepatology and Nutrition suggests that relatives of patients with CD or other autoimmune diseases should also be screened for the same conditions. Mass screening for CD is currently not recommended[3]. At present, there are no relevant guidelines for CD in the Chinese population; however, a strategy similar to that followed in Europe could be adopted.

CD is caused by gluten in susceptible subjects, however, its etiology is not fully understood. With the increasing prevalence of CD, researchers have begun to consider environmental risk factors that may trigger autoimmunity in the small intestine[34]. *H. pylori* is one of the most common chronic bacterial infections worldwide and can cause severe gastroduodenal diseases[35]. Both *H. pylori* infection and CD



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Figure 1 Flow chart of the patient enrollment. IgA: Immunoglobulin A; HIV: Human immunodeficiency virus; AIDS: Acquired immunodeficiency syndrome.

involve systemic humoral and local inflammatory immune responses. Chronic gastric infections that can induce duodenal ulcers and affect the systemic immune response may trigger autoimmunity in the small intestine[36]. Whether *H. pylori* infection can prevent or induce CD remains debatable. Epidemiological studies have investigated the association between *H. pylori* infection and CD. However, these studies reported conflicting results[37-39]. The variability in the results may be due to the different prevalence of *H. pylori* infection in different populations and the identification of patients who have not yet demonstrated clinically significant CD. There are no reports on the relationship between CD and *H. pylori* infection in northwest China.

We evaluated the relationship between *H. pylori* infection and CD and found that *H. pylori*-positive CD patients demonstrated more severe mucosal damage than *H. pylori*-negative CD patients (Marsh grades 2 and 3) (P = 0.018). This finding is similar to that of Gungor *et al*[40]. However, it has been reported that in individuals without CD, *H. pylori* infection itself can cause duodenal mucosal damage [41]. In a study by Konturek *et al*[42], the prevalence of *H. pylori* infection was higher in patients with CD than in controls[42]. Previous studies have shown that *H. pylori* infection can prevent the development of CD[43,44]. This association may be related to the genetic factors of CD and/or *H. pylori*, virulence of *H. pylori*, and immunopathology involved. In addition to altering the acidity and content of gastric juice, *H. pylori* directly interacts with the immune system and increases intestinal permeability [45].

In patients with CD, biopsy usually shows villous atrophy, crypt hyperplasia, and inflammation. However, some serologically positive individuals can have normal intestinal mucosa, but many of these patients later develop CD, which is sometimes termed "latent CD"[46]. In these atypical cases, more than 95% of anti-tTG IgA-positive patients may be sensitive to glutenin[47]. Our study found that the grades of five patients who presented with Marsh grade 1 in 2016–2019 improved to Marsh grade 0 on gastroscopy after following a GFD for at least half a year. Therefore, we speculate that early initiation of a GFD can improve the condition of patients with anti-tTG IgA-positive "latent CD." The healing rates of patients often differ significantly, and the older the patient at the time of the first diagnosis, the slower the intestinal healing process and the higher the possibility of nonreactive CD. There is no relevant research on "latent CD" in China and more extensive screening and follow-up are necessary. Early diagnosis of CD can reduce the long-term and persistent damage caused by gluten to the intestinal tract and whole body, thus resulting in better patient prognosis. Common causes of CD-related deaths are intestinal non-Hodgkin's lymphoma and small-bowel cancers[48]. Refractory CD (RCD) is a major cause of poor prognosis. RCDs can be divided into types I (RCD I) and II (RCD II). The phenotype of intraepithelial lymphocytes (IELs) is abnormal in RCD II patients and normal in RCD I patients. Approximately 50%–60% of patients with RCD II develop EATL within 5 years after diagnosis[49]. Both are (pre)malignant complications of CD. Patients with RCD II and EATL often have more severe malnutrition due to intestinal malabsorption and hypermetabolism[50]. No patients with RCD or EATL were found in this study; however, Marsh grades were positively correlated with patient age. Therefore, patients with CD who have significant weight loss or are elderly should be screened for CD using GI

endoscopy.

Study limitations

To the best of our knowledge, this is the first study to comprehensively analyze the clinical and pathological characteristics of Chinese patients with CD and evaluate the association of CD with *H. pylori* infection. Our study not only bridges the gap in relevant research in the Chinese population but also provides reference values for the diagnosis and treatment of CD. However, this study has several limitations. The subjects were patients with GI symptoms in the hospital, which may have resulted in a selection bias. HLA-DQ2 and HLA-DQ8 genotypes were not identified in our study; therefore, further research on the relationship between these genotypes and the pathological types of CD is warranted.

CONCLUSION

Among people with GI symptoms in northwest China, the prevalence of CD is higher in the Uyghur and Kazak populations. Therefore, physicians should be aware of the risk of developing CD in regional populations. *H. pylori* infection may be related to CD severity, which warrants further study.

ARTICLE HIGHLIGHTS

Research background

Research on celiac disease (CD) in Northwest China is still in its infancy. At present, large sample data on the epidemiological, clinical, and pathological characteristics of CD are limited.

Research motivation

This study reports the epidemiological, clinical, and pathological characteristics of CD and its association with *Helicobacter pylori* (*H. pylori*) infection, and aims to provide useful information for clinical diagnosis and treatment of CD.

Research objectives

To investigate the epidemiological, clinical, and pathological characteristics of CD in northwest China.

Research methods

The clinical data of 2884 patients with gastrointestinal (GI) symptoms were retrospectively analyzed. Total immunoglobulin A and anti-tissue transglutaminase (tTG) immunoglobulin A (IgA) levels were examined for all patients. Gastroscopy and colonoscopy were performed in patients with positive anti-tTG IgA and deficient total IgA levels. Atrophy of the duodenal and ileal villi was examined, and histopathological examinations were performed. The modified Marsh–Oberhuber classification system was used to grade villous atrophy in the duodenum or distal ileum. Patient *H. pylori* infection status was compared in terms of clinical presentation and Marsh grade. Statistical analyses were performed using t-test or chi-square test.

Research results

The detection rate of CD was significantly higher in Kazakhs (4.39%) than in Uygurs (2.19%), Huis (0.71%), and Hans (0.55%). The main symptoms of CD were chronic diarrhea, anorexia, anemia, fatigue, weight loss, sleep disorders, osteopenia, and osteoporosis. The body mass index of CD patients was significantly lower than that of non-CD patients. Endoscopy revealed crypt hyperplasia and/or duodenal villous atrophy, which mainly manifested as nodular mucosal atrophy, grooves, and fissures. The difference in *H. pylori* infection rates was not statistically significant between CD and non-CD patients, but was significantly different among CD patients with different Marsh grades. Patients with *H. pylori* infection were more commonly found with Marsh grade 2 and more patients without *H. pylori* had Marsh grade 3b.

Research conclusions

Among people with GI symptoms in Northwest China, the prevalence of CD is higher in the Uygur and Kazak populations. Physicians should be aware of the risk of CD in the regional population. *H. pylori* infection may be related to the severity of CD, which warrants further study.

Research perspectives

H. pylori infection may be related to the severity of CD, which warrants further study.

FOOTNOTES

Author contributions: Wang M and Gao F designed the study; Wang M, Kong WJ, and Lu JJ acquired the data and drafted the article; Hui WJ and Liu WD analyzed and interpreted the data; Cui M and Sun ZZ made a pathological diagnosis; Feng Y, Li ZQ, Shi T and Gao F revised the article critically for important intellectual content; all the authors approved the version to be published.

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Country/Territory of origin: China

ORCID number: Man Wang 0000-0003-2726-7218; Wen-Jie Kong 0000-0003-1480-9583; Yan Feng 0000-0002-0185-3457; Jia-Jie Lu 0000-0001-6919-3486; Wen-Jia Hui 0000-0001-6055-1339; Wei-Dong Liu 0000-0002-7384-6651; Zi-Qiong Li 0000-0001-8449-6506; Tian Shi 0000-0002-7173-9502; Mei Cui 0000-0001-6547-3827; Zhen-Zhu Sun 0000-0002-8046-626X; Feng Gao 0000-0002-6798-1814.

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LETTER TO THE EDITOR

Near-infrared fluorescence imaging guided surgery in colorectal surgery

Sung Uk Bae

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Sung Uk Bae, Department of Surgery, School of Medicine, Dongsan Medical Center, Keimyung University, Daegu 700-712, South Korea

Corresponding author: Sung Uk Bae, PhD, Professor, Department of Surgery, School of Medicine, Dongsan Medical Center, Keimyung University, 194 Dongsan-Dong, Jung-Gu, Daegu 700-712, South Korea. sabiston0000@hanmail.net

Abstract

Near infrared fluorescence using indocyanine green is beneficial for visual assessment of blood vessels, blood flow, and tissue perfusion, sentinel lymph node biopsy, lymph node road mapping, identification of the vascular system round the major vessels, and the detection of ureters in order to reduce the risk of iatrogenic ureteral lesions in colorectal surgery.

Key Words: Fluorescence; Enhanced reality; Anastomotic leak; Lymph node; Anastomosis

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Core Tip: Near infrared fluorescence technique using indocyanine green can be used in estimation of intestinal vascularization to detect areas of poor perfusion for preventing anastomotic leakage, the visualization of sentinel lymphatic drainage and peritoneal metastases, and the detection of ureters in order to reduce the risk of iatrogenic ureteral lesions in colorectal surgery. Additionally, this technique can be used in identifying suspected lymph nodes and preventing their incomplete dissection during lateral pelvic lymph node dissection and D3 Lymphadenectomy for rectal cancer and right-sided colon cancer, respectively, and in identification of the vascular system round the major vessels.

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TO THE EDITOR

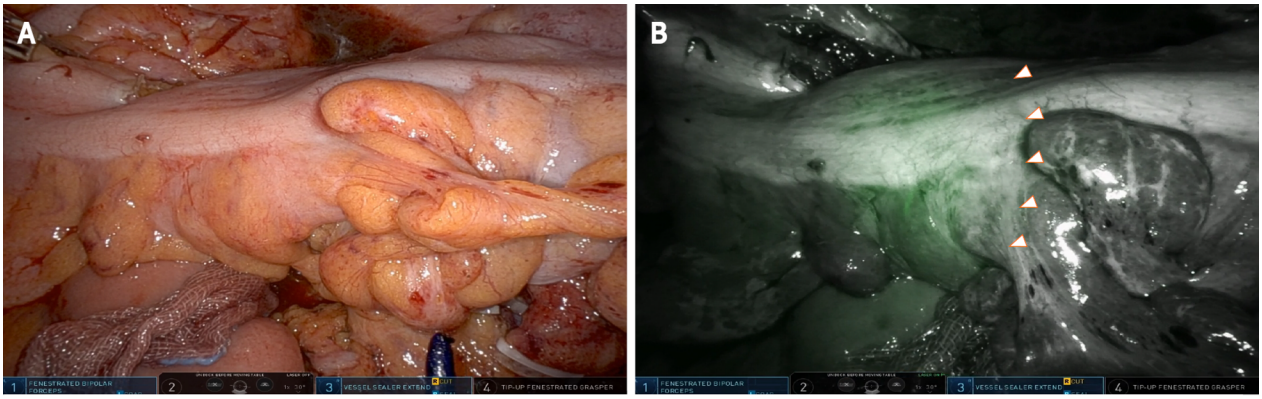
Indocyanine green (ICG) emits an infrared signal when excited by laser light in situ, which can be detected with near infrared fluorescence (NIF) camera. NIF imaging uses laser technology to activate an intravenously delivered agent, ICG, which rapidly binds to plasma proteins. This allows ICG to remain predominantly in visual assessment of blood vessels, blood flow, and tissue perfusion, sentinel lymph node biopsy and lymph node road mapping[1,2].

In this issue of World Journal of World Journal of Gastroenterology, the review article by Zocola *et al*[3] highlights the role of NIF in colorectal surgery. They reviewed the literature regarding NIF for three main indications including the estimation of intestinal vascularization to detect areas of poor perfusion for preventing anastomotic leakage, the visualization of sentinel lymphatic drainage and peritoneal metastases, and the detection of ureters in order to reduce the risk of iatrogenic ureteral lesions in colorectal surgery.

NIF in conjunction with ICG allows for visualization of the microcirculation before formation of the anastomosis, thereby allowing the surgeon to choose the point of transaction at an optimally perfused area (Figure 1). Zocola *et al*[3] intensively reviewed the role of NIF in the intraoperative bowel viability assessment to prevent anastomotic leaks. They divided the retrospective cohort study and prospective randomized controlled study and reviewed the effectiveness of NIF in reducing anastomotic leakage.

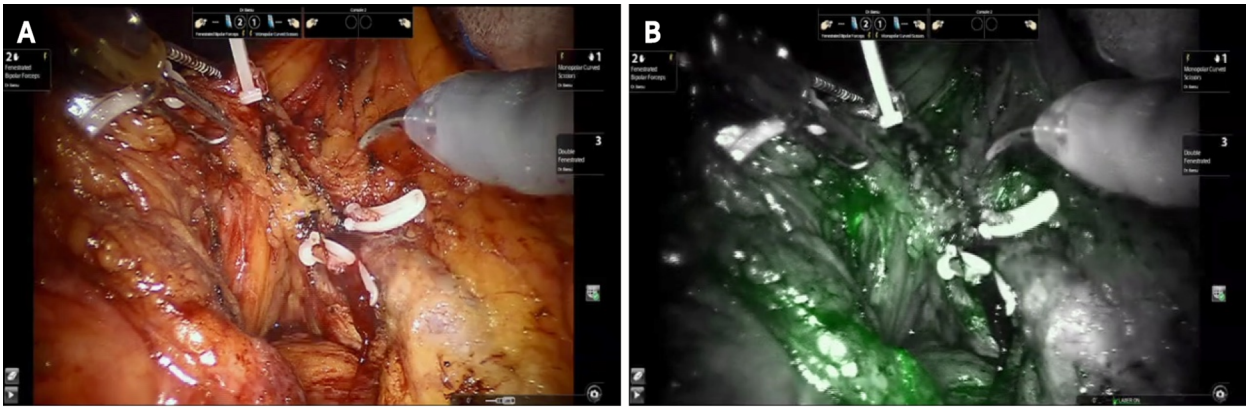
Regarding the role of NIF with ICG to detect metastatic lymph node, Zocola *et al*[3] reviewed studies on the identification of sentinel lymph node and mapping additional lymph nodes outside of the proposed resection margins to achieve radical lymphadenectomy for curative surgery (Figure 2). In addition to the studies mentioned by Zocola *et al*[3], I would like to mention some recent studies related to this issue. One of the issues related to radical lymphadenectomy in colorectal cancer is lateral pelvic node dissection (LPND), and recent introductions and data on this procedure using NIF have been reported. Kim *et al*[4] demonstrated a novel application of NIF using ICG during robotic total mesorectal excision (TME) with LPND to identify suspected lateral pelvic lymph nodes and prevent their incomplete dissection. They injected ICG at a dose of 2.5 mg around the tumor transanally before surgery and NIF imaging–guided robotic TME with lateral pelvic lymphadenectomy allowed the surgeon to identify lymph nodes and lymphatic flow of rectal cance. Zhou *et al*[5] compared patients who underwent TME and LPND with NIF technique (n = 12) and patients who received conventional TME and LPND without NIF-guided imaging (n = 30). They reported that the NIF group had significantly lower intraoperative blood loss (55.8 ± 37.5 mL *vs* 108.0 ± 52.7 mL, P = 0.003) and a significantly larger number of lateral pelvic nodes harvested (11.5 ± 5.9 *vs* 7.1 ± 4.8, P = 0.017), and lateral pelvic lymph nodes from two patients in the NIF group remained during LPND. Additionally, Park *et al*[6] and Bae *et al*[7] used NIF technique for colorectal cancer surgery is D3 Lymphadenectomy, especially in right-sided colon cancer. Park *et al*[6] injected ICG around the tumor for visualization of lymphatic flow and lymph nodes and demonstrated the numbers of apical lymph nodes (14 *vs* 7, P < 0.001) and total harvested lymph nodes (39 *vs* 30, P = 0.003) were significantly higher in the NIF group than in the conventional group.

When injected intravenously, ICG rapidly binds to plasma proteins and remains predominantly in the vasculature. Although there was no mention in the review article, NIF angiography can be used in identification of the vascular system (Figure 3). ICG can be easily injected into the blood circulation during surgery, when the blood vessels are exposed, to allow direct visual observation. Bae *et al*[8,9] included 11 patients who underwent a robotic TME with preservation of the left colic artery for rectal cancer using NIF technique. The optimal point of division was then chosen by the surgeon under NIF imaging that facilitated the identification of the left colic branch of the inferior mesenteric artery (IMA). In addition, NIF imaging was used for the identification of the collateral vessels (Arc of Riolan) around the inferior mesenteric vein in their study. The left colic artery branches mainly at the Griffith point (watershed), which is located in the splenic bend where the left branch of the middle colic and the ascending branch of the left colic join. This area is vulnerable to injury and ischemia during surgery due to poor blood supply. For this reason, great care must be taken not to interfere with the bifurcation of the left colic artery. Real-time identification of collateral vessels using NIF technology can help implement safe low ligation of the IMA while preventing damage to these vessels. For now, it remains a linear graded outcome that requires subjective interpretation of the demarcation point between sufficient and insufficient perfusion and perfusion is assessed is based on a subjective qualitative impression of the surgeon. Quantitative analysis of NIF images is desirable but not currently available in robotic or laparoscopic systems. Son *et al*[10] performed quantitative evaluation of colon perfusion patterns using NIF angiography to find the most reliable predictive factor of anastomotic complications after laparoscopic colorectal surgery. They found that the fluorescence slope, T1/2MAX, and time ratio were related with anastomotic complications and those complications were significantly correlated with the novel factor time ratio (> 0.6) as the most reliable predictor of perfusion and anastomotic complications. Recently, Han *et al*[11] compared the changes in perfusion status between high tie and low tie through quantitative evaluation of ICG using NIF technique. They demonstrated that T_max increased and Slope_max decreased significantly in the high tie group after IMA ligation, whereas the intensity of perfusion status (F_max), which indicates the intensity of perfusion, did not change according to the level of IMA ligation. They suggested that the speed of blood perfusion could be more delayed after



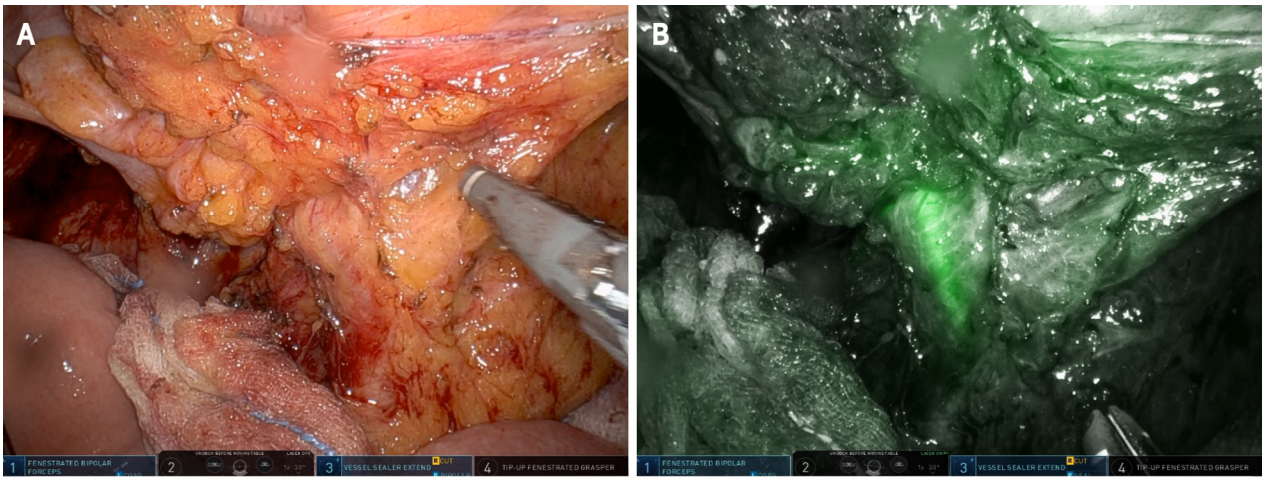
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Figure 1 Near infrared fluorescence in conjunction with indocyanine green allowing visualization of the microcirculation before development of the colorectal anastomosis. A: A white light image before visualizing the ischemic zone of the sigmoid colon using excited fluorescence; B: An intraoperative near infrared fluorescence image after visualizing the ischemic zone of the sigmoid colon using excited fluorescence.



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Figure 2 Mapping of additional lymph nodes outside the proposed resection margins to achieve curative radical lymphadenectomy in robot-assisted right hemicolectomy. A: A white light image after D3 Lymphadenectomy around superior mesenteric vessels; B: A near infrared fluorescence image after visualizing the remained lymph nodes after lymphadenectomy using excited fluorescence.



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Figure 3 Robot-assisted lymph node dissection around the inferior mesenteric artery with preservation of the left colic artery using near infrared fluorescence imaging. A: Dissection around the root of the inferior mesenteric artery (white light image); B: A near infrared fluorescence image visualizing the left colic artery using excited fluorescence.

high tie than low tie, but the intensity of perfusion was similar between high and low ligation of IMA. There are still a lot of questions and debates to be discussed, but we believe that the NIF technique will play an important role in improving the clinical and oncologic outcomes of colorectal surgery.

FOOTNOTES

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Country/Territory of origin: South Korea

ORCID number: Sung Uk Bae 0000-0002-7876-4196.

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Editorial Board Member of *World Journal of Gastroenterology*, Yasir M Khayyat, FACG, FRCPC, Professor, Department of Medicine, Faculty of Medicine, Umm Al-Qura University, Alawali District, Makkah 24381-8156, Saudi Arabia. ymkhayyat@uqu.edu.sa

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REVIEW

Locoregional therapies and their effects on the tumoral microenvironment of pancreatic ductal adenocarcinoma

Thomas Lambin, Cyril Lafon, Robert Andrew Drainville, Mathieu Pioche, Frédéric Prat

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Thomas Lambin, Cyril Lafon, Robert Andrew Drainville, LabTAU, INSERM, Centre Léon Bérard, Université Lyon 1, Univ Lyon, Lyon 69003, France

Thomas Lambin, Mathieu Pioche, Department of Gastroenterology, Hospices Civils de Lyon, Edouard Herriot Hospital, Lyon 69008, France

Frédéric Prat, Service d'Endoscopie Digestive, Hôpital Beaujon, Assistance Publique-Hôpitaux de Paris, Clichy 92110, France

Frédéric Prat, INSERM U1016, Institut Cochin, Université de Paris, Paris 75014, France

Corresponding author: Frédéric Prat, MD, Doctor, Service d'Endoscopie Digestive, Hôpital Beaujon, Assistance Publique-Hôpitaux de Paris, 100 Bd du Général Leclerc, Clichy 92110, France. Frederic.prat@aphp.fr

Abstract

Pancreatic ductal adenocarcinoma (PDAC) is expected to become the second leading cause of death from cancer by 2030. Despite intensive research in the field of therapeutics, the 5-year overall survival is approximately 8%, with only 20% of patients eligible for surgery at the time of diagnosis. The tumoral microenvironment (TME) of the PDAC is one of the main causes for resistance to antitumoral treatments due to the presence of tumor vasculature, stroma, and a modified immune response. The TME of PDAC is characterized by high stiffness due to fibrosis, with hypo microvascular perfusion, along with an immunosuppressive environment that constitutes a barrier to effective antitumoral treatment. While systemic therapies often produce severe side effects that can alter patients' quality of life, locoregional therapies have gained attention since their action is localized to the pancreas and can thus alleviate some of the barriers to effective antitumoral treatment due to their physical effects. Local hyperthermia using radiofrequency ablation and radiation therapy - most commonly using a local high single dose - are the two main modalities holding promise for clinical efficacy. Recently, irreversible electroporation and focused ultrasound-derived cavitation have gained increasing attention. To date, most of the data are limited to preclinical studies, but ongoing clinical trials may help better define the role of these locoregional therapies in the management of PDAC patients.

Key Words: Pancreatic ductal adenocarcinoma; Tumoral microenvironment; Stroma; Hyperthermia; Radiation therapy; High-intensity focused ultrasound

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Core Tip: The prognosis of pancreatic ductal adenocarcinoma is poor, with a 5-year survival rate of approximately 8%. This is mainly due to an unfavorable tumoral microenvironment (tumor vasculature, stroma, and immune response). Locoregional therapies can alleviate some barriers to effective antitumoral treatment. This review explores the action of locoregional treatments on pancreatic cancer, with a specific focus on hyperthermia, radiation therapy, high-intensity focused ultrasound, and irreversible electroporation. After a description of the particularities of the tumoral microenvironment of pancreatic cancer, the effects of these treatments on the tumoral microenvironment and implications for future management of patients are discussed.

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INTRODUCTION

Pancreatic ductal adenocarcinoma (PDAC) is one of the most common types of pancreatic cancer[1]. Given the rapidly increasing incidence of PDAC in recent decades, it is expected to become the second leading cause of cancer death by 2030 in the United States and other industrialized countries[2]. The 5-year survival rate is approximately 8% following diagnosis[3], with only 20% of the patients eligible for surgery[4]. Among the patients who undergo surgery, the 5-year survival rate is only 20% due to rapid recurrence and metastasis development[5]. Systemic treatments are associated with various types of adverse events that result in a poor quality of life for patients[6] and are often inefficient due to the characteristics of the PDAC tumor microenvironment (TME), which protects tumor cells from chemotherapies and immunotherapies. The TME is increasingly being considered a potential target of choice to improve outcomes for PDAC. Locoregional treatments, such as hyperthermia (HT) with microwave ablation or radiofrequency ablation (RFA), radiation therapy (RT), irreversible electroporation (IRE), and high-intensity focused ultrasound (HIFU) therapy, are gaining increasing attention for their ability to specifically target the tumor while limiting deleterious systemic adverse events and may often be used in combination with anticancerous drugs. Among other effects, locoregional therapies can induce changes in the structure, components, and properties of the TME that may help alleviate some of the barriers to successful treatment. In this review, we will summarize the characteristics of the TME in PDAC and describe the effects of locoregional therapies on vasculature, stroma, and immune response. We will pay special attention on characterizing the TME for clinical applications (Table 1).

PDAC MICROENVIRONMENT

Although most solid tumors develop specific interactions with their host *via* neoangiogenesis, the creation of a supporting network of cells and extracellular matrix, and some form of immunomodulation, PDAC remains one of the most stroma-rich cancers, with 90% of its tumor mass composed of fibroblasts and their products[7]. The PDAC stroma is composed of an acellular and cellular compartment. The acellular compartment consists of an extracellular matrix rich in collagen, fibronectin, laminin, integrins, glycosaminoglycan, matrix metalloproteinase and secreted protein acids and is rich in cysteine[1,8] with cytokines and growth factors[9]. The cellular component is composed of pancreatic stellate cells and immune cells, such as macrophages, mast cells, lymphocytes, and plasma cells[1]. Pancreatic stellate cells, which are resident cells of the pancreas, acquire an activated myofibroblast-like phenotype when activated and are assumed to be the main regulators of TME extracellular matrix production. With the tumoral microvasculature, all these elements form the TME. Interactions between acellular and cellular components of the TME are key factors in PDAC progression[10]. In PDAC, the TME has been associated with tumoral progression, metastasis dissemination, and resistance to chemotherapy by various mechanisms[8]. The abundant extracellular matrix increases intratumoral interstitial pressure[11] and acts as a barrier for drug delivery with compression of blood vessels. Tumor stiffness can also have a direct effect on chemosensitivity: *in vitro*, mechanically compressed PDAC spheroids (displaying high stiffness) are less sensitive to gemcitabine than free spheroids (without any mechanical compression), whereas there is no difference in Hoechst dye penetration between compressed and free spheroids, suggesting a therapeutic effect of compression independent of the sole

Table 1 Locoregional therapies and their main effects on the tumoral microenvironment of pancreatic ductal adenocarcinoma

	Vasculature	Stroma	Immune response
Hyperthermia	Increased blood flow and vascular permeability. Recruitment of bradykinin and histamin. Increased iNOS.	Destructuration of collagen fibers. Reduction of CAF. Reduction of tumor stiffness.	Promotes APC activation. Increased infiltrating CD8+. Increased pro-inflammatory cytokines. Abscopal effect (RFA).
Radiation therapy	Reduced blood perfusion. Destructuration of microvessels with thickening vessel walls. Platelet aggregation. Microthrombus formation. Increased HIF-1 and VEGF. Increased vascular permeability.	Accumulation of extracellular matrix proteins. Increased stromal cells (fibroblasts). Thickened and stiffened tissue. Loss of hyaluronic acid. Collagen remodeling. Modification of CAF population.	Release of tumor antigens (DAMPs) ≥ APC presentation and CD8+ activation. Increased peptide availability and T cell repertoire. Release of inflammatory cytokines, CD8+, and CD4+ cells. Increased adhesion molecules (VCAM-1, ICAM-1). T cells homing. Increased PDL-1.
HIFU (mechanical effect)	Reduced blood perfusion and microvascular density.	Disruption of the collagen matrix.	Released DAMPs ≥ T cell activation. Induction of Th1 inflammation. Increased CD8+/Treg ratio.

iNOS: Inducible nitric oxide synthase; CAF: Carcinoma-associated fibroblasts; APC: Antigen presenting cell; RFA: Radiofrequency ablation; DAMPs: Damage-associated molecular patterns; HIFU: High-intensity focused ultrasound.

penetration of gemcitabine into the spheroid cells[12]. One hypothesis to explain this phenomenon is that mechanical stress decreases cell proliferation, which may alter the efficacy of chemotherapies targeting proliferating cells[12]. Intratumoral stiffness itself can modify intracellular signaling pathways and promote epithelial-mesenchymal transition, leading to tumoral progression and chemoresistance [13]. In PDAC, the microvascular density is generally low and leaky. Combined with mechanical forces caused by the dense stroma and tumoral growth that compresses the vessels, limited perfusion can result, which is responsible for hypoxia and low nutrient availability along with low anticancer drug delivery[1,14]. On the immune response side, the TME in PDAC is characterized by a reduced number of cytotoxic T cells, along with an increase in M2 macrophages, N2 neutrophils, and T-regulatory cells at the tumor site, which all contribute to an immunosuppressive environment[15].

Current studies suggest that the TME is an attractive target in the management of PDAC. Provenzano *et al*[11] showed that the administration of an enzymatic agent allowing the deletion of stromal hyaluronic acid (PEGPH20) in a murine model of PDAC led to normalized intratumoral interstitial pressure, expansion of the tumoral microvasculature, and increased survival *in vivo* when combined with gemcitabine compared to gemcitabine alone. However, caution should be taken since other studies have shown that the presence of some TME elements serves to prevent cancer progression and should therefore not be suppressed indistinctively[16,17]. In a mouse model of PDAC, as well as in patients with pancreatic cancer, a decreased bulk of aSMA + myofibroblasts was associated with poor prognosis and reduced overall survival[17].

HT

HT is a therapeutic procedure used to increase the intratumoral temperature. There are various ways to increase temperature: for superficial tumors, local HT can be applied by means of antennas or applicators that emit microwaves (microwave ablation) or radio waves (radiofrequency ablation or RFA) placed at the surface of the tumor with an intervening medium. Interstitial and endocavitary HT are used for small tumors in which an intratumoral implantation of the antenna can deliver various types of waves: microwaves, radiofrequency, ultrasound, heat sources, or laser fibers. Regional HT and partial body HT are more suitable for deep seated tumors, such as PDAC, that can be heated by antennas placed in rings around the patient. Whole-body HT is dedicated to the treatment of metastatic tumors[18]. HT can be generated by an external source, such as hot air or infrared radiation, or an internal source, such as magnetic nanoparticles, which can be deposited in the region of interest and then exposed to a magnetic field, leading to an increase in temperature and allowing localized heating [magnetic hyperthermia (MHT)][14]. Nanoparticles can also be used for photothermal therapy (PTT), during which a laser can activate nanophotoabsorbers. MHT and PTT are both nanoparticle-based HT treatments[19,20].

Hyperthermia and tumoral vasculature

HT is thought to increase blood flow and vascular permeability with an increase in antitumor effects. A study by Miyamoto *et al*[21] evaluated the effects of mild HT on the efficacy and accumulation of an anti-EGFR agent (cetuximab) in various xenograft mouse models of pancreatic cancer. Using a water bath, a temperature of 37 °C or 41 °C was applied to the tumor and allowed to decrease the tumoral volume compared to mice exposed to a standard ambient temperature of 25 °C (control). This was accompanied by an increase in cetuximab accumulation in cancer cells by 2.5- to 5-fold, depending on

the model studied. Of note, this effect was also observed when stromal dense tissue derived from surgically resected pancreatic cancer was transplanted into mice. This effect may be due to an increase in blood flow with an increase in tumor vessel permeability[22]. Indeed, another study showed that mild HT in a mouse model of pancreatic cancer could induce an 11-fold increase in blood perfusion in a reversible manner during heating and a 3-fold increase after the end of HT treatment along with an increase in vasculature permeability and an enhanced extravasation of macromolecules[23]. In a xenograft mouse model of breast cancer, non invasive radiofrequency (RF) produced increased transport and perfusion of fluorescent tracers into the tumors at temperatures below 41°C, whereas vessel deformation and blood coagulation were observed when the temperature reached 44°C[24]. The mechanism through which HT increases blood perfusion may be linked to a relaxation of smooth muscle following an increase in nitric oxide synthetized by endothelial cells. In a study by Song *et al* [25], the content of inducible nitric oxide synthase (iNOS) was evaluated in a murine model of fibrosarcoma following HT. No iNOS was detectable before HT treatment, while an increase in iNOS was observed 3 h after HT and remained detectable 24 h after treatment. Additionally, HT increases the recruitment of bradykinin and histamine molecules responsible for vessel dilation and the recruitment of capillaries[26].

Hyperthermia and stromal architecture

HT has been shown to disrupt the stromal architecture. In a study by Piehler *et al*[14], Achilles tendons (mainly composed of type I collagen) were exposed to various regimens of either extrinsic or ion-oxide nanoparticle-based MHT. The amount of intact collagen fibers decreased with the application of HT, with only 10% of collagen fibers intact after 1 h of the 42 °C regimen and almost complete degradation after 1 h of the 50 °C or 70 °C regimen. Mild HT applied to spheroids of pancreatic cancer cells (Panc1) and fibroblasts (WI 38) significantly decreased the amount of intact collagen fibers, with a coinciding decrease in spheroid volume and cell viability by apoptotic and necrotic processes[14]. Local HT through the use of a photothermal agent combined with a photothermal-chemotherapeutic agent (Abraxane@$Mose_2$) and subsequently irradiated by a laser beam could disrupt tissue architecture and reduce the number of carcinoma-associated fibroblasts (CAFs), subsequently enhancing the efficacy of Abraxane in a mouse model of PDAC[27]. Similarly, in a mouse model of cholangiocarcinoma, a nanoheater used for PTT (multifunctional iron oxide nanoflowers decorated with gold particles) with high uptake by CAFs produced a significant depletion of CAFs as well as a reduction in tumor stiffness followed by significant tumor regression[28]. In another study by Marangon *et al*[29], tumor stiffness was monitored following PTT in squamous cell carcinoma in mice. Shear wave elastrography revealed a transient and reversible increase in tumor stiffness after thermal ablation or mild HT, followed by a return to its initial value within 24 h of laser exposure in the case of thermal ablation or a reduced level for mild HT. Additionally, while increased tumor stiffness was observed in untreated mice, the stiffness in the treated group was stable over time. In the same study, second harmonic generation was used to evaluate the effect of PTT on collagen structure and revealed a destructuration of collagen fibers in the vicinity of heated carbon nanotubes.

In several mouse models of pancreatic cancer displaying various levels of stroma formation, the concomitant application of mild hyperthermia and cetuximab induced a more significant antitumoral effect on stroma-rich models[21]. *In vitro*, noninvasive RF has been shown to affect molecular transport in a 3D model of PDAC with increased diffusion of DAPI fluorescence in spheroids following RF compared to no treatment[30]. In a xenograft mouse model of squamous cell carcinoma, the combination of MHT and doxorubicin demonstrated a more efficient reduction in tumor growth than doxorubicin alone[31]. In the same study, the space between collagen fibers was determined following MHT: while there were no differences between the control group and the group injected with nanocubes without exposure to a magnetic field (no HT), there was an increase in the interfibrillar space between the group injected and exposed to the magnetic field compared to the injected group without exposure to the magnetic field[31].

Hyperthermia and immune response

Data on the effect of HT on the immune response in PDAC are scarce, but the literature is abundant for other types of cancer. HT promotes antigen presenting cell (APC) activation and antigen-specific naïve CD8+ T cell differentiation, allows CD4+ T cells to shift towards the Th1 phenotype, and transforms regulatory T cells (Tregs) into Th17 cells[32]. In a mouse model of PDAC, RFA induced an increase in infiltrating CD8+ T cells and a decrease in Treg cells but showed no difference in the proportion of infiltrating CD4+ lymphocytes[33]. HT has been shown to induce chemokine production, such as CCL21, combined with adhesion factors (selectin, integrin, ICAM-1), thus allowing an increase in the interactions between lymphocytes and endothelial cells and the homing of lymphocytes[34-37]. In parallel, HT induces the production of various proinflammatory cytokines, such as IL6[38]. HT induces immunogenic cell death through various mechanisms[32], such as triggering DNA damage that produces mutations in tumor cell genes, which generate neoantigens that stimulate the T cell-based immune response[32,39]. HT can also generate damage-associated molecular patterns (DAMPs), of which heat shock proteins (HSP) are the most important but also include molecules such as calreticulin, HMGB1 or ATP. HSPs are chaperones that participate in the presentation of the chaperoned antigen to

the MHC-1 complex of dendritic cells, thus allowing antigen-specific T-cell activation[34,35,40]. High levels of HSP are associated with poor prognosis in parallel to an enhanced immune response[32]. Membrane HSP has been found to be a tumor-specific target for natural killer cells, whereas extracellular HSP can be considered a potent adjuvant to facilitate tumor antigen presentation and the induction of antitumor immunity[32,41,42]. More specifically, HSP70 has been shown to induce tumor cell proliferation in a mouse model of PDAC by activating AKT-mTOR signaling[33]. HSP60 has been shown to induce IFNg secretion and T cell upregulation[43]. In a murine model of PDAC, a study by Lin *et al*[44] found that the maximum HSP synthesis was achieved at 43 °C, corresponding with an increased antitumor immune response. Beyond this temperature, both the release of HSP and the associated immune response decreased[34,44]. The accumulation of neoantigens, secondary to mutations and DAMPs, favors the activation of dendritic cells, allowing the transformation of the tumoral immunosuppressive microenvironment by inhibiting Treg cells and promoting tumor-infiltrating lymphocyte maturation[32].

The so-called "abscopal effect" has often been invoked to suggest that an immunomodulating mechanism had to take place when the local treatment of a malignant tumor - most commonly the use of RT - results in a response at a distant location[45,46]. In a PDAC mouse model implanted with tumors on both flanks, Fei *et al*[47] tried to determine whether RFA on one flank's tumor could affect the untreated tumor located on the other side. After RFA on one side, the immune response on the opposite side showed an increase in CD8+/PD-1+ T cells, along with suppression of immunosuppressive components of the tumor microenvironment (*i.e.*, Tregs, tumor-associated macrophages, and tumor-associated neutrophils). Additionally, immune checkpoints such as PD-1 and LAG3 were upregulated in distant (untreated) T cells after one-sided RFA. Similarly, Gameiro *et al*[48] found that RFA induced local immunogenic modulation at the tumor surface in a model of colon adenocarcinoma, and the combination of RFA with vaccine therapy eradicated both primary and secondary tumors. Finally, in a clinical study for 10 patients with locally advanced pancreatic cancer (LAPC) that evaluated the immune response following coagulation necrosis-inducing RFA ablation, an increase in CD4+, CD8+, and effector memory T cells along with IL 6 was seen[49].

RT

RT uses an ionizing radiation beam (X-rays) whose energy is deposited in water along its path, leading to the formation of free radicals (reactive oxygen species or reactive nitrogen species) that oxidize molecular targets, provoking a dysregulation of cellular functions. These free radicals target DNA, leading to single- or double-strand breaks[50]. Today, there is no consensus on the role of RT in PDAC. The LPA07 trial did not show any improvement to the tumor in a small number of fractions to minimize the impact on the surrounding organs.

Radiation therapy and tumor vasculature

The effect of RT on tumor vasculature has been widely explored in various types of cancers. RT has been shown to have many direct or indirect effects on endothelial cells[50], and these effects are dependent on the dose received and the radiation schedule[51]. High single doses of radiation have been shown to cause vascular damage with reduced blood perfusion and hypoxia[52]. RT induces changes in tumor vasculature by destructuring microvessels and thickening vessel walls, thus reducing vessel lumen, all of which favor atherosclerosis. RT also induces platelet aggregation and microthrombus formation with an increase in inflammatory cell adhesion to endothelial cells[53]. RT can regulate and stabilize the level of HIF-1, leading to the production of VEGF, which is responsible for endothelial cell proliferation and increased survival. RT can directly upregulate the expression of a_vb_3 integrins[54] and adhesion proteins [50]. In pancreatic cells, HIF-1 has been shown to induce the sonic hedgehog protein, leading to the formation of a stroma-rich microenvironment[55]. In a rodent model of pancreatic tumor, a single high dose (SHD) of radiation led to temporary vascular dysfunction along with enhanced expression of HIF-1, which could be restored after 14 d. However, vascular permeability was higher in irradiated tumors 14 d after RT[56]. Similarly, a study by Lee *et al*[57] evaluated the effect of an SHD of radiation *vs* a fractionated regimen of radiation, which showed increased perfusion ability of tumor vessels following SHD, whereas fractionated RT had no effect. Mechanisms were further studied and showed that vessels treated with SHD-RT had lower pericyte coverage; increased vessel perfusion could therefore be due to an increased leakage of immature vessels, and the surviving vessels after SHD-RT might favor the penetration of small molecule drugs.

RT and stroma

RT induces chronic inflammation, leading to fibrosis through the accumulation of extracellular matrix proteins and an increase in stromal cells such as fibroblasts[58], which thicken and stiffen the tissue[51, 59]. Fibrosis formation depends on the dose of radiation received. For example, in a 3D model of mammary cancer stroma, increasing RT doses resulted in a reduction in fibroblast proliferation and activation along with a modest increase in matrix stiffness[60]. RT induces a loss of hyaluronic acid

along with a remodeling of collagen and a modification of CAF population[50]. Protease activity is also altered with an upregulation of MMP2[61], which is responsible for an increase in tumor invasiveness [58]. *In vitro* studies showed that human lung fibroblasts develop an irreversible senescent phenotype after exposure to a radiation dose higher than 10 Gy, while lower doses induced reversible DNA damage without growth arrest[51,62,63]. Senescent fibroblasts can release proteolytic enzymes, cytokines, growth factors, and ROS, creating a protumorogenic environment[49,57,64]. Similarly, *in vitro*, the coculture of ionizing radiation-exposed CAFs with pancreatic cancer cells enhanced the invasion-promoting capacity of CAFs, induced a high secretion of CXCL12 (a chemokine implicated in hematopoietic stem cell maintenance and cell migration) by CAFs, and promoted pancreatic cell migration, invasion, and epithelial-mesenchymal transition[65].

RT and immune response

RT has been shown to modulate the immune response by various mechanisms, the first of which is the release of tumor antigens, whereby DAMPs allow APC presentation and CD8+ activation followed by cell death, called immunogenic cell death. RT can also increase peptide availability and activate mTOR, leading to an increase in the MHC-1 protein subunit and an increase in the T cell repertoire[66]. RT induces the release of inflammatory cytokines such as IFN *via* the cGAS-STING pathway, which is activated by DNA damage caused by RT[67]. Adhesion molecules are also upregulated, with an increase in VCAM-1 and ICAM-1 leading to increased infiltration of lymphocytes to tumor cells and affinity binding to CD3+ cells[68]. Finally, RT facilitates homing of T cells to the TME by upregulating chemokines such as CXCL16[69]. In a murine orthotopic pancreatic cancer model, irradiated tumors displayed increased CD8+ and CD4+ cells, with a high single dose of RT being more efficient in recruiting CD8+ T lymphocytes than fractionated RT. However, fractionated RT induced more infiltration of myeloid-derived suppressor cells than high-dose RT[57]. *In vitro*, RT increased the expression of PDL-1 in a Jack/stat1-dependent manner[70]. Evidence to date suggests that immunotherapy such as anti-CTLA-4 or anti-PD-1 in PDAC has disappointing results or displays efficacy only in patients with PDAC who test positive for mismatch repair deficiency or microsatellite instability-high (MSI-h) due to the poorly immunogenic nature of PDAC[71-74]. Some data suggest that the combination of RT with immunotherapy could be a future approach to overcome this limitation. In a study by Lee *et al*[57], the combination of SHD-RT with anti-PD1 increased the delivery of anti-PD1 in a murine orthotopic mouse model of PDAC (UN-KC-6141), which is consistent with the increased tumor perfusion observed *in vivo* following RT. The survival of mice receiving a combined treatment of anti-PD1/SHD-RT was significantly improved compared to that of mice receiving anti-PD1 or SHD-RT alone. Splenocytes isolated from mice treated with the combination therapy showed increased cytotoxicity specifically toward UN-KC-6141 cells. In addition, while the combined group was free of peritoneal tumors, all of the control, SHD-RT, and anti-PDL1 alone groups bore metastases. This encouraging result is in accordance with another *in vivo* PDAC mouse model study by Fujiwara *et al*[75] reporting increased survival following a combination of anti-PD1 therapy and RT.

HIFU

HIFU is a noninvasive therapeutic technique using a focused ultrasound beam to create either thermal effects or a mechanical effect called cavitation at the focal point. With respect to thermal effects, due to the focal concentration of energy delivery, HIFU is capable of producing rapid coagulation necrosis with limited inflammatory response and minimal damage to the TME outside the focal zone, inside of which the TME is destroyed. Otherwise, the effects of HT on the TME have been described above in a dedicated section. With respect to the mechanical effect of HIFU, acoustic cavitation can be defined as the initiation, growth, oscillation, and collapse of gas bubbles inside a medium due to high tensile acoustic pressures that exceed cohesion forces between molecules. When exposed to an acoustic field, a bubble will oscillate radially (regime of stable cavitation) and possibly collapse (regime of inertial cavitation). At the tissue level, stable cavitation can stretch tight junctions and allow the extravasation of molecules from the vascular to interstitial space, making the plasma cell membrane transiently permeable and allowing for the internalization of molecules. Comparatively, inertial cavitation is more violent and may induce irreversible membrane disruption and cell implosion or hemorrhage in tissues [76]. HIFU is regularly used in prostate cancer or in the management of uterine fibroids[77,78] but also in the management of PDAC, although it is much less common. HIFU has been suggested to improve quality of life and alleviate pain in patients with a metastatic course of their disease[79].

To date, few preclinical studies have evaluated cavitation as a potentiator of chemotherapy with promising results. A previous study from our group evaluated the impact of various inertial cavitation intensities combined with gemcitabine on the viability of PDAC spheroids composed of both KPC pancreatic cancer cells and activated fibroblasts designed to mimic the tumor stroma[80]. Even if this model was far from a PDAC tumor, it possessed some of its essential features, including the presence of activated fibroblasts, the production of extracellular matrix and a dense intercellular arrangement. This work demonstrated that inertial cavitation decreased the viability of spheroids exposed to cavitation

and gemcitabine compared to either cavitation alone or gemcitabine alone. Moreover, gemcitabine had no impact on fibroblast viability, whereas the effect of chemotherapy on the viability of PDAC cells was enhanced when combined with cavitation. Of note, the effects of gemcitabine toxicity were less evident in spheroids composed of both KPC cells and fibroblasts compared to those composed of KPC cells only, which is consistent with the protective effect of the TME and supports the benefit of the combination[80].

In 2015, Li *et al*[81]. showed in KPC mice that cavitation with pulsed HIFU enhanced the intratumoral concentration of doxorubicin by 4.5-fold compared to controls, with an increase in doxorubicin concentration when cavitation was high and sustained. Of note, there were no differences when pulsed HIFU was delivered during or before doxorubicin administration. On the pulsed HIFU-treated tumors, macroscopic evaluation revealed hemorrhagic areas, while microscopic evaluation showed disorientation and separation of the collagen matrix with fraying of collagen fibrils. A study by Huang *et al*[82] evaluated the impact of cavitation induced with an ultrasound contrast agent (microbubble) in a mouse model of pancreatic cancer. Blood perfusion evaluated by contrast-enhanced ultrasound imaging revealed a decrease in blood flow within the tumor after cavitation treatment compared to pretreatment measurements, whereas blood perfusion of nontumoral tissue was not impacted. Immunostaining of blood vessels also showed decreased expression of CD31 and reduced microvascular density in the cavitation group.

On the immunotherapy side, the mechanical effects of HIFU have been shown to induce subcellular fragmentation, leading to the release of DAMPs that are subsequently presented to dendritic cells[83] and trigger cytotoxic T cell activation[84]. Pulsed HIFU or low-intensity HIFU have been shown to drive Th1 inflammation, to stimulate localized cell recruitment factors and tumor cell surface immunogenic proteins, and increase the CD8+/Treg ratio[85]. However, these data come from non-PDAC tumor types.

IRREVERSIBLE ELECTROPORATION

IRE is a nonthermal ablative therapy using a direct high voltage current with a short pulse length to increase cell membrane permeability, resulting in permanent cell death with minimal thermal deposition[86-88]. IRE is applied by placing two or more electrodes in the tumor or around it[89] and can be used intraoperatively, laparoscopically, or percutaneously. IRE induces damage only to the cell membrane and has no effect on protein denaturation, blood flow, and connective tissue[88] and was first described for the treatment of human pancreatic cancer in 2012[90].

Studies on the specific effects of IRE on the PDAC stroma are scarce. One study by Bhutiani *et al*[91] described an increase in gemcitabine delivery to the tissue located in the electroporation area in mice treated by IRE compared to untreated mice. Even if mechanistic explanations were not explored, this effect may be attributable to IRE-related alteration of the stroma. TME modulation following IRE is also characterized by a transient increase in microvascular density and an increase in tumor blood vessel permeability along with a softening of the extracellular matrix can lead to an increase in T cell infiltration[92]. IRE can induce microvessel endothelial cell apoptosis with microvessel thrombosis *in vivo*[93]. In a xenograft mouse model of PDAC, alterations of tumor microstructure were described following IRE in which acute coagulative necrosis and thrombosis were visible throughout the treated tumor volume after IRE, whereas minimal thrombosis was observed in the control group (no treatment). Using transmission electron microscopy, microvessel endothelial apoptosis and microvessel thrombosis were visualized, and magnetic resonance imaging (MRI) analysis revealed a significant increase in water diffusion after IRE, with a reduction in diffusion-weighted MRI images reflecting an increase in diffusion (water mobility) in the tissue after IRE[93].

From an immunologic point of view, available data are limited: a mouse study by Yang *et al*[94] described an increase in calreticulin after IRE, suggesting an induction of immunogenic cell death, with an increase in the intratumoral expression of CD8+ cells and GrB (granules of enzymes expressed by cytotoxic lymphocytes) when IRE was combined with a dendritic cell vaccine. In the same study, stromal fibrosis formation was not modified following IRE. In another study by White *et al*[95], IRE was found to induce an increase in macrophage, T cell, and neutrophil infiltration within the tumor.

CLINICAL PERSPECTIVES

Hyperthermia

In accordance with mouse studies showing an increase in drug delivery with hyperthermia, a recent systematic review evaluated the clinical benefit of HT (regional, intraoperative, or whole-body HT) combined with chemotherapy, RT or both in 248 patients. Out of 14 studies, 6 showed a longer median overall survival in the HT group compared to the control group, with an 11.7 mo median survival *vs* 5.6 mo. The response rate was also higher in the HT groups[96]. These encouraging results have prompted

randomized clinical trials to more clearly demonstrate any benefit of this therapeutic approach. A phase II study (HEATPAC-NCT02439593) is currently recruiting to compare deep locoregional HT administered with a microwave system (Aim 40-43 °C for 60 min) with chemotherapy *vs* chemotherapy alone in LAPC. The results from this study could provide a practical assessment of the efficacy of HT in PDAC[97]. Other current studies are summarized in Table 2.

RFA in PDAC has been reported in small exploratory series for tumor debulking rather than for complete tumor ablation because safety margins are needed to avoid thermal damage to surrounding structures[98]. Following RFA combined with chemotherapy, overall survival ranges from 19 to 25.6 mo [98,99]. There is a lack of randomized studies assessing the place of RFA in the management of LAPC. The PELICAN trial (NCT03690323) is planned to evaluate whether the combination of chemotherapy and RFA improves overall survival compared to chemotherapy alone in patients with LAPC without any progression after 2 mo of systemic treatment[100] (Table 2).

Endoscopic application of RFA, which is already feasible, is an attractive approach because of its minimal invasiveness. The active component is a 19G needle that has a tip equipped with an electrode to be placed in the lesion under endoscopic ultrasound (EUS) guidance. This approach has been proven safe and feasible in small-sized studies of patients with unresectable PDAC[101-103]. Nevertheless, larger prospective studies are needed. EUS-RFA clinical trials are ongoing (Table 2), and it would be interesting to evaluate EUS-RFA as an alternative to RT in LAPC patients with an objective response to chemotherapy who retain criteria against surgical resection.

RT

Stereotactic body radiotherapy (SBRT) is increasingly being explored for the management of PDAC in combination with anticancerous drugs, especially for LAPC. An open-label phase 2 multicenter study by Herman *et al*. evaluated the combination of gemcitabine plus SBRT in patients with LAPC, showing a good safety profile[104]. Similarly, 39 patients who underwent FOLFIRINOX followed by SBRT seemed to have an increased chance of undergoing radical surgery[105]. SBRT in combination with immune therapy is also being studied. In a phase I study by Xie *et al*[106], a combination of immune therapy (durvalumab ± tremelimumab) with SBRT in metastatic PDAC showed a favorable safety profile but only a modest clinical efficacy. Of note, none of the responders were MSI-h. While these results are interesting, further exploration is required, and many clinical trials are underway to evaluate the combination of SBRT or RT with anticancerous drugs (Table 2). A challenge for the use of SBRT is the required placement of fiducials to facilitate the delivery of radiation, which can be made difficult by respiratory movements and the vicinity of other organs[107]. These fiducials can be placed percutaneously when not impeded by surrounding organs or in a more invasive fashion, surgically. EUS-guided placement of fiducial also appears to be a promising method with a high rate of technical success and a reasonable rate of adverse events[91,108], but randomized studies are needed.

HIFU

Cavitation generated by HIFU is a very attractive method with a high potential to disrupt the stroma, thus overcoming the barrier to efficient drug delivery and stimulating the immune response in preclinical works. To date, there are no published clinical trials. However, one upcoming clinical trial (NCT04146441) of HIFU combined with chemotherapy (FOLFIRINOX) will determine whether focused ultrasound can increase drug uptake and overcome chemoresistance (Table 2). In a minimalist approach of ultrasound-induced enhancement of chemotherapy, 10 patients were enrolled in a phase I clinical trial to receive gemcitabine combined with low intensity ultrasound and microbubbles as an ultrasound contrast agent programmed to favor sonoporation, with encouraging results in terms of the number of chemotherapy cycles tolerated and median overall survival when compared to 63 historical controls receiving only chemotherapy[109].

HIFU is also a very attractive approach to increase the intratumoral temperature and increase drug delivery. In a monocentric retrospective study among 523 patients, a combination of HIFU with gemcitabine appeared to produce better overall survival than standard CT in unresectable PDAC[110]. The PanDox study is a phase I study that plans to evaluate whether HIFU can increase the amount of drug delivery (doxorubicin or heat-sensitive doxorubicin) within the tumor in 18 patients with unresectable PDAC (NCT04852367, Table 2).

Challenges in the method of ultrasound delivery still need to be addressed, since extracorporeal delivery to the deeply seated pancreas with gas interposition could be challenging. We are currently working on an endoscopic device that could overcome these limitations and noninvasively deliver cavitation at any part of the pancreas. The endoscopic approach to HIFU delivery, foreseen by our team some time ago[111], has also been recently studied in a porcine model[112].

Irreversible electroporation

After the landmark study by Martin *et al*[90] and subsequent large series of intraoperative applications [86], less invasive percutaneous IRE has shown promising results in terms of efficacy. A nonrandomized prospective single-center case series by Ma *et al*[113] evaluated the efficacy of a combination of percutaneous IRE with gemcitabine compared with gemcitabine alone. The combination increased the

Table 2 Ongoing studies in locoregional therapies used alone or in combination with chemotherapy or immunotherapy for pancreatic ductal adenocarcinoma

	Clinical trial number	Study name	Number of patients	Status	Country
Hyperthermia	NCT04858009	Hyperthermic intraperitoneal chemotherapy for the treatment of pancreatic cancer and peritoneal metastasis.	40	Not yet recruiting	United States
	NCT04889742	HT enhanced reirradiation of loco-regional recurrent tumors (HETERERO).	100	Recruiting	Germany
	NCT04310111	EUS-RFA for unresectable pancreatic cancer.	18	Recruiting	China
	NCT03218345	EUS-guided RFA for pancreatic neoplasms.	30	Recruiting	China
	NCT02439593	Concurrent HT and chemoradiotherapy in LAPC: phase II study (HEATPAC).	78	Recruiting	Switzerland
	NCT04164992	EUS-RFA of not-resectable pancreatic cancer.	15	Not yet recruiting	Italy
	NCT03690323	Pancreatic locally advanced irresectable cancer ablation (PELICAN).	228	Recruiting	Netherlands
	NCT04156087	Progression-free survival after microwave ablation plus durvalumab and tremelimumab for unresectable LAPC (MIMIPAC).	20	Recruiting	Belgium
Radiation therapy	NCT04361162	Nivolumab + ipilimumab + radiation in microsatellite stable pancreatic cancer.	30	Recruiting	United States
	NCT01972919	Magnetic resonance guided, dose-escalated RT + chemotherapy in pancreatic cancer.	23	Recruiting	United States
	NCT03374293	Combination of radiation therapy and anti-PD-1 antibody in treating patients with pancreatic cancer.	21	Recruiting	China
	NCT03492671	Testing the combination of two approved chemotherapy drugs and radiation prior to surgery in localized pancreatic cancer.	30	Recruiting	United States
	NCT04975516	Standard of care chemotherapy with or without SBRT for the treatment of oligometastatic pancreatic cancer.	50	Not yet recruiting	United States
	NCT04327986	Immune checkpoint inhibitor m7824 and the immunocytokine m9241 in combination with SBRT in adults with advanced pancreas cancer.	52	Recruiting	United States
	NCT03991962	Phase II study to evaluate modified folfirinox and SBRT in nonmetastatic unresectable pancreatic adenocarcinoma.	28	Recruiting	United States
	NCT02128100	Effects of folfirinox and SBRT for advanced pancreatic cancer.	28	Recruiting	United States
	NCT04089150	Mfolfirinox and SBRT for pancreatic cancer with high risk and locally advanced disease.	120	Not yet recruiting	Australia
	NCT04172532	Testing the addition of a new anticancer drug, m3814 (peposertib), to radiation therapy for localized pancreatic cancer.	24	Recruiting	United States
HIFU	NCT04146441	Ultrasound-enhanced uptake of chemotherapy in patients with inoperable PDAC.	30	Recruiting	Norway
	NCT04852367	PanDox: targeted doxorubicin in pancreatic tumors.	18	Not yet recruiting	England
Irreversible electroporation	NCT03257150	A study of the use of IRE in pancreatic ductal cancer.	47	Recruiting	Canada
	NCT03105921	IRE (nanoknife) for the treatment of pancreatic adenocarcinoma.	20	Recruiting	France
	NCT03899636	A pivotal study of safety and effectiveness of nanoknife IRE for stage 3 pancreatic cancer (direct).	528	Recruiting	United States
	NCT02822716	IRE for inoperable hepatic and pancreatic malignancy.	35	Recruiting	China
	NCT02343835	Antitumor immunity induced by IRE of unresectable pancreatic cancer.	20	Recruiting	China
	NCT03484299	Chemotherapy and IRE in the treatment of advanced pancreatic adenocarcinoma.	20	Recruiting	United States
	NCT04835402	Electroporation potentiated immunotherapy in cancer (EPIC-1).	16	Recruiting	Denmark

NCT04276857	Systemic therapy with a loco-regional treatment in patients with LAPC (smart).	27	Not yet recruiting	Canada
NCT04212026	IRE followed by nivolumab in patients with metastatic pancreatic cancer.	15	Recruiting	Switzerland
NCT04612530	Panfire-3 trial: assessing safety and efficacy of IRE + nivolumab + CpG for metastatic pancreatic cancer.	18	Recruiting	Netherlands
NCT02041936	Outcomes of ablation of unresectable pancreatic cancer using the nanoknife IRE system.	12	Recruiting	United States
NCT04310553	An open-label, multicenter, prospective study of IRE (nanoknife) combined with RT and chemotherapy in patients with LAPC.	240	Recruiting	China
NCT04093141	Chemotherapy followed by IRE in patients with unresectable LAPC (chemofire-2).	30	Recruiting	Denmark
NCT03614910	Ablation of unresectable LAPC with IRE system.	30	Recruiting	United States

HT: Hyperthermia; EUS: Endoscopic ultrasound; RFA: Radiofrequency ablation; LAPC: Locally advanced pancreatic cancer; RT: Radiation therapy; SBRT: Stereotactic body radiation therapy; PDAC: Pancreatic ductal adenocarcinoma; IRE: Irreversible electrotherapy.

overall survival from the time of diagnosis by 3-fold and nearly doubled the progression-free survival. In a *post hoc* comparison of data derived from a prospective IRE-FOLFIRINOX cohort and a retrospective FOLFIRINOX-only cohort, van Veldhuisen *et al*[114] found that the combination (30 LAPC patients) increased the time to progression compared to standard therapy (22 patients). Lin *et al*[115] showed promising results with IRE combined with allogenic natural killer cell immunotherapy with an increase in progression-free survival and overall survival. However, a multicenter prospective study by Ruarus *et al*[116] (PANFIRE II) described a high rate of adverse events in patients undergoing percutaneous IRE, with 29 out of 50 participants experiencing adverse events, 21 of which were major, and 2 deaths, including one clearly related to IRE. Thus, this procedure can be considered a high-risk procedure that requires the selection of patients who will benefit the most from the treatment. This high rate of adverse events, along with a relative cumbersomeness to set up, has limited the spread of this technique. New application methods are needed to overcome these issues. Many clinical trials are ongoing to better understand the benefits of combining IRE with chemotherapy or immunotherapy (Table 2).

CONCLUSION

The TME is one of the major causes of therapeutic resistance in PDAC. Fibrosis-related stiffness, hypomicrovascular perfusion, and an immune suppressive microenvironment are, within the limits of current knowledge, key determinants of this resistance. While systemic chemotherapies and immunotherapies have disappointing results and are responsible for adverse events resulting in poor quality of life, locoregional therapies can specifically target the tumor area with limited effects on surrounding tissues but significant impacts on the TME. Local HT using RFA and radiotherapy using local SHD are the two main modalities currently holding promise for clinical efficacy, but IRE and focused ultrasound-derived cavitation are also gaining increasing attention as treatments for PDAC. These techniques influence the tumor stroma, microvasculature, and immune environment and response (Table 1). To date, most of the data are preclinical with some promising results. Clinical trials are underway (Table 2) and will allow the scientific community to have a more precise idea of the interest in using these treatment options alone or in combination with systemic therapies.

FOOTNOTES

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Country/Territory of origin: France

ORCID number: Thomas Lambin 0000-0002-9437-0154; Cyril Lafon 0000-0003-1550-970X; Robert Andrew Drainville 0000-0003-2922-9522; Mathieu Pioche 0000-0002-6482-2375; Frédéric Prat 0000-0002-6018-0491.

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MINIREVIEWS

Management of incidentally discovered appendiceal neuroendocrine tumors after an appendicectomy

José Luis Muñoz de Nova, Jorge Hernando, Miguel Sampedro Núñez, Greissy Tibisay Vázquez Benítez, Eva María Triviño Ibáñez, María Isabel del Olmo García, Jorge Barriuso, Jaume Capdevila, Elena Martín-Pérez

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José Luis Muñoz de Nova, Elena Martín-Pérez, Department of General and Digestive Surgery, Hospital Universitario de La Princesa, Madrid 28006, Spain

José Luis Muñoz de Nova, Elena Martín-Pérez, Department of Surgery, Universidad Autónoma de Madrid, Madrid 28029, Spain

Jorge Hernando, Jaume Capdevila, Gastrointestinal and Endocrine Tumor Unit, Medical Oncology Department, Vall d´Hebron University Hospital, Vall d´Hebron Institute of Oncology, Barcelona 08035, Spain

Miguel Sampedro Núñez, Department of Endocrinology and Nutrition, Hospital Universitario de La Princesa, Madrid 28006, Spain

Greissy Tibisay Vázquez Benítez, Department of Pathology, Hospital Universitario Puerta de Hierro, Madrid 28222, Spain

Greissy Tibisay Vázquez Benítez, Department of Pathology, Universidad Autónoma de Madrid, Madrid 28029, Spain

Eva María Triviño Ibáñez, Department of Nuclear Medicine, Virgen de las Nieves University Hospital, Granada 18014, Spain

María Isabel del Olmo García, Department of Endocrinology and Nutrition, Hospital Universitario i Politècnic La Fe, Valencia 46023, Spain

Jorge Barriuso, Division of Cancer Sciences, School of Medical Sciences, Faculty of Biology, Medicine and Health, University of Manchester, Manchester M13 9PL, United Kingdom

Jorge Barriuso, Department of Medical Oncology, The Christie NHS Foundation Trust, Manchester M20 4BX, United Kingdom

Corresponding author: José Luis Muñoz de Nova, MD, PhD, Associate Professor, Surgeon, Department of General and Digestive Surgery, Hospital Universitario de La Princesa, Diego de León, 4th Floor, Madrid 28006, Spain. jmunoz@salud.madrid.org

Abstract

Appendiceal neuroendocrine tumors (aNETs) are an uncommon neoplasm that is relatively indolent in most cases. They are typically diagnosed in younger patients than other neuroendocrine tumors and are often an incidental finding after an

appendectomy. Although there are numerous clinical practice guidelines on management of aNETs, there is continues to be a dearth of evidence on optimal treatment. Management of these tumors is stratified according to risk of locoregional and distant metastasis. However, there is a lack of consensus regarding tumors that measure 1-2 cm. In these cases, some histopathological features such as size, tumor grade, presence of lymphovascular invasion, or mesoappendix infiltration must also be considered. Computed tomography or magnetic resonance imaging scans are recommended for evaluating the presence of additional disease, except in the case of tumors smaller than 1 cm without additional risk factors. Somatostatin receptor scintigraphy or positron emission tomography with computed tomography should be considered in cases with suspected residual or distant disease. The main point of controversy is the indication for performing a completion right hemicolectomy after an initial appendectomy, based on the risk of lymph node metastases. The main factor considered is tumor size and 2 cm is the most common threshold for indicating a colectomy. Other factors such as mesoappendix infiltration, lymphovascular invasion, or tumor grade may also be considered. On the other hand, potential complications, and decreased quality of life after a hemicolectomy as well as the lack of evidence on benefits in terms of survival must be taken into consideration. In this review, we present data regarding the current indications, outcomes, and benefits of a colectomy.

Key Words: Neuroendocrine tumors; Carcinoid tumor; Appendiceal neoplasms; Colectomy; Neoplasm grading; Treatment outcome

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Core Tip: In this review, we analyze the features to consider when appendiceal neuroendocrine tumor is found after an appendectomy. We critically analyze the main indications for a completion right hemicolectomy and the risk-benefit ratio.

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INTRODUCTION

More than 80% of appendiceal neuroendocrine tumors (aNETs) are diagnosed incidentally in appendectomy specimens and are found in approximately 0.5% to 1% of all appendectomies[1]. These neoplasms have several characteristic features that differ from other gastroenteropancreatic neuroendocrine tumors (GEP-NETs). They usually progress indolently and are diagnosed in younger patients other NETs; the majority are detected in the third or fourth decade of life while other NETs are usually diagnosed close to the sixth decade of life[2-5]. Nevertheless, these tumors do occasionally have an aggressive course with liver and mesenteric lymph node metastasis (LNM)[6]. In a recent review of patients included in the Surveillance, Epidemiology, and End Results database, the five-year survival rates for aNETs are 97.4%, 88.6%, and 27.4% for localized, regional, and distant disease, respectively. Non-metastatic aNETs had the highest overall survival rate of all GEP-NETs[7].

Surgery is curative in most cases[3]. However, controversy arises when deciding whether an appendectomy alone is sufficient or whether the patient will achieve better outcomes with a completion right hemicolectomy (CRH) after an initial appendectomy. The main purpose of a CRH is to complete the regional lymph node dissection. These nodes are involved in 6% to 9% of cases[8]. Both the North American Neuroendocrine Tumor Society[9] and European Neuroendocrine Tumor Society (ENETS)[10] guidelines suggest a tailored approach to these patients based on features such as tumor size, margin status, mesoappendix infiltration, vascular and lymphatic invasion, and tumor grade. In tumors smaller than 1 cm, an appendectomy is indicated whereas in those larger than 2 cm, a CRH is recommended. In tumors between 1 and 2 cm, CRH should be considered when there are affected margins in tumors located at the base, when there is invasion of the mesoappendix that measures greater than 3 mm, or when there are other risk factors. However, several studies have challenged these recommendations, mainly in tumors smaller than 2 cm, arguing that CRH offers no benefits in terms of survival in tumors smaller than 2 cm[8,11]. Furthermore, in addition to the potential postoperative complications, a colectomy could lead to a poorer quality of life for these patients.

This narrative review critically evaluates the management of these patients based on evidence in the current literature with a special focus on the indications for and outcomes of a CRH.

WHAT ARE THE FEATURES ANALYZED IN THE HISTOPATHOLOGICAL DIAGNOSIS?

A careful histopathological evaluation of the surgical specimen will provide crucial information for determining management. aNETs are epithelial neoplasms that likely arise from neuroendocrine cells, including enterochromaffin cell neuroendocrine tumors, L-cell NETs, and tubular NETs. Their pathogenesis is largely unknown. Their hypothetical origins include neuroendocrine cells within the mucosal crypts or subepithelial neuroendocrine cells, especially in enterochromaffin cell NETs[12,13].

Appendiceal neoplasms include well-differentiated aNETs (classified as low grade-G1, intermediate grade-G2, or high grade-G3 according to proliferative rate), poorly differentiated neuroendocrine carcinoma, and mixed neuroendocrine-non neuroendocrine neoplasms (MINEN). Between 70% to 75% of neuroendocrine neoplasms in the appendix are well-differentiated NETs. Goblet cell adenocarcinoma is no longer considered a subtype of aNET[13,14]. Goblet cell adenocarcinoma and MINEN will not be discussed further in this review.

Macroscopically, most of these cases are found incidentally on the tip of the appendix after an appendectomy for acute appendicitis. They are usually yellowish nodules and most are smaller than 2 cm in diameter (only 8% to 19% are larger than 2 cm)[15,16].

Microscopically, well-differentiated aNETs include:

Enterochromaffin cell appendiceal neuroendocrine tumors (EC-NETs): This is the most common subtype. EC-NETs usually appear as uniform polygonal cells arranged in nests or a glandular pattern with a fibrotic stromal response. Necrosis and mitosis are uncommon. Immunohistochemistry techniques demonstrate positivity to chromogranin A (CgA), synaptophysin, and serotonin production in EC cells[15,17] (Figure 1).

L-cell NETs: These tumors have trabecular or glandular growth patterns. L-cell NETs produce glucagon-like peptide 1 and proglucagon-derived peptides. L-cell NETs express chromogranin B rather than CgA[14].

Tubular NETs: These tumors represent < 10% of all aNETs[8] and must be distinguished from adenocarcinoma NOS and goblet cell adenocarcinoma[14].

Whether a tumor is an EC or L-cell NET is usually not specified on pathology reports as this distinction has no prognostic or therapeutic implications[14,18].

Poorly differentiated aNETs are rare and are microscopically similar to other intestinal neuroendocrine carcinomas[15,17,19].

The staging of aNETs is mainly based on tumor size and serosal or mesoappendix invasion. The pathology report should also include pTNM staging (according to either American Joint Committee on Cancer classification[20], ENETS classification[10], or both), margin status, and vascular and lymphatic vessel involvement (Table 1). Mesoappendix invasion is usually associated with a higher rate of vascular and lymphatic vessel involvement[10,15,17].

WHAT ADDITIONAL TESTS ARE RECOMMENDED IN CASES OF ANET?

After an incidental diagnosis of aNET, the main purpose of additional tests is to assess the presence of residual locoregional disease or distant metastasis in order to determine postoperative staging.

Biochemical tests

There are no clear benefits to measuring any specific biomarker in aNETs[10]. Although CgA is a widely used biomarker for evaluating and following-up on GEP-NETs, its role in aNETs is unclear. CgA levels are usually within normal range in NETs with a low proliferative potential, which are the majority of aNETs[21]. It has been suggested that its measurement may only be useful in advanced cases[10]. Carcinoid syndrome is uncommon at the time of diagnosis[2], but when it is suspected, determination of urinary 5-hydroxyindoleacetic acid (5-HIAA) could be useful[10].

Endoscopy

The usefulness of performing an endoscopy after an incidental diagnosis of aNETs seems to be negligible unless the tumor infiltrates the cecum[10].

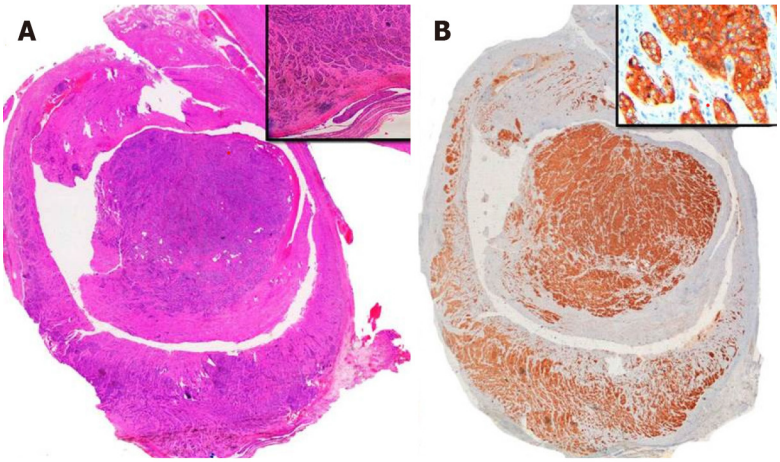
Conventional imaging tests

After a complete resection of an incidentally diagnosed well-differentiated aNET measuring < 1 cm, no further diagnostic testing is required. However, controversy remains regarding tumors that measure between 1 and 2 cm, since these tumors rarely have LNM. To evaluate the presence of lymphatic involvement or distant metastasis, an abdominal computed tomography (CT) or magnetic resonance

Table 1 TNM staging for appendiceal neuroendocrine tumor according to European Neuroendocrine Tumor Society and American Joint Committee on Cancer classification

	ENETS	AJCC
T0	No evidence of primary tumor	No evidence of primary tumor
T1	Tumor ≤ 1 cm with infiltration of submucosa and muscularis propia	
T1a		Tumor ≤ 1 cm
T1b		Tumor 1-2 cm
T2	Tumor ≤ 2 cm with infiltration of the submucosa, muscularis propia and/or minimal (≤ 3 mm) infiltration of the subserosa and/or mesoappendix	Tumor 2-4 cm or with extension into the cecum
T3	Tumor > 2 cm and/or extensive (> 3 mm) infiltration of the subserosa and/or mesoappendix	Tumor > 4 cm or with extension into the ileum
T4	Tumor with infiltration of the peritoneum and/or other neighboring organs	Tumor with perforation of the peritoneum or invasion of other adjacent structures
N0	No regional lymph node metastasis	No regional lymph node metastasis
N1	Locorregional lymph node metastasis	Locorregional lymph node metastasis
M0	No distant metastasis	No distant metastasis
M1	Distant metastasis	Distant metastasis

ENETS: European Neuroendocrine Tumor Society; AJCC: American Joint Committee on Cancer.



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Figure 1 Histological images. A: Well-differentiated aNET that infiltrate the entire wall of the appendix and focally infiltrate the adjacent fat, affecting the surgical margin; B: Immunohistochemical techniques reveal positivity for synaptophysin and CgA.

imaging (MRI) scan is recommended. These tests should also be considered for tumors > 2 cm or for those with mesoappendix infiltration or vascular and lymphatic vessel invasion. In these cases, somatostatin receptor scintigraphy (SRS) or a positron emission tomography with computed tomography (PET/CT) scan should be considered[10].

Nuclear medicine imaging

There are a multitude of articles on the applications of SRS in GEP-NETs, however, only sporadic cases of aNET have been described in this body of literature[22,23]. SRS using either indium-111 or technetium-99m (including single photon emission CT) or a PET scan using gallium-68-labeled somatostatin analogs (SSAs) in combination with a CT scan can be considered in cases in which curative resection is not completely assured or when distant metastasis is suspected[24]. In these cases, published studies suggest that SRS is useful for detecting residual disease[23,25] and that SRS results could modify management in 20% to 25% of patients[23,26], mainly for those with high proliferative activity.

Positron emitting radiopharmaceuticals such as gallium-68-labeled peptides or 18F-fluorodopamine are now preferred for the diagnosis of well-differentiated GEP-NETs, particularly those smaller than 1

cm[10]. These PET radiopharmaceuticals provide greater resolution, faster results, shorter imaging time, and 3D visualization. However, due to their cost and availability, their use is not yet widespread. 18F-2-fluoro-2-deoxy-glucose PET/CT is recommended for detecting poorly differentiated or heterogeneous NETs[27]. Furthermore, even though these radiopharmaceuticals have been widely studied in other GEP-NETs, to date no works have evaluated their usefulness specifically in aNETs.

INDICATIONS IN THE LITERATURE FOR A COMPLETION RIGHT COLECTOMY

Most cases of aNET are patients with a tumor < 1 cm in the distal third of the appendix. The presence of risk factors associated with more extensive disease, such as serosal or mesoappendix invasion, are usually associated with tumors greater than 2 cm[2].

After a CRH, residual disease or lymph node involvement is present in 0% to 40% of cases[2,8,28]. A recent review by Webb *et al*[11] of patients in the National Cancer Database treated with surgery found that, unlike other types of appendiceal neoplasms, in aNET, the presence of regional LNM does not reduce overall survival. Since most studies supporting the indication of a CRH are based on completing a lymph node dissection, the claim that this procedure improves patient survival is highly questionable.

The main indications for CRH are based on the following evidence:

Tumor size: Some recent studies suggest that size is the main factor related to the occurrence of LNM [1,8,15,29,30]. As stated above, controversy arises in aNETs that are greater than 2 cm and those from 1 to 2 cm, which represent from 3% to 7% and from 20% to 25% of cases, respectively[8,28]. The 10-year survival rate according to size is 100%, 92%, and 91% for < 1 cm, 1 to 2 cm, and > 2 cm, respectively[31]. Although the indication is most likely a continuum, it has been suggested that 2 cm may be the optimal cut-off point for presence of LNM[1]. Current guidelines recommend this size for considering a CRH after an appendectomy and to consider CRH for tumors from 1 to 2 cm when other risk factors are present. In a recent meta-analysis, the rate of LNM was 12.1%, 38.5%, and 61% for tumors measuring < 1 cm, 1 to 2 cm, and > 2 cm, respectively[32]. A reduction in the cut-off point for a colectomy from 15 mm to 13 mm regardless of other features has been proposed due to the possible presence of LNM, but no studies have found any benefit in terms of recurrence or survival[5,28].

Mesoappendix invasion: Its incidence ranges from 23% to 39%[5,8,28]. There is controversy in the literature regarding its prognostic relevance. Some studies have found no effects on the recurrence rate [33] or presence of LNM[1,8], while others suggest that the disease behaves more aggressively in these patients[17,31]. A recent publication found that presence of mesoappendix invasion entails a higher risk of LNM and suggests an optimal cut-off point of 1 mm for indicating a CRH, which is substantially smaller than what has been suggested in previous reports[28]. However, these data should be interpreted cautiously because they were calculated based on patients who underwent a right colectomy according to ENETS guidelines indications and thus, the sample is biased. Furthermore, no patients with mesoappendix invasion in that study had recurrence. A recent meta-analysis reported a LNM rate of 30.3% when mesoappendix invasion was present and 26.2% when it was absent (OR, 1.4; 95%CI: 0.8-2.4)[32]. Also, in some works, the increased risk of LNM found on the univariate analysis disappeared on the multivariate analysis, with only size remaining as a relevant factor[5,8].

Positive margins: Although this may seem uncommon, a French multicenter study that included patients treated in non-referral centers reported a rate of 8%[15]. Given the possibility that the tumor could remain in the cecum area, most authors recommend CRH in this scenario[8,28].

Vascular and lymphatic vessel involvement: This feature can be found in 11% to 18% of patients[5,8,28]. It could be particularly relevant in patients with tumors < 2 cm, in whom it could reflect a metastatic potential similar to tumors measuring > 2 cm[5,34]. On the contrary, some studies have not described a higher LNM rate, but the low incidence of this finding could limit statistical significance[1,8]. Perineural invasion has been reported in 18% of patients[8], but there is no data to suggest more aggressive disease when it is present[32].

Tumor grade: Almost all aNETs are G1. Less than 15% have been described as G2 and the existence of G3 and poorly differentiated aNETs is anecdotal[1,28]. Although some works do not show a higher LNM rate in patients with a grade higher than G1[8], many others describe a significant risk in up to 90% of G2 neoplasms[28,35].

SURGICAL TECHNIQUE

The main purpose of a right hemicolectomy after an appendectomy is not only to resect residual local tumor, but also to complete the regional lymph node dissection. Nodal spread of aNETs is usually through the ileocolic vessels, a territory with few anatomical variations. Laparoscopic dissection of this territory is quite a standard approach to treat right side colonic cancer. In the absence of gross central nodal involvement, a laparoscopic approach seems to be safe and could provide some benefits, namely a shorter recovery time.

OUTCOMES AFTER A COMPLETION RIGHT HEMICOLECTOMY

Postoperative complications

Patients who undergo CRH for aNETs are younger and have fewer comorbidities than typical patients with colon cancer. Nevertheless, the rate of major complications after a colectomy for aNETs ranges from 5% to 15%[28,36,37]. A systematic review and meta-analysis conducted by Ricci *et al*[30] found that when performing a CRH in aNETs > 2 cm, the number needed to treat was five, while the number needed to harm was six, suggesting that the risk was similar to the possible benefit.

Survival and recurrence

It is important to emphasize that in general, these patients have an excellent prognosis, with survival rates close to 100% at 10 years of follow-up. Table 2 summarizes the literature on recurrence and survival rates in this type of tumor[38-41].

Although several studies agree that 2 cm is a reliable cut-off point for a colectomy, others have shown that a simple appendectomy offers comparably good results in tumors > 2 cm[29]. For example, in the classic work by Moertel *et al*[42], only one patient treated with an appendectomy out of 12 experienced recurrence, and it occurred 29 years after the initial appendectomy. This patient then underwent a right hemicolectomy, after which he remained disease-free 17 years later. Such data could lead us to speculate whether there is a decrease in overall survival by waiting to confirm the presence of lymphadenopathy instead of performing "prophylactic" CRH. Similarly, Groth *et al*[43] performed an appendectomy alone on 34 of 122 patients with aNETs > 2 cm and there was no difference in survival compared to patients who underwent a right hemicolectomy. Controversy mainly arises in patients with aNETs between 1 to 2 cm. In these patients, all studies suggest that survival is similar after either an appendectomy or a CRH.

While most studies report features associated with LNM and recommend CRH when its presence is possible, there is a dearth of data regarding its influence on patient survival. A recent meta-analysis did not find any difference in disease-specific survival at five or ten years in patients with or without LNM (100% in both groups for five years and 95.6% *vs* 99.2% for ten years)[32]. It is clear the extremely long course of these tumors makes it difficult to evaluate overall survival.

An interesting study on how surgical technique may influence patient survival is a review of the National Cancer Database performed by Heller *et al*[44]. Their work described 3198 cases of aNETs and found that 32.4% of those smaller than 2 cm were treated with a right hemicolectomy and 31.5% of those larger than 2 cm were treated with a simple appendectomy. There were no differences in survival between the groups according to surgical procedure.

Quality of life

In addition to questions regarding a survival benefit after CRH in aNETs < 2 cm, its influence on patients' quality of life must also be considered. This topic has recently been evaluated in a multicenter study from five ENETS centers of excellence in which the health-related quality of life European Organization for Research and Treatment of Cancer-QLC-C30 was administered to 79 patients with aNET[29]. While the patient group did not have lower scores compared to matched healthy controls, patients who underwent CRH (30 patients) had worse scores on social functioning, diarrhea, and financial difficulties compared to patients treated only with an appendectomy (49 patients). Additionally, no benefit in disease-free survival was observed after CRH.

FOLLOW-UP

There are no specific recommendations based on randomized trial data on follow-up after resection of an aNET and no adjuvant therapy is recommended after complete resection of a well-differentiated midgut NET. Several international guidelines include strategies for follow-up based on tumor size and the surgery performed[9,10,45].

Patients with tumors < 1 cm or from 1 to 2 cm without poor prognostic factors do not generally require further routine surveillance and tests should only be ordered if they are clinically indicated[9,45]. ENETS guidelines extend this recommendation to patients with tumors treated with a hemicolectomy without evidence of lymph node involvement[10].

Patients with tumors from 1 to 2 cm with poor prognostic factors who do not undergo a hemicolectomy or those with tumors > 2 cm should be followed-up on every three to six months in the first year after resection and then every six to 12 mo for at least seven years. Due to the slow growth pattern of these tumors, lifelong monitoring for potential recurrence must be provided[9,10].

In the case of candidates for surveillance, follow-up should consist of a medical history and physical examination. In addition, tumor markers (including 5-HIAA and CgA) and abdominal imaging by means of a CT or MRI scan should be considered. The role of a colonoscopy or transabdominal ultrasound has not been established in these patients[9,10,45] (Table 3).

Table 2 Summary of published data on appendiceal neuroendocrine tumor recurrence and survival

Author (Yr)	*n*	Recurrence rate	Specific disease survival rate	Reported follow-up
Tsikitis *et al*[38], 2012	982	-	95.6%	5-yr rate
Volante *et al*[17], 2013	138	-	97.1%	86.5 mo (1 - 267)
Mosquera *et al*[39], 2017	418	-	95.7%	5-yr rate
Sarchekeh *et al*[40], 2017	118	-	97.5%	10-yr rate
Pawa *et al*[41], 2017	215	0	99.05%	10-yr rate
Alexandraki *et al*[32], 2020	136	2.2%	100%	10-yr rate
Brighi *et al*[5], 2020	435	0%	98.5%	Median follow-up not provided, but at least 20% longer than 10 yr
Alabraba *et al*[1], 2021	102	1%	99%	6.2 yr (0.8-27.8)
Holmager *et al*[28], 2021	335	0%	100%	66 mo (1-250)

Table 3 Follow-up recommendations according to European Neuroendocrine Tumor Society and North American Neuroendocrine Tumor Society

Tumor characteristics	Surgery	Follow-up
Size < 1 cm	Appendectomy	No
Any size	Hemicolectomy (no lymph node involvement)	No (consider follow-up if tumor size > 2 cm)
Size 1-2 cm with poor prognostic factors	Appendectomy	History and physical examination every three to six months for the first year and then every six to 12 months
Any size	Hemicolectomy (lymph node involvement)	Consider tumor markers and abdominal imaging tests

SRS imaging is not routinely recommended for restaging in the initial follow-up after resection with curative intent and should be performed only for restaging at the time of clinical or laboratory progression without progression on conventional imaging tests[46].

TREATMENT IN ADVANCED DISEASE

The initial evaluation for patients with metastatic relapse or progression should include conventional imaging tests (CT or MRI scan), functional imaging tests with SRS-PET, and an assessment of carcinoid syndrome. All patients with advanced aNETs should be referred to a center with experience in neuroendocrine neoplasms and evaluated by a multidisciplinary tumor board.

Systemic treatment strategies for advanced or metastatic disease are similar to what is indicated for other midgut NETs. SSAs are usually the first-choice systemic therapy for symptomatic control in functional tumors. Antiproliferative activity has been demonstrated in two placebo-controlled randomized trials: The PROMID study with octreotide LAR 30 mg/28 d[47] and the CLARINET study with lanreotide autogel 120 mg/28 d[48].

Patients with a positive SRS functional imaging test are potential candidates for peptide receptor radionuclide therapy after progression on somatostatin analogs. Currently, lutetium 177 is approved for GEP-NETs in light of data from the NETTER-1 trial on midgut NETs[49].

Treatment with targeted therapies is based on the mTOR inhibitor everolimus. The RADIANT-4 trial confirmed the efficacy of everolimus in non-functioning NETs of gastrointestinal and pulmonary origin, including midgut NETs[50].

In addition, for patients who need regional control of liver metastasis due to carcinoid syndrome or for disease control in cases with liver-limited disease, locoregional therapies should be evaluated. Liver-directed therapies include ablative techniques such as percutaneous radiofrequency ablation or particle embolization with or without cytotoxic agents or radioactive microspheres. Chemotherapy is not routinely recommended and is reserved only for patients with progressive disease along with other strategies or for patients with rapidly progressing disease[51].

DISCUSSION

A hemicolectomy after the incidental finding of an aNET during an appendectomy is currently indicated depending on the stratified risk of relapse. There is consensus on not performing a hemicolectomy in tumors that measure < 1 cm, as these patients are considered cured and do not even require follow-up. On the other hand, a hemicolectomy should always be considered for tumors that measure > 2 cm.

Indications for tumors between 1-2 cm are where the controversy lies. Current guidelines give recommendations based on risk factors beyond tumor size: mesoappendix invasion, positive margins, vascular and lymph node involvement, and tumor grade. However, these recommendations are not supported with enough high-quality evidence.

A decision regarding a hemicolectomy in an aNET between 1 and 2 cm should be discussed by a multidisciplinary tumor board in expert centers. The opinions of pathologists, surgeons, gastroenterologists, endocrinologists, radiologists, medical oncologists, and nuclear medicine specialists should be taken into consideration before making a recommendation. Long-term issues related to a hemicolectomy should be discussed with the patient, particularly with those who are younger.

This narrative review aims to examine current evidence that is mainly based on clinical guidelines, providing the framework for a multidisciplinary tumor board discussion. The main limitation is a lack of prospective studies, an unmet need that should be addressed in the future. Indeed, at present, the SurvivApp study aims to analyze distant metastasis and long-term outcomes in patients after complete resection of an aNET. It aims to recruit 700 participants over ten years and divide them into two cohorts of retrospective and prospective cases. Its primary objectives are related to tumors measuring 1-2 cm and include clinically relevant relapse, clinically relevant mortality, and frequency of distant metastasis [52].

CONCLUSION

Decisions related to the indication of a hemicolectomy and follow-up should be made by a multidisciplinary tumor board at expert centers in order to offer each patient an individualized treatment approach. The factors that should be discussed include tumor size, mesoappendix invasion, positive margins, vascular and lymphatic vessel involvement, and tumor grade. Prospective studies regarding optimal treatment for aNETs are an unmet need in the NET field and should be addressed in the future.

FOOTNOTES

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Country/Territory of origin: Spain

ORCID number: José Luis Muñoz de Nova 0000-0003-1439-5632; Jorge Hernando 0000-0002-0929-7360; Miguel Sampedro Núñez 0000-0002-0089-4046; Greissy Tibisay Vázquez Benítez 0000-0002-9442-9064; Eva María Triviño Ibáñez 0000-0001-6604-6608; María Isabel del Olmo García 0000-0002-4278-2624; Jorge Barriuso 0000-0002-5641-9105; Jaume Capdevila 0000-0003-0718-8619; Elena Martín-Pérez 0000-0002-4933-738X.

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ORIGINAL ARTICLE

Basic Study

Jianpi Qingchang Bushen decoction improves inflammatory response and metabolic bone disorder in inflammatory bowel disease-induced bone loss

Ya-Li Zhang, Qian Chen, Lie Zheng, Zi-Wei Zhang, Yu-Jun Chen, Yan-Cheng Dai, Zhi-Peng Tang

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Ya-Li Zhang, Qian Chen, Zi-Wei Zhang, Yu-Jun Chen, Zhi-Peng Tang, Institute of Digestive Diseases, LongHua Hospital, Shanghai University of Traditional Chinese Medicine, Shanghai 200032, China

Lie Zheng, Department of Gastroenterology, Traditional Chinese Medicine Hospital of Shaanxi Province, Xi'an 710003, Shaanxi Province, China

Yan-Cheng Dai, Department of Gastroenterology, Shanghai Traditional Chinese Medicine-Integrated Hospital, Shanghai University of Traditional Chinese Medicine, Shanghai 200082, China

Corresponding author: Zhi-Peng Tang, MD, PhD, Chief Doctor, Professor, Institute of Digestive Diseases, LongHua Hospital, Shanghai University of Traditional Chinese Medicine, No. 725 South Wanping Road, Shanghai 200032, China. zhipengtang@sohu.com

Abstract

BACKGROUND

Bone loss and osteoporosis are commonly described as extra-intestinal manifestations of inflammatory bowel disease (IBD). Jianpi Qingchang Bushen decoction (JQBD) is a prescription used in clinical practice. However, further studies are needed to determine whether JQBD regulates the receptor activator of nuclear factor kappa B (NF-κB) (RANK)/receptor activator of NF-κB ligand (RANKL)/osteoprotegerin (OPG) pathways and could play a role in treating IBD-induced bone loss.

AIM

To evaluate the therapeutic effect of JQBD in IBD-induced bone loss and explore the underlying mechanisms.

METHODS

An IBD-induced bone loss model was constructed by feeding 12 6-to-8-wk-old interleukin-10 (IL-10)-knockout mice with piroxicam for 10 d. The mice were randomly divided into model and JQBD groups. We used wild-type mice as a control. The JQBD group was administered the JQBD suspension for 2 wk by gavage, while the control and model groups were given normal saline at the corresponding time points. All mice were killed after the intervention. The effect

of JQBD on body weight, disease activity index (DAI), and colon length was analyzed. Histopathological examination, colon ultrastructure observation, and micro-computed tomographic scanning of the lumbar vertebrae were performed. The gene expression of NF-κB, tumor necrosis factor-α (TNF-α), IL-1β, IL-6, and IL-8 in the colon was evaluated by real-time polymerase chain reaction. Colon samples were assessed by Western blot for the expression of RANKL, OPG, RANK, and NF-κB proteins.

RESULTS

The model group lost body weight, had a shorter colon, and showed a dramatic increase in DAI score, whereas JQBD had protective and therapeutic effects. Treatment with JQBD significantly improved inflammatory cell infiltration and reduced crypt abscess and ulcer formation. Three-dimensional imaging of the vertebral centrum in the model group revealed a lower bone mass, loose trabeculae, and "rod-shaped" changes in the structure compared to the control group and JQBD groups. The bone volume/total volume ratio and bone mineral density were significantly lower in the model group than in the control group. JQBD intervention downregulated the NF-κB, TNF-α, IL-1β, IL-6, and IL-8 mRNA expression levels. The RANKL and OPG protein levels were also improved.

CONCLUSION

JQBD reduces inflammation of the colonic mucosa and inhibits activation of the RANK/RANKL/OPG signaling pathway, thereby reducing osteoclast activation and bone resorption and improving bone metabolism.

Key Words: Inflammatory bowel disease; Osteoporosis; Jianpi Qingchang Bushen decoction; Inflammation; Bone metabolism

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Core Tip: Bone loss and osteoporosis are commonly described as extra-intestinal manifestations of inflammatory bowel disease (IBD). Jianpi Qingchang Bushen decoction (JQBD) is a prescription used in clinical practice. This study aimed to evaluate the therapeutic effect of JQBD in IBD-induced bone loss and explore the underlying mechanisms. An IBD-induced bone loss model was constructed by feeding interleukin-10-knockout mice with piroxicam. JQBD reduced inflammation of the colonic mucosa and inhibited activation of the receptor activator of nuclear factor kappa B (NF-κB)/receptor activator of NF-κB ligand/osteoprotegerin signaling pathway, thereby reducing osteoclast activation and bone resorption and improving bone metabolism.

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INTRODUCTION

Inflammatory bowel disease (IBD) is a chronic, relapsing immune-mediated inflammatory disease of the gastrointestinal tract, which includes Crohn's disease (CD) and ulcerative colitis (UC)[1,2]. Its main clinical manifestations are diarrhea, abdominal pain, bloody stool, and various degrees of systemic symptoms[3,4]. Nearly 50% of IBD patients show at least one kind of extra-intestinal symptom during their lifetime, among which musculoskeletal damage is the most common[5-7]. In recent years, increasing attention has been paid to the study of osteopenia and osteoporosis caused by IBD. Osteoporosis is a systemic bone disease characterized by low bone mass and the destruction of bone microstructure. It increases bone fragility, making it prone to fractures. Osteoporosis is one of the more common extraintestinal manifestations in IBD patients[8]. Studies have shown that the risk of osteoporosis is significantly higher in IBD patients than in healthy controls[9,10], exposing them to a higher risk of fracture. The risk of fracture in IBD patients is 40% higher than that in healthy controls, seriously affecting their quality of life[11]. The search for a suitable treatment to reduce pain and the economic burden has become a priority of international medical and social establishments.

The pathogenesis of osteoporosis in IBD patients has not been fully elucidated. Many factors affect bone metabolism. Recent studies have shown that intestinal inflammation is one of the most important factors leading to osteoporosis in IBD patients. Inflammation is involved in the pathophysiological process of bone loss in patients with IBD[12-14]. Therefore, controlling intestinal inflammation could help to prevent the occurrence and progression of osteoporosis. The receptor activator of nuclear factor kappa B (NF-κB; RANK), its ligand (receptor activator of NF-κB ligand, RANKL), and the soluble decoy receptor osteoprotegerin (OPG) orchestrate resorption of osteoclastic bone and play a key role in the common pathogenic pathway between gut inflammation and bone loss[15]. RANK/RANKL/OPG signaling is a key regulator of osteoclast biology and bone metabolism and an important pathway regulating osteoclast differentiation[16-18]. Stanisławowski *et al*[19] reported that patients with UC have higher local expression of serum OPG mRNA and protein than healthy participants[19]. Krela-Kaź mierczak *et al*[20] reported an increase in the OPG/RANKL ratio in CD patients[20]. According to a recent study, elevated osteocyte tumor necrosis factor (TNF)-α, interleukin (IL)-6, RANKL, and OPG correspond to higher osteoclast surfaces and a lower bone formation rate in an IBD model induced with dextran sodium sulfate[21]. No effective preventive or treatment modality exists for osteoporosis in IBD patients. A great deal of interest has been generated to evaluate the mechanism by which natural products exert their beneficial effects in the gastrointestinal tract[22]. Traditional Chinese medicine (TCM) has good effects on IBD patients with osteoporosis. Spleen and kidney insufficiencies and dampness-heat are important components of IBD-associated osteoporosis pathogenesis. Treatment is aimed at invigorating the spleen, clearing the intestine, and tonifying the kidney. Thus, Jianpi Qingchang Bushen decoction (JQBD) was established under the guidance of TCM theory. JQBD is a prescription developed by our team to treat IBD patients with osteoporosis. Our previous study reported that JQBD-medicated serum promotes osteoclast apoptosis by downregulating Bcl-2 and upregulating the expression of the Bax protein[23]. According to TCM theory, the kidney stores the essence and this can be transformed into the bone marrow to nourish the bones and strengthen the skeleton. Thus, a kidney deficiency can cause osteoporosis. Tonifying the kidneys regulates bone metabolism to alleviate osteoporosis. Modern pharmacological studies have confirmed that *Psoralea corylifolia* Linn, *Alpinia oxyphylla* Miq, and their extracts improve bone metabolism to prevent bone loss and are commonly used to treat osteoporosis[24-27].

In this study, the potential pharmacodynamic mechanism of JQBD for treating IBD-induced osteoporosis was studied *in vivo* for the first time. An IBD-induced osteoporosis model was constructed by treating interleukin (IL)-10-knockout mice with piroxicam. JQBD was given as an intervention, and its effects on the inflammatory response and bone metabolism were observed to provide a theoretical basis for the clinical prevention and treatment of osteoporosis in IBD patients.

MATERIALS AND METHODS

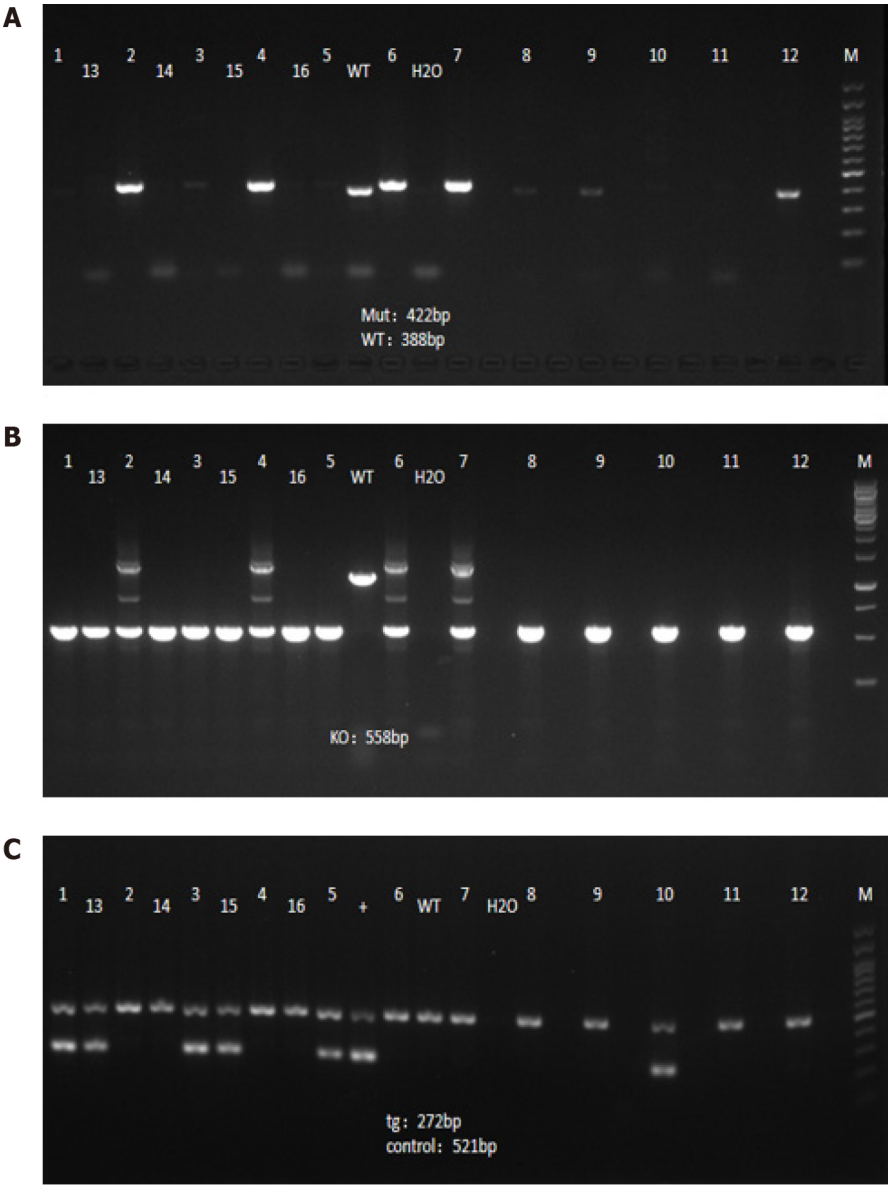
TCM preparation and identification of the phytochemicals

JQBD was prepared by the TCM pharmacy of Longhua Hospital, Shanghai University of TCM. It was composed of eight herbal species, including *Astragalus mongholicus* Bunge 30 g, *Codonopsis pilosula* (Franch.) Nannf. 15 g, *Alpinia oxyphylla* Miq. 12 g, *Cullen corylifolium* (L.) Medik. 9 g, *Portulaca oleracea* L. 30 g, *Sanguisorba officinalis* L. 15 g, *Aucklandia costus* Falc. 6 g, and *Glycyrrhiza glabra* L. 6 g. The components were soaked, boiled twice, filtered, concentrated, freeze-dried into a powder, and stored at −20 °C. Ultra-performance liquid chromatography quadrupole time-of-flight mass spectrometry (UPLC/Q-TOF/MS) was used to analyze the samples and identify 44 phytochemicals in JQBD. The MS chromatograms of the JQBD negative and positive ion modes are shown in Supplementary Figure 1. The chemical information of the identified phytochemicals is shown in Supplementary Table 1. Quality control measures were performed according to the guidelines of the Chinese State Food and Drug Administration.

Animal model and drug intervention

Six IL-10-knockout C57BL/6 mice were produced by the Shanghai Nanfang Research Center for Model Organisms (SNRCMO; Shanghai, China) and were maintained and bred in a specific-pathogen-free animal room at 23 ± 3 °C, 35%–45% relative humidity, and a 12 h/12 h light/dark regime at the Laboratory Animal Center of the Shanghai University of Traditional Chinese Medicine. Food and water were provided *ad libitum*. The Animal Ethics Committee of the Shanghai University of Traditional Chinese Medicine approved this study (PZSHUTCM191108004).

The IL-10-knockout mice were bred, and 70 F6-generation mice underwent gene detection using tail tip samples. IL-10 knockout was detected in 30 of these mice. We used wild-type mice as controls, which were the progeny of IL-10 +/- mating and bred at the same facility. The gene detection results are shown in Figure 1. The IBD-induced osteoporosis model was constructed by feeding the IL-10-knockout mice 200 ppm piroxicam for 10 d[28]. The mice were randomly divided into IL-10-knockout controls (model group) and IL-10-knockout mice with JQBD intervention (JQBD group). Six mice were included in each group (three males and three females). The JQBD group was administered 16.5 g/kg/d of the



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Figure 1 Gene detection results in F6-generation mice. A: Interleukin (IL)-10 conditional knockout; B: IL-10 knockout; C: Dppa3-cre.

JQBD suspension by gavage, while the control and model groups were given the same volumes of normal saline at the corresponding time points. The intervention was conducted for 14 consecutive days. All mice were killed after the intervention, and blood samples and colon and bone tissues were harvested.

Daily record of observational parameters

The mental state, coat color, activity, body weight, diet, fecal traits, occult blood, and gross bloody stool were observed and recorded daily from the beginning of modeling. The disease activity index (DAI) score was used to evaluate colitis severity in the mice daily following the grading scheme presented in Table 1[29].

Measurement of colon length and colon histopathological examination

Dead mice were dissected to isolate the colon. The colons were placed on filter paper, and their length was measured with a ruler.

The colon samples were fixed in 4% paraformaldehyde solution, dehydrated, paraffin-embedded, sliced, and stained with hematoxylin and eosin (HE).

Ultrastructural observation of the colon

Part of each colon was fixed in 2.5% glutaraldehyde within 1 min, dehydrated, embedded, sliced, and stained with 3% uranium acetate and lead citrate. The colon ultrastructure was observed by

Table 1 Calculation of disease activity index score

Score	Weight loss	Stool consistency	Bleeding
0	Normal	Normal (well-formed pellets)	Not observed
1	1%-5%	Normal (well-formed pellets)	Not observed
2	6%-10%	Loose (pasty stools that do not stick to the anus)	Occult
3	11%-15%	Loose (pasty stools that do not stick to the anus)	Occult
4	> 15%	Diarrhea (liquid stools that stick to the anus)	Gross bleeding

transmission electron microscopy (TEM).

Specimen preparation and micro-computed tomography

After the mice were killed, the intact lumbar vertebrae were separated, and the residual soft tissue was removed. The bone tissue was fixed in a 75% ethanol solution pending the experiment. All specimen assessments were performed on the lumbar vertebra by μ-CT80 micro-computed tomography (CT) (SCANCO Medical AG, Bassersdorf, Switzerland). The scanning resolution was 18 μm *per* layer, followed by three-dimensional (3D) reconstruction. The morphometric analysis included bone volume/total volume (BV/TV) ratio, trabecular number (Tb.N), trabecular thickness (Tb.Th), trabecular separation (Tb.Sp), connectivity-density (Conn-Dens), and bone mineral density (BMD).

Real-time polymerase chain reaction detection of NF-κB, TNF-α, IL-1β, IL-6, and IL-8 mRNA expression levels

Total RNA was extracted from the colon tissues with TRIzol, and the RNA concentration was determined. cDNA was reverse transcribed following the Takara kit instructions and was detected on the ABI StepOne Plus real-time fluorescence quantitative polymerase chain reaction (RT-qPCR) instrument. The primers were designed and synthesized by Shanghai ShineGene Molecular Biotechnology Co., Ltd. (Shanghai, China) (Table 2) and verified using the BLAST program. A 20-μL RT-qPCR reaction system was configured, and the reaction amplification was carried out following the kit instructions. β-actin was used as the internal reference, fold-change was estimated by the $2^{-\Delta\Delta Ct}$ method, and the mRNA expression level was compared between the groups. The assay was repeated three times.

Detecting the effect of JQBD on RANKL, OPG, RANK, and NF-κB protein expression in colon tissue by Western blot

We added 1 mL of RIPA Lysis Buffer and 10 μL of a protease inhibitor mixture to a homogenization tube and precooled it on ice; 50 μg of colon tissue was weighed and homogenized repeatedly in the tubes six times until no obvious tissue was seen in the homogenate. The homogenate was centrifuged (Eppendorf 5417R, Eppendorf) at 4 °C and 15000 × *g* for 15 min, and the supernatant was placed in another centrifugation tube. A small volume of the supernatant was taken, and the protein concentration was determined by the bicinchoninic acid method. Samples of 30 μg were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to a membrane, and sealed. TBST-diluted RANKL, OPG, RANK, and NF-κB antibodies (1:1000) were added, and the membrane was incubated overnight at 4 °C. After the incubation, the membrane was washed and incubated for 1 h with gentle shaking at room temperature with fluorescence-labeled secondary antibody diluted 1:3000. TBST was used to rinse the membranes. An enhanced chemiluminescence kit (reagents A and B were mixed at equal volumes) was placed on the surface of the polyvinylidene fluoride (PVDF) membrane and incubated in the dark at room temperature for 1 min. The PVDF membrane was placed on the operating table of a Western blot imager, and a strip image was acquired. The gray values of the bands were analyzed using ImageJ analysis software.

Statistical analysis

Statistical analyses were performed using GraphPad Prism, version 8.0 software (GraphPad Software Inc., La Jolla, CA, United States). Data are expressed as the mean ± SD. Differences between groups were assessed by one-way analysis of variance. A P value < 0.05 was considered significant.

RESULTS

General condition of the mice

The control mice showed a flexible response, agile movement, glossy fur, pink paws, active foraging, normal water intake, and mostly globular feces that were negative for occult blood. The mice in the

Table 2 List of primers used in this study

Gene	Primers(5'-3')	Primer length
NF-κB	Forward GTGGAGGCATGTTCGGTAGTG	195
	Reverse TCTTGGCACAATCTTTAGGGC	
TNF-α	Forward CCCTCCAGAAAAGACACCATG	183
	Reverse CACCCCGAAGTTCAGTAGACAG	
IL-1β	Forward GCTTCAGGCAGGCAGTATCA	196
	Reverse TGCAGTTGTCTAATGGGAACG	
IL-6	Forward AAATGATGGATGCTACCAAACTG	137
	Reverse CTCTGGCTTTGTCTTTCTTGTTATC	
IL-8	Forward TAGTGTTGAGCATGAAAAGCCTC	152
	Reverse TGACTTCACTGGAGTCCCGTAG	

NF-κB: Nuclear factor-kappaB; TNF-α: Tumor necrosis factor-α; IL: Interleukin.

model group were lazy, with a reduced appetite, dirty and sparsely erected fur, white paws, soft feces, and perianal body hair stained with loose stools. Some had bloody stools, occasional anal peeling, perianal blood, feces strongly positive for occult blood, and decreased body weight. The JQBD group mice displayed a better mental status and they had glossy hair, only slightly white paws, feces not as soft, only occasional bloody stools, lower positive occult blood rate, and less lost weight than the model group (Figure 2).

Weight change and DAI scores

The weights of the mice in the model and JQBD groups on day 10 of the experiment were significantly lower ($P < 0.05$) than that of the control group. The weight in the JQBD group was higher than that in the model group ($P < 0.05$). The weight in all groups on day 14 was higher than that at the start of the experiment, but the weight increase in the model and JQBD groups was less evident than that in the control group ($P < 0.05$). The weight of the model group was significantly lower than that of the other two groups ($P < 0.05$). Higher DAI scores were observed in the model and JQBD groups. The DAI score of mice in the model group was higher than that of mice in the control group ($P < 0.05$), which decreased significantly in response to JQBD treatment on day 14 ($P < 0.05$) (Figure 3).

Morphological changes in the colonic mucosa

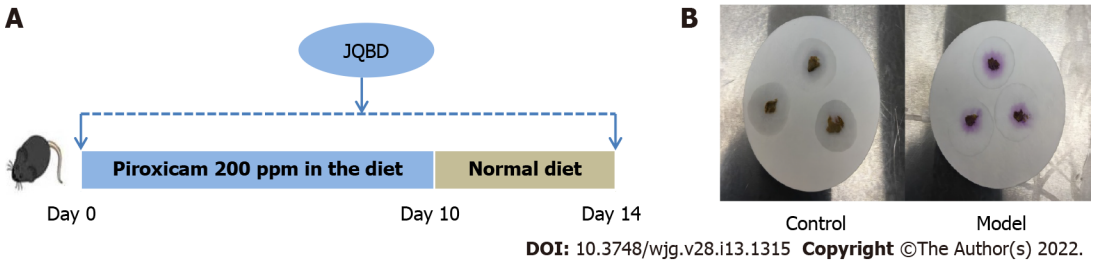
The colon wall in the control group was smooth, red, and resilient. In the model group, it was thickened, stiff, hyperemic, edematous, and erosive, with some irregular ulcers and a brittle texture. The colonic mucosa hyperemia and edema in the JQBD group were less severe than those in the model group with significantly reduced ulcer formation. The colon in the model group was significantly shorter than that in the control ($P < 0.001$) and the JQBD ($P < 0.05$) groups.

Histological evaluation of the colonic mucosa

The colonic mucosal epithelium in the control group was intact and continuous, the glands were orderly arranged, the goblet cells were visible, the blood vessels and fibrous interstitium in the lamina propria and muscle layer were normal, and inflammatory cell infiltration was rarely observed. Necrosis, erosion, disordered gland arrangement, different degrees of inflammatory cell infiltration, a reduced number of goblet cells, and ulcer formation were observed in the colonic mucosa of mice in the model group, suggesting successful IBD modeling in these mice. JQBD provided partial protection to the mucosa, so it was less damaged, showing mild to moderate hyperemia and edema. Lower inflammatory cell infiltration and ulcer formation rates were noted, and the glands were arranged in an orderly manner (Figure 4).

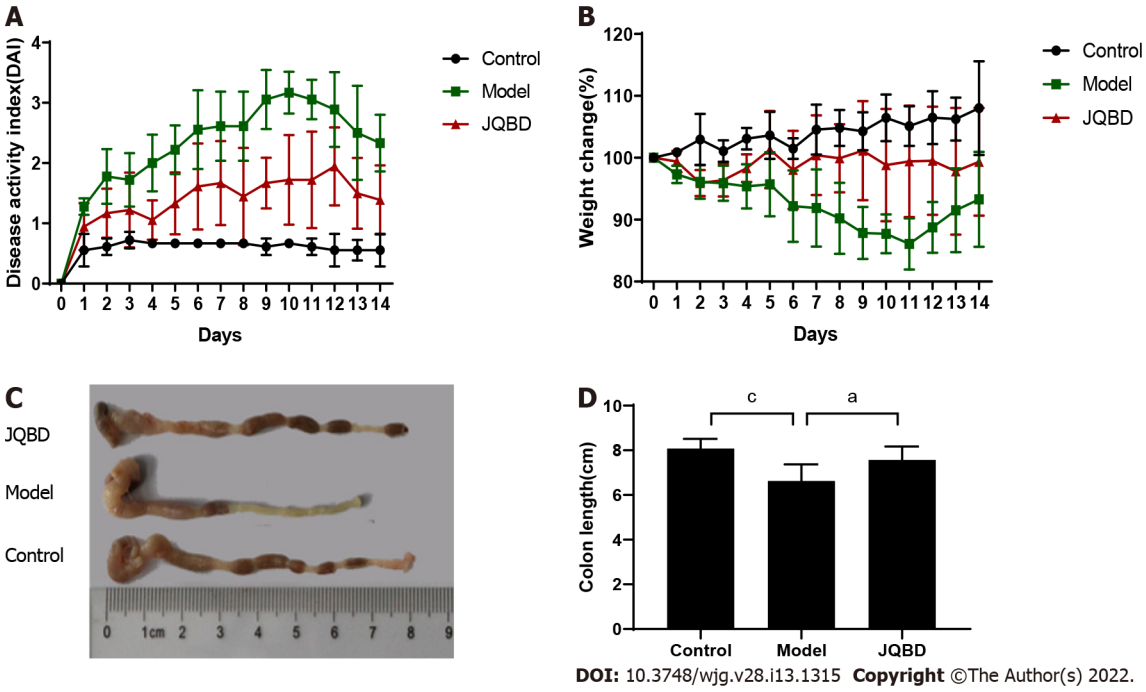
Bone metabolic parameters on micro-computed tomography

The 3D imaging of the vertebral centrum in the model group revealed a lower bone mass, loose trabeculae, and "rod-shaped" changes in the structure compared to the control group. Treatment with JQBD resulted in a higher bone mass, tighter trabecular bone gaps, and higher connectivity than those in the model group. The quantitative micro-CT results showed that the BV/TV ratio and BMD decreased in the model group. The Conn-Dens and Tb.Th were similar in the normal and model groups. Although BV/TV and BMD were significantly lower in the model group than in the control group, these values



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Figure 2 Animal experimental flow and fecal occult blood test. A: An experimental bone loss inflammatory bowel disease model was induced by peroral administration of piroxicam for 10 d in interleukin-10$^{-/-}$ mice. Normal saline or Jianpi Qingchang Bushen decoction (JQBD; 16.5 g/kg/d) was given intragastrically to the control/model groups and JQBD group, respectively (n = 6, each); B: Fecal occult blood test of control and model groups: The control group was negative, and the model group was strongly positive for occult blood. JQBD: Jianpi Qingchang Bushen decoction Group.



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Figure 3 General condition of the mice. A: Disease activity index scores gauged daily (n = 6 *per* group); B: Body weight measured daily (n = 6 *per* group); C and D: Colon length measurement and graph presenting the statistical analysis results. Data are presented as the mean ± SD. $^{a}P < 0.05$; $^{b}P < 0.01$; $^{c}P < 0.001$ (n = 6 *per* group).

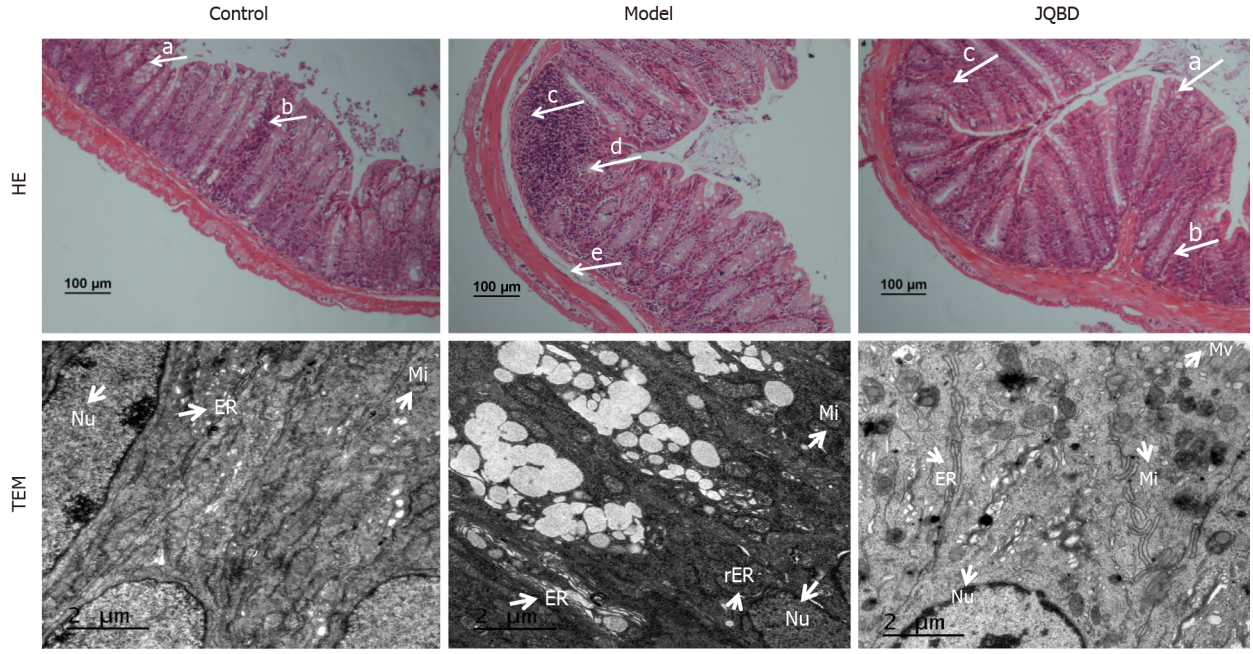
were similar in the JQBD, control, and model groups (Figure 5).

Structural changes in the colonic epithelial cells as observed by TEM

The TEM images illustrated intact colon cells in the control group, with no increase in intercellular space. The endoplasmic reticulum had a reticular structure and was bundled, the cavity was not expanded, many ribosomes were attached to it, and numerous mitochondria were observed. In the model group, the organelles were significantly swollen and the endoplasmic reticulum cavity was significantly expanded, attaining different shapes and sizes, with many vacuoles. Some of the organelles fused into clusters. A microvillar structure was partially observed in the JQBD group. The mitochondria were slightly swollen, the number of epithelial cells increased, and the cell structure was more intact than in the model group. The endoplasmic reticulum cavity was slightly expanded (Figure 4).

Effect of JQBD on NF-κB, TNF-α, IL-1β, IL-6, and IL-8 gene expression

The expression of the five genes was verified by RT-qPCR. The mRNA expression levels of NF-κB, TNF-α, IL-1β, IL-6, and IL-8 were significantly upregulated in the colon of the model group compared to the control group. JQBD intervention downregulated the mRNA expression levels of these genes, suggesting that JQBD improved the mucosal inflammatory response (Figure 6).



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Figure 4 Histological evaluation of the colonic mucosa following hematoxylin and eosin staining (× 100) and ultrastructure of the colonic epithelium by transmission electron microscopy (× 6000). Arrows indicate goblet cells (a), crypts (b), inflammatory cells infiltration (c), epithelium surface erosion (d), and submucosal oedema (e). Control: Control group; Model: Model group; JQBD: Jianpi Qingchang Bushen decoction Group; Nu: Nucleus; Mi: Mitochondrial; ER: Endoplasmic Reticulum; rER: Rough endoplasmic reticulum; Mv: Microvillus.

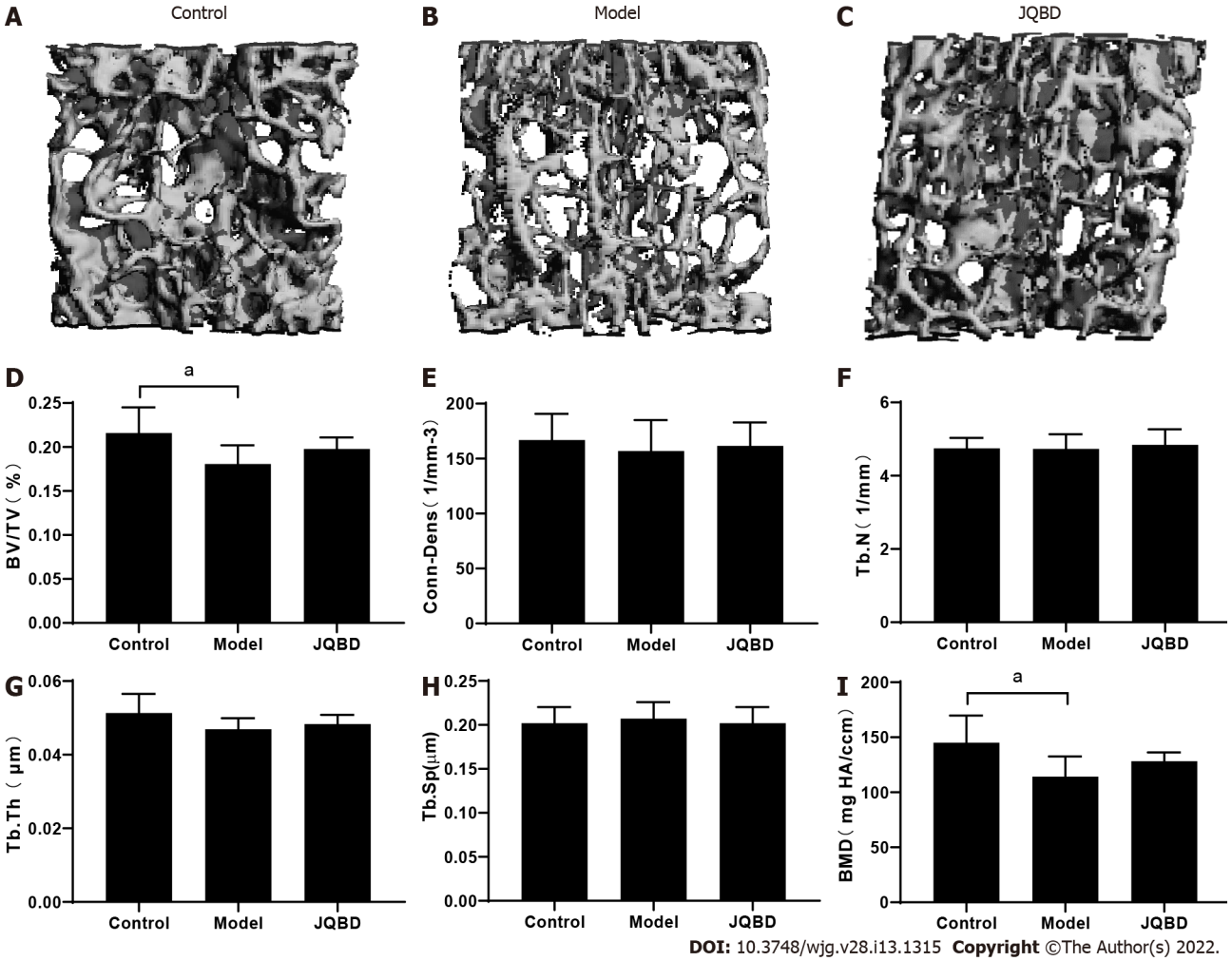
Effect of JQBD on RANKL, OPG, RANK, and NF-κB protein expression

The RANKL, OPG, RANK, and NF-κB protein levels were measured by Western blot. RANKL and OPG protein expression was significantly upregulated in the model group colon and was markedly downregulated following JQBD intervention. Although the RANK and NF-κB protein expression levels were significantly higher in the model group than in the control group, they were similar between the model and JQBD groups. Taken together, these data indicate that JQBD improved the mucosal inflammatory response and restrained the RANK/RANKL/OPG signaling pathway during the development of bone loss in IBD (Figure 7).

DISCUSSION

IBD is a chronic, non-specific, intestinal inflammatory disease with unclear etiology and common digestive system clinical presentation. The disease is protracted and difficult to cure, and the symptoms easily recur. IBD affects multiple systems, including the bones and calcium deposits. Studies have shown that IBD patients have a lower bone mass than healthy people[30-33]. The risk of osteoporosis and subsequent fractures is significantly higher in IBD patients[34,35], and it is the main cause of mobility problems and decreased quality of life in these patients[36]. Osteoporosis is a common and easily overlooked extra-intestinal manifestation in IBD patients. Early diagnosis and prevention of osteoporosis in IBD patients are very important. Therefore, we explored the possible pathogenesis of IBD-induced osteoporosis and evaluated the effectiveness of a promising drug in the present study. We propose that JQBD may have a protective effect against IBD-induced osteoporosis through the RANK/RANKL/OPG signaling pathway.

We established a bone loss IBD model in IL-10-knockout mice by peroral administration of piroxicam. IL-10-knockout mice are commonly used as an animal model to study IBD[37,38]. When exposed to non-steroidal anti-inflammatory drugs, such as piroxicam, these animals rapidly develop colitis that persists for a long time[39,40]. Holgersen *et al*[15] reported that they orally fed IL-10 knockout mice piroxicam for 12 d before the bone loss and trabecular bone structural changes occurred[15]. We report similar results in IL-10-knockout mice following 10 d of oral piroxicam administration. Our micro-CT data show that the BV/TV ratio and BMD were significantly lower in the model mice than in the controls. The 3D simulation map suggested that bone mass was significantly lower in the model group than in the control group and that the trabecular bone structure was lost, with visible fractures and destruction of the bone microstructure. The model group exhibited bone loss and significant colitis, similar to another report[41]. Our results preliminarily confirm the abnormal bone metabolism in the model group, suggesting successful modeling.

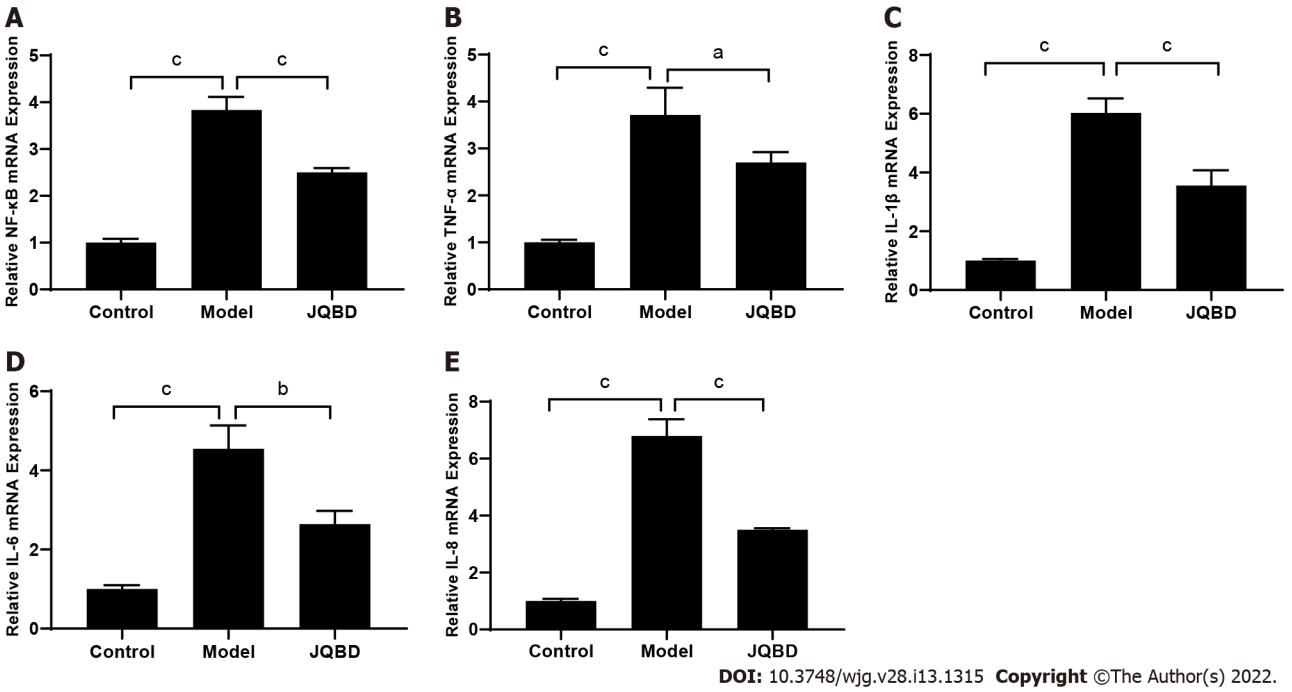


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Figure 5 Three-dimensional reconstruction of the lumbar spine trabecular structure in mice and micro-computed tomographic analyses of the lumbar vertebral metaphysis. Bone volume to total volume (BV/TV) ratio, Conn-Dens, trabecular number (Tb.N), trabecular thickness (Tb.Th), trabecular separation (Tb.Sp), and bone mineral density (BMD) were obtained for all mice. A: Control; B: Model; C: JQBD; D: BV/TV; E: Conn-Dens; F: Trabecular number; G: Trabecular thickness; H: Trabecular separation; I: Bone mineral density. Control: Control group; Model: Model group; JQBD: Jianpi Qingchang Bushen decoction Group; BV/TV: Bone volume to total volume ratio; Conn-Dens: Connectivity-density; Tb.N: Trabecular number; Tb.Th: Trabecular thickness; Tb.Sp: Trabecular separation; BMD: Bone mineral density. Data are presented as the mean ± SD. $^{a}P < 0.05$. ($n = 6$ *per* group).

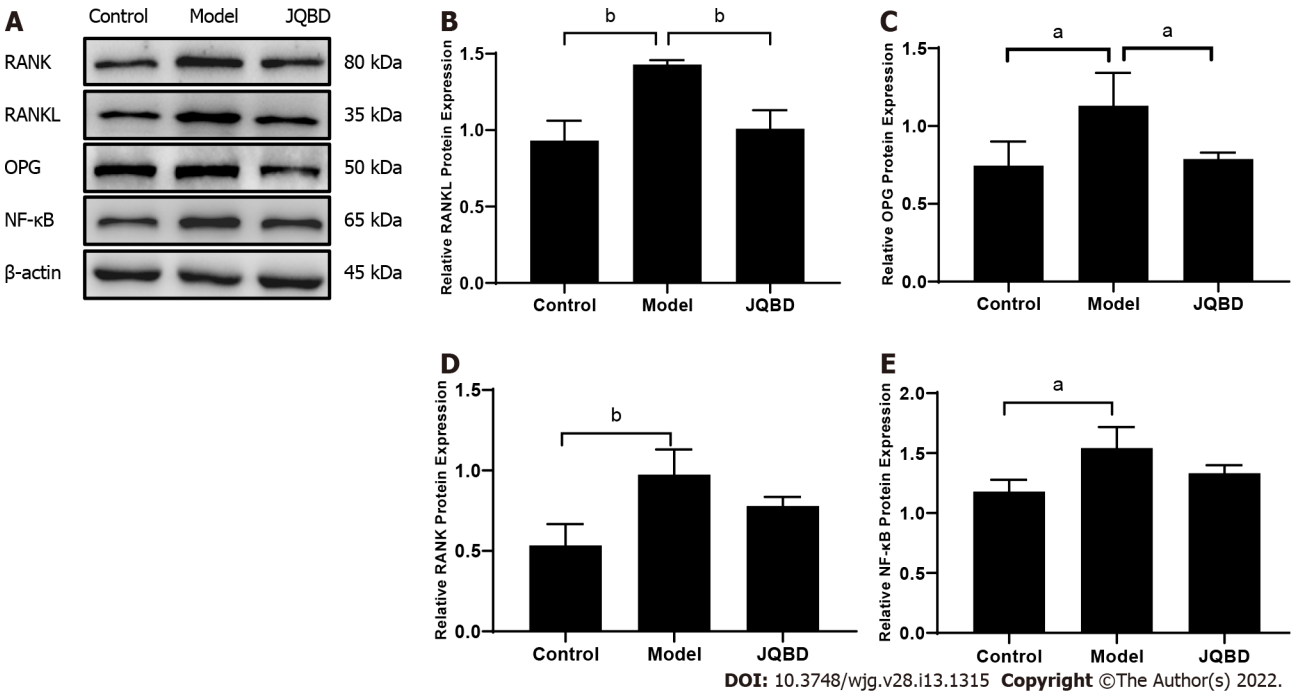
Our data show that the JQBD was protective against IBD-induced bone loss by inhibiting inflammation. Evidence shows that intestinal inflammation is involved in the pathophysiology of osteoporosis in IBD[42-44]. Expression of NF-κB, TNF-α, IL-1β, IL-6, and IL-8 mRNA in colonic tissue from the model group increased significantly, confirming that pro-inflammatory cytokines are involved in the occurrence of experimental colitis in piroxicam-induced IL-10-knockout mice with IBD. The administration of the JQBD reduced the levels of pro-inflammatory cytokines and decreased colonic inflammation. Several studies have evaluated the pathophysiology of IBD and its association with osteoporosis. Bone loss was initially attributed to the use of drugs, such as corticosteroids. However, it was later found that BMD decreases in IBD patients even without drug treatment, suggesting that IBD might be involved in the pathophysiology of bone loss, and chronic inflammation is the primary determinant[45-47].

We evaluated the curative effects of JQBD on the bone-loss IBD model mice and confirmed that the RANK/RANKL/OPG signaling pathway was involved in the process. The RANKL, OPG, RANK, and NF-κB protein levels increased significantly in colon tissue from the model group. Their expression levels were downregulated after JQBD intervention, suggesting that JQBD inhibits activation of the RANK/RANKL/OPG signaling pathway. Several pro-inflammatory cytokines, including IL-1β, TNF-α, IL-6, and IL-8 increase in IBD and have been shown to stimulate osteoclast differentiation[48]. Studies have suggested that the abnormal intestinal tract immune response in IBD leads to injury and inflammation of the intestinal mucosa, activation of the RANK /RANKL/OPG signaling pathway, changes in the bone conversion rate, osteoclast activation, and bone loss, leading to IBD-induced osteoporosis[21,49,50]. These results highlight the interactions between inflammation and the RANK/RANKL/OPG signaling pathway during the development of osteoporosis in IBD. JQBD reduced inflammation of the colonic mucosa and inhibited activation of the RANK/RANKL/OPG signaling pathway (Figure 8).



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Figure 6 Effect of Jianpi Qingchang Bushen decoction on nuclear factor-kappaB, tumor necrosis factor-α, interleukin-1β, interleukin-6, and interleukin-8 gene expression. The relative expression of these genes was quantified by real-time fluorescence quantitative polymerase chain reaction. A: NF-κB; B: TNF-α; C: IL-1β; D: IL-6; E: IL-8. Control: Control group; Model: Model group; JQBD: Jianpi Qingchang Bushen decoction Group; NF-κB: Nuclear factor-kappaB; TNF-α: Tumor necrosis factor-α; IL: Interleukin. Data are presented as the mean ± SD. $^{a}P < 0.05$; $^{b}P < 0.01$; $^{c}P < 0.001$ ($n = 3$ *per* group).

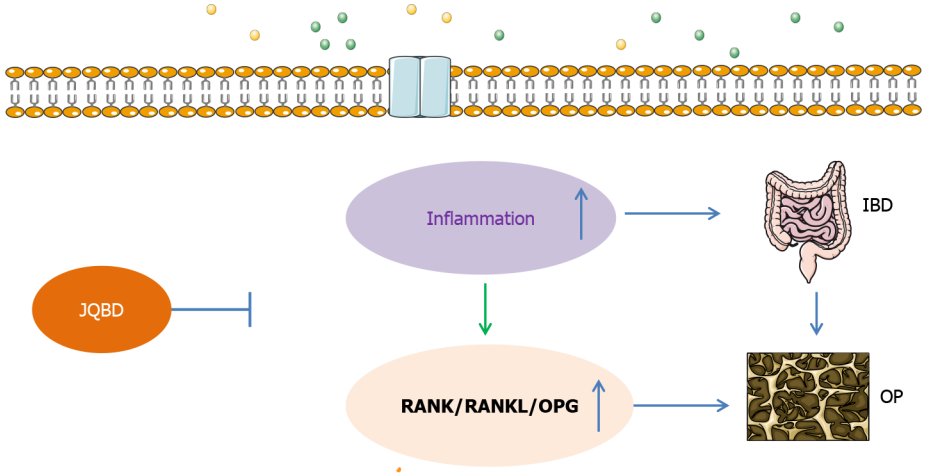


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Figure 7 Effect of Jianpi Qingchang Bushen decoction on receptor activator of nuclear factor κB ligand, osteoprotegerin, receptor activator of nuclear factor kappa B, and nuclear factor-kappaB protein expression. A: Protein expression quantified by Western blot; B: RANKL; C: OPG; D: RANK; E: NF-κB. Control: Control group; Model: Model group; RANK: Receptor activator of nuclear factor kappa B; RANKL: Receptor activator of nuclear factor κB ligand; JQBD: Jianpi Qingchang Bushen decoction Group. Data are presented as the mean ± SD. $^{a}P < 0.05$; $^{b}P < 0.01$; $^{c}P < 0.001$ ($n = 3$ *per* group).

Taken together, our present study data demonstrate that the JQBD improved IBD-induced osteoporosis *via* the RANK/RANKL/OPG signaling pathway.

This study primarily focused on the mechanism by which JQBD regulates the RANK/RANKL/OPG signaling pathway and improves IBD-induced osteoporosis. Our investigation has only preliminarily discussed the topic at the animal level and had several limitations. First, we considered using future



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Figure 8 Possible mechanisms of bone loss in inflammatory bowel disease. IBD: Inflammatory bowel disease; OPG: Osteoprotegerin; RANK: Receptor activator of nuclear factor kappa B; RANKL: Receptor activator of nuclear factor κB ligand; JQBD: Jianpi Qingchang Bushen decoction Group.

emerging methods, such as systems pharmacology, to systematically explore the potential efficacy of JQBD in the treatment of IBD-induced osteoporosis. Such a study would include related experimental verification at the cell and animal levels. Second, the basic mechanism behind IBD-induced osteoporosis needs to be further explored. We will perform long-term observations in future experiments by extending the experimental period to 1 or 2 mo. We will also investigate the activation of the RANK/RANKL/OPG signaling pathway in bone tissues. Third, changes in intestinal microecology play an important role in the healing of the mucosa. Some studies have reported that an increase in the intestinal microbial bifidobacteria promotes healing of the colonic mucosa, increases the IL-10 level, and reduces the IL-6 and TNF-α levels[51,52]. The intestinal flora was not determined in this study. Thus, whether JQBD regulates intestinal microecology is worthy of further study.

CONCLUSION

JQBD plays a role in treating IBD-related bone metabolic abnormalities by inhibiting colonic mucosal inflammation, promoting mucosal healing, and inhibiting activation of the RANK/RANKL/OPG signaling pathway, osteoclast formation, and bone resorption.

ARTICLE HIGHLIGHTS

Research background

In recent years, increasing attention has been paid to the study of osteopenia and osteoporosis caused by inflammatory bowel disease (IBD). Osteoporosis is one of the more common extra-intestinal manifestations in IBD patients. No effective preventive or treatment modality exists for osteoporosis in IBD patients. Therefore, we explored the possible pathogenesis of IBD-induced osteoporosis and evaluated the effectiveness of a promising drug in the present study.

Research motivation

Jianpi Qingchang Bushen decoction (JQBD) is a prescription developed by our team to treat IBD patients with osteoporosis. In this study, the potential pharmacodynamic mechanism of JQBD for treating IBD-induced osteoporosis was studied *in vivo*.

Research objectives

The effects of JQBD on the inflammatory response and bone metabolism were observed to provide a theoretical basis for the clinical prevention and treatment of osteoporosis in IBD patients.

Research methods

An IBD-induced osteoporosis model was constructed by treating interleukin-10-knockout mice with piroxicam. JQBD was given as an intervention, and its effect on the inflammatory response and bone metabolism was observed.

Research results

Our data show that JQBD was protective against IBD-induced bone loss by inhibiting inflammation. The receptor activator of nuclear factor kappa B (NF-κB) ligand (RANKL), osteoprotegerin (OPG), receptor activator of NF-κB (RANK), and NF-κB protein levels increased significantly in colon tissue from the model group. Their expression levels were downregulated after JQBD intervention.

Research conclusions

We evaluated the curative effects of JQBD on the bone-loss IBD model mice and confirmed that the RANK/RANKL/OPG signaling pathway is involved in the process.

Research perspectives

This study primarily focused on the mechanism by which JQBD regulates the RANK/RANKL/OPG signaling pathway and improves IBD-induced osteoporosis.

FOOTNOTES

Author contributions: Zhang YL and Chen Q contributed equally to this work, and both performed the majority of research; Dai YC, Zheng L, Zhang ZW, and Chen YJ performed the research and analyzed the data; Tang ZP designed and coordinated the research; Zhang YL and Chen Q wrote and revised the paper; all authors read and approved the final manuscript.

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Institutional review board statement: The study was reviewed and approved by the Animal Ethics Committee of the Shanghai University of Traditional Chinese Medicine, No. PZSHUTCM191108004.

Conflict-of-interest statement: The authors declare that there are no conflicts of interest related to this study.

Data sharing statement: The datasets generated during and/or analyzed during the current study will be available upon request from the principle investigator. The shared data will only be allowed to be used by the applicant for scientific studies. No commercial activities are allowed.

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

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Country/Territory of origin: China

ORCID number: Ya-Li Zhang 0000-0002-8987-3558; Qian Chen 0000-0002-1598-2735; Lie Zheng 0000-0002-0918-0728; Zi-Wei Zhang 0000-0001-6557-4062; Yu-Jun Chen 0000-0001-9731-1400; Yan-Cheng Dai 0000-0002-3571-077X; Zhi-Peng Tang 0000-0001-5695-8072.

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ORIGINAL ARTICLE

Basic Study

Comparison of the performance of MS enteroscope series and Japanese double- and single-balloon enteroscopes

Jin-Hua Liu, Dan-Yang Liu, Yong-Feng Yuan, Xue-Jun Sun, Shu-Mei Shan

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Jin-Hua Liu, Xue-Jun Sun, Department of General Surgery, The First Affiliated Hospital of Xi'an Jiaotong University, Xi'an 710061, Shaanxi Province, China

Jin-Hua Liu, Department of General Surgery, Affiliated Dalian Municipal Friendship Hospital of Dalian Medical University, Dalian 116001, Liaoning Province, China

Dan-Yang Liu, Department of Endocrinology, Affiliated Dalian Municipal Friendship Hospital of Dalian Medical University, Dalian 116001, Liaoning Province, China

Yong-Feng Yuan, College of Computer Science and Technology, Harbin Institute of Technology, Harbin 150001, Heilongjiang Province, China

Shu-Mei Shan, General Manager Office, Dalian Ming Sheng Technology Development Co., Ltd., Dalian 116001, Liaoning Province, China

Corresponding author: Xue-Jun Sun, PhD, Full Professor, Department of General Surgery, The First Affiliated Hospital of Xi'an Jiaotong University, No. 277 West Yanta Road, Xi'an 710061, Shaanxi Province, China. sunxy@mail.xjtu.edu.cn

Abstract

BACKGROUND

Small intestine disease endangers human health and is not easy to locate and diagnose.

AIM

To observe the effect of the MS series of small intestine endoscopes on the gastrointestinal tract, the changes in serum gastrin levels and intestinal tissue, and the time required for the examination.

METHODS

In vivo experiments in 20 Living pigs were conducted, Bowel preparation was routinely performed, Intravenous anesthesia with propofol and ketamine was applied, the condition of the small intestine was observed and the detection time of the MS series of small intestine endoscopes were recorded, The changes in intestinal tissue using the MS series of small intestine endoscopes observed and compared before and after the examination, Venous blood (3-5 mL) from pigs was collected before and after the experiment; changes in intestinal tissue after use of the MS series of small intestine endoscopes observed after examination. After

completion of each type of small intestine endoscope experiment, the pigs were allowed to rest and the next type of small intestine endoscope experiment was performed after 15 days of normal feeding. The detection time data of the single-balloon small intestine endoscope and double-balloon small intestine endoscope were collected from four hospitals.

RESULTS

One case of *Ascarislumbricoides*, one of suspected Crohn's disease, one small intestinal diverticulum and one anesthesia accident were observed in pigs. The small intestine showed no differences in the MS series of small intestine endoscopes and there were no differences in serum gastrin between the groups ($P > 0.05$). The time required for inspection was recorded, and the overall detection time for the Japanese small intestine endoscopes was approximately 1.68 ± 0.16 h.

CONCLUSION

Intestinal ascariasis is a common disease in pigs. Some pigs have abnormal intestinal variation. After continuous upgrade and improvement, the MS-3 and MS-4 small intestine endoscope appear superior in terms of detection time.

Key Words: Intestinal ascariasis; Intestinal variation; Crohn's disease; Serum gastrin; Small intestine endoscope

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Core Tip: There are many types of hormones and their contents are extremely small (mostly in the range of nanograms and even picograms).They are neither the energy source of the body nor structural substances of the human body. However, they play essential roles in coordinating physiological processes such as metabolism and growth/development by transmitting information. Research on the changes in gastrin before and after the use of MS series of small intestine endoscope can provide theoretical support for the safe application of small intestine endoscopes.

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INTRODUCTION

The small intestine is the longest organ in the digestive system, and the average length varies among different species[1-3]. Thus, the precise treatment of small intestine disorders remains a challenge for clinicians[4-6]. In 2003, Yamamoto *et al*[7] from Jichi Medical University (Tokyo, Japan), together with Fuji Photo Optical Co., Ltd., developed a double-balloon endoscope and applied it in clinical practice[8, 9]. The double-balloon small intestine endoscope was developed by Yamamoto *et al*[10-12] in Japan. During double-balloon small intestine endoscopy, an over tube with a balloon on top is added to the original push-type endoscope, along with the addition of a balloon at the top of the master camera. Small intestine endoscopy can be completed by alternately pushing-pulling the balloons at the overtube and the master camera. The single-balloon small intestine endoscope is a small bowel endoscope designed by Olympus, Japan, in an attempt to avoid patent infringement[13]. The hook-pull action of the front-end of its master camera replaces the master camera of the double-balloon small intestine endoscope.

In 2005, we also initiated research and development on the MS series of small intestine endoscopes. The past ten years have witnessed the evolution of five generations of our small intestine endoscope. The MS-1 small intestine endoscope is a negative-pressure device that is advanced into the small intestine in a paralleled alternating manner, and has a long detection time. Based on the design of the MS-1 small intestine endoscope, the MS-2 small intestine endoscope was designed with two delivery devices, along with fixing rings and a metal slide. The MS-3 small intestine endoscope abandoned the fixing rings and metal slide and fixed the power and transmission parts together. The MS-4 small intestine endoscopeused a different fixation method to avoid the risk of bowel perforation under extreme working conditions. This new type of small intestine endoscopehad increased stability and speed during the examination, with fewer slippage events. The detection time was not significantly different between the MS-4 small intestine endoscope and MS-3 small intestine endoscope. The design of our latest MS-5 small intestine endoscope has been finalized, and its development is in progress.

The Japanese double-balloon small intestine endoscope has gradually been accepted by patients with the development of painless anesthesia. In this study, we aimed to analyze the detection time of the MS series of small intestine endoscopes and the detection time of the single-balloon small intestine endoscope (SBE) and double-balloon small intestine endoscope (DBE), investigated the possible reasons for the prolonged detection time of small intestine endoscopy.

MATERIALS AND METHODS

Experimental study

In vivo experiments were conducted in 20 Living adult pigs weighing 100 ± 20 kg. The animals were fed with water for two days and fasted for four hours before each experiment. Bowel preparation was routinely performed and each type of MS small intestine endoscope was checked before the experiment. Intravenous anesthesia with propofol and ketamine was applied. Following intravenous anesthesia, oxygen was provided to the pig *via* a nasal catheter at 3 L/min, and the vital signs were continuously monitored. The MS series of small intestine endoscopes was performed by a single endoscopist. When the auxiliary power tube was lubricated with paraffin oil, the camera was inserted into the main auxiliary power tube. Whether the endoscope could slide smoothly through the power tube was examined. After entering the upper part of the small intestine at 20 cm, the auxiliary power system was opened, the MS-1 small intestine endoscope used a double drive power pipe, the auxiliary power catheters were worked alternately; MS-2 to MS-4 small intestine endoscopes used four drive power pipes, MS-1 to MS-3 small intestine endoscopes worked with negative pressure power and MS-4 small intestine endoscope worked with positive pressure power, the auxiliary power catheters of group 1 and group 3 were pushed 40-50 cm to the distal end, group 1 and group 3 were given power catheter pressure or balloon dilatation, then the power catheters of groups 1 and 3 were pulled back to the starting position of groups 2 and 4. When the auxiliary power catheters of groups 2 and 4 were pushed 40-50 cm to the distal end, the auxiliary power catheters of groups 2 and 4 were injected with pressure, and then the power catheters of groups 2 and 4 were pulled back to the proximal end of groups 1 and 3. At the same time, the catheter was pulled back to the back of the catheter in groups 1 and 3, and then the catheter in groups 1 and 3 was pushed forward again. When the tube was folded or if the camera was difficult to insert, the extension distance of the power arm and the operating frequency were adjusted according to the insertion depth. The condition of the small intestine was observed and the detection times of MS-2 to MS-4 small intestine endoscopes were recorded. The changes in intestinal tissue using the MS series of small intestine endoscopes were observed and compared before and after the MS series of small intestine endoscopes. Venous blood (3-5 mL) from the pigs was collected before and after the experiment, and the changes in intestinal tissue after the MS series of small intestine endoscopes were observed after the examination. The MS series of small intestine endoscopes was assisted by a nurse during camera insertion. Using the auxiliary power system, the endoscopist repeatedly pushed the power device to continuously push into the small intestine to the distal end of the camera, thus completing the examination of the entire small intestine. After the examination, venous blood was collected again when anesthesia had worn off. After the completion of each type of small intestine endoscopy experiment, the pigs were allowed to rest, the time to return to normal diet and the time to return to normal stool were recorded, and the next type of small intestine endoscope experiment was performed after 15 days of normal feeding. After the experiment, the live pigs returned to their normal living state.

Clinical data

The detection times of the MS series of small intestine endoscopes were measured. Data on the detection times of SBE and DBE in the following four hospitals were analyzed: (1) Guangdong Provincial People's Hospital: 40 patients including 26 males and 14 females underwent Japanese DBE; (2) Shengjing Hospital Affiliated to China Medical University: From January 2009 to November 2013, DBE (Fujino EN-450P5 Double-Balloon small intestine endoscope, Japan) was performed in 78 patients (including 41 males and 37 females). No definite lesions were found before the procedure; (3) Affiliated Hospital of Zunyi Medical College: Small intestine endoscopy (SIF-Q260 type, Olympus, Japan) was performed in 17 patients with clinically suspected small bowel diseases from January to October 2010; (4) Zhongshan Hospital Affiliated to Fudan University: SBE (SIF-Q260, Olympus, Japan) was performed in 148 patients with suspected small bowel diseases from June 2009 to March 2011.

Observational indicators

To observe the effect of the MS series of small intestine endoscopes on gastrointestinal tract, the changes in serum gastrin levels and intestinal tissue were observed and compared before and after the MS series of small intestine endoscopes examination. The time required for the examination was recorded.

Statistical analysis

Statistical analysis was performed using the SPSS software package 21.0. The measurement data are expressed as mean ± standard deviation (X ± SD).

RESULTS

The experiment duration was 3 mo. One case of *Ascarislumbricoides* (Figure 1), one of suspected Crohn's disease (Figure 2), one small intestinal diverticulum (Figure 3) and one anesthesia accident were observed in pigs.

The serum gastrin level was 22.36 ± 2.88 pg/mL before the MS-1 small intestine endoscope examination, and was 22.33 ± 2.98 pg/mL after the examination. There was no difference in theselevels before and after the MS-1 small intestine endoscope experiment (P > 0.05), and there were no abnormalities in the intestinal tract. The time required for inspection was 2.30 ± 0.15 h. After completion of the MS-1 small intestine endoscope experiment, the pigs returned to their normal diet after 4 h and defecation returned to normal after one day.

The serum gastrin value was 21.88 ± 3.18 pg/mL before the MS-2 small intestine endoscope examination, and was 22.08 ± 2.96 pg/mL after the examination. There was no difference in these levels before and after the MS-2 small intestine endoscope experiment (P > 0.05), and there were no abnormalities in the intestinal tract. The time required for inspection was 1.50 ± 0.30 h. After completion of the MS-2 small intestine endoscope experiment, the pigs returned to their normal diet after 4 h and defecation returned to normal after one day.

The serum gastrin value was 22.28 ± 3.06 pg/mL before the MS-3 small intestine endoscope examination, and was 21.78 ± 3.76 pg/mL after the examination. There was no difference between these levels before and after the MS-3 small intestine endoscope experiment (P > 0.05). *Ascarislumbricoides* was found in one case, small intestinal diverticulum was found in one case and suspected Crohn's disease was found in one case. The time required for inspection was 0.67 ± 0.33 h. After completion of the MS-3 small intestine endoscope experiment, the pigs returned to their normal diet within 3 h and defecation returned to normal within one day. The pig with *Ascarislumbricoides* infection was isolated and treated with albendazole and ivermectin for one week. Small intestinal diverticulum and suspected Crohn's disease were not treated.

Serum gastrin level was 22.30 ± 3.08 pg/mL before the MS-4 small intestine endoscope examination, and was 21.66 ± 3.93 pg/mL after the examination. There was no difference in these levels before and after the MS-4 small intestine endoscope experiment (P > 0.05). The small intestinal diverticulum and the suspected Crohn's disease were as before. The time required for inspection was 1.00 ± 0.13 h. One experimental pig had an anesthesia accident due to excessive ketamine application in the later stage of the MS-4 small intestine endoscope experiment, and the other 19 pigs returned to their normal diet 3 h after the MS-4 small intestine endoscope experiment, and defecation returned to normal after 0.5 days. No *Ascarislumbricoides* was detected in the MS-4 small intestine endoscope group. All 20 experimental pigs completed the relevant experiments, including one case which developed cardiac arrest due to excessive infusion of anesthetics, and in the other 19 cases no abnormalities were found (Table 1).

There were no differences in serum gastrin between the groups (P > 0.05).

A total of 283 small intestine endoscopic procedures (including 165 SBE sessions and 118 DBE sessions) were performed in the four hospitals. The detection time was 1.36 ± 0.07 h for SBE and 2.01 ± 0.25 h for DBE, and the overall detection time for the Japanese small intestine endoscopes was approximately 1.68 ± 0.16 h.

In Guangdong Provincial People's Hospital, the average detection time for DBE in 40 patients was 147 ± 28.5min. In Shengjing Hospital Affiliated to China Medical University, 78 patients successfully completed DBE, and the average examination time was 91.54 ± 22.59 min. In the Affiliated Hospital of Zunyi Medical College, the duration of SBE ranged from 30 to 80 min (mean: 45 min) in 17 patients, among whom one patient had an accidental bowel perforation, which improved after surgical repair. In Zhongshan Hospital Affiliated to Fudan University, 148 patients underwent 166 sessions of SBE, with an average duration of 83.4 min (range: 11–180min). The detection time was 1.36 ± 0.07 h for SBE and 2.01 ± 0.25 h for DBE, and the overall detection time of the Japanese small intestine endoscopes was approximately 1.68 ± 0.16 h (Table 2).

DISCUSSION

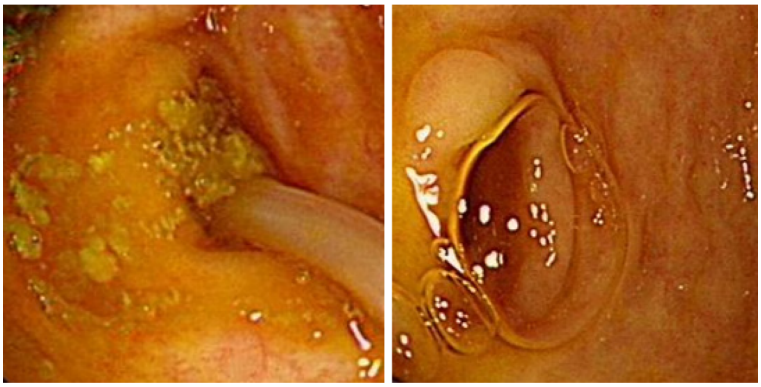
The small intestine includes the duodenum, jejunum and ileum, and each part has no clear boundary [14]. Microscopic observations can only be preliminarily judged by color, mucosal annular folds, small intestinal villus density, intestinal wall lymphatic follicles and so on[15].The main physiological function of the small intestine is digestion and absorption[16]. In addition to continuous digestion by gastric juice, pancreatic juice and bile in the small intestine, the mucosal glands of the small intestine can

Table 1 Detection time and serum gastrin level of MS series of small intestine endoscopes

Type	*n*	Serum gastrin level	Detection time (h)
MS-1 enteroscope	20	Before: 22.36 ± 2.88 pg/mL; After: 22.33 ± 2.98 pg/mL	2.30 ± 0.15
MS-2 enteroscope	20	Before: 21.88 ± 3.18 pg/mL; After: 22.08 ± 2.96 pg/mL	1.50 ± 0.30
MS-3 enteroscope	20	Before: 22.28 ± 3.06 pg/mL; After: 22.33 ± 2.98 pg/mL	0.67 ± 0.33
MS-4 enteroscope	19	Before: 21.30 ± 3.08 pg/mL; After: 21.66 ± 3.93 pg/mL	1.00 ± 0.13

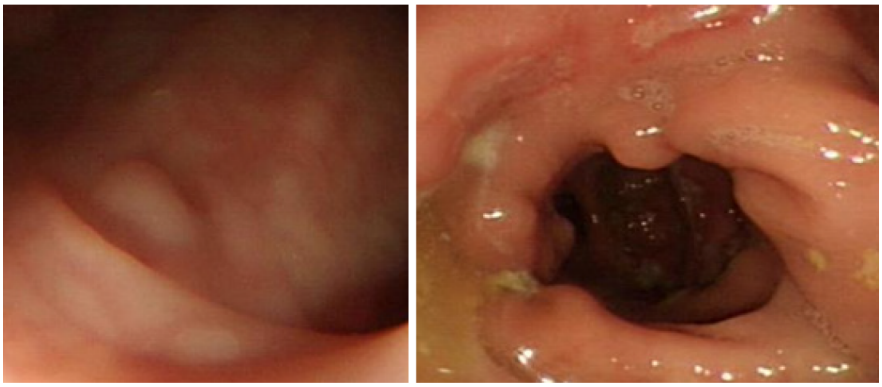
Table 2 Detection time of the Japanese small intestine endoscopes

Type	*n*	Detection time (h)
EN-450P5/20 (double-balloon enteroscope)	118	2.01 ± 0.25
SIF-Q260 (single-balloon enteroscope)	165	1.36 ± 0.07
Overall	283	1.68 ± 0.16



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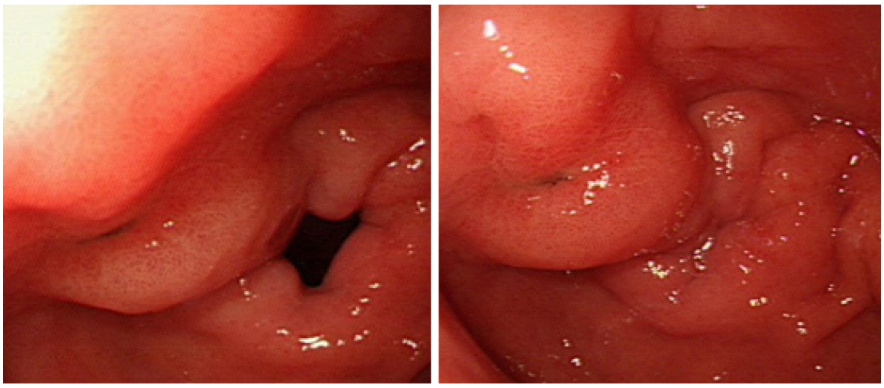
Figure 1 Ascarislumbricoides.



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Figure 2 Suspected Crohn's disease.

also secrete a variety of gastrointestinal hormones, including gastrin, trypsin and cholecystokinin. Hormones are chemical substances produced by endocrine cells and are highly efficient in transmitting information[17-20]. There are many types of hormones and their levels are extremely small (mostly in the range of nanograms and even picograms)[21]. They are neither the energy source of the body nor structural substances in the human body. However, they play essential roles in coordinating physiological processes such as metabolism and growth/development by transmitting information. Research on the changes of gastrin before and after the MS series of small intestine endoscopes examination can provide theoretical support for the safe application of small intestine endoscopes.



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Figure 3 Small intestinal diverticulum.

Analysis of the changes in gastrin may help determine the impact of small intestine endoscopes on the internal environment of the digestive system, thus research and development of small intestine endoscopes are important.

In the experiments performed in the present study, we found that the MS series of small intestine endoscopes had little effect on the gastrointestinal tract, and the experimental animals soon returned to normal. At the same time, we also found that diseases are often found in living organisms, such as *Ascarislumbricoides*, suspected Crohn's disease and small intestinal diverticulum[22-24]. *Ascaris-lumbricoides* can be cured quickly by the combined application of albendazole and ivermectin[25].At present, there isno good solution for small intestinal diverticulum and small intestinal tumor[26,27]. In the future, the precise treatment of lesions may be realized through small intestine endoscopes and laparoscopy.

The incidence rate of gastrointestinal diseases has reached 20% in China. The incidence rate of small intestinal diseases is approximately 2.5% of digestive system diseases. The number of people with small intestinal diseases in China is about 1.4 billion × 20% × 2.5% ≈ 7 million every year, these people were diagnosed by capsule endoscopy, double balloon small intestine endoscopy and single balloon small intestine endoscopy[28,29]. Each year, many patients attend tertiary general hospitals for hospital screening layer by layer to choose surgical treatment. However, due to the lack of small intestine endoscope locations, some patients have incomplete resection, intestinal leakage and intestinal obstruction. If these patients have the money to continue treatment, they could attend Nanjing Army General Hospital, which is a laminar flow ward. With regard to small intestinal tumors requiring surgery, although they only account for 5% of gastrointestinal tumors (the 7th edition of Huang Jiasi's Surgery), they cannot be ignored.

The past ten years have witnessed our efforts in the research and development of the MS series of small intestine endoscopes. From the type MS-1 to MS-5, the performance of our devices has been constantly improved, and the detection time has been gradually shortened. The MS-1 small intestine endoscope is a negative-pressure device that is advanced into the small intestine in a paralleled alternating manner, and the detection time is long. In 2008, we adjusted the design to develop the MS-2 small intestine endoscope based on the patented drawings of the pipette small intestine endoscope, and its detection time was slightly shorter when compared with the MS-1 small intestine endoscope. However, slippage of the small intestine endoscope persisted due to the extremely poor stabilities of the fixing rings and the metal slide, making the MS-2 small intestine endoscope unfeasible for clinical applications. In 2010, we further optimized our design and developed the MS-3 small intestine endoscope based on the patented design of the built-in straw-type small intestine endoscope. This new-generation device had a shorter detection time than the MS-2 small intestine endoscope. It had higher stability and speed and fewer slippage events during small intestine endoscopy; however, negative-pressure perforation occurred in some experiments. Two articles describing the MS-3 small intestine endoscope were published in the Chinese Journal of Digestive Endoscopy and Chinese Journal of Gastrointestinal Surgery, respectively, and our research on this device also won the third prize of the Dalian Science and Technology Progress Award in 2012.In 2012, by adopting a new working principle, we developed the MS-4 type based on the patented design of the catheter balloon-type small intestine endoscope. The MS-4 small intestine endoscope has significantly higher stability and speed, along with fewer slippage events[30]. The detection time of the MS-4 small intestine endoscope did not decrease compared with the MS-3 small intestine endoscope. Notably, the MS-4 small intestine endoscope has obtained the production license from Liaoning Provincial Food and Drug Administration. In 2018, we completed the design of the MS-5 small intestine endoscope based on the latest and most practical design. In this new endoscope, we optimized the structure of the auxiliary power system using a four-drive design. Relevant studies have been carried out since then.

Our team is trying to further optimize the MS series of small intestine endoscopes, particularly to shorten the detection time of small intestine endoscopy. The clinical application of these endoscopes will facilitate the examination, diagnosis, and treatment of gastrointestinal diseases and solve existing problems. To perform new types of surgery, a small intestine endoscope is combined with laparoscopic resection of intestinal lesions. This reduces open surgery, avoids intestinal adhesions or intestinal obstruction caused by open surgery, increases the diagnostic accuracy and cure rate, and thus further improves the capability and quality of medical services.

CONCLUSION

Intestinal ascariasis is a common disease in pigs. Some pigs have abnormal intestinal variations. After continuous upgrade and improvement, the MS-3 and MS-4 small intestine endoscopes appear superior in terms of detection time.

ARTICLE HIGHLIGHTS

Research background

Small intestine disease endangers human health and is not easy to locate and determine. The fundamental reason for this is that there is no appropriate small intestine endoscopic equipment for treatment. The MS-5 small intestine endoscope should be introduced as soon as possible to benefit patients.

Research motivation

To alleviate the suffering of patients and reduce intestinal adhesions and intestinal obstruction.

Research objectives

To assess the data on the fifth generation small intestine endoscope should be fast-tracked because there is no blind spot in the examination, diagnosis and treatment of small intestinal diseases.

Research methods

Experimental animals and patients underwent routine bowel cleaning before the examination, which was performed using small intestine endoscopes.

Research results

The average detection time for the MS-1, MS-2, MS-3 and MS-4 small intestine endoscope was 2.30 ± 0.15 h, 1.50 ± 0.30 h, 0.67 ± 0.33 h and 1.00 ± 0.13 h, respectively. The detection time was 1.36 ± 0.07 h for SBE and 2.01 ± 0.25 h for DBE, and the overall detection time for the Japanese small intestine endoscopes was approximately 1.68 ± 0.16 h.

Research conclusions

After continuous upgrade and improvement, the MS-4 small intestine endoscope showed superior detection time.

Research perspectives

Improve the diagnosis and treatment of small intestinal diseases, solve practical problems, and reduce the number of cases of open surgery to improve the quality of medical treatment in the future.

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FOOTNOTES

Author contributions: Liu JH, Liu DY and Sun XJ designed the research; Liu JH, Liu DY, Yuan YF, Sun XJ and Shan SM performed the research; Liu JH, Liu DY and Sun XJ contributed new reagents/analytic tools; Liu JH analyzed the data; Liu JH and Sun XJ wrote the paper.

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Institutional review board statement: The study was reviewed and approved by the Academic Committee of Dalian Friendship Hospital Institutional Review Board [(Approval No. YY-XS-2019-001-1]).

Institutional animal care and use committee statement: All procedures involving animals were reviewed and approved by the Institutional Animal Care and Use Committee of the Ethics Committee of Dalian Friendship Hospital (IACUC protocol number: [Protocol No. YY-LL-2020-015]).

Informed consent statement: All study participants, or their legal guardian, provided informed written consent prior to study enrollment.

Conflict-of-interest statement: Xue-Jun Sun has received fees for serving as a speaker for The First Affiliated Hospital of Xi'an Jiaotong University; Jin-Hua Liu has received research funding from Liaoning Provincial Natural Science Foundation; Yong-Feng Yuan is an employee of Harbin Institute of Technology; Shu-Mei Shan owns stocks and/or shares in Dalian Mingsheng Technology Development Company; Jin-Hua Liu and Dan-Yang Liu owns patent Built-in Straw type small intestine endoscope: China, 200920351611.5 [P]. 2010-09-08; and Catheter balloon type small intestine endoscope: China, 200920351612.X [P]. 2010-09-08.

Data sharing statement: Technical appendix, statistical code, and dataset available from the corresponding author at URL. Participants gave informed consent for data sharing.

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Country/Territory of origin: China

ORCID number: Jin-Hua Liu 0000-0002-5818-2834; Dan-Yang Liu 0000-0002-6169-5390; Yong-Feng Yuan 0000-0002-2182-3789; Xue-Jun Sun 0000-0003-0178-5551; Shu-Mei Shan 0000-0001-8568-3880.

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Basic Study

c-MET immunohistochemical expression in sporadic and inflammatory bowel disease associated lesions

Grant Halliday, Ross J Porter, Catherine J Black, Mark J Arends, Shahida Din

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Grant Halliday, Catherine J Black, Department of Pathology, Western General Hospital, NHS Lothian, Scotland EH4 2XU, United Kingdom

Ross J Porter, Centre for Inflammation Research, Queen's Medical Research Institute, University of Edinburgh, Edinburgh EH16 4TJ, United Kingdom

Ross J Porter, Shahida Din, Edinburgh Inflammatory Bowel Disease Unit, Western General Hospital, NHS Lothian, Edinburgh EH4 2XU, United Kingdom

Mark J Arends, Cancer Research UK Edinburgh Centre, Institute of Cancer & Genetics, Western General Hospital, University of Edinburgh, Edinburgh EH4 2XU, United Kingdom

Corresponding author: Shahida Din, BSc, FRCP, MBChB, PhD, Consultant Physician-Scientist, Edinburgh Inflammatory Bowel Disease Unit, Western General Hospital, NHS Lothian, Anne Feguson Building, Edinburgh EH4 2XU, United Kingdom. sdin@exseed.ed.ac.uk

Abstract

BACKGROUND

Post-colonoscopy colorectal cancer (CRC) rates for patients with inflammatory bowel disease (IBD) are unacceptably high. During colonoscopy, an intravenous fluorescent anti-c-MET probe may improve endoscopic detection of lesions. However, c-MET expression in IBD lesions is poorly defined, limiting translational studies.

AIM

To comprehensively define c-MET expression in sporadic and IBD-associated colorectal carcinogenesis.

METHODS

c-MET expression was immunohistochemically assessed in 319 formalin-fixed paraffin-embedded tissue specimens, colonoscopically or surgically retrieved between 1994-2017. Tissue included: 30 normal colorectal biopsies, 30 hyperplastic polyps (HP), 31 sessile serrated lesions (SSL), 55 tubular/tubulovillous adenomas with low (TA-LGD, n = 32) or high grade dysplasia (TA-HGD, n = 23), 26 sporadic (s)-CRCs, 16 quiescent IBD biopsies, 11 active/inflamed IBD biopsies, 18 IBD-associated dysplastic lesions (IBD-dys), and 102 IBD-CRCs. Expression was scored by two independent observers as: 0 = absent, 1 = weak, 2 = moderate or 3 = strong. Mann-Whitney U and Kruskal-Wallis tests were used to assess significan-

ce.

RESULTS

Positive epithelial cytoplasmic and membranous c-MET expression was observed in all tissues, indicating there is ubiquitous expression in the colorectum. c-MET expression was weak in normal colonic epithelium compared with each of the sporadic colonic lesions, including TA-LGD (P < 0.001), TA-HGD (P = 0.004), HP (P < 0.001), SSL (P < 0.001), and s-CRC (P < 0.001). Specifically, in sporadic (non-IBD) lesions, expression was stronger in TA-LGD compared with normal mucosa (P < 0.001), and stronger in s-CRC compared with TA-HGD (P = 0.004). However, there was no significant difference between TA-LGD and TA-HGD (P = 0.852). Further, there was no difference in c-MET expression between HP and SSL (P = 0.065). In IBD, expression was weaker in quiescent colonic mucosa compared with inflamed colonic mucosa (P < 0.001). There was no difference between inflamed colonic mucosa and IBD-dys (P = 0.512) or IBD-CRC (P = 0.296). However, expression was stronger in IBD-dys (P < 0.001) and IBD-CRC (P < 0.001) compared with quiescent IBD colonic mucosa.

CONCLUSION

The characterisation of c-MET expression suggest that an intravenous probe may improve the endoscopic detection of lesions in both non-IBD patients and IBD patients with quiescent disease.

Key Words: Inflammatory bowel diseases; Colorectal cancer; Surveillance; Detection; c-MET; Immunohistochemistry

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Core Tip: During colonoscopy, an intravenous fluorescent anti-c-MET probe may improve endoscopic detection of dysplasia and cancer. However, c-MET expression in inflammatory bowel disease (IBD) lesions is poorly defined, limiting translational studies. We demonstrate that stronger immunohistochemical c-MET expression is associated with dysplasia and cancer in both sporadic and IBD-associated lesions. Therefore, c-MET expression could be exploited clinically to enhance endoscopic detection of pre-malignant lesions and cancer, particularly in IBD where post-colonoscopy colorectal cancer rates are unacceptably high.

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INTRODUCTION

Patients with inflammatory bowel disease (IBD) have an increased risk of developing colorectal cancer (CRC), with poorer survival compared with the general population[1,2]. The British Society of Gastroenterology currently recommends high-definition surveillance ileocolonoscopy ± chromoendoscopy, with targeted biopsies starting 8-years after the onset of IBD symptoms[3]. Despite this, IBD post-colonoscopy CRC rates - defined as a diagnosis of cancer or high-grade dysplasia > 6 mo to 3 years following a colonoscopy that was negative for cancer - are unacceptably high, at 28%-45%[4,5]. One challenge in identifying dysplastic and malignant lesions in IBD is the morphological changes associated with flat lesions which are difficult to detect endoscopically. With an ageing population and rising global burden of IBD[6], there is an increasing requirement for endoscopic surveillance indicating an urgent clinical need to improve the endoscopic detection of IBD-associated dysplasia and cancer.

c-MET is a receptor tyrosine kinase (encoded by the *MET* gene on chromosome 7q21-31) overexpressed at a protein level in a variety of human primary tumours, including in the colorectum where it is associated with the sporadic adenoma-carcinoma pathway[7-11]. In 2015, Burggraaf and colleagues published a first-in-human study demonstrating that an intravenous injection of a fluorescently labelled peptide with a high affinity for c-MET was safe, well tolerated in humans, and could improve the detection of colonic polyps in a high-risk asymptomatic patient cohort[12]. Since then, translational data have emerged in support of this technology for sporadic CRC[13,14].

The original data from Burggraaf and colleagues stated that there were several lesions visible with fluorescence assisted colonoscopy that were not identified during first or second pass conventional

white light colonoscopy. These small lesions were mostly < 6 mm and non-polypoid[12]. Given this, we hypothesise that this technology may be especially useful for identifying IBD-associated lesions with similar flat morphology. However, the clinical utility of c-MET is unclear in the setting of chronic inflammation and injury to the colonic mucosa, as c-MET has been reported to be upregulated in tissue repair[15].

While the efficacy of an *in vivo* c-MET probe has never been investigated in the setting of human IBD, there are some murine data. The azoxymethane (AOM)/dextran sulphate sodium (DSS) mouse model is commonly used to simulate colitis-associated carcinogenesis in the laboratory[16]. Using this model, Tao and colleagues reported that there was an accumulation of fluorescence from their c-MET probe (Crizotinib and MPA, a water-soluble cyanine dye, covalently conjugate *via* PEG4) in colonic lesions, while there was minimal fluorescence in adjacent tissue[17]. While an acceptable surrogate model, AOM/DSS lesions are not identical to those seen in human disease. Further, Tao *et al*[17] resected colons for imaging *in vitro*, rather than assessment by *in situ* fluorescence colonoscopy. Nonetheless, these data are encouraging and warrant investigation in human IBD: there are a paucity of histopathological studies that define c-MET expression in human IBD and IBD-associated carcinogenesis.

To address this need, this study comprehensively defines the expression of c-MET in a large cohort of 319 paraffin-embedded tissue sections, representing the spectrum of both sporadic and IBD-associated colorectal carcinogenesis. Our results suggest that c-MET could be exploited clinically to enhance detection of potentially malignant lesions in IBD.

MATERIALS AND METHODS

Patient tissue selection

217 formalin fixed paraffin embedded (FFPE) human tissue specimens, colonoscopically or surgically retrieved between January 2000 to December 2017, were identified from the Edinburgh Pathology database. Tissue included: 30 normal colorectal biopsies, 30 hyperplastic polyps (HP), 31 sessile serrated lesions (SSL), 55 tubular/tubulovillous adenomas with low (TA-LGD, n = 32) or high (TA-HGD, n = 23) grade dysplasia, 26 sporadic colorectal adenocarcinomas (s-CRC), 16 quiescent IBD biopsies, 11 active/inflamed IBD biopsies, and 18 conventional IBD-associated dysplastic lesions (IBD-dys)-15 were considered low-grade and 3 were considered high-grade. A tissue microarray comprising 102 IBD-associated CRC (IBD-CRC) cores from 43 patient tumours, retrieved from surgical resection specimens between 1994 and 2011, was also used. For all tissue, the original haematoxylin and eosin (HE) diagnostic slide, case history and pathology report were reviewed by two expert gastrointestinal pathologists (MJA, CJB) to ensure consensual agreement and accurate tissue diagnosis. All selected cases were anonymised. Ethical approval was obtained from Lothian NRS Bioresource Research Tissue Bank (15/ES/0094; SR148, SR389, SR400 and SR588).

Immunohistochemistry

Immunohistochemistry (IHC) was performed using an optimised protocol. In summary, 3μm tissue sections were cut by microtomy from each selected FFPE block and floated onto positively charged glass slides. Tissue was oven-dried overnight. Sections were deparaffinised and underwent heat-mediated antigen retrieval in pH6 citrate buffer for 15 min (BOND Epitope Retrieval Solution 1, Leica Biosystems, United Kingdom). After 5 min of peroxidase blocking, tissue was stained for 15 min with recombinant monoclonal anti-MET (c-MET) antibody (ab51067, clone EP1454Y, Abcam, United Kingdom) at a 1:200 dilution, on Leica Bond-III and BONDMAX autostainers (Leica Biosystems, United Kingdom). Primary antibody detection used the BOND Polymer Refine Detection Kit (Leica Biosystems, United Kingdom).

Interpretation of IHC staining

Two expert gastrointestinal pathologists (MJA, CJB) independently assessed c-MET expression as: 0=no staining, 1+ = weak intensity staining, 2+ = moderate intensity staining, and 3+ = strong intensity staining. Epithelial cytoplasmic and epithelial membranous immunopositivity was ubiquitously equivalent in this study which meant one representative score was assigned to each biopsy. Discrepant scores were resolved by discussion and consensus reached. Use of a histoscore was not considered appropriate in small dysplastic lesions. Scoring methodology and staining intensity agreement came from comparison with internal control normal tissue adjacent to the lesions, and previously published staining intensities[7,9-12,14,18]. Negative control slides were used to allow observers to account for background staining (which was negligible due to IHC optimisation).

Statistical analysis

Statistical analysis was performed using IBM®SPSS® V25.0.0.1 and GraphPad Prism software. Associations between ordinal and categorical variables were assessed using exact two-tailed Mann-Whitney U and Kruskal-Wallis tests. Scores of 0, 1+, 2+ and 3+ were thus compared and statistical significance was determined at $P \leq 0.05$.

RESULTS

c-MET is ubiquitously expressed in the colorectum, with increased expression in colonic dysplastic and other lesions

Positive epithelial cytoplasmic and membranous c-MET expression was observed in all tissues, indicating there is ubiquitous expression in the colorectum. As anticipated, c-MET expression was weak in normal colonic epithelium compared with each of the sporadic colonic lesions, including TA-LGD ($P < 0.001$), TA-HGD ($P = 0.004$), HP ($P < 0.001$), SSL ($P < 0.001$), and s-CRC ($P < 0.001$) (Figure 1).

Increased c-MET expression is associated with sporadic colonic dysplasia and adenocarcinoma

c-MET expression was stronger in TA-LGD compared with normal colonic mucosa ($P < 0.001$), and stronger in s-CRC compared with TA-HGD ($P = 0.004$). There was no significant difference in c-MET expression between TA-LGD and TA-HGD ($P = 0.852$).

There is no significant difference in c-MET expression between hyperplastic polyps vs sessile serrated lesions

Given the association between c-MET expression and malignancy, we investigated whether there was a difference between hyperplastic polyps (with low malignant potential) and sessile serrated lesions (with higher malignant potential). There was no statistically significant difference in c-MET expression between these lesions ($P = 0.065$).

c-MET shows increased expression in IBD-dysplasia and IBD-cancer compared with quiescent, but not actively inflamed, IBD mucosa

Given the association between c-MET expression and sporadic colorectal carcinogenesis, we assessed whether this was also true for IBD-associated dysplasia and cancer, where detection of dysplasia and cancer is more challenging. c-MET expression was weak in quiescent IBD mucosa compared with active / inflamed IBD ($P < 0.001$). There was no difference in c-MET expression between inflamed IBD mucosa and IBD-dys ($P = 0.512$) or IBD-CRC ($P = 0.296$). However, c-MET expression was stronger in IBD-dys ($P < 0.001$) and IBD-CRC ($P < 0.001$) compared with quiescent IBD mucosa. There was no difference between IBD-dys and IBD-CRC ($P = 0.673$) (Figure 2).

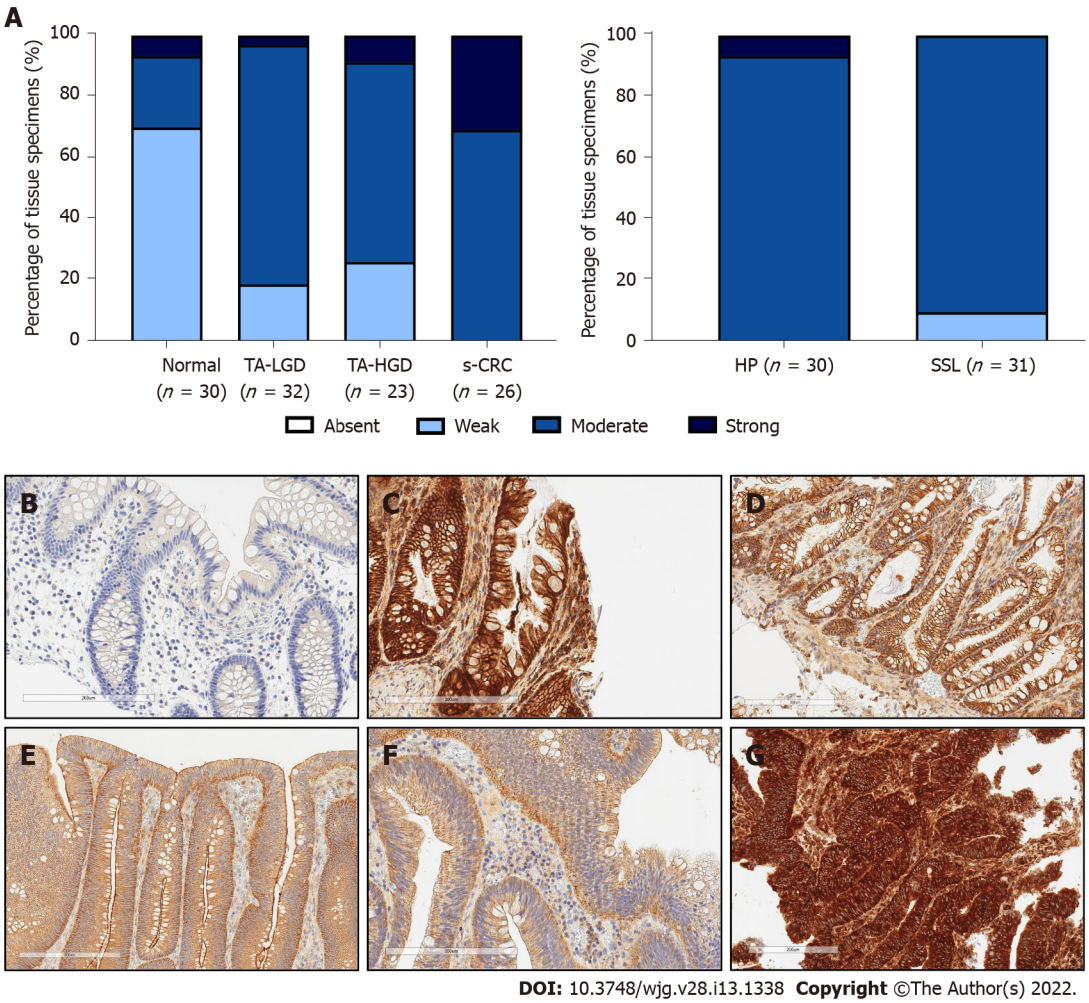
DISCUSSION

This immunohistochemistry study of 319 human tissue specimens provides a comprehensive overview of c-MET expression in both sporadic and IBD-associated colorectal carcinogenesis (Supplementary Table 1). We demonstrate c-MET expression in the colorectum, with an increase in expression in sporadic dysplasia and s-CRC. In IBD-associated lesions we report increased c-MET expression in IBD-dysplasia and IBD-CRC compared with quiescent, but not actively inflamed IBD mucosa.

Previous histopathological studies report overexpression of c-MET in sporadic CRC, associated with tumour invasion, metastasis, local recurrence, and poor overall survival[7-10]. Less data exist for pre-cancerous lesions. However, studies suggest that c-MET expression is increased across the adenoma-dysplasia-carcinoma sequence[11]. These data are in keeping with our comprehensive assessment of sporadic lesions; there is an increase in c-MET expression from normal colonic mucosa to dysplasia to colorectal adenocarcinoma.

Gayyed and colleagues reported increased c-MET expression in colonic polyps with HGD compared with LGD[11]. Our study reported no statistically significant difference between these LGD and HGD groups, using a rigorous approach to achieve full diagnostic agreement from two expert gastrointestinal pathologists, based upon review of the HE slide, and all available clinico-pathological data. The proposed use for c-MET is not necessarily to discriminate LGD from HGD; instead, it is as an *in vivo* probe to improve endoscopic detection of colonic lesions. Both lesions had stronger c-MET expression compared with normal mucosa suggesting that either lesion would be positively identified at colonoscopy allowing targeted biopsy for histopathological assessment.

Identification of SSL from HP is useful as the former have higher malignant potential which is not fully appreciated during endoscopic assessment[19]. Joshi and colleagues report increased c-MET expression in SSL compared with HP, using immunofluorescence on FFPE SSL ($n = 17$) and HP ($n = 10$) tissue. This was also observed by Wu *et al*[13]. In our study, there was no statistically significant difference between SSL ($n = 31$) and HP ($n = 30$) ($P = 0.065$). One reason could be that we used IHC whereas previous studies used immunofluorescence. There has been inconsistent reporting of SSLs; due to previous high inter-observer variability, lack of robust diagnostic criteria and prior misclassification of hyperplastic polyps[19-21]. As previously discussed, our study ensured accurate polyp sub-classification (through consensual agreement by two expert gastrointestinal pathologists) and it is reassuring both lesions had increased expression compared with normal mucosa, as this infers both lesions would



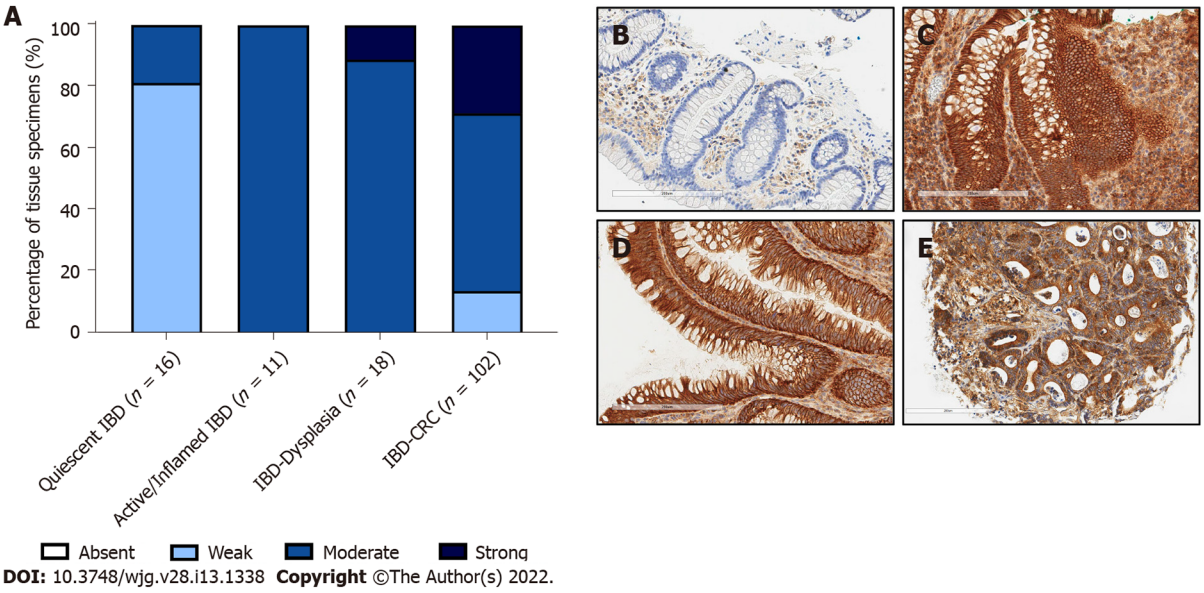
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Figure 1 Expression intensity of c-MET in sporadic colonic lesions. A: Semi-quantitative assessment of c-MET expression intensity throughout sporadic colorectal carcinogenesis; B: Representative photomicrograph of normal colonic mucosa; C: Representative photomicrograph of hyperplastic polyp; D: Representative photomicrograph of sessile serrated lesion; E: Representative photomicrograph of tubular/tubulovillous adenoma with low grade dysplasia; F: Representative photomicrograph of tubular/tubulovillous adenoma with high grade dysplasia; G: Representative photomicrograph of sporadic colorectal adenocarcinoma. Tissue provided by Lothian NRS Bioresource. Brightfield photomicrographs taken at X20 magnification. TA-LGD: Tubular/tubulovillous adenoma with low grade dysplasia; TA-HGD: Tubular/tubulovillous adenoma with high grade dysplasia; s-CRC: Sporadic colorectal adenocarcinoma; HP: Hyperplastic polyp; SSL: Sessile serrated lesion.

be positively identified for histopathological assessment and SSL would thus not be missed.

Our study found no difference in c-MET expression between inflamed IBD mucosa, IBD-dys and IBD-CRC. This is in agreement with the IHC study from Harpaz *et al*[18]. A key challenge is detecting dysplasia in the context of inflammation-both endoscopically and histopathologically. While this is a major priority, it is based upon the assumption that the post-colonoscopy CRC rate in IBD is related to active inflammation which reduces the ability to detect dysplasia. Therefore, we need to improve detection across the board. While an inability to distinguish between inflamed IBD mucosa, IBD-dys and IBD-CRC could thus be perceived as a barrier to clinical translation, our study reports lower expression of c-MET in quiescent IBD mucosa. Therefore, careful selection of patients could help identify a cohort which would benefit from the use of such an adjunct in detecting subtle lesions during surveillance colonoscopy. Objective biomarkers such as faecal calprotectin ± serum C-reactive protein levels could indicate whether a patient is more likely to have quiescent disease. Indeed, The British Society of Gastroenterology recommend surveillance colonoscopy in patients during quiescent phases of disease where possible[3]. Careful protocol optimisation will minimise any background fluorescence, due to microscopic histological inflammation, which may be present in endoscopically normal and/or quiescent mucosa in patients with IBD.

There are several practical questions that now need to be answered to determine the clinical viability of introducing a c-MET probe into IBD surveillance programs: (1) Is a c-MET probe safe and well tolerated in IBD patients; (2) Does inflammation confound endoscopic assessment of colonic lesions using a fluorescent c-MET probe; (3) Can a c-MET probe be optimised to differentiate between background quiescent/non-inflamed colonic mucosa and IBD-associated lesions, as assessed by fluorescence colonoscopy; (4) Can pre-screening patients using symptom questionnaires, faecal calpro-



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Figure 2 Expression intensity of c-MET in inflammatory bowel disease-associated lesions. A: Semi-quantitative assessment of c-MET expression intensity throughout inflammatory bowel disease (IBD)-associated colorectal carcinogenesis; B: Representative photomicrograph of quiescent IBD mucosa; C: Representative photomicrograph of active/inflamed IBD mucosa; D: Representative photomicrograph of IBD-associated dysplastic lesions; E: Representative photomicrograph of IBD-associated colorectal cancer. Tissue provided by Lothian NRS Bioresource. Brightfield photomicrographs taken at X20 magnification. IBD: Inflammatory bowel disease; CRC: Colorectal cancer.

tectin ± serum C-reactive protein accurately identify quiescent disease, resulting in low false-positive fluorescence; and (5) Does a c-MET probe offer benefit compared with standard care or other advanced endoscopy techniques for the detection of IBD lesions: for example, can the probe detect endoscopically 'invisible' or flat dysplasia (*i.e.* dysplasia seen only histopathologically on a random biopsy of macroscopically normal mucosa)?

CONCLUSION

In this comprehensive study, we have robustly defined the expression of c-MET in both sporadic and IBD-associated colorectal carcinogenesis. These data provide a platform for clinical studies to investigate the efficacy of an *in vivo* c-MET probe to enhance the endoscopic detection of colonic lesions during IBD surveillance colonoscopy. Such an application would be especially important for identifying IBD-associated dysplasia, to reduce the high post-colonoscopy CRC rate within a pre-selected cohort of patients with quiescent disease.

ARTICLE HIGHLIGHTS

Research background

Patients with inflammatory bowel disease (IBD) are more likely to develop colorectal cancer (CRC) compared with the general population, and surveillance colonoscopy is therefore performed at defined intervals to identify pre-malignant lesions. Despite this, IBD post-colonoscopy CRC rates remain unacceptably high. One key challenge is endoscopically identifying the more subtle and flat lesions associated with dysplasia and cancer in IBD.

Research motivation

There is an urgent need to improve endoscopic detection of pre-malignant lesions, especially in patients with IBD. Recent studies have suggested that an intravenously administered fluorescent probe against c-MET protein may improve the detection of sporadic colorectal lesions-specifically small non-polypoid lesions of similar morphology to IBD-associated (pre-) malignant lesions. However, most data come from murine studies or sporadic disease, and there are lack of immunohistochemical data defining c-MET expression in IBD-associated colonic lesions. This is limiting translational studies.

Research objectives

This study was designed to systematically assess the immunohistochemical expression of c-MET in both sporadic and inflammatory bowel disease-associated colonic lesions.

Research methods

c-MET expression intensity was semi-quantitatively assessed after immunohistochemically staining formalin-fixed paraffin-embedded tissue specimens with an anti-c-MET antibody. Tissue had been colonoscopically or surgically retrieved from patients with and without IBD between 1994-2017, and included normal colonic mucosa, hyperplastic polyps, sessile serrated lesions, tubular/tubulovillous adenomas with low or high grade dysplasia, sporadic-CRC, quiescent IBD mucosa, inflamed IBD mucosa, IBD-associated dysplastic lesions, and IBD-associated CRC.

Research results

There was ubiquitous expression of c-MET in normal colonic mucosa, as well as in sporadic and IBD lesions. c-MET expression intensity was similar between low *vs* high grade dysplasia, and between hyperplastic polyps *vs* sessile serrated lesions. However, c-MET expression was stronger in sporadic dysplasia and cancer compared with normal colonic mucosa. Similarly, c-MET expression was stronger in IBD-associated dysplastic and malignant lesions compared with quiescent IBD mucosa. There was no difference in c-MET expression between inflamed IBD mucosa and IBD-associated dysplasia or malignant lesions.

Research conclusions

c-MET expression intensity is stronger in dysplastic and malignant lesions compared with normal colonic epithelium and quiescent IBD mucosa. These data provide a platform to allow future studies to investigate whether an intravenous anti-c-MET probe could help endoscopically identify dysplasia and malignancy, particularly within surveillance colonoscopy programmes for IBD patients where post-colonoscopy CRC rates are unacceptably high.

Research perspectives

Further study is needed to determine whether histopathological expression correlates with mucosal expression at endoscopy in the context of IBD. The ability of such a probe to improve the endoscopic detection of colorectal lesions and reduce the post-colonoscopy CRC rate should then be assessed, in patients with quiescent IBD.

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FOOTNOTES

Author contributions: Porter RJ and Halliday G contributed equally to this work; Porter RJ analysed data, wrote the first draft of the manuscript and reviewed and edited the final manuscript; Halliday G performed experiments, contributed to writing the first manuscript draft and reviewed and edited the manuscript; Arends MJ and Black CJ provided histopathological expertise, contributed to study design, performed experiments and reviewed and edited the final manuscript; Din S was the principal investigator for this study, conceptualised and designed the study, analyzed data and reviewed and edited the manuscript; all authors have read and approved the final manuscript.

Institutional review board statement: Ethical approval was obtained from Lothian NRS Bioresource Research Tissue Bank (15/ES/0094; SR148, SR389, SR400 and SR588).

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Country/Territory of origin: United Kingdom

ORCID number: Grant Halliday 0000-0002-1044-0857; Ross J Porter 0000-0001-5043-186X; Catherine J Black 0000-0001-6938-8128; Mark J Arends 0000-0002-6826-8770; Shahida Din 0000-0003-2855-3400.

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ORIGINAL ARTICLE

Retrospective Study

Increased prognostic value of clinical–reproductive model in Chinese female patients with esophageal squamous cell carcinoma

Dong-Yun Zhang, Jian-Wei Ku, Xue-Ke Zhao, Hai-Yan Zhang, Xin Song, Hong-Fang Wu, Zong-Min Fan, Rui-Hua Xu, Duo You, Ran Wang, Ruo-Xi Zhou, Li-Dong Wang

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Dong-Yun Zhang, Xue-Ke Zhao, Xin Song, Zong-Min Fan, Rui-Hua Xu, Duo You, Ran Wang, Li-Dong Wang, State Key Laboratory of Esophageal Cancer Prevention & Treatment and Henan Key Laboratory for Esophageal Cancer Research of the First Affiliated Hospital, Zhengzhou University, Zhengzhou 450052, Henan Province, China

Dong-Yun Zhang, Hong-Fang Wu, Department of Pathology, Nanyang Medical College, Nanyang 473061, Henan Province, China

Jian-Wei Ku, Department of Endoscopy, The Third Affiliated Hospital, Nanyang Medical College, Nanyang 473061, Henan Province, China

Hai-Yan Zhang, Department of Pathology, The First Affiliated Hospital, Nanyang Medical College, Nanyang 473061, Henan Province, China

Duo You, Department of Medical Oncology, Affiliated Cancer Hospital of Zhengzhou University, Henan Cancer Hospital, Zhengzhou 450052, Henan Province, China

Ruo-Xi Zhou, Department of Biology, University of Richmond, Richmond, VA 23173, United States

Corresponding author: Li-Dong Wang, MD, PhD, Professor, State Key Laboratory of Esophageal Cancer Prevention & Treatment and Henan Key Laboratory for Esophageal Cancer Research of the First Affiliated Hospital, Zhengzhou University, No. 40 Daxue Road, Zhengzhou 450052, Henan Province, China. ldwangpaper2018@126.com

Abstract

BACKGROUND

In China, it has been well recognized that some female patients with esophageal squamous cell carcinoma (ESCC) have different overall survival (OS) time, even with the same tumor-node-metastasis (TNM) stage, challenging the prognostic value of the TNM system alone. An effective predictive model is needed to accurately evaluate the prognosis of female ESCC patients.

AIM

To construct a novel prognostic model with clinical and reproductive data for Chinese female patients with ESCC, and to assess the incremental prognostic value of the full model compared with the clinical model and TNM stage.

METHODS
A new prognostic nomogram incorporating clinical and reproductive features was constructed based on univariatie and Cox proportional hazards survival analysis from a training cohort (n = 175). The results were recognized using the internal (n = 111) and independent external (n = 85) validation cohorts. The capability of the clinical–reproductive model was evaluated by Harrell's concordance index (C-index), Kaplan–Meier curve, time-dependent receiver operating characteristic (ROC), calibration curve and decision curve analysis. The correlations between estrogen response and immune-related pathways and some gene markers of immune cells were analyzed using the TIMER 2.0 database.

RESULTS
A clinical–reproductive model including incidence area, age, tumor differentiation, lymph node metastasis (N) stage, estrogen receptor alpha (ESR1) and beta (ESR2) expression, menopausal age, and pregnancy number was constructed to predict OS in female ESCC patients. Compared to the clinical model and TNM stage, the time-dependent ROC and C-index of the clinical–reproductive model showed a good discriminative ability for predicting 1-, 3-, and 5-years OS in the primary training, internal and external validation sets. Based on the optimal cut-off value of total prognostic scores, patients were classified into high- and low-risk groups with significantly different OS. The estrogen response was significantly associated with p53 and apoptosis pathways in esophageal cancer.

CONCLUSION
The clinical–reproductive prognostic nomogram has an incremental prognostic value compared with the clinical model and TNM stage in predicting OS in Chinese female ESCC patients.

Key Words: Esophageal squamous cell carcinoma; Female; Nomogram; Prognosis; Estrogen receptor

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Core Tip: In China, some female patients with esophageal squamous cell carcinoma (ESCC), even with the same tumor-node-metastasis (TNM) stage, have significantly different overall survival (OS) time. The prognostic value of the TNM system has been challenged due to its unsatisfactory discriminative ability. A new prognostic nomogram that combines clinical and reproductive features was developed and validated in this study. Compared with the clinical model and TNM stage, clinical–reproductive model has incremental prognostic value in predicting OS in Chinese female patients with ESCC, which can help clinicians to make individual treatment and medical decisions.

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INTRODUCTION

Esophageal squamous cell carcinoma (ESCC) is highly invasive malignancy in China with a 5-year overall survival (OS) rate of only 10%-20% in patients with advanced disease[1]. In addition to pathological features, reproductive factors such as menopausal status, estrogen receptors, and pregnancy number are also correlated with clinical outcome in female ESCC patients[2,3]. The increased risk of esophageal cancer is associated with a decrease in estrogen level[4]. An epidemiological study of menopausal hormone therapy (MHT) confirmed that patients who have been using MHT have a reduced risk of ESCC[5]. Previous research also supports the protective effects of female hormones on the risk of ESCC[6].

The most common prognostic evaluation system for ESCC mainly depends on the tumor node metastasis (TNM) staging system of the American Joint Committee on Cancer, which has been adopted in the United States since 1959[7,8]. Recently, the prognostic value of the TNM system has been challenged due to its unsatisfactory discriminative ability[9]. Relevant studies have shown significant differences in OS in female ESCC patients even with the same TNM stage[3]. Combined with other clinical prognostic factors such as age and tumor differentiation, individual prognosis can be more

Table 1 Distribution of the clinical and reproductive factors for female patients with esophageal squamous cell carcinoma in primary training, internal and external validation cohorts (mean ± SD)

Characteristics	Primary training cohort (%)	Internal validation cohort (%)	External validation cohort (%)
Total	175 (61.2)	111 (38.8)	85 (100)
Diagnose age			
< 50	21 (12.0)	16 (14.4)	10 (11.8)
50-60	59 (33.7)	47 (42.3)	35 (41.2)
> 60	95 (54.3)	48 (43.2)	40 (47.1)
Age	61.35 ± 7.99	60.25 ± 7.97	60.15 ± 8.50
Incidence area			
High	138 (78.9)	89 (80.2)	64 (75.3)
Low	37 (21.1)	22 (19.8)	21 (24.7)
Tumor location			
Upper	34 (19.4)	19 (17.1)	11 (12.9)
Middle	113 (64.6)	79 (71.2)	66 (77.6)
Lower	28 (16.0)	13 (11.7)	8 (9.4)
Differentiation			
Higher	11 (6.3)	8 (7.2)	3 (3.5)
Moderate	101 (57.7)	67 (60.4)	51 (60.0)
Lower	63 (36.0)	36 (32.4)	31 (36.5)
T stage			
T1/T2	64 (36.6)	32 (28.8)	26 (30.6)
T3/T4	111 (63.4)	79 (71.2)	59 (69.4)
N stage			
N0	86 (49.1)	60 (54.1)	46 (54.1)
N1-N3	89 (50.9)	51 (45.9)	39 (45.9)
Therapy methods			
Operation	164 (93.7)	106 (95.5)	82 (96.5)
Others	11 (6.3)	5 (4.5)	3 (3.5)
Family history			
No	116 (66.3)	66 (59.5)	56 (65.9)
Yes	59 (33.7)	45 (40.5)	29 (34.1)
ESR1			
Negative	49 (28.0)	41 (36.9)	29 (34.1)
Positive	126 (72.0)	70 (63.1)	56 (65.9)
ESR2			
Negative	63 (36.0)	39 (35.1)	24 (28.2)
Positive	112 (64.0)	72 (64.9)	61 (71.8)
Menarche age			
< 14	28 (16.0)	23 (20.7)	12 (14.1)
14-16	85 (48.6)	45 (40.5)	41 (48.2)
> 16	62 (35.4)	43 (38.7)	32 (37.6)
Menopausal age			

< 46	48 (27.4)	19 (17.1)	23 (27.1)
46-55	47 (26.9)	36 (32.4)	20 (23.5)
> 55	80 (45.7)	56 (50.5)	42 (49.4)
Pregnancy number			
< 4	61 (34.9)	40 (36.0)	30 (35.3)
4-6	86 (49.1)	52 (46.8)	43 (50.6)
> 6	28 (16.0)	19 (17.1)	12 (14.1)

T: Tumor invasion depth; N: Lymph node metastasis; ESR1: Estrogen receptor alpha; ESR2: Estrogen receptor beta.

accurately predicted[10,11]. An effective predictive model is needed for female ESCC to precisely assess the clinical outcome.

A nomogram is a graphical representation tool based on statistical predictive modeling[12]. So far, no nomogram including reproductive factors has been constructed to predict OS in female ESCC. The current study was designed to identify independent prognostic factors based on univariatie and Cox proportional hazards survival analysis, and then develop and validate a new prognostic nomogram incorporating clinical and reproductive characteristics to predict 1-, 3-, and 5-years OS in female ESCC. We also aimed to establish whether the nomogram model could provide more accurate prognostic prediction than the clinical model and TNM stage.

MATERIALS AND METHODS

Study population

This retrospective study was approved by the Ethics Committee of the First Affiliated Hospital of Zhengzhou University and Institutional Review Board of the First Affiliated Hospital of Nanyang Medical College. The documentation of informed consent was waived due to anonymity of the participants. In the multicenter study, the analysis was performed on the two independent cohorts of female patients with ESCC (Cohort 1, 500 000 esophageal and gastric cardiac carcinoma database of First Affiliated Hospital of Zhengzhou University[3]; Cohort 2, First Affiliated Hospital of Nanyang Medical College). Patients who were pathologically diagnosed with ESCC, underwent surgery, and had detailed follow-up information where eligible. Patients who received preoperative therapy (radiotherapy and/or chemotherapy) and lost clinical and reproductive information were excluded.

Baseline characteristics and clinical outcome

Clinicopathological factors, including age at diagnosis, incidence area, tumor location, tumor differentiation, tumor invasion depth (T), lymph node metastasis (N), metastasis, and therapy methods were collected from the medical records. Reproductive factors such as menarchal age, menopausal status, menopausal age, pregnancy number, and biomarker levels [estrogen receptor alpha (ESR1) and estrogen receptor beta (ESR2)] were also recorded. All eligible patients were followed-up every 3 mo *via* telephone interview or outpatient review. Patients were followed to the latest lost-to-follow-up time (December 31, 2020) or time of death. In our study, the primary clinical outcome was OS which was defined with reference to our previous study[3].

Construction and validation of the nomogram

Factors affecting OS in univariate analysis ($P < 0.20$) were included in a Cox proportional hazards regression for multivariable analysis[13]. Hazard ratio and 95% confidence interval were assessed. After testing for collinearity, a prognostic nomogram was constructed to predict 1-, 3-, and 5-years OS for female ESCC. The discrimination was evaluated using the time-dependent receiver operating characteristic (ROC) and concordance index (C-index) calculated by bootstrapping with 1000 resamples[14]. Calibration was examined *via* calibration plots. Decision curve analysis (DCA) was conducted to evaluate the clinical application and net benefit at different threshold probabilities[15]. Patients in the study were separated into high- or low-risk groups based on the best cut-off point of total prognostic score (TPS) that was decided by X-Tile software[16]. Survival curves were plotted using the Kaplan–Meier analysis. Sub-analysis was conducted to confirm the potential correlations between risk score and OS among different subgroups in the primary training cohort.

Correlations with immune-related pathways and gene markers of immune cells

We analyzed the correlations between estrogen response and immune-related pathways such as p53

Table 2 Comparison of three models in primary training, internal validation, and external validation sets

	Primary training set		Internal validation set		External validation set	
	C-index (95%CI)	*P* value	C-index (95%CI)	*P* value	C-index (95%CI)	*P* value
Full model	0.701 (0.655-0.746)	Ref.	0.684 (0.619-0.748)	Ref.	0.672 (0.609-0.734)	Ref.
Clinical-model	0.629 (0.578-0.681)	0.000	0.593 (0.531-0.655)	0.009	0.589 (0.514-0.663)	0.014
TNM stage	0.638 (0.576-0.699)	0.013	0.552 (0.462-0.641)	0.011	0.560 (0.462-0.657)	0.033

Full model: Pregnancy number + menopausal age + estrogen receptor alpha + estrogen receptor beta + N stage + differentiation + diagnose age + incidence area; Clinical model: N stage + differentiation + diagnose age+ incidence area; CI: Confidence interval; TNM stage: Tumor-node-metastasis stage; N: Lymph node metastasis.

pathway and apoptosis pathway in esophageal cancer from The Cancer Genome Atlas (TCGA) by R packages. We also assessed the associations of ESR1 and ESR2 levels with gene markers of tumor-infiltrating immune cells, including B cells, tumor-associated macrophages (TAMs), M1 macrophages, M2 macrophages, and neutrophils by TIMER 2.0 (http://timer.comp-genomics.org/).

Statistical analysis

All statistical analyses were conducted using R language (version 4.1.0). Baseline characteristics of female ESCC patients were summarized. Student's *t* test was used to analyze the continuous variables, and categorical variables were compared with the χ^2 test and Fisher's exact bilateral test. Survival curves were conducted by using the Kaplan–Meier method, and OS was analyzed with a log-rank test. The rms, Hmisc, survival, survcomp, and ggplot2 packages were used for analysis. $P < 0.05$ (two-sided) was considered statistically significant.

RESULTS

Patient characteristics

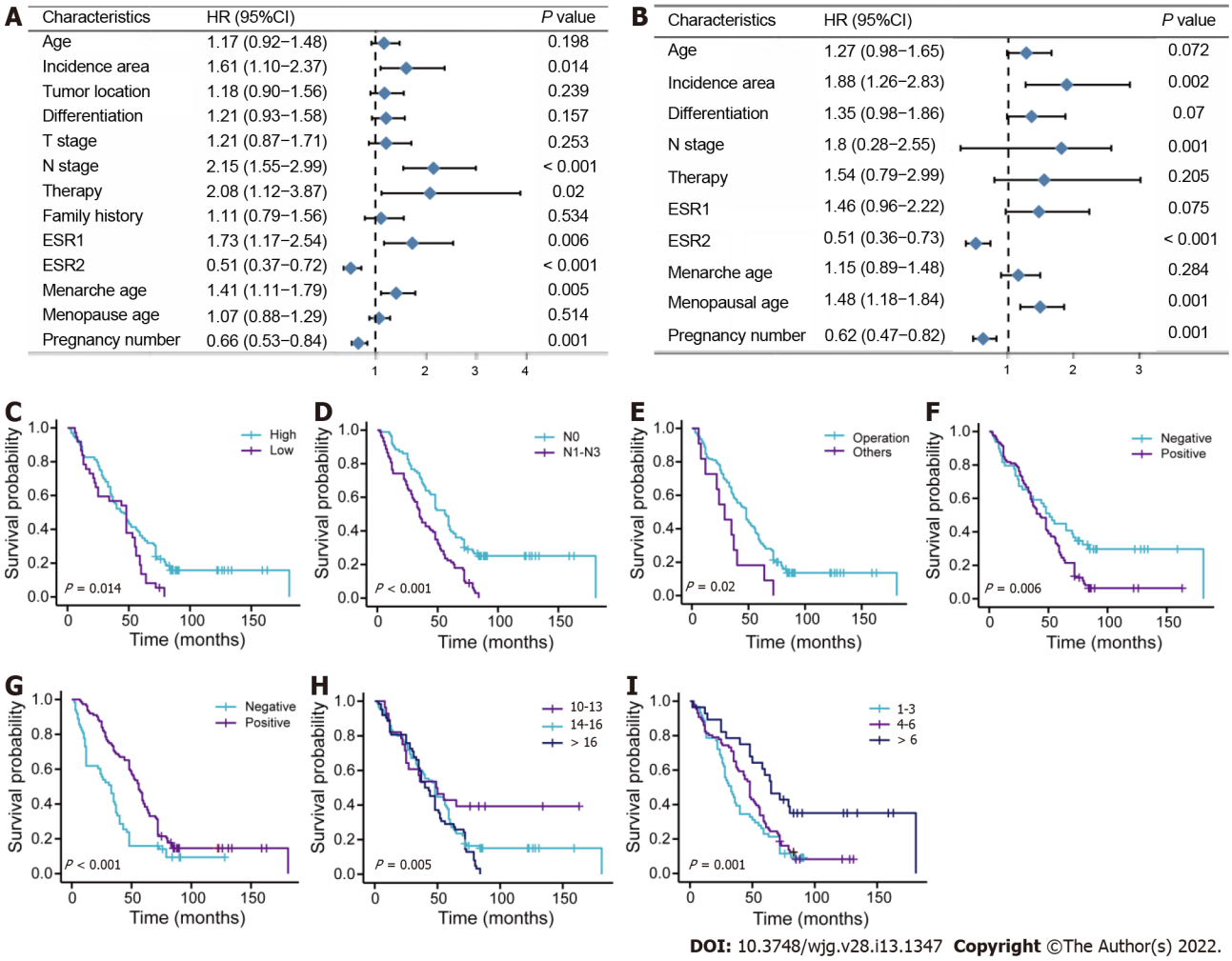
In Cohort 1, all 286 eligible patients were randomly divided into two sets according by computer-generated random numbers: 175 (60%) (mean age, 61.35 years; range, 41–78 years) were randomly assigned to a primary training set; 111 (40%) (mean age, 60.25 years; range, 42–81 years) to an internal validation set. An independent dataset (n = 85) from Cohort 2 was established to be an external validation cohort. The mean age was 60.15 years with a range of 42–80 years. The median survival time in the primary training cohort was 47.59 mo and the 1-, 3-, and 5-years OS rates were 84.0%, 58.9% and 31.4%, respectively. In the internal validation cohort, the median survival time was 45.63 mo, and the 1-, 3-, and 5-years OS rates were 85.6%, 64.0% and 31.5%, respectively. The median survival time of the external validation cohort was 39.94 mo, and the 1-, 3-, and 5-years OS rates were 84.7%, 56.5% and 23.5%, respectively. The detailed distribution of baseline characteristics of the primary training, internal validation, and external validation datasets are shown in Table 1.

Prognostic roles of clinical and reproductive variables

The prognostic role of each variable in OS was tested in the training cohort (Figure 1). Incidence area (P = 0.014; high *vs* low area), N stage ($P < 0.001$; N0 *vs* N1–N3), therapy methods (P = 0.02; operation *vs* other methods), ESR1 expression level (P = 0.006; negative *vs* positive), ESR2 level ($P < 0.001$; negative *vs* positive), menarche age (P = 0.005; > 16 years *vs* < 13 years), and pregnancy number (P = 0.001; > 6 *vs* < 4–6) were significantly associated with OS in the univariate analysis (Figure 1A). Data were also represented using Kaplan–Meier curves (Figure 1C-I). Parameters associated with $P < 0.20$ based on univariate analysis and relevant clinical factors were entered into a Cox proportional hazards regression model, which included age, incidence area, tumor differentiation, N stage, therapy, ESR1, ESR2, menarche age, menopausal age, and pregnancy number (Figure 1B). No evidence of problematic multicollinearity was found.

Building and performance of prognostic nomogram

To address patient prognosis, we identified a nomogram model for the prognosis prediction of female ESCC patients at 1-, 3-, and 5-years OS in the primary training cohort. Incidence area, diagnose age, differentiation, N stage, ESR1 expression, ESR2 expression, menopausal age, and pregnancy number were finally included in the full model (Figure 2). Pregnancy number contributed to the highest points in the model, followed by menopausal age, N stage, differentiation, and ESR2 status. Incidence area, age and ESR1 Level had a minor impact on OS. To investigate whether the clinical–reproductive model had incremental prognostic value for individualized OS prediction, a clinical model was also constructed by



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Figure 1 Survival analysis for female patients with esophageal squamous cell carcinoma in primary training cohort. A: Univariate analysis; B: Multivariate analysis; C-I: Survival curves for patients with incidence area, lymph node metastasis, surgery methods, estrogen receptor alpha, estrogen receptor beta, menarche age, and pregnancy number, respectively. T: Tumor invasion depth; ESR1: Estrogen receptor alpha; ESR2: Estrogen receptor beta; N: Lymph node metastasis.

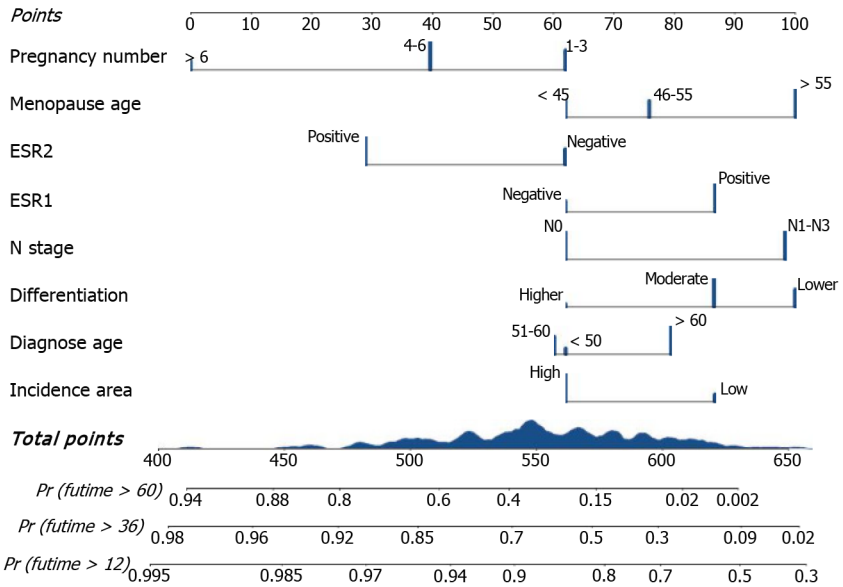
only including incidence area, age, differentiation, and N stage.

To further reveal the prognostic prediction of the full model, patients were categorized into high- and low-risk groups based on the optimal cut-off value of TPS in the primary training set. The relationships among risk scores and survival status of patients are shown in Figure 3A. Kaplan–Meier survival curves revealed that patients with higher risk scores had significantly poorer OS (0.28, 95%CI: 0.20–0.40, P < 0.001, Figure 3B). Using the same cut-off values, we identified the same risk groups in both internal and external validation sets with OS represented by Kaplan–Meier curves (0.47, 95%CI: 0.30–0.73, P = 0.001; 0.54, 95%CI: 0.34–0.85, P = 0.009, respectively, Figure 3C–F). In addition to highly differentiated patients and patients who received other treatments (surgery plus postoperative radiotherapy, surgery plus postoperative chemotherapy), the clinical–reproductive model also maintained a good and stable prediction performance among other subgroups (Figure 4).

Calibration and validation of clinical–reproductive model

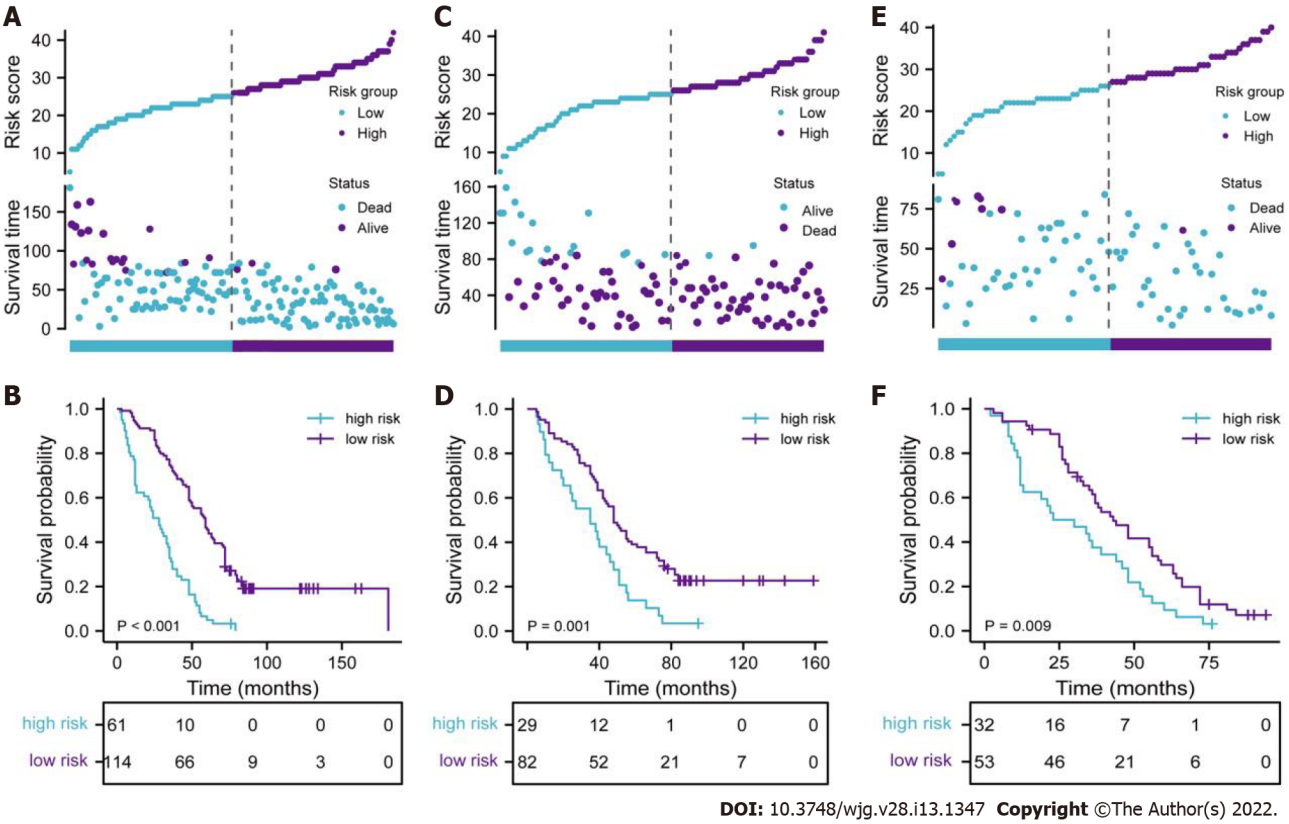
The Harrell C-indexes are summarized in Table 2 for the full model, clinical model, and TNM stage in the training, internal and external validation sets. For OS prediction, the clinical model achieved a C-index of 0.629 (95%CI: 0.578–0.681) in the training set, with internal and external validation C-index of (0.593, 95%CI: 0.531–0.655, and 0.589, 95%CI: 0.514–0.633, respectively). After integrating the clinical with reproductive risk factors, the C-index of the full model increased to 0.701 (95%CI: 0.655–0.746, P = 0.000) in the training set, 0.684 (95%CI: 0.619–0.748, P = 0.009) in the internal validation, and 0.672 (95%CI: 0.609–0.734, P = 0.014) in the external validation set. The C-indexes for OS were also higher than those for TNM stage in all of the three cohorts (P = 0.013, 0.011 and 0.033, respectively) (Table 2).

The calibration suggested that the OS prediction at 1-, 3-, and 5-years was well matched to the actual outcomes in the primary training cohort (Figure 5A). The time-dependent ROC curve also demonstrated that the full model showed a good performance in predicting OS in the training set (Figure 5B). The AUC at 1-, 3-, and 5-years was 0.792, 0.738 and 0.789, respectively. These discoveries were verified in



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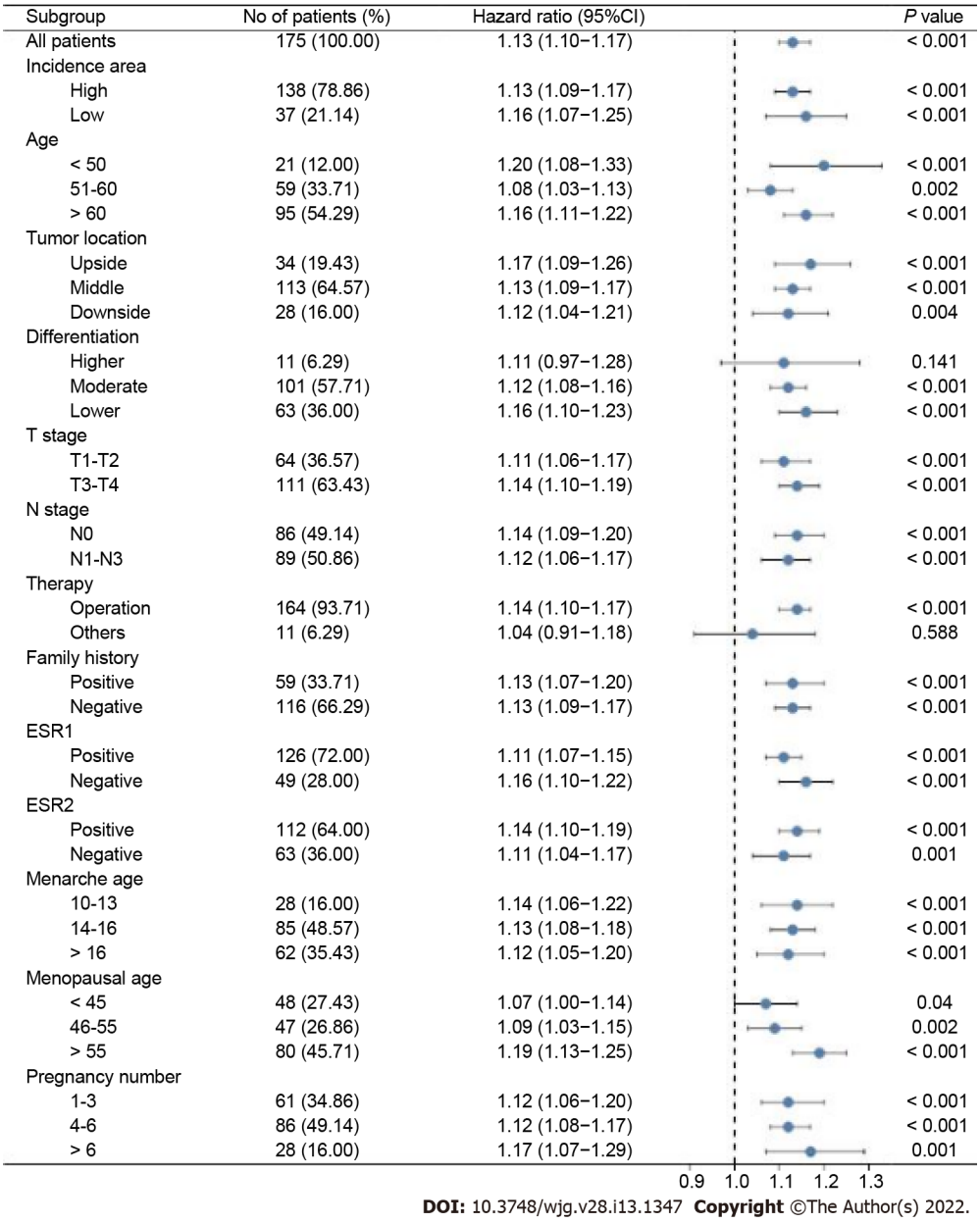
Figure 2 The Clinical-reproductive model for predicting 1-, 3-, and 5-yr overall survival probability in female patients with esophageal squamous cell carcinoma. ESR1: Estrogen receptor alpha; ESR2: Estrogen receptor beta; N: Lymph node metastasis.



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Figure 3 Evaluation of full model performance in the primary training set and confirmation based on both internal and external validation sets. A, C, and E: Distribution of risk scores and survival status in primary training, internal and external validation sets, respectively; B, D, and F: Kaplan-Meier survival curves in primary training, internal and external validation sets, respectively.

two validation sets (Figure 5C–F). Compared with the clinical model and TNM stage, the predicted value of the full model was in good agreement with the OS at 1 year (Figure 6A), 3 years (Figure 6B), and 5 years (Figure 6C). The full model also confirmed the better discrimination for OS at 1 year (AUC: 0.792 *vs* 0.719 *vs* 0.744; Figure 6D), 3 years (AUC: 0.738 *vs* 0.631 *vs* 0.635; Figure 6E), and 5 years (AUC: 0.789 *vs* 0.629 *vs* 0.640; Figure 6F) OS in the primary training cohort. DCA for 18 mo OS prediction showed that the clinical-reproductive model yielded a larger net benefit than either clinical model or



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Figure 4 Stratified analysis of the clinical-reproductive model in different subgroups. T: Tumor invasion depth, N: Lymph node metastasis; ESR1: Estrogen receptor alpha; ESR2: Estrogen receptor beta.

TNM stage when the threshold probability was > 0.10 (Figure 7).

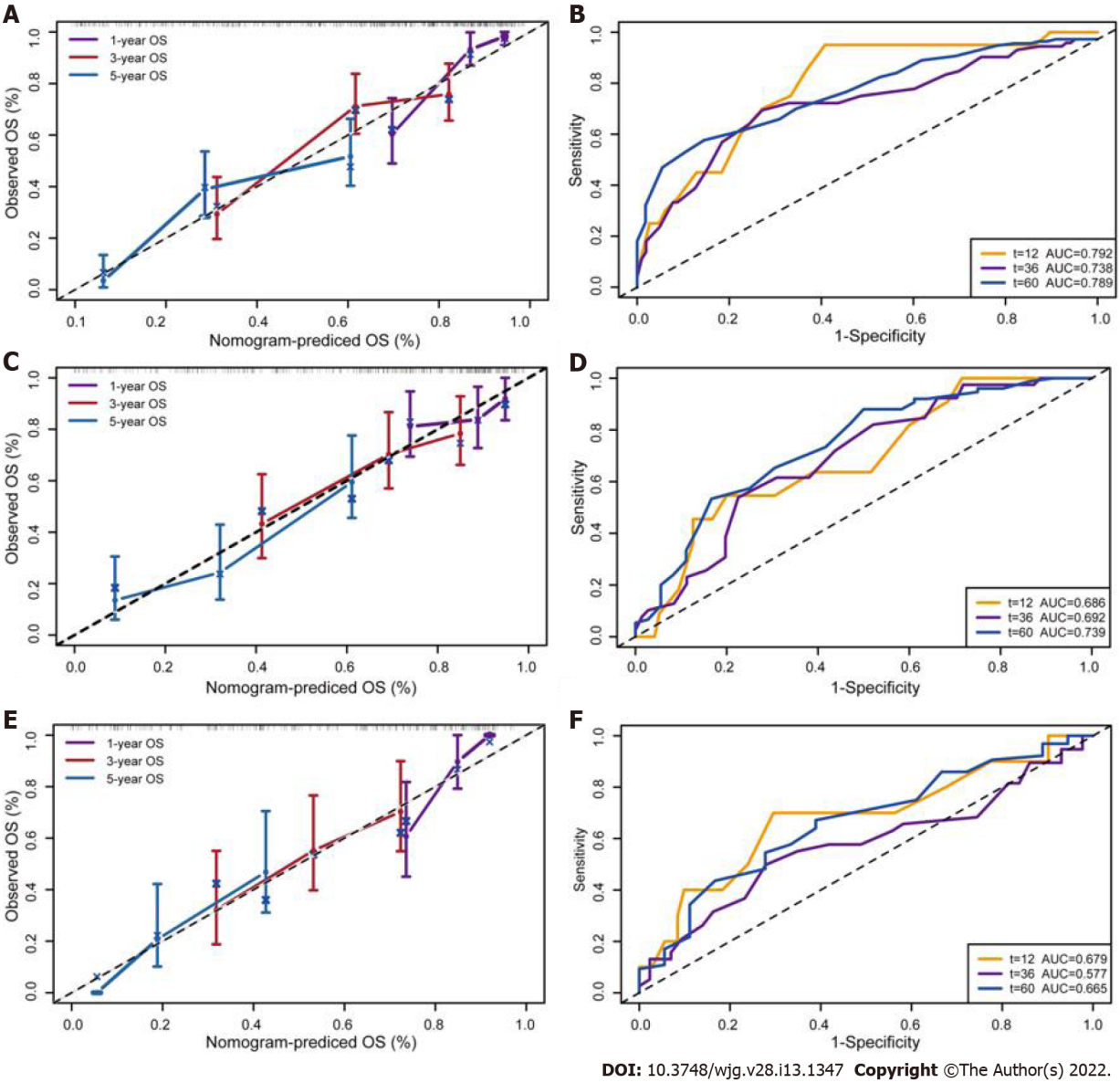
Correlations with immune-related pathways and gene markers of immune cells

The estrogen response was positively correlated with p53 pathway (r = 0.436, P < 0.001), and apoptosis pathway (r = 0.245, P < 0.001) (Supplementary Figure 1). ESR1 and ESR2 Levels were positively correlated with some biomarkers of immune cells or their subsets (Supplementary Table 1).

Clinical relevance of clinical–reproductive model

The relationships between the full model and clinical parameters are shown in Figure 8. In terms of TNM stage, female patients with III/IV stage ESCC had significantly higher risk scores than patients with I/II stage disease had (P < 0.001, Figure 8A). The risk scores were also higher for patients with than those without lymph node metastasis (P < 0.001, Figure 8B). No significant difference was found in patients with different T stages (P = 0.152, Figure 8C).

To improve the predictive performance and clinical utility of the full model, we transferred the data and formulae to a user-friendly website. Figure 9 shows a snapshot of web-based nomogram that is available on predictbcos.shaws.cn: https://female-escc-predictor.shinyapps.io/DynNomapp/. Visitors can select values from the drop-down list according to the circumstance of clinical and reproductive factors, and then click the "predict" button to predict the OS rates in Chinese female ESCC.



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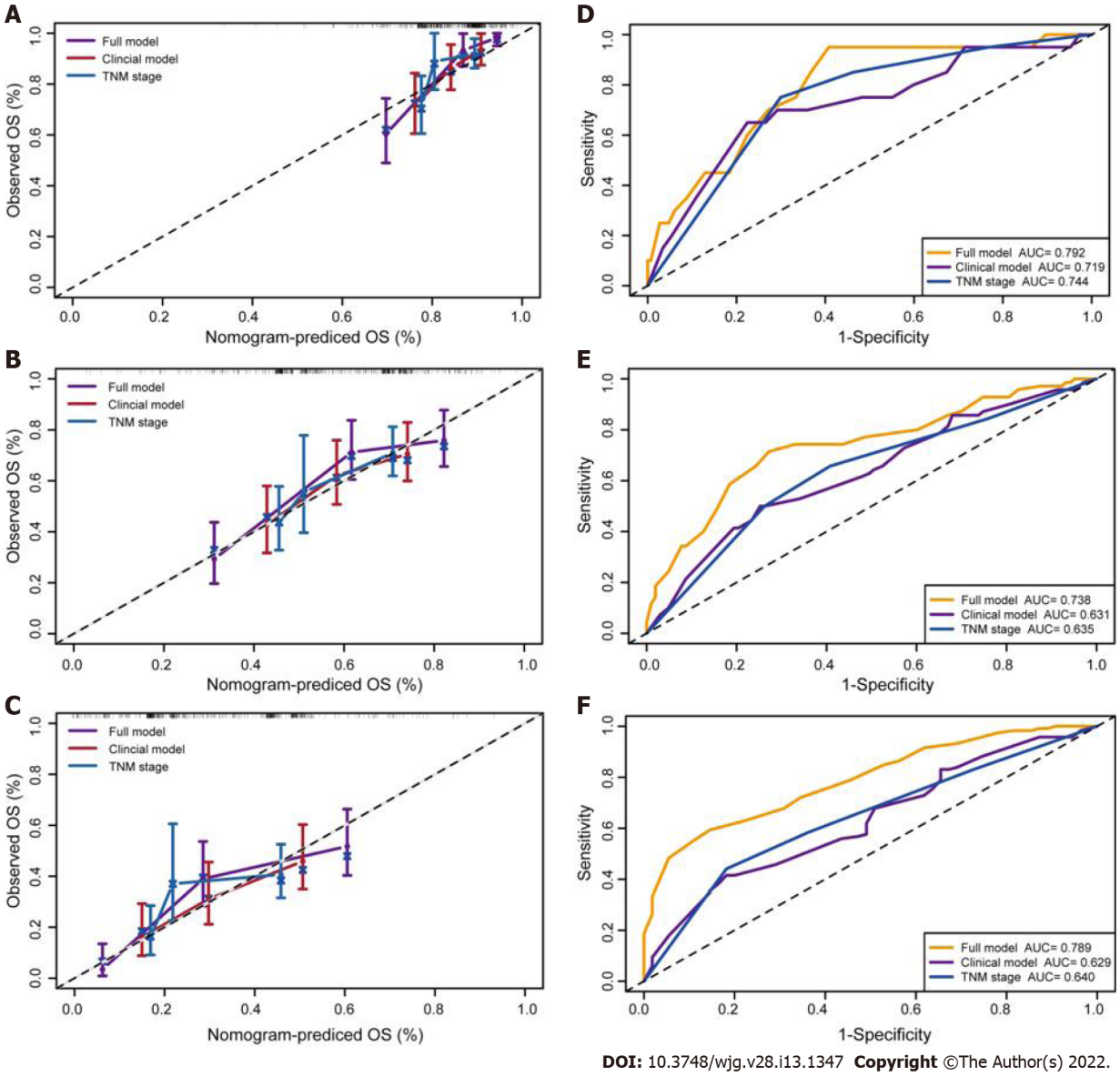
Figure 5 Calibration curves and time-dependent receiver operating characteristic curves for validation of clinical-reproductive model in the primary training, internal and external validation cohorts. A and B: Calibration curves for predicting 1-, 3-, and 5-yr overall survival and time-dependent receiver operating characteristic (ROC) curves in primary training cohort; C and D: Calibration curves and time-dependent ROC curves in internal validation cohort; E and F: Calibration curves and time-dependent ROC curves in external validation cohort. OS: Overall survival.

DISCUSSION

Although advances have been achieved in the prevention and treatment of ESCC in the past few decades, the prognosis of ESCC is still poor with high mortality rates[17,18]. Traditionally, the TNM staging system has been used to predict the prognosis of many cancers. However, accumulated studies have reported that the prognostic predictive probability of clinical nomograms is more accurate than that of TNM stage due to the incorporation of all known significant prognostic factors of individual patients[19-21]. To date, nomograms have been widely applied in clinical prognostic evaluation for several cancers[22-24].

We constructed and validated a clinical–reproductive prognostic model for assessing the added value of reproductive factors over existing risk factors in female ESCC. Our results revealed that reproductive factors have independent prognostic value with respect to clinical parameters for individualized OS prediction in female ESCC. Subgroup analysis showed that the prognosis of female patients in the low-risk group was significantly better than that in the high-risk group. Our results of discrimination, calibration and DCA curves also showed that the clinical–reproductive model had enhanced prognostic value compared with clinical model and TNM stage.

To further explore the potential molecular mechanism of increased prognostic value of our clinical–reproductive model in female ESCC, we analyzed the correlations of estrogen response with

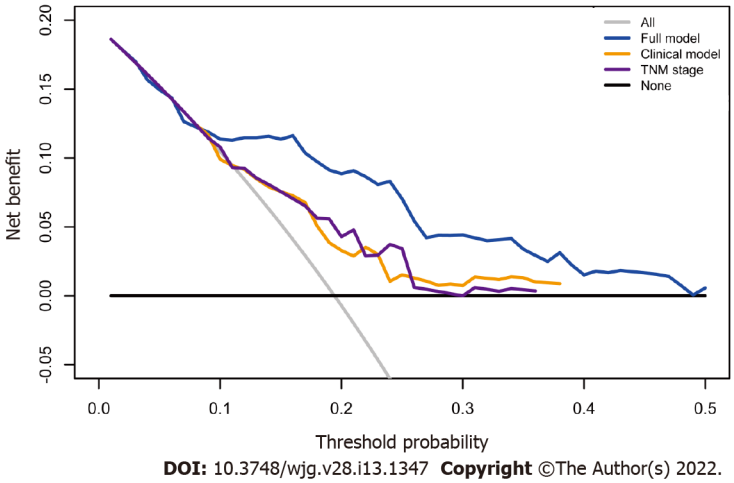


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Figure 6 Calibration curves and time-dependent receiver operating characteristic curves of 1-, 3-, and 5-yr overall survival prediction for the full model, clinical model and tumor-node-metastasis stage in the primary training set. A-C: Calibration curves for predicting 1-, 3-, and 5-yr overall survival (OS) in primary training set, respectively; D-F: Time-dependent receiver operating characteristic curves of 1-, 3-, and 5 yr OS prediction in primary training cohort, respectively. Full model: Pregnancy number + menopausal age + estrogen receptor alpha + estrogen receptor beta + N stage + differentiation + diagnose age + incidence area; Clinical model: N stage + differentiation + diagnose age + incidence area; TNM stage: Tumor-node-metastasis stage.

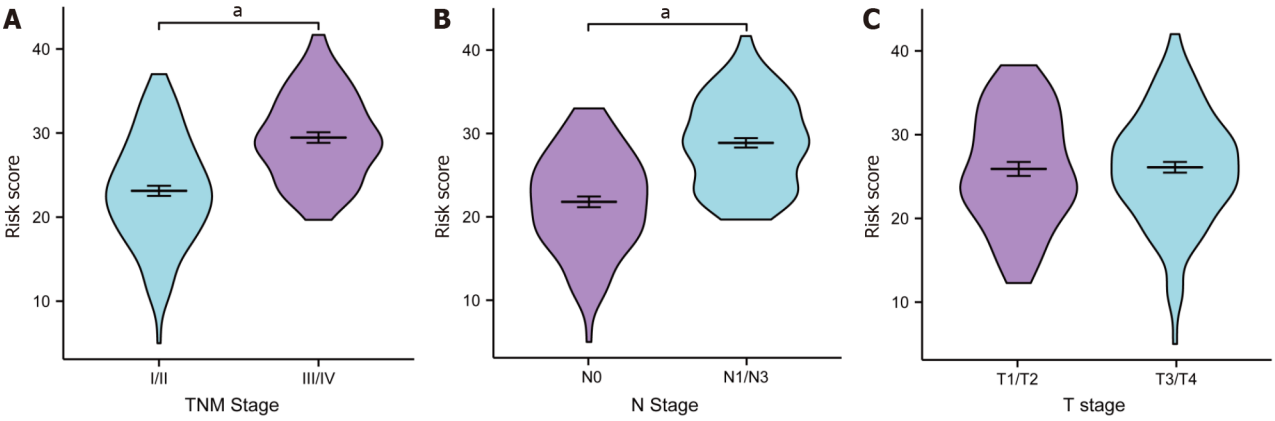
immune-related pathways and biomarker genes of immune cells in esophageal cancer. An in vitro study has found that estrogen mediates the apoptosis of esophageal cancer cells by interacting with ERs[25]. The p53 signaling pathway serves as a major barrier to prevent the occurrence and progression of cancer [26,27]. Our results revealed that estrogen response could prolong clinical outcome by promoting the p53 pathway and inducing apoptosis. ESCC expresses ERs and estrogen plays a protective role through ERs[28]. Overexpression of ERs is associated with prognosis and female ESCC patients with higher ESR2 expression have a better prognosis and those with higher ESR1 seem to have shorter survival[3]. In the present study, the expression levels of some biomarker genes in immune cells were positively correlated with ESR1 and ESR2 levels. Significant correlations were found between ESR2 and PTGS2 (Prostaglandin-Endoperoxide Synthase 2, biomarker of M1 macrophages), ESR1 and CD163, VSIG4 (V-Set And Immunoglobulin Domain Containing 4), MS4A4A (Membrane Spanning 4-Domains A4A, biomarker of M2 macrophages). It is well known that different cell subtypes may have distinct roles in the immune microenvironment. M1 macrophages showed an antitumor effect after being activated by Th1 cytokines, while M2 macrophages showed protumor activity[29]. This may partly explain the survival difference in female ESCC patients with different expression levels of ESR1 and ESR2.

Several studies have developed some prognostic nomograms to predict prognosis in ESCC. These models provide useful tools to stratify the risk and predict survival probability in ESCC. However, the patients in these studies were mainly extracted from the Surveillance, Epidemiology, and Final Results



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Figure 7 Decision curve analysis for full model, clinical model, and tumor node metastasis stage. Black line: All patients were dead. Gray line: None of patients was dead. Full model: Pregnancy number + menopausal age + estrogen receptor alpha + estrogen receptor beta + N stage + differentiation + diagnose age + incidence area; Clinical model: N stage + differentiation + diagnose age+ incidence area; TNM stage: Tumor-node-metastasis stage.



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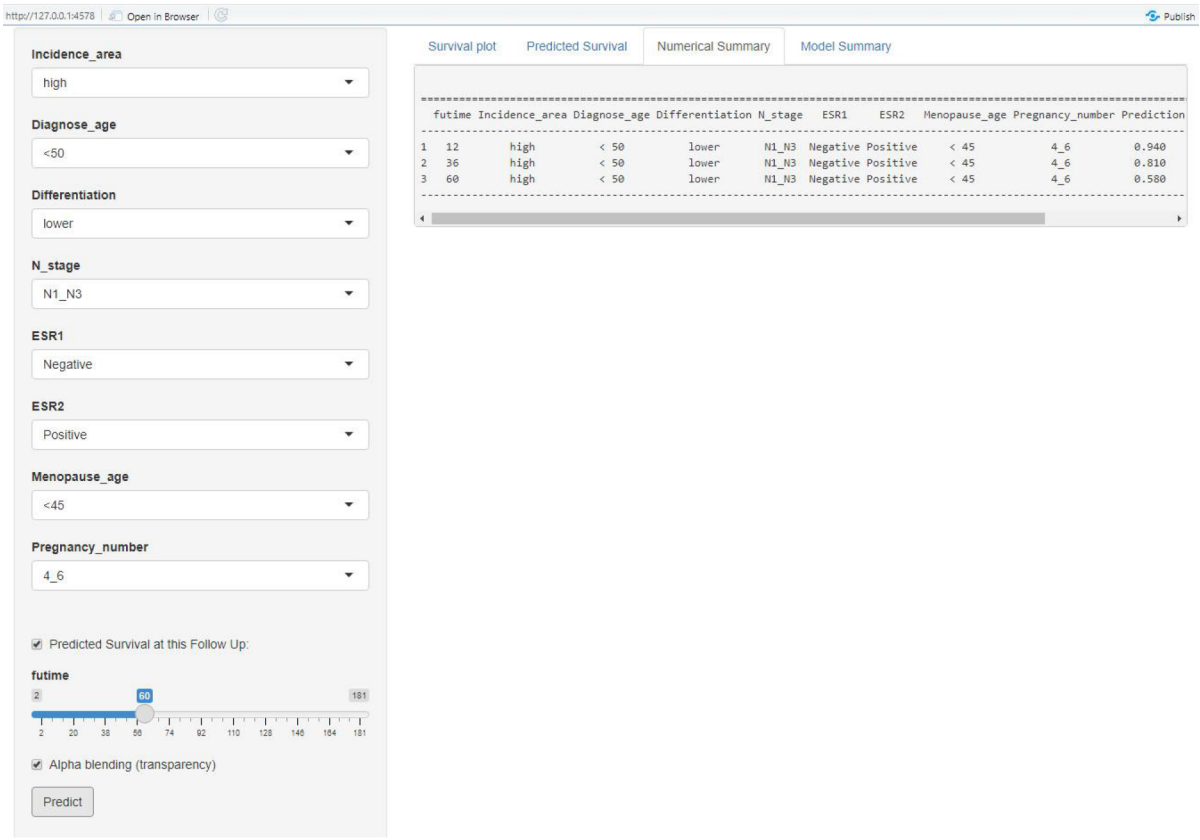
Figure 8 Clinical relevance of full model. Distribution of the risk score based on different tumor-node-metastasis (TNM) stage, lymph node metastasis (N) stage, and tumor invasion depth (T) stage in the primary training cohort. A: TNM stage; B: N stage; C: T stage. [a]*P* < 0.001. T: Tumor invasion depth, N: Lymph node metastasis; TNM stage: Tumor-node-metastasis stage.

(SEER) database in the United States, and these models were not entirely suitable for predicting OS for Chinese female ESCC due to the ethnic difference. In our study, the internal C-index of our model was 0.701, which is higher than two previously released models (0.67 and 0.66 for both nomograms)[30,31]. Although the C-index of the Yu *et al*[32] model was higher (0.749), the nomogram can only be used to predict the clinical outcome of T1 esophageal cancer patients with positive lymph nodes. A recent study on breast cancer showed that user-friendly online prognostic tools could greatly improve patient care [33]. There is no such online tool available for female ESCC. We implemented our nomogram in an online webserver, which means that clinicians can easily use it to predict prognosis. For example, a 46-year-old menopausal ESCC patient with five pregnancies showed a poorly differentiated tumor with positive lymph node metastasis, and the tumor was ESR1 negative and ESR2 positive. The predicted results by the website show that the OS rates at 1-, 3-, and 5-years were 0.94, 0.81 and 0.58, respectively.

There were some limitations to our research. First, our analysis may have been affected by bias and loss of follow-up due to the retrospective nature of the study. Second, we must be careful to extrapolate our findings to patients of other races because most of our patients were of Han nationality. Finally, the in-depth molecular mechanisms of reproductive factors in progression and prognosis of female ESCC depend on further experimental studies to elucidate.

CONCLUSION

We developed and verified a prognostic nomogram with clinical and reproductive factors to improve



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Figure 9 Screenshot from the web-based nomogram for predicting 1-, 3-, and 5-yr overall survival for female patients with esophageal squamous cell carcinoma. N: Lymph node metastasis; ESR1: Estrogen receptor alpha; ESR2: Estrogen receptor beta.

the accuracy of prognostic prediction in Chinese female patients with ESCC. Compared with the clinical model and TNM stage, the full model has increased prognostic value and can help clinicians to make individual treatment and medical decisions.

ARTICLE HIGHLIGHTS

Research background

Nomogram has been widely used and proved to be more accurate than the tumor-node-metastasis (TNM) staging system for predicting prognosis in different cancers. In China, female patients with esophageal squamous cell carcinoma (ESCC), even with the same TNM stage, had distinct overall survival (OS) difference, which requires an effective prediction model to accurately evaluate the prognosis.

Research motivation

Several studies have developed some prognosis nomogram models, which provide useful tools for predicting OS probability in patients with esophageal cancer. The included patients were mainly extracted from the Surveillance, Epidemiology, and Final Results (SEER) database in United States. Due to the ethnic difference, the models are not entirely applicable to perform the OS prediction for Chinese female ESCC.

Research objectives

The purpose of the study was to develop and validate a clinical-reproductive model for predicting OS in Chinese female patients with ESCC, and to further explore whether the model had higher prognostic value than the clinical model and TNM stage.

Research methods

A new prognostic nomogram incorporating clinical and reproductive characteristics was constructed in the primary training cohort and verified in the internal and external validation cohorts. The

performance of the full model was evaluated by Kaplan-Meier curve, time-dependent receiver operating characteristic (ROC) curve, Harrell's concordance index (C-index), calibration curve and decision curve analysis.

Research results

The clinical-reproductive model incorporated incidence area, age, differentiation, N stage, estrogen receptor alpha expression, estrogen receptor beta expression, menopausal age, and pregnancy number. Compared to the clinical model and TNM stage, the ROC curve and C-index indicated good discriminative ability of the full model for predicting 1-, 3-, and 5-years OS in the primary, internal, and external validation sets.

Research conclusions

A clinical-reproductive nomogram for OS prediction in Chinese female ESCC was developed and validated in the present study, which showed superior survival prediction than the clinical model and TNM stage.

Research perspectives

The clinical–reproductive model has incremental prognostic predictive value in Chinese female ESCC, which may be beneficial to individualized treatment and medical decision-making.

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FOOTNOTES

Author contributions: Wang LD and Zhang DY designed and wrote the paper; Ku JW, Xu RH, Wang R, Wu HF, and Fan ZM performed the data collection, interpretation and follow-up; Zhang DY, Zhao XK, Zhou RX, and Song X contributed to the data analysis; all authors performed the final approvlement.

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Country/Territory of origin: China

ORCID number: Dong-Yun Zhang 0000-0001-5885-9238; Jian-Wei Ku 0000-0002-0015-4662; Xue-Ke Zhao 0000-0002-9036-6342; Hai-Yan Zhang 0000-0001-5611-2034; Xin Song 0000-0002-7680-4908; Hong-Fang Wu 0000-0001-7713-4523; Zong-Min Fan 0000-0003-0087-3410; Rui-Hua Xu 0000-0002-6914-2168; Duo You 0000-0003-1035-8375; Ran Wang 0000-0002-2588-5760; Ruo-Xi Zhou 0000-0001-5461-0400; Li-Dong Wang 0000-0001-5103-8226.

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META-ANALYSIS

Generic and disease-specific health-related quality of life in patients with Hirschsprung disease: A systematic review and meta-analysis

Veerle Huizer, Naveen Wijekoon, Daniëlle Roorda, Jaap Oosterlaan, Marc A Benninga, LW Ernest van Heurn, Shaman Rajindrajith, Joep PM Derikx

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Veerle Huizer, Daniëlle Roorda, LW Ernest van Heurn, Joep PM Derikx, Department of Pediatric Surgery, Emma Children's Hospital, Amsterdam UMC, University of Amsterdam and Vrije Universiteit Amsterdam, Amsterdam Reproduction and Development, Amsterdam 1105 AZ, Netherlands

Naveen Wijekoon, Department of Surgery, University of Colombo and Department of Paediatric Surgery, Lady Ridgeway Hospital for Children, Colombo 00800, Sri Lanka

Daniëlle Roorda, Jaap Oosterlaan, Marc A Benninga, Department of Pediatrics, Emma Children's Hospital, Amsterdam UMC, University of Amsterdam, Emma Neuroscience Group, Amsterdam Reproduction and Development, Amsterdam 1105 AZ, Netherlands

Shaman Rajindrajith, Department of Pediatrics, Lady Ridgeway Hospital for Children, Colombo 00800, Sri Lanka

Corresponding author: Danielle Roorda, MD, MSc, Research Scientist, Department of Pediatric Surgery, Emma Children's Hospital, Amsterdam UMC, University of Amsterdam and Vrije Universiteit Amsterdam, Amsterdam Reproduction and Development, Meibergdreef 9, Amsterdam 1105 AZ, Netherlands. d.roorda@amsterdamumc.nl

Abstract

BACKGROUND

Patients with Hirschsprung disease (HD) are at risk of persistent constipation, fecal incontinence or recurrent enterocolitis after surgical treatment, which in turn may impact physical and psychosocial functioning. Generic health-related quality of life (HRQoL) and disease-specific health-related quality of life are relevant outcome measures to assess the impact of HD on the QoL of these patients.

AIM

To summarize all available evidence on HRQoL of patients with HD after surgery and the impact of possible moderating factors.

METHODS

Pubmed, Web of Sciences, PsycInfo and Embase were searched with search terms related to 'Hirschsprung disease', 'Pediatrics' and 'Quality of life'. Mean and standard deviation of generic HRQoL overall and domain scores were extracted from each study, as well as data describing potential factors associated with QoL. Random effect models were used for meta-analytic aggregation of generic HRQoL

scores. Meta-regression was used to assess the relationship between patient and clinical characteristics and generic HRQoL. Disease-specific HRQoL outcomes of patients with HD were systematically reviewed.

RESULTS

Seventeen articles were included in the systematic review (n = 1137 patients) and 15 in the quantitative meta-analysis (n = 1024 patients). Four studies reported disease-specific HRQoL. Patient's age ranged between 0 and 21 years. Meta-analytic aggregation showed a non-significantly impaired generic HRQoL (d = -0.168 [95%CI: -0.481; 0.145], P = 0.293, I^2 = 94.9) in patients with HD compared to healthy controls. Physical (d = -0.042 [95%CI: -0.419; 0.335], P = 0.829, I^2 = 95.1), psychosocial (d = -0.159 [95%CI: -0.458; 0.141], P = 0.299, I^2 = 93.6) and social HRQoL (d = -0.092 [95%CI: -0.642; 0.457], P = 0.742, I^2 = 92.3) were also not significantly lower compared to healthy controls. There was no relation between health-related outcomes and the sex of the patients and whether generic HRQoL was measured by parental proxy or self-report. Disease-specific complaints of patients with HD impaired physical HRQoL, but not psychosocial and social HRQoL.

CONCLUSION

In this systematic review and meta-analysis, no evidence was found for impaired generic HRQoL in patients with HD compared to healthy controls, neither for moderating effects of sex, parental proxy or self-report.

Key Words: Hirschsprung disease; Health-related quality of life; Meta-analysis; Systematic review; Pediatrics

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Core Tip: Patients with Hirschsprung disease are at risk of constipation, fecal incontinence or enterocolitis after surgery, which in turn may negatively impact physical and psychosocial functioning. In this meta-analysis of 17 available studies representing 1137 patients, we found no evidence that patients with Hirschsprung disease have lower generic health-related quality of life (HRQoL) compared to healthy controls. Generic HRQoL was not dependent of sex of patients and not dependent of whether quality of life was measured by self-report or by parental proxy-report. Disease-specific complaints of patients with HD impaired physical HRQoL, but not psychosocial and social HRQoL.

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INTRODUCTION

Hirschsprung disease (HD) is a congenital anomaly with an incidence of approximately 1 in 5000 live births, characterized by a lack of ganglionic cells in the enteric nervous system of a distal segment of the gastrointestinal tract[1-3]. Affected neonates typically present with failure to pass meconium followed by obstructive defecation problems[2]. In most patients, the aganglionosis extends no further proximally than the rectum or rectosigmoid, which is defined as short segment disease[4]. In some patients aganglionosis is more severe and extends further proximal, with extension to the complete colon or small intestine in 5%-10%[5]. Definitive surgical management for HD involves resection of the affected bowel segment and restoration of bowel continuity with a straight anastomosis or a pouch[5]. Even after surgical resection of the aganglionic segment, it may take years for patients to acquire normal bowel function and continence[2]. In addition, patients are at risk of long-term disease-specific problems, including persistent constipation (11%-16%)[1,6-11], fecal soiling or incontinence (7%-48%)[1,6-11] or recurrent episodes of enterocolitis (0%-33%)[1,6-11]. HD can therefore be regarded as a chronic bowel condition, which may severely impact daily functioning.

According to the WHO, quality of life (QoL) can be described as a person's subjective evaluation of their position in life in the context of their culture and value systems[12]. Health-related QoL (HRQoL) describes a person's subjective evaluation of their physical and mental health[13]. HRQoL can be considered as a multidimensional construct and its evaluation generally relies on the patient's subjective

evaluation of well-being and functioning in different aspects and dimensions of well-being and functioning, together resulting in an overall construct[14]. Instruments that measure HRQoL can be generic and disease-specific[15]. Instruments that measure generic HRQoL can be used to measure HRQoL both in healthy and ill children and can be used for the comparison of HRQoL accros different conditions and settings, whereas disease-specific HRQoL instruments only measure HRQoL in patients with a certain condition, and are typically better in detecting changes in HRQoL over time[15]. For patients with HD the disease-specific questionnaire HAQL (Hirschsprung disease / Anorectal malformation Quality of Life) has been developed[4].

There is growing interest in HRQoL of patients with HD, as shown by the increasing number of studies reporting on generic and disease-specific HRQoL in these patients. The current body of evidence is inconsistent about whether HRQoL and functional outcomes are lower in patients with HD compared to healthy controls[6,7,11,15,16]. The available literature suggests evidence for several factors to be related to HRQoL and functional outcomes, including patient age[1,3,17], the use of parental proxy *vs* self-report[18], length of aganglionosis[19], type of surgical procedure and anastomosis technique used in surgery[20], postoperative complications[20-25], presence of a stoma[26-28] and syndromal anomalies [29]. Thus far the available evidence on generic HRQoL and disease-specific HRQoL among HD patients has not been systematically reviewed and aggregated precluding clear conclusions to be drawn.

The primary aim of this systematic review and meta-analysis was to summarize all available evidence on differences in generic health-related quality of life between patients with HD and normative data. Secondary aim was to summarize available evidence on disease-specific health-related quality of life of patients with HD. Third aim was to study patient and clinical factors that could explain differences in generic HRQoL between patients and normative data, including sex, age, the use of parental proxy *vs* self-report, length of aganglionosis, operation technique, postoperative complications, presence of stoma and syndromal anomalies using meta-regression.

MATERIALS AND METHODS

This meta-analysis was conducted according to the PRISMA guidelines[30].

Eligibility criteria

The following eligibility criteria were used: (1) The sample consisted of patients diagnosed with HD and a medical history of surgical resection of the affected bowel segment; (2) The majority of the patients included in the study had the age between 0 and 18 years old at assessment; (3) Generic HRQoL was measured as outcome; (4) Studies had an observational or case-control design and used HRQoL measures for which normative data is available, or compared patients with HD with normative data or a healthy control group; and (5) The study reported original data, with a minimum sample size arbitrarily set at $n = 10$. Studies were excluded if the full text was only available in a language other than English.

Search and selection

Pubmed, Web of Sciences, PsycInfo and Embase were searched using entry terms related to: 'Hirschsprung disease', 'Pediatrics' and 'Quality of life'. The full search strategy can be found in the Supplementary Material. Reference lists of included articles were checked for additional studies matching our eligibility criteria. Screening of title, abstract and full-text of the studies was performed by two independent researchers (N.W. and V.H.) using the online software Covidence[31]. Initial disagreements on study selection were discussed and in case of persistent conflicting judgement, a third party (D.R.) was consulted to reach consensus.

Data extraction

As primary outcome measurement, the mean scores and corresponding standard deviation of generic HRQoL were extracted by one researcher (V.H.). Additionally, the following characteristics were extracted from each study: domain scores (physical, psychosocial and social HRQoL), publication year, study design, sample size, type of questionnaire used to assess HRQoL, mean age of patients, percentage of male patients , percentage of measurements with parental proxy and self-report, percentage of patients with short aganglionic bowel segment, type of surgery (percentage of patients operated using the Duhamel technique and percentage of patients operated using Transanal Endorectal Pull-through [TEPT] technique), percentage of patients with postoperative complications, percentage of patients with a stoma present, and percentage of patients who had a syndromal comorbidity. If required data was not reported, authors were requested by email to provide additional data. In case of non-response of the author after one reminder and when available data was not sufficient for adequate analysis, the study was excluded from the analyses. In case a study reported median generic HRQoL scores, these were recalculated to mean scores[32,33], or else the median was considered to be the best approximation of the mean. For those studies that did not include a control group, questionnaire specific local age- and sex-matched normative values were used and compared with the reported outcomes of patients with HD[34-39].

Quality assessment

The Newcastle-Ottawa Scale (NOS) was used to assess quality of the studies in this meta-analysis[40]. According to the manual, we adapted the scoring system to our study design. A detailed description of the adjustment can be found in the Supplementary Material. The included studies were rated on a 9-point rating scale by two of the authors (N.W. and V.H.), based on aspects of participant selection (4 points), group comparability (2 points) and outcome assessment (3 points). Quality of studies was considered good, fair and poor using AHRQ (Agency for Health Research and Quality) criteria[41]. Rating discrepancies were resolved by consensus.

Statistical analysis

Statistical analysis was performed using CMA (Comprehensive Meta-Analysis)[42]. The standardized mean difference (expressed as Cohen's d) of generic HRQoL and domain scores between patients with HD and healthy control or normative populations were calculated for each study. Effect sizes d of 0.2, 0.5 and 0.8 were taken as thresholds to define small, medium and large effects, respectively[43]. For all outcomes, heterogeneity of effect sizes was quantified using I^2 statistic. Heterogeneity was regarded small ($I^2 \leq 0.25$), moderate ($0.25 < I^2 < 0.50$) or large ($I^2 \geq 0.50$), according to Higgins[44]. In case of moderate or large heterogeneity, random effects models were used.

Individual study findings were presented in forest plots and aggregated into summary estimates of standardized mean differences for: (1) Generic HRQoL; (2) Physical HRQoL; (3) Psychosocial HRQoL; and (4) Social HRQoL. In case a study reported on more than one outcome, within study findings were pooled into an weighted estimate on these predefined domains, using a built-in function in CMA. A detailed description of which items in different HRQoL-measurements were pooled into domain scores can be found in the Supplementary Material. In case a study reported no generic HRQoL score, the mean of at least two reported domain scores was considered as a reflection of the generic HRQoL.

Publication bias analysis included visual inspection of funnel plots and calculation of Egger's intercept. Robustness of the calculated aggregated effect sizes against the influence of publication bias was assessed with fail safe N. An effect was considered robust when fail safe N > 5k+10[45].

To explore sources of heterogeneity, subgroup analyses were performed to assess differences in each of the HRQoL outcomes between: (1) Studies reporting on different age groups ([< 12 years], [12-16 years] and [16+ years]); (2) Studies using different questionnaires to assess HRQoL (for example assessment by the CHQ *vs* PedsQL); (3) Studies that used a control group and studies that used normative reference data; and (4) Studies that reported generic HRQoL scores and studies in which a generic score was constructed from domain scores. Sensitivity analysis was used to explore the influence of study quality on summary estimates of all HRQoL outcomes. The moderating effect of sex and parental proxy *vs* self-report on generic HRQoL scores was explored using univariate meta-regression which was only conducted in case of at least 10 observations.

Narrative analysis of disease-specific HRQoL

Since the HAQL is a disease-specific questionnaire, a quantitative comparison with healthy controls could not be made. Designating one cohort as reference group is random and was therefore not considered to be meaningful in this context. Thus pooled summary estimates for the HAQL scores were made and findings within individual studies, in term of domains on which relatively lower disease-specific HRQoL scores were reported, were narratively described.

RESULTS

The flowchart of the study search and selection process is provided in Figure 1. Our search yielded 334 unique records, of which 17 studies (n = 1141 patients) were included in the current systematic review and of which 15 studies contributed data to the meta-analysis.

Study characteristics

Table 1 summarizes the characteristics of the 17 included studies. Of the 17 studies, 15 studies measured generic HRQoL and four studies measured disease-specific HRQoL. The PedsQL was most frequently used to assess generic HRQoL (in 10 of 15 studies). All four studies used the HAQL to assess disease-specific HRQoL. Four out of 17 studies used a controlled study design. Patients' ages ranged from 0 to 21 years.

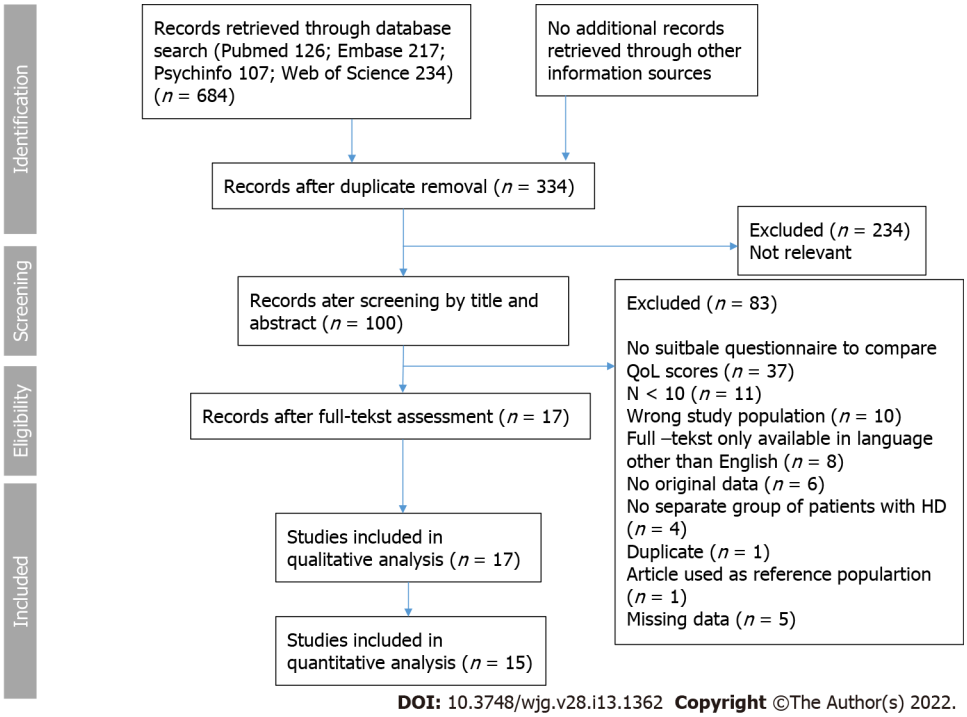
Generic Health-related Quality of Life

Generic HRQoL: Fifteen (15) studies (n = 1024 patients) were included in the meta-analysis comparing patients with HD to normative data or controls on generic HRQoL. A significantly lower generic HRQoL was found for patients with HD in 4 out of 15 studies, whereas 3 out of 15 studies found a significantly higher generic HRQoL in patients. Meta-analytic aggregation showed a non-significantly impaired generic HRQoL (d = -0.168 [95%CI: -0.481; 0.145], P = 0.293, I^2 = 94.9) in patients with HD

Table 1 Characteristics of included studies

Ref.	Study design	Sample size	Self-report	Parental proxy report	HRQoL instrument	Age range (yr)	Mean age (yr)	Sex (% male)	Operation-technique	Segment-length (% short)	Syndromal comorbidity (%)	Stoma present (%)	Postoperative complications (%)
Amin *et al* [50], 2018	Cohort	46	0	46	PedsQL	0-18	4.2	71		80			
Baayen *et al*[70], 2017	Cohort	50	50	0	HAQL	12-16						6	
Cavusoglu *et al*[46], 2012	Case-control	12	6	12	PedsQL	2-12	4.7	84					
Collins *et al*[26], 2017	Cohort	60	0	60	PedsQL	2-10	6.4	82	13% Duhamel 80% Trans-anal PT	78	12	13	
Espeso *et al*[21], 2019	Cohort	63			HAQL	6-18	10.8	70	22% Duhamel 78% Trans-anal PT	79	0	0	44
Hartman *et al*[53], 2007	Cohort	121	121	0	HAQL / TACQoL	8-16	11.4	82		69	0	4	
Hartman *et al*[49], 2008	Cohort	152	152	0	TACQoL	8-16	11.6	80		68	0	3	
Khalil *et al* [47], 2015	Cohort	53	0	53	PedsQL	5-7	5.9	70					3
Lane *et al* [52], 2016	Cohort	118	0	118	PedsQL	0-17							
Meinds *et al*[17], 2019	Cohort	150	0	150	CHQ-CF87	8-17		79	62% Duhamel 16% Trans-anal PT	84		0	31
Mills *et al* [25], 2008	Cohort	51	37	44	PedsQL	3-21	9.8	82	15% Duhamel 77% Trans-anal PT	66	6		
Nah *et al* [33], 2018	Case-control	44	21	42	PedsQL	2-20	9.1	75		73			
Neuvonen *et al*[23], 2017	Case-control	39	27	12	PedsQL	0-17		75	96% Trans-anal PT	84	24	5	47
Roorda *et al*[61], 2018	Cohort	18 /14	7	7	HAQL / CHQ-CF87 / CHQ-PF50	4-17		77	67% Duhamel	0	13	6	
Sood *et al* [48], 2018	Cohort	58	38	58	PedsQL	11-18	14.5	84	7% Duhamel 86% Trans-anal PT	84	12	7	28
Townley *et al*[67], 2019	Cohort	56	0	56	PedsQL	0-13	5.4	80	84% Duhamel 16% Trans-anal PT	77		14	
Xi *et al* [50], 2018	Case-control	50	0	50	TACQoL	6	6.0	76	100% Trans- anal PT				

HRQoL: Health-related quality of life; HAQL: Hirschsprung disease/anorectal malformation quality of life.



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Figure 1 PRISMA flowchart of study selection.

compared to healthy controls (Figure 2). Visual interpretation of the funnel plot and Egger's test (t = 0.841; P = 0.416) suggested that there was no indication of publication bias, and the aggregated effect was also not robust with a fail safe N of 9 studies.

Physical HRQoL: Twelve (12) studies (n = 774 patients) were included in the meta-analysis comparing patients with HD to normative data or controls on physical HRQoL. A significantly lower physical HRQoL was found for patients with HD in 3 out of 12 studies, whereas 3 out of 12 studies found a significantly higher physical HRQoL in patients. Meta-analytic aggregation showed a non-significantly lower physical HRQoL (d = -0.042 [95%CI: -0.419; 0.335], P = 0.829, I^2 = 95.1) in patients with HD compared to healthy controls (Figure 2). Visual interpretation of the funnel plot and Egger's test (t = 1.391; P = 0.194) suggested that there was no indication of publication bias, and the aggregated effect was also not robust with a fail safe N of 0 studies.

Psychosocial HRQoL: Thirteen (13) studies (n = 924 patients) were included in the meta-analysis of comparing patients with HD to normative data or controls on psychosocial HRQoL. A significantly lower psychosocial HRQoL was found for patients with HD in 6 out of 13 studies, whereas 4 out of 13 studies found a significantly higher psychosocial HRQoL in patients. Meta-analytic aggregation showed a non-significantly lower psychosocial HRQoL (d = -0.159 [95%CI: -0.458; 0.141], P = 0.299, I^2 = 93.6) in patients with HD compared to healthy controls (Figure 2). Visual interpretation of the funnel plot and Egger's test (t = 0.476; P = 0.643) suggested that there was no indication of publication bias, and the aggregated effect was also not robust with a fail safe N of 18 studies.

Social HRQoL: Five (5) studies (n = 308 patients) were included in the meta-analysis comparing patients with HD to normative data or controls on social HRQoL. A significantly lower social HRQoL was found for patients with HD in 1 out of 5 studies, whereas 1 out of 5 studies found a significantly higher social HRQoL in patients. Meta-analytic aggregation showed a non-significantly lower social HRQoL (d = -0.092 [95%CI: -0.642; 0.457], P = 0.742, I^2 = 92.3), in patients with HD compared to healthy controls (Figure 2). Visual interpretation of the funnel plot and Egger's test (t = 0.554; P = 0.618) suggested there was no indication of publication bias, and the aggregated effect was also not robust with a fail safe N of 0 studies.

Patient and clinical factors explaining differences in generic HRQoL

Table 2 summarizes the results from the subgroup analyses. Meta-analytic effects for generic HRQoL did not significantly differ between age groups. There was an influence of the type of questionnaire used, as meta-analytic aggregation of studies using the CHQ showed a significant medium-sized impairment in generic HRQoL in patients with HD compared to normative data or controls, whereas meta-analytic aggregation of studies using the TACQoL or PedsQL showed no significant differences. Meta-analytic aggregation also showed no significant differences between studies comparing patients to

Table 2 Differences in health-related quality of life scores in subgroup analyses

Factor associated with HRQoL		Cohen's d [95%CI], *P* value[1]	*Q* value, *P* value[2]
Age	[0-12 yr]	*d* = -0.206 [-0.721, 0.310], *P* = 0.434	Q = 1.727, *P* = 0.422
	[12-16 yr]	*d* = 0.609 [-0.492, 1.711], *P* = 0.278	
	[16+ yr]	*d* = -0.033 [-1.170, 1.103], *P* = 0.954	
Type of questionnaire	PedsQL	*d* = -0.043 [-0.281, 0.195], *P* = 0.724	Q = 6.370, *P* = 0.041[a]
	TACQoL	*d* = -0.562 [-2.271, 1.147], *P* = 0.519	
	CHQ-CF87	*d* = -0.412 [-0.575,-0.249], *P* < 0.001[b]	
Reference data	Normative scores	*d* = 0.028 [-0.278, 0.334], *P* = 0.859	Q = 1.768, *P* = 0.184
	Selected controls	*d* = -0.793 [-1.965, 0.378], *P* = 0.184	
Overall HRQoL scores	Reported overall HRQoL scores	*d* = -0.440 [-0.817,-0.063], *P* = 0.022[a]	Q = 4.078, *P* = 0.043[a]
	Constructed overall HRQoL scores	*d* = 0.245 [-0.303, 0.792], *P* = 0.381	

[1]The difference in scores between patients with Hirschsprung disease and normative or control groups was calculated for each study and expressed as the standardized difference in means (Cohen's d) and aggregated across studies.
[2]A Q-test was done to test for significant heterogeneity between groups.
[a]*P* < 0.05.
[b]*P* < 0.01. HRQoL: Health-related quality of life.

normative reference data and studies comparing patients with constructed control groups. The complexity of the construct of generic HRQoL was expressed by the difference that was seen when comparing the effects sizes of studies reporting generic HRQoL scores and studies for which a generic HRQoL score was derived from the average of domain scores. Meta-analytic aggregation showed a significant medium-sized impairment in generic HRQoL when only studies were included that reported generic HRQoL scores, whereas meta-analytic aggregation showed no significant effect when only studies were included in which a generic HRQoL score was constructed from the average of the reported domain scores. There was no relationship between the percentage of male patients in studies and the individual study's effect sizes for generic HRQoL (b = 0.308, P = 0.631, R^2 = 0.00). Also the percentage of measurements with self-report in studies was not significantly associated with effect sizes for generic HRQoL (b = 0.331, P = 0.467, R^2 = 0.00). Based on the amount of observations, statistical power was too small to test the influence of age, length of aganglionosis, operation technique, postoperative complications, presence of stoma and syndromal anomalies on generic HRQoL of patients with HD.

Disease-specific health-related quality of life

Disease-specific HRQoL was reported in 4 studies (n = 252 patients). Hartman *et al*[49] only reported an overall QoL score, with 5 domain scores constructed into a single HAQL score (18.3 ± 1.7), which represented a good disease-specific HRQoL, but none of the other studies with the HAQL used this total disease-specific HRQoL score, making it impossible to compare these findings to those obtained in other studies. The domain scores of the remaining 3 studies were aggregated to construct summary estimates for the HAQL scores, of which an overview is presented in Table 3. Physical symptoms impacted disease-specific physical HRQoL negatively in all 3 studies, which is similar to the findings reported by Hanneman *et al*[4], where physical symptoms had the lowest mean rank score. Across all studies, diarrhea was the second factor to negatively impacted disease-specific HRQoL, and – to a lesser extent - fecal incontinence. The domains laxative diet, constipating diet, constipation, urinary continence, social functioning, emotional functioning and body image had no significant negative impact on disease-specific HRQoL. In summary, all studies showed an impairment on disease-specific physical HRQoL. Psychosocial and social HRQoL were not negatively influenced by disease-specific complaints in all four studies.

Quality of the evidence

The majority of the studies in the current systematic review were of good quality (10 out of 17 studies, 59%), whereas one study was of fair and 6 studies were of poor quality according to the NOS (Table 4). Meta-analytic estimates of generic HRQoL did not differ significantly between studies of good, fair and poor quality (Q = 2.220; P = 0.330), neither did meta-analytic estimates of physical HRQoL (Q = 4.391; P = 0.111) and psychosocial HRQoL (Q = 2.705; P = 0.259). However, meta-analytic estimates of social HRQoL did differ significantly between studies of good, fair and poor quality (Q = 9.285; P = 0.010). More specifically, social HRQoL was rated higher in the one study of low quality (d = 0.589 [95%CI:

Table 3 Summary estimates Hirschsprung disease / Anorectal malformation Quality of Life scores

Summary estimates HAQL scores	Aggregated HAQL domain scores[21,61,70][1]	Hanneman *et al*[4], 2001
Domain	Mean (SD)	Mean rank
Laxative diet	88.41 (19.63)	64.67
Constipating diet	91.67 (17.39)	63.77
Diarrhea	73.32 (26.86)	61.22
Constipation	83.33 (36.51)	64.75
Urinary continence	94.82 (13.57)	65.72
Fecal incontinence	78.70 (24.47)	68.58
Social functioning	93.77 (15.40)	65.33
Emotional functioning	84.42 (19.92)	65.11
Body image	84.70 (20.08)	65.48
Physical symptoms	69.33 (19.09)	58.52

[1]The average of mean scores was calculated across domain scores of each studies and aggregated into a weighted mean (SD). HAQL: Hirschsprung disease/anorectal malformation quality of life.

Table 4 Study quality assessment according to the Newcastle-Ottawa Scale criteria

Ref.	Selection	Comparability	Outcome	Assessment
Amin *et al*[50], 2018	2 points		2 points	Poor
Baayen *et al*[70], 2017	1 point		2 points	Poor
Cavusoglu *et al*[46], 2012	3 points	2 points	2 points	Good
Collins *et al*[26], 2017	3 points	1 point	3 points	Good
Espeso *et al*[21], 2019	1 point		2 points	Poor
Hartman *et al*[53], 2007	2 points		2 points	Poor
Hartman *et al*[49], 2008	3 points	1 point	3 points	Good
Khalil *et al*[47], 2015	2 points		3 points	Poor
Lane *et al*[52], 2016	2 points		3 points	Poor
Meinds *et al*[17], 2019	3 points	1 point	2 points	Good
Mills *et al*[25], 2008	3 points	1 point	2 points	Good
Nah *et al*[33], 2018	3 points	2 points	2 points	Good
Neuvonen *et al*[23], 2017	3 points	2 points	2 points	Good
Roorda *et al*[61], 2018	2 points	1 point	2 points	Fair
Sood *et al*[48], 2018	3 points	1 point	3 points	Good
Townley *et al*[67], 2019	3 points	1 point	3 points	Good
Xi *et al*[50], 2018	3 points	2 points	3 points	Good

0.317; 0.861], $P < 0.001$, $n = 1$), compared to those of fair and good quality.

When including only studies of good quality, meta-analytic aggregation showed a medium-sized significantly impaired generic HRQoL in patients compared to normative data or controls (d = -0.342 [95%CI: -0.665; -0.019], $P = 0.038$). When including only studies of good quality for psychosocial HRQoL, meta-analytic aggregation showed a medium-sized significantly impaired psychosocial HRQoL (d = -0.339 [95%CI: -0.656; -0.022], $P = 0.036$), whereas meta-analytic aggregation of only studies with good quality showed no significant differences between patients and normative data or controls for physical HRQoL (d = -0.199 [95%CI: -0.527; 0.129], $P = 0.235$) and social HRQoL (d = -0.322 [95%CI: -1.033; 0.390], $P = 0.376$).

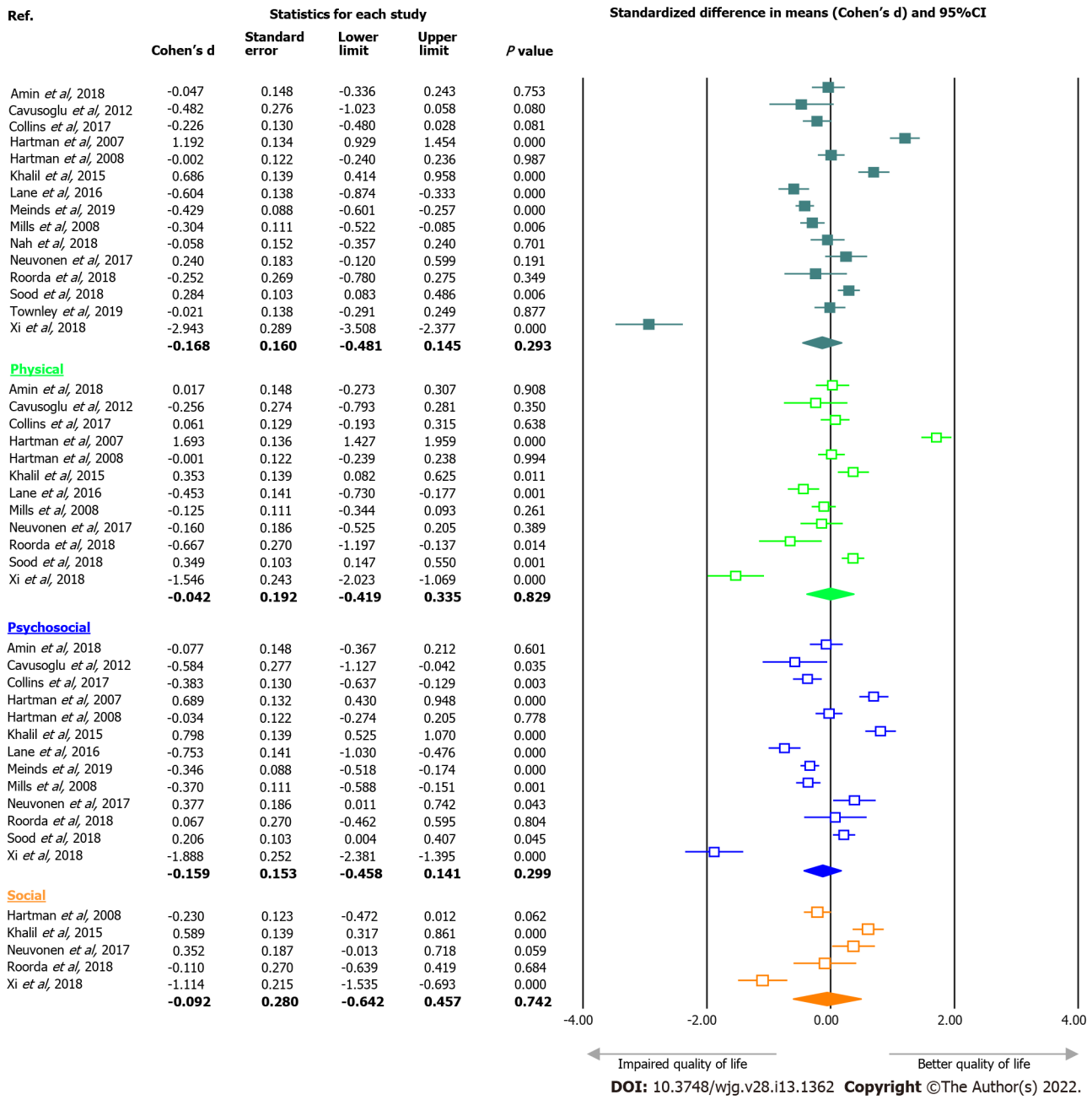


Figure 2 Forest plots of total health-related quality of life (HRQoL), physical HRQoL, psychosocial HRQoL and social HRQoL of patients with Hirschsprung disease compared to normative data.

DISCUSSION

The findings of this systematic review and meta-analysis suggest that generic HRQoL is not impaired in patients with HD compared to healthy controls and that physical HRQoL is most impaired as a result of disease-specific complaints. The high heterogeneity in HRQoL findings among the included studies implies that there are underlying factors moderating the HRQoL of patients with HD. Our findings indicated that generic HRQoL is not influenced by sex and type of respondent (parental proxy or self-report) and that generic HRQoL did not differ significantly between different age groups. The quantity of the available evidence did not allow to test for the moderating effect of length of aganglionosis, operation technique, postoperative complications, presence of a stoma and the presence of a syndromal anomalies on generic HRQoL.

Findings in several studies have shown no differences in HRQoL between male and female patients, which also corresponds to our findings[26,46-48]. Furthermore, there is evidence suggesting that HRQoL varies with increasing age and is influenced by factors like coping strategies[23,48,49] and psychological changes accompanying life events, including puberty[11,16,25,26,49-54]. Adolescents may have less ability to adapt their lifestyle because of the influence of peer pressure and the wish to adapt to their peers and not be different from their peers. Adult patients and parents of patients with HD are

more capable to adapt their lifestyle, which may result in better HRQoL in children and adults patients, compared to adolescent patients. Findings in previous studies have shown that parents tend to overestimate problems and impairments in patients with chronic diseases[41,55-58]. This in turn may be influenced by their cultural, social and educational background[39,46,53,59]. Also anxiety in parents of patients with HD may play an important role as well[54,60].

The severity of HD may also impact HRQoL. In this meta-analysis we could not test for the relation between length of disease and generic HRQoL. Findings from earlier studies are inconsistent[26,48,61,62], but suggest that HRQoL may not so much be dependent on length of disease itself, but with factors associated with differences in length of aganglionosis, including occurrence of obstructive defecation problems, postoperative complications and the operation technique that was used[49,53,61,63]. Also, current evidence is inconclusive on which type of the operation technique is associated with better HRQoL outcome[63-66]. Frequently reported disease-specific sequelae including obstipation[1,11,16,23,25,26,48,49,53,61,67,68], fecal incontinence[1,11,16,23,25,26,48,49,53,61,67,68] and the presence of a stoma [1,11,16,23,25,26,48,49,53,61,67] have shown to be associated with impaired HRQoL.

Previous studies have suggested that patients with HD who have an associated syndrome, may have lower HRQoL than patients with non-syndromal HD. In particular Down syndrome is known to be associated with lower QoL[29,69]. Patients with an associated syndrome unfortunately were underrepresented in the studies included in this meta-analysis, as these patients were often excluded from questionnaire surveys because of mental retardation that is associated with syndromes. Therefore, the influence of having an associated syndrome on HRQoL outcome could not be assessed in this study.

Our findings indicate that overall HRQoL estimates cannot simply be calculated by taking the average of domain scores, suggesting different weight of different aspects of health on the overall evaluation of HRQoL. This may be explained by differences in the items based on which the domain scores are constructed, which varies between questionnaires. But it may also reflect that HRQoL is a challenging multidimensional construct to measure. Some dimensions of health may have more influence on overall HRQoL than others, which may be based on the extent to which functioning in that domain is limited, but may also be under the influence of personal beliefs, goals and coping strategies.

The relationship between functional outcome and HRQoL outcome also remains subject to debate. Based on the available evidence in the current study, the relationship between functional outcome and HRQoL could not be tested. Our findings on disease-specific HRQoL suggest that some disease-specific symptoms, in particular diarrhea and fecal incontinence, may impair physical functioning of patients with HD rather than psychosocial and social functioning. Differences in outcome may be explained by differences in selection of cohorts between the few studies describing disease-specific HRQoL, as some cohorts included patients with Anorectal Malformation (ARM) and another cohort consisted only of patients with a severe form of HD: Total Colonic Aganglionosis (TCA)[61,70].

Heterogeneity in findings on generic HRQoL outcome may be explained by methodological aspects. Although we found no differences in HRQoL scores between studies that compared HRQoL findings of patients with HD to a normative cohort, and studies that compared HRQoL findings with a selected cohort of controls, sensitivity analysis showed that when aggregating only studies of good quality, overall and psychosocial HRQoL of patients with HD is significantly lower compared to healthy controls. This suggests our main findings on overall and psychosocial HRQoL may have overestimated overall and psychosocial HRQoL outcomes in patients with HD, resulting in a smaller difference compared to healthy controls.

Strengths and limitations

This is the first meta-analysis that summarizes the available evidence on HRQoL outcome of patients with HD. Our findings must be interpreted in the light of a few limitations. First, we could not test whether HRQoL is moderated by length of aganglionosis, operation technique, postoperative complications, presence of stoma and the presence of an associated syndrome, because of the small number of studies with complete data of study- and patient characteristics. Although for a few factors the moderation on HRQoL findings was tested in this study, this was done by meta-regression or group-comparison of meta-analytic findings. This type of analysis does not allow for the calculation of direct relations but calculates relations between studies' averages or proportions and studies effect sizes. Second, there was large heterogeneity in HRQoL findings among the small amount of included studies, resulting in non-robust effects in this study. Third, the evidence of this meta-analysis is based on small, cross-sectional studies, which makes it impossible to assess longitudinal trends in HRQoL, and to assess the influence of transitions in life including puberty and adolescence on HRQoL. Our findings showed no linear relationship between age and HRQoL, but this relation may not be linear. The subgroup analysis did not indicate significant differences between age groups, but is limited by the randomness of the cut-off values used to group the patients into age groups. A last limitation of this meta-analysis is that different instruments to measure HRQoL make different estimations, we aimed to correct for this by including the difference of patients with Hirschsprung disease with normative data in the meta-analysis, which is less vulnerable to a bias introduced by variance between instruments than directly including scores.

Risk of bias

There was a risk of selection bias, as some included studies consisted of a cohort with patients with anorectal malformations and patients with Hirschsprung disease, and also because patients with Down syndrome were underrepresented. Although we tried to limit the influence of information bias by including only studies that used questionnaires that had shown to have adequate construct validity to measure HRQoL, there was heterogeneity in HRQoL outcomes assessed by the different instruments. Differences in the design of the questionnaires, the amount of detail assess with the different items and constitution of domain scores may account for this. There was no evidence of an influence of publication bias on our findings based on Egger's intercept and visual inspection of funnelplots (presented in the Supplementary Material), although findings were not very robust with low fail safe N's.

CONCLUSION

In this systematic review and meta-analysis, no evidence was found for impaired HRQoL outcome in patients with HD compared to healthy controls, neither for the moderating effect of sex, parental proxy or self-report on HRQoL outcome. Physical functioning was most impaired by disease-specific complaints. To further study the longitudinal trends in HRQoL and determinants of HRQoL in patients with HD, we need longitudinal studies that assess the relationship between patient characteristics, functional outcomes and HRQoL.

ARTICLE HIGHLIGHTS

Research background

Patients with Hirschsprung disease are at risk for disease-specific sequelae, that in turn may impair health-related quality of life (HRQoL).

Research motivation

An increasing number of cohort studies describe health-related quality of life in patients with Hirschsprung disease, but no previous study has systematically reviewed and quantitatively analyzed all available evidence on HRQoL.

Research objectives

To summarize all available evidence on HRQoL in patients with Hirschsprung disease and to study moderating factors of HRQoL.

Research methods

A systematic review and meta-analysis in accordance with the PRISMA guidelines was conducted. After search in Pubmed, Web of Sciences, PsycInfo and Embase and selection, 17 studies met eligibility criteria and were included in this study. Random effect models were used for meta-analytic aggregation of generic HRQoL scores. Meta-regression was used to assess the relationship between patient and clinical characteristics and generic HRQoL. Disease-specific HRQoL outcomes of patients with HD were systematically reviewed.

Research results

Seventeen articles were included in the systematic review (n = 1137 patients) and 15 in the quantitative meta-analysis (n = 1024 patients). Patient's age ranged between 0 and 21 years. Meta-analytic aggregation showed a non-significantly impaired generic HRQoL in patients with HD compared to healthy controls. Physical, psychosocial and social HRQoL were also not significantly lower compared to healthy controls. There was no relation between health-related outcomes and the sex of the patients and whether generic HRQoL was measured by parental proxy or self-report. Disease-specific complaints of patients with HD impaired physical HRQoL, but not psychosocial and social HRQoL.

Research conclusions

In this systematic review and meta-analysis, no evidence was found for impaired generic HRQoL in patients with HD compared to healthy controls, neither for moderating effects of sex, parental proxy or self-report.

Research perspectives

Future studies should focus on longitudinal trends in HRQoL and on the relation between funtional outcome and HRQoL.

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FOOTNOTES

Author contributions: Huizer V, Roorda D, Derikx JPM and Rajindrajith S designed the study and designed data collection; Huizer V and Wijekoon N performed study selection, performed study quality assessment, collected data, carried out the initial analyses, drafted the initial manuscript and revised the manuscript; Roorda D supervised data collection, statistical analyses and critically reviewed the manuscript; Derikx JPM, Rajindrajith S, Benninga MA, van Heurn LWE and Oosterlaan J reviewed the manuscript; all authors approved the final manuscript as submitted and agree to be accountable for all aspects of the work.

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Country/Territory of origin: Netherlands

ORCID number: Veerle Huizer 0000-0001-9466-9861; Naveen Wijekoon 0000-0002-6721-5546; Daniëlle Roorda 0000-0001-9740-4957; Jaap Oosterlaan 0000-0002-0218-5630; Marc A Benninga 0000-0001-9406-9188; LW Ernest van Heurn 0000-0002-8001-1222; Shaman Rajindrajith 0000-0003-1379-5052; Joep PM Derikx 0000-0003-0694-7679.

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LETTER TO THE EDITOR

Endoscopic resection for early gastric cancer: Towards a global understanding

Alba Panarese

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Alba Panarese, Department of Gastroenterology and Digestive Endoscopy, Central Hospital, Taranto 74123, Italy

Corresponding author: Alba Panarese, MD, Director, Department of Gastroenterology and Digestive Endoscopy, Central Hospital, 1 Francesco Bruno Street, Taranto 74123, Italy. albapanarese@libero.it

Abstract

Gastric cancer is widespread globally, and disease diagnosis is accompanied by high mortality and morbidity rates. However, prognoses and survivability have improved following implementation of surveillance and screening programs, which have led to earlier diagnoses. Indeed, early diagnosis itself supports increased surgical curability, which is the main treatment goal and guides therapeutic choice. The most recent Japanese guidelines for endoscopic submucosal dissection and endoscopic mucosal resection for early gastric cancer consider the degree of endoscopic curability in relation to the characteristics of the gastric lesions. In clinical practice, the management approach for both prevention and treatment should be similar to that of colon lesions; however, unlike the established practices for colorectal cancer, the diagnostic and therapeutic pathways are not shared nor widespread for gastric cancer. Ultimately, this negatively impacts the opportunity to perform an endoscopic resection with curative intent.

Key Words: Early gastric cancer; Artificial intelligence; Malignancy; *Helicobacter pylori*; Autoimmune gastritis; Dysplasia

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Core Tip: Gastric cancer accounted for 5.6% of all new global cancer cases in 2020 and 7.7% of all cancer deaths. It's generally high mortality and morbidity rates highlight the need for early detection, to increase the curability of surgical treatment. In countries where gastric cancer screening programs exist, endoscopic curability is possible because gastroscopy with magnification and chromoendoscopy can detect gastric lesions at an early stage. It is necessary to support screening programs more widely to achieve the successful implementation of the common strategies of prevention, diagnosis and treatment, thereby reducing the incidence of advanced gastric cancer around the world.

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TO THE EDITOR

The review by Young *et al*[1], which offers a broad overview of endoscopic diagnosis and treatment of gastric dysplasia and early cancer with the prospect of future implications, was received with great interest. The authors reported that endoscopic treatment of precancerous lesions and early gastric cancer reduces rates of advanced carcinoma and avoids the requirement for surgery. However, there are relevant differences between the East and West for data related to the practice of endoscopic resection and the histological definitions of lesions[1].

Gastric cancer accounts for a significant proportion of cancer-related morbidity and mortality worldwide[2,3], and early detection is required to reduce the rates of each. The risk of progression from low-grade and high-grade dysplasias detected by biopsy to gastric cancer is high. For this reason, guidelines suggest endoscopic removal of any dysplastic lesion[4,5]. However, the incidence of early gastric cancer differs among countries, particularly for those in the Eastern and Western regions of the world[2,3,6]. In European countries, for example, early-stage gastric cancer accounts for less than 10% of diagnosed cases, which is much less than that in Asian countries where endoscopic screening is practiced. It has been reported that endoscopic screening may reduce the risk of death from gastric cancer without affecting gastric cancer incidence[7]. In fact, in the East, gastric cancer screening programs allow early diagnosis and consequently increase the curability of subsequent treatment by surgical resection. Likewise, endoscopic resection allows endoscopic curability, and through these collective clinical efforts, morbidity and mortality of gastric cancer can continue to decrease[3].

Therefore, advances in endoscopic imaging and resection techniques as well as improved endoscopist training are very important. Yet the maximal benefits will only be realized if these efforts are carried out alongside expanded implementation of screening and surveillance gastroscopies because gastric cancer originates from precancerous lesions. Intriguingly, this pathogenic pattern is similar to that of colon cancer, whereby the cancer arises from polypoid and non-polypoid lesions of the colon[8].

Our ongoing Italian multicenter prospective observational study on endoscopic submucosal dissections of early gastric cancer has, so far, enrolled 32 cases in 18 mo in 12 centers. A comparison with other studies is not possible at this time for several reasons, namely among them unavoidable alterations to the clinical routine caused by the coronavirus disease 2019 pandemic. However, it appears that in addition to technological and diagnostic advances and improved skills of endoscopists, the presence of screening programs could be important to further identify and enroll patients who are at-risk. This represents a similar trend to that experienced in the colon cancer screening programs.

The ongoing high incidence of gastric cancer, especially in the youngest generations, due to the persistent spread of *Helicobacter pylori* (*H. pylori*) infection, makes gastric cancer a major public health challenge. Currently, the mortality rate for total gastric cancer deaths is higher than that of either breast cancer or colon cancer[3,9]. Since it is important to achieve a common vision for eradicating *H. pylori* on a global scale, the additional application of both *H. pylori* antibody titer and pepsinogen levels together may further promote the effectiveness of gastric cancer screening programs[10].

The new Japanese Guidelines for Endoscopic Submucosal Dissection and Endoscopic Mucosal Resection for Early Gastric Cancer (Second Edition)[11] define the indications for endoscopic treatment in relation to curability and according to the risk of lymph node metastases, based on current scientific evidence. Absolute, expanded and relative indications for the endoscopic treatment of gastric lesions and levels of curability ("endoscopic curability A: curative resection," "endoscopic curability B" and "endoscopic curability C")[11] are the starting point for prospective confirmatory studies in both Eastern and Western countries. These studies should determine the 5-year survival when these screening programs are implemented.

FOOTNOTES

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Country/Territory of origin: Italy

ORCID number: Alba Panarese 0000-0002-6931-2171.

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LETTER TO THE EDITOR

Therapeutic drug monitoring in inflammatory bowel disease: At the right time in the right place

Brindusa Truta

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Brindusa Truta, Internal Medicine, Johns Hopkins University, Baltimore, MD 21210, United States

Corresponding author: Brindusa Truta, MD, Assistant Professor, Internal Medicine, Johns Hopkins University, 1830 E Monument Street, Room 426, Baltimore, MD 21210, United States. brindusa_73@yahoo.com

Abstract

Therapeutic drug monitoring (TDM) was one of most sought-after objective tools to determine therapeutic efficiency of different biologics and its role in the management of patients with inflammatory bowel disease (IBD) was regarded with great anticipation. But implementation of the TDM in clinical practice was challenged by several factors including uncertainty of the optimal cut-off values, assay variable sensitivity in detecting drug levels and antibodies and, most importantly, individual pharmacokinetics. While reactive TDM was embraced in clinical practice as a useful tool in assessing lack of response to therapy, the utility of proactive TDM in managing IBD therapy is still challenged by the lack of consistency between evidence. Described here, there are four groups of IBD patients for whom proactive TDM has the potential to greatly impact their therapeutic outcomes: Patients with perianal Crohn's disease, patients with severe ulcerative colitis, pregnant women with IBD and children. As the future of IBD management moves towards personalizing treatment, TDM will be an important decision node in a machine learning based algorithm predicting the best strategy to maximize treatment results while minimizing the loss of response to therapy.

Key Words: Therapeutic drug monitoring; Inflammatory bowel disease; Biologics; Crohn's disease

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Core Tip: While reactive therapeutic drug monitoring (TDM) was embraced in clinical practice as an important tool for assessing lack of response to biologics, existent evidence inconsistently supports the proactive use of TDM in managing inflammatory bowel disease (IBD) therapy. Exceptions are made for patients with severe ulcerative colitis and perianal Crohn's disease (fistula) for whom TDM has consistently shown to improve clinical outcome, pregnant women with IBD for whom TDM has the potential to play a decisive role in withholding therapy and for children, for whom proactive TDM was found to increase steroid free clinical remission. Future studies are needed to define the real value of TDM in management of IBD.

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TO THE EDITOR

The article presented by Albader *et al*[1] titled "Therapeutic drug monitoring in inflammatory bowel disease: The dawn of reactive monitoring" addresses a controversial topic in clinical practice: The role of therapeutic drug management in patients with inflammatory bowel disease (IBD).

Therapeutic drug monitoring (TDM) was as one of most sought-after objective tools to determine therapeutic efficiency of different biologics. Around one third of patients are primary non-responders and 25%-50% who respond, lose response over time [secondary loss of response (sLOR)][2,3]. Clinicians investigated different techniques to early detect, prevent and overcome sLOR in their patients including serologic and fecal biomarkers, capsule endoscopy and imaging. TDM was regarded with great hope. But, as recognized by Albader *et al*[1], implementation of TDM in clinical practice was challenged by few factors including uncertainty of the optimal cut-off values, assay variable sensitivity in detecting drug levels and antibodies and, most importantly, individual pharmacokinetics influenced by severity of the disease and body weight[4-6].

The studies presented in this review, of which the majority are retrospective and targeting anti-tumor necrosis factors (TNFs), have controversial results regarding the utility of TDM in management of IBD. This controversy arises in part due to the differences in study design including different outcomes: Clinical, endoscopic, histologic response and/or cost efficiency but also due to timing of TDM implementation proactive *vs* reactive to sLOR. The author concluded that it is "difficult to prove that proactive TDM is associated with better therapeutic outcomes" but it should be considered an addition to the other tools already routinely used in practice including biomarkers (calprotectine), imaging, capsule endoscopy[1].

There are few situations that should be discussed as exempt from this conclusion.

In patients with perianal Crohn's disease (CD), closure of the fistula have been consistently shown to require higher trough level of infliximab (≥ 10 μg/mL) (IFX) than the level considered optimal for luminal CD disease (3–7 μg/mL)[6]. This finding seems to be true for both induction and maintenance phase[7,8]. It needs to be recognized that most of the studies reporting on the anti-TNF levels in perianal CD are retrospective in design[7-9]. The results of the prospective randomized controlled trial of adults with perianal fistulizing CD and optimized therapeutic IFX levels (PROACTIVE Trial) currently evaluates the benefit on clinical, radiological, patient-reported outcomes and economic costs of a higher than standard IFX[10].

In patients with moderate to severe ulcerative colitis, a higher than 30 μg/mL IFX level after the induction phase and a detectable drug level at 54 wk has been associated with greater clinical and endoscopy improvement in the post-hoc analysis of 728 patients who participated to ACT-1 and ACT-2 clinical trials[11]. This higher level is also associated with lower colectomy rates and hospitalization (OR: 9.3, $P < 0.001$)[12] when compared with patients with standard IFX level. Patients with severe inflammation have lower tissue anti-TNF levels than those in remission[13] likely due to increased clearance, although drug clearance depends on other additional factors such as albumin level, body mass and gender[14,15]. For these patients, proactive TDM may represent the rescue technique for clinical improvement and colectomy sparing.

TDM may be useful in managing anti-TNF therapy in IBD pregnancy where concerns of intrauterine fetal exposure has been raised, as the data showed higher than therapeutic levels for children of mothers who continue biologics beyond second trimester than for those of mothers who stopped biologics early in pregnancy[16]. Since mother's IFX trough levels increased during pregnancy by 4.2 μg/mL *per* trimester ($P = 0.02$), it has been suggested that late second trimester trough level of biologic may determine timing and dose of biologic agent in the third trimester[17,18].

Although withholding biologic therapy in the third trimester has been associated with increased risk of flaring in pregnancy[19], this approach may be considered safe with TDM in a well-defined group of patients once there is a clear understanding of drug pharmacokinetics and determinants of flaring in pregnancy. TDM may be considered in children with intrauterine drug exposure to decide the timing of safe administration of life virus vaccines. Current guidelines recommend avoiding any live vaccinations for at least 6 mo following delivery unless serum levels in the infant are undetectable[20].

Pediatric IBD represents a special group of patients, where the limited therapeutic armamentarium and challenges in balancing drug safety and efficiency created a critical need for drug monitoring[21]. Pro-active TDM showed to increase corticoid-free clinical remission in children with CD treated with Adalimumab (ADL) compared with reactive monitoring (PAILOT study)[22] and sustained clinical remission in children with CD, ulcerative colitis or IBD-unclassified treated with either IFX or ADL therapy[23]. In addition, model outcomes indicated that proactive TDM *vs* reactive TDM for ADL may provide higher quality-adjusted life-years at lower cost in pediatric CD patients[24].

In comparison with proactive TDM, the utility of reactive TDM has received a greater consensus in guiding therapy for those patients who lost response and where either dose intensification or change to an alternative therapy may be necessary. The utility of reactive TDM have been extended to recently introduced biologics and oral small molecules[25].

As the future of IBD management moves towards personalizing treatment, TDM will play an important role in the algorithm of machine learning based models that predict best strategy to optimize treatment outcomes while minimizing the sLOR to therapy.

FOOTNOTES

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Country/Territory of origin: United States

ORCID number: Brindusa Truta 0000-0003-4319-9278.

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1499 Comments on "Effect of type 2 diabetes mellitus in the prognosis of acute-on-chronic liver failure patients in China"

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ABOUT COVER

Editorial Board Member of *World Journal of Gastroenterology*, Sung-Chul Lim, MD, PhD, Professor, Head, Department of Pathology, Chosun University Hospital, 365 Pilmun-daero, Dong-gu, Gwangju 61453, South Korea. sclim@chosun.ac.kr

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EDITORIAL

Hepatocellular adenoma: Where are we now?

Xi Wang, Xuchen Zhang

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Xi Wang, Xuchen Zhang, Department of Pathology, Yale University School of Medicine, New Haven, CT 06520, United States

Corresponding author: Xuchen Zhang, MD, PhD, Associate Professor, Department of Pathology, Yale University School of Medicine, 310 Cedar Street, PO Box 208023, New Haven, CT 06520, United States. xuchen.zhang@yale.edu

Abstract

Hepatocellular adenoma (HCA) is a benign hepatocellular neoplasm, commonly occurs in young women with a history of oral contraceptive use. Complications including hemorrhage and malignant transformation necessitate the need for a thorough understanding of the underlying molecular signatures in this entity. Recent molecular studies have significantly expanded our knowledge of HCAs. The well-developed phenotype-genotype classification system improves clinical management through identifying "high risk" subtype of HCAs. In this article, we attempt to provide updated information on clinical, pathologic and molecular features of each subtype of HCAs.

Key Words: Hepatocellular adenoma; Subtype; Pathology; Classification; Hepatocellular carcinoma

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Core Tip: Hepatocellular adenoma (HCA) has been well recognized as a benign liver neoplasm with risks of hemorrhage and malignant transformation. Studies revealed that different HCA subtypes with specific genetic mutation and pathologic findings are associated with different clinical features. Currently HCAs are classified into at least 5 major subtypes, involving 4 different pathways driving HCA pathogenesis: Hepatocyte nuclear factor 1A, interleukin-6/the Janus kinase/signal transducer and activator of transcription, β-catenin, and Sonic hedgehog pathway.

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INTRODUCTION

Hepatocellular adenoma (HCA) is an uncommon benign liver neoplasm with two major complications: Hemorrhage and malignant transformation. Epidemiological data from the United States and European countries have revealed HCAs occur mainly in young to middle-aged women (median ages: 36-38 years, female/male ratio of 8:1), often with a history of long-term use of oral contraceptives (OCPs)[1,2]. Besides the well-known risk factor of exogenous estrogen exposure, other risk factors, such as androgen use, obesity, fatty liver disease, glycogen storage disease (GSD), especially GSD type 1, hepatic vascular disorders and other genetic disorders are also associated with the occurrence of HCA[2-6]. However, epidemiological data from Asia, where the use of OCPs and the incidence of obesity are lower, are unclear. Limited studies from Taiwan, China and Japan showed a male predominance in patients with HCA[7-11]. Further exploration of these epidemiological differences is needed to better understand the pathogenesis of HCA.

Historically, HCA was thought to be a single group of tumor. Recent advances in HCA clinicopathologic features and molecular biology have not only enhanced our understanding of the disease pathogenesis, but also significantly transitioned into the daily practice of pathology-morpho-molecular correlation. It is known now that HCA is a heterogenous group of liver tumors with heterogenous etiology, clinical presentation, risk of malignant transformation or hemorrhage, radiologic findings, histopathologic features, clinical management strategies and underlying molecular changes. In the current review, we will provide an overview of the current knowledge of how HCA histomorphology correlates to its underlying molecular changes, as well as discuss the controversies in some HCA variants.

HCA: MORPHO-MOLECULAR CLASSIFICATION

Currently, HCAs are classified into hepatocyte nuclear factor 1A *(HNF-1A)* inactivated HCA (H-HCA), inflammatory HCA (I-HCA), β-catenin activated HCA (b-HCA), as well as unclassified HCA (U-HCA) based on underlying molecular changes in 3 different pathways driving benign hepatocytic proliferation: HNF-1A, interleukin-6/the Janus kinase/signal transducer and activator of transcription (IL-6/JAK/STAT), and β-catenin signaling. A new subtype of Sonic hedgehog HCA (shHCA) has been recently described[2], which still needs further characterization. The different mutations in HCA identified to date are summarized in Table 1.

H-HCA

As the first identified subtype, *HNF-1A*-inactivated HCA was proposed by a French group in their pioneer work published in 2002. In an attempt to search for tumor suppressor gene in HCA, Bluteau *et al*[12] genotyped DNA from HCAs and confirmed the bi-allelic inactivation of *HNF-1A* gene in a subgroup of tumors. HNF-1A is an important transcription factor regulating hepatocytes differentiation. It is mainly expressed in pancreatic beta cells, intestine, and liver, that plays an important role in the regulation of glycolipid metabolism[13,14]. Specifically, HNF-1A positively regulates *FABP1* gene, which encodes liver fatty acid binding protein (L-FABP). As a result of *HNF-1A* inactivated mutation, expression of L-FABP is downregulated, along with diffuse steatosis due to dysregulated lipogenesis.

As expected, *HNF-1A*-inactivated HCA histomorphologically shows diffuse marked steatosis (Figure 1A). Immunohistochemical stain for L-FABP is negative (Figure 1B), indicating the functional loss of *HNF-1A* gene. Of note, some H-HCAs may not show steatosis and some other subtype HCAs such as I-HCAs can be seen with marked steatosis. Therefore, steatosis alone should not be used as a sole feature to diagnose H-HCAs. This subtype of HCA approximately composes of 30%-35% of overall HCAs and is mainly due to somatic mutation. Typical H-HCA can also be detected in magnetic resonance imaging (MRI), with diffuse and homogenous signal dropout on T1-weighted images, due to massive fat component[15]. In the clinical aspect, cases of familial liver adenomatosis (greater than 10 adenomas in the liver) have been consistently linked to the germline mutation of *HNF-1A*[16], which is also the genotype in "Maturity onset diabetes of the young, type 3 (MODY3)". Thus, detection of liver adenomatosis with H-HCA is suggested to start family screen for familial adenomatosis, MODY3 diabetes, and the *HNF-1A* germline mutation[17]. Although H-HCA is not usually associated with malignant transformation, hepatocellular carcinoma (HCC) has been reported to arise in the settings of sporadic HCAs[18] as well as those in patients with hepatic vascular disorders or MODY3[19], especially in female patients with multiple lesions without significant steatosis and presence of myxoid change, peliosis and sinusoidal dilatation[3].

I-HCA

Accumulating evidence suggests a critical role of inflammatory response in tumorigenesis, including STAT signaling pathway in the development of breast and lung cancer[20]. Similarly, a subtype of inflammatory epithelial tumors has been described in the family of HCA, involving the IL-6/JAK/STAT3 signaling pathway[21]. IL-6 belongs to the IL-6 cytokine family, which binds to the ligand-

Table 1 Morpho-molecular features of the subtypes of hepatocellular adenoma

	Key pathogenesis	Histology	Immunohistochemical stains	Clinical features
H-HCA	*HNF1A* inactivating mutation: Negative regulation of glycolipid metabolism and L-FABP	Marked steatosis	L-FABP: Negative	Associated with maturity onset diabetes of the young (MODY3) and familial hepatic adenomatosis
I-HCA	IL-6/JAK/STAT3 pathway mutations: (1) Constitutive activation of inflammatory pathway; and (2) Upregulation of acute reactants and hepatocellular proliferation	(1) Inflammatory infiltration; (2) Pseudoportal tracts; (3) Ductular reaction; and (4) Sinusoidal dilatation/peliosis	SSA and CRP: Diffuse positive	Obesity, metabolic syndrome, glycogen storage disease, high alcohol consumption, inflammatory syndrome
b-HCA	(1) *CTNNB1* gene mutation: Activation of signaling pathway and upregulation of targeted genes including GS; and (2) Level of activation depends on mutation loci (Exon 3 Non-S45: Strong activation; S45: Weak activation, and T41: Moderate activation; Exon 7/8: Weak activation)	More atypical features: Pseudoacini and mild cytologic atypia	(1) β-catenin: Aberrant nuclear expression; and (2) GS: Positive	More in men, anabolic steroids use, glycogen storage disease, high risk of malignant transformation, and high risk of bleeding (exon 7/8)
b-IHCA	Share the features of both b-HCA and I-HCA			
shHCA	*INHBE-GLI1* gene fusion: Constitutive activation of Sonic hedgehog pathway	Hemorrhage	PTGSD and ASS1	High risk of bleeding and obesity
Unclassified	Not other specified			
Uncommon subtypes	Myxoid HCA, pigmented HCA, atypical HCA, I-ICA in cirrhotic liver			

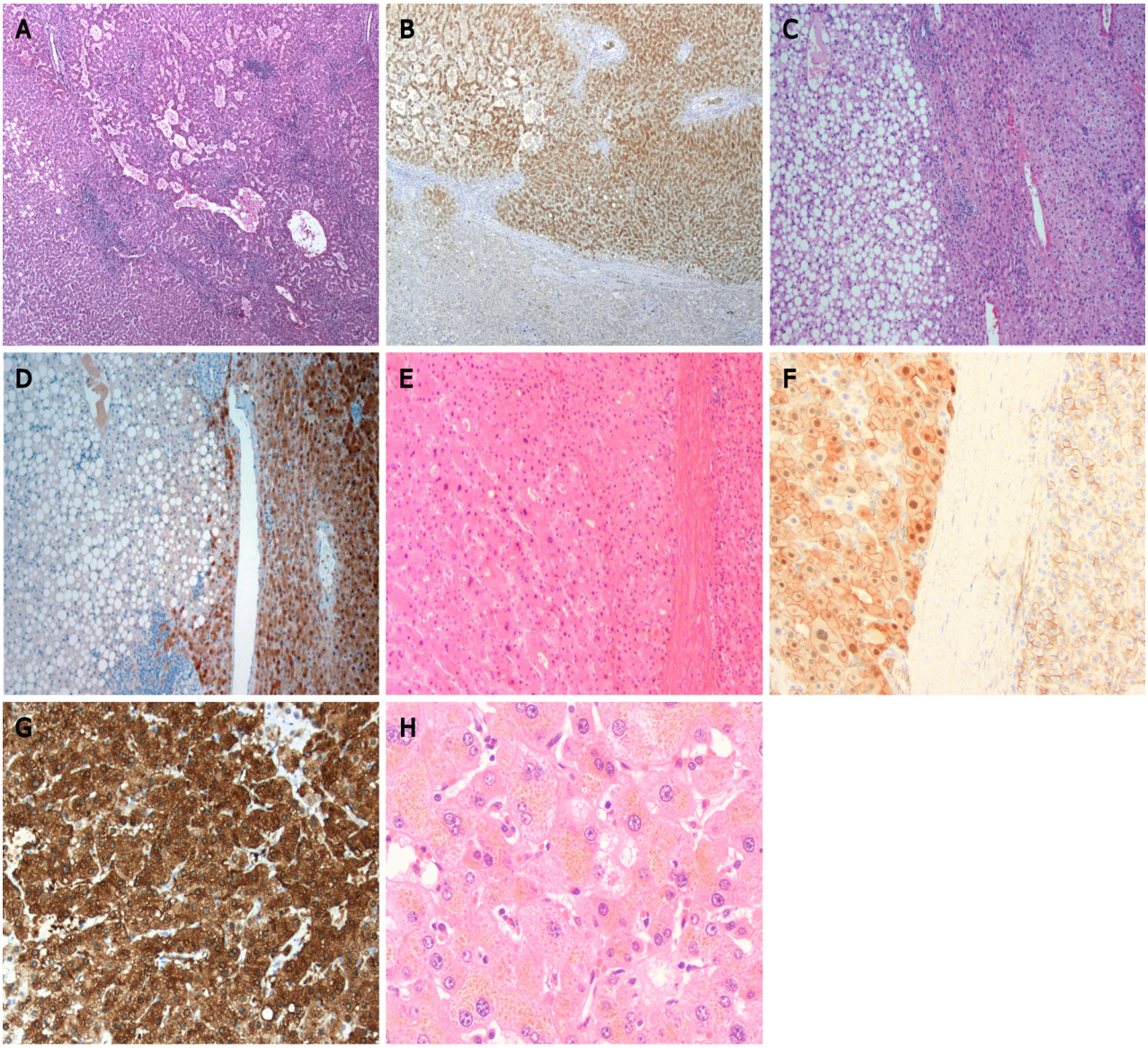
HNF1A: Hepatocyte nuclear factor 1A; L-FABP: Liver fatty acid binding protein; SSA: Serum amyloid A; CRP: C-reactive protein; CTNNB1: Catenin beta 1; GS: Glutamine synthetase; INHBE-GLI1: Inhibin beta E chain/glioma-associated oncogene 1; PTGDS: Prostaglandin D synthase; ASS1: Argininosuccinate Synthase 1; HCA: Hepatocellular adenoma; I-HCA: Inflammatory HCA; H-HCA: *HNF1A*-inactivated HCA; shHCA: Sonic hedgehog HCA; b-HCA: β-catenin mutated HCA.

specific receptor gp130 to initiate the downstream signaling. This activates downstream signaling pathways such as the Src-homology 2 domain-containing tyrosine phosphatase 2 (SHP2)-Ras-ERK, JAK1/2-STAT3, mosaic G-protein alpha-subunit (GNAS), and the phosphatidylinositol-3-kinase/Akt and the mechanistic target of rapamycin (PI3K-AKT-mTOR), and further the expression of targe genes that regulate cell survival, proliferation and angiogenesis[2,22]. Rebouissou *et al*[21] demonstrated that 60% of I-HCA harbored small in-frame deletions of *IL6ST* gene (encodes the signaling co-receptor gp130), resulting in the constitutive activation of IL-6 signaling and hepatocellular proliferation in the absence of ligand binding. The remaining I-HCAs are linked to other mutations that belong to IL-6/JAK/STAT3 family. The inflammatory response is well-observed histologically[23], characterized by inflammatory infiltration of lymphocytes, plasma cells and neutrophils, ductular reaction, and sinusoidal dilatation/peliosis (Figure 1C). Immunohistochemical stain is remarkable for diffuse overexpression of acute-phase inflammatory protein such as serum amyloid A (SAA) and C-reactive protein (CRP) (Figure 1D).

Clinically, I-HCA is frequently associated with obesity, metabolic syndrome and high alcohol consumption. Furthermore, the risk of developing HCA in patients with GSD is high. The HCAs in patients with GSD are mainly I-HCAs (52%), followed by b-HCAs (28%) and U-HCAs (20%), but never H-HCAs[5]. A few I-HCA cases had also been reported to induce systemic AA amyloidosis[24]. Typical I-HCA also has unique MRI features, with hyperintensity on T2-weighted images due to sinusoidal dilatation[15]. This subtype approximately composes of 35%-45% of overall HCAs, and about 10% also have β-catenin activation. While the mutation alone is not associated with malignant transformation, it is important to remember that I-HCA can co-exist with β-catenin mutation, the latter of which can transform into HCC[2].

b-HCA

Aberrant Wnt/β-catenin signaling has been identified underlying pathogenesis of many diseases including the well-known familial adenomatous polyposis[25]. In the liver, it also plays an essential role in regulating various cellular events including differentiation, proliferation, survival and others. Not surprisingly, β-catenin (cadherin-associated protein) beta 1 (*CTNNB1*) gene mutations have been reported in around 20%-40% of HCC cases. Chen *et al*[26] detected interstitial deletions in the *CTNNB1* gene in HCAs, indicating the dysregulation of Wnt/β-catenin as a possible preneoplastic pathway for hepatocellular proliferation. Exome sequencing study[27] further subclassified into two types: Exon 3



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Figure 1 Histologic features of hepatocellular adenoma. A: Hepatocyte nuclear factor 1A (HNF-1A) inactivated hepatocellular adenoma showing marked steatosis (Hematoxylin-eosin stain, original magnification 200 ×); B: HNF-1A inactivated hepatocellular adenoma showing loss expression of liver fatty acid binding protein in tumor component (Immunohistochemical stain, original magnification 200 ×); C: Inflammatory hepatocellular adenoma showing marked sinusoidal dilatation and pseudoportal tract with inflammatory infiltrate and ductular reaction (Hematoxylin-eosin stain, original magnification 200 ×); D: Inflammatory hepatocellular adenoma showing strong and diffuse expression of C-reactive protein in tumor component (Immunohistochemical stain, original magnification 200 ×); E: β-catenin activated hepatocellular adenoma showing mild cytologic atypia and pseudoacini (Hematoxylin-eosin stain, original magnification 200 ×); F: β-catenin activated hepatocellular adenoma showing nuclear expression of β-catenin in tumor component (Immunohistochemical stain, original magnification 400 ×); G: β-catenin activated hepatocellular adenoma showing strong and diffuse expression of glutamine synthetase in tumor component (Immunohistochemical stain, original magnification 200 ×); H: Pigmented hepatocellular adenoma showing marked cytoplasmic lipofuscin (Hematoxylin-eosin stain, original magnification 400 ×).

and exon 7/8 with distinct canonical and non-canonical mutations respectively. In the canonical pathway, inactive β-catenin is attached with E-cadherin at cell membrane to maintain cell-cell adhesion. Cytoplasmic β-catenin is sequestered by a "destruction protein complex", then phosphorylated, and further recognized by E3 ubiquitin, eventually degraded in the cytoplasm by proteasomes. The overall net effect is low β-catenin levels within the cells. In the activate states or upon WNT binding, the phosphorylation activity is inhibited and the disheveled protein complex becomes disintegrated. As a result, β-catenin accumulates in the cytoplasm and translocates into the nucleus to regulate downstream target gene expression, such as a glutamate-ammonia ligase, which codes for glutamine synthase (GS) and leucine-rich-repeat containing G protein-coupled receptor-5 (LGR5). Thus, the overall net effect is abnormally high expression level of β-catenin within the nucleus and cytoplasm[28]. HCAs with β-catenin mutation are divided into two groups based on the mutation loci in the *CTNNB1* coding for β-catenin: β-catenin exon 3 mutated HCA and β-catenin exon 7/8 mutated HCA[29].

b-HCA exon 3: Approximately 10%-15% of HCAs harbored canonical mutations in *CTNNB1* gene exon 3. The level of β-catenin pathway activation further depends on the specific mutations: Large in-frame deletions, D32-S37 deletions and T41 exon 3 mutations are associated with high level, whereas S45 exon 3 mutations are associated with low level of activation[29]. Comparing with other HCA subtypes, this subtype HCA has more atypical features including mild cytologic atypia, cholestasis, and/or pseudoacinar formation (Figure 1E), which sometimes can be difficult to distinguish from well differentiated HCC. Of note, no specific histologic features can be reliably used to predict a diagnosis of b-HCA. Immunohistochemical stains for β-catenin shows aberrant nuclear and cytoplasmic expression (Figure 1F). While the interpretation of β-catenin expression can be challenging, a better surrogate marker, GS, is also widely used. A recent study has revealed that diffuse homogenous GS staining pattern was strongly associated with exon 3 non-S45 mutation (Figure 1G). Whereas, a diffuse heterogenous GS staining pattern with strong positivity at the tumor periphery indicated exon 3 S45 mutation [30].

Clinically, β-catenin exon 3 mutated HCA is more frequent in men than other subtypes and is more often associated with androgen exposure. In addition, this subtype especially with large in-frame deletions is associated with a high risk of malignant transformation. It is hypothesized that *CTNNB1* exon 3 mutation is the earliest genetic alteration, while additional mutations such as telomere reverse transcriptase (TERT) promoter mutation is involved in the final step of transition from HCA to HCC [27].

b-HCA exon 7/8: A smaller subset of HCAs (5%-10%) harbor non-canonical mutations of *CTNNB1* gene in exon 7 and 8, which is exclusive to mutations in exon 3. In comparison with exon 3 mutations, mutations in exon 7 and 8 result in a weak activation of β-catenin. A focal patchy GS staining pattern in the tumor with strong positivity at the tumor periphery by immunohistochemistry can indicate exon 7/8 mutation[30]. This subtype is associated with a low risk of malignant transformation. A recent study demonstrated that b-HCA exon 7/8 is significantly associated with tumor hemorrhage[31].

β-catenin activated I-HCA

While β-catenin mutations are nearly exclusive to *HNF-1A* mutations, they can be associated with altered JAK/STAT pathway and demonstrate inflammatory features. If the I-HCA shows beta-catenin activation, then a diagnosis of I-HCA with beta-catenin activation can be rendered [β-catenin activated I-HCA (b-IHCA)], which shares the histopathologic features of both b-HCA and I-HCA, and carries malignant transformation potential similarly. It is thus important to continue workup for β-catenin inactivation when a diagnosis of I-HCA is made.

shHCA

ShHCA is a recently recognized subtype of HCA with sonic hedgehog pathway activation[2]. This subtype represents approximately 4% of overall HCAs and was previously classified as unclassified HCA due to the lack of mutations in typical HCA genes. Genetic studies demonstrated that this type of HCA is caused by inhibin beta E subunit (*INHBE*) gene and *GLI1* gene fusion (INHBE-GLI1). The Hedgehog pathway is a complex signal transduction pathway including 4 main components: The ligand Hedgehog, the receptor Patched, the signal transducer Smoothened and the effector transcription factor, Gli[32]. It is not only crucial for embryogenesis of liver, but also plays a role in liver regeneration. Evidence has shown that the Hedgehog pathway is dormant in healthy adult liver, while significantly activated after liver injury. INHBE is a growth factor belonging to the transforming growth factor-beta family. It is highly expressed in the liver and regulates hepatocellular growth and differentiation. As a result, the INHBE-GLI1 fusion leads to uncontrolled activation of sonic hedgehog pathway due to overexpression of transcription factor GLI1.

Clinically, shHCAs are more frequently seen in women and are associated with higher body mass index and/or OCP use. While there are no specific histopathologic features in this HCA subtype, it seems that prostaglandin D synthase and argininosuccinate synthase 1 (ASS1), particularly ASS1 positivity by immunohistochemistry is a hallmark of shHCA[2,33]. Although ASS1 may be expressed in other HCA subtypes with hemorrhage and thought to be a marker of hemorrhage[34], a recent study showed ASS1 expression did not correlate with HCA hemorrhagic complications[35]. Interestingly, higher risk of hemorrhage has been consistently observed both histologically and clinically in shHCA[2, 31]. This serious complication warrants a thorough understanding of pathogenesis of shHCA for better diagnosis and clinical management.

Unclassified HCA

Even though extensive studies have been conducted to explore molecular features of HCAs, approximately 5%-10% remain unclassified. These HCAs lack distinct histopathologic features and any specific molecular abnormality[2].

UNUSUAL SUBTYPES AND CONTROVERSIES

In addition, uncommon HCA subtypes have been well documented, which do not fit well into the current classification.

Pigmented HCA

Pigmented HCAs are a heterogenous group of HCAs with different genetic mutations, which contain pigment deposition of lipofuscin (Figure 1H) as confirmed by electron microscopy[36]. Although H-HCA is the commonest subtype, other subtypes such as b-HCA, I-HCA, b-IHCA, and unclassified HCA also can be seen in pigmented HCAs[36-38]. Pigmented HCAs often show histologic atypia with higher risk malignant transformation. Besides the heavy deposition of lipofuscin in tumor cells, lipofuscin pigment deposition is commonly seen in hepatocytes of the background livers[36]. Although unclear, the underlying biology may be related to dysregulation of autophagy resulting in lipofuscin accumulation, which could contribute to carcinogenesis including the liver[39,40].

Myxoid HCA

Myxoid HCAs are characterized by the deposition of myxoid materials between the hepatic cords within the tumor[41-44]. In addition to loss of L-FABP expression and/or *HNF-1A* mutation as documented in all myxoid HCAs, a recent study identified recurrent mutations in genes within the protein kinase A (PKA) pathway or in genes that regulate the PKA pathway, such as GNAS, CDKN1B (p27) and RNF123, in myxoid HCAs[44]. Myxoid HCAs are often seen in individuals with older age and carry a high risk of malignant transformation[3,44]. It is still controversial whether this is a rare variant of H-HCA with additional mutations or a distinct subtype of HCAs.

Atypical/borderline HCA/hepatocellular neoplasm of uncertain malignant potential

HCA, as described above, has potential of malignant transformation that sometimes can be challenging to distinguish from well-differentiated HCC, especially in biopsy specimens. Various terms, such as atypical HCA, borderline HCA, atypical hepatocellular neoplasm, well-differentiated hepatocellular neoplasm with atypical or borderline features, and hepatocellular neoplasm of uncertain malignant potential (HUMP) have been used for hepatocellular neoplasms that demonstrate features atypical for HCA but insufficient for an unequivocal diagnosis of HCC. So far there are no widely accepted criteria in diagnosing this entity, however, these clinical (male, females > 50 years or < 15 years) and pathologic (focal cytological atypia, small cell change, pseudoacini, focal reticulin network loss, presence of β-catenin activation or *CTNNB1* mutations) features have been consistently used when an atypical/borderline HCA (A-HCA)/HUMP is diagnosed[45-47]. A recent study showed that greater than 60% HCAs would be re-classified as A-HCAs/HUMPs using the above suggested criteria. Furthermore, in this study A-HCA/HUMP does not seem to correlate in patients with or without synchronous or metachronous HCC[45]. The high rate of HCAs placed in the category of A-HCA/HUMP, particularly in resected tumors may cause confusion to clinicians in managing those tumors. Molecular study of *TERT* promoter mutations, a marker of HCC, may be useful to distinguish A-HCA/HUMP from well-differentiated HCC and predict the risk of malignant transformation. Studies have demonstrated that *TERT* promoter mutations have been identified in 17% of A-HCAs/HUMPs compared with 50%–60% of HCC[48]. Further study to refine the widely accepted criteria of diagnosing A-HCA/HUMP and to predict its malignant behavior by combining the clinical, pathological and molecular features is warranted.

I-HCA in cirrhotic livers

HCAs typically arise in the livers without significant fibrosis. The background liver can be histologically normal or have steatosis or steatohepatitis or other genetic and vascular disorders that have been recognized as risk factors for developing HCAs. Thus, a solid mass arising in a cirrhotic liver is generally not considered as HCA. However, rare HCAs with SAA positivity or harboring activating mutations in *IL6ST* or *STAT3* same as I-HCAs have been described in metabolic syndrome and/or alcoholic cirrhosis[49,50]. Of note, some cirrhotic nodules and HCCs can be positive for CRP and SAA, the two important I-HCA markers. Furthermore, activation of IL-6/gp130/STAT3-dependent pathway is involved in the development of liver fibrosis[51]. Therefore, classifying those SAA-positive cirrhotic nodules with *IL6ST* or *STAT3* mutations as I-HCAs is thought to be premature[52]. Additional data are warranted to confirm whether HCA can arise in a background liver with cirrhosis.

MANAGEMENT

Guidelines from both the American College of Gastroenterology[53] and the European Association for Study of the Liver[54] recommend a surgical resection when HCAs are > 5 cm, since they have a higher potential for hemorrhage and malignant transformation. The adoption of a genotype-phenotype classi-

fication is increasingly relevant to clinical decision-making given growing evidence that the risk of complications is likely dependent on HCA subtype and gender. Currently, the treatment of HCA is based on HCA subtype and gender[17,54,55]. Briefly, surgery is recommended for all men with HCAs since they carry a high risk of malignant transformation. For women, after the initial 6 mo lifestyle management including the discontinuation of OCPs and control of body weight, the management of HCAs is based on the size and HCA subtypes. For tumors persistently > 5 cm, or increasing in size after lifestyle change, irrespective of their subtypes, resection or curative treatment is indicated. For tumors < 5 cm of the H-HCA subtype, or those that are either inflammatory or β-catenin negative on biopsy, conservative management is recommended. Of note, surgical resection is recommended for b-HCAs, b-IHCAs and A-HCAs/HUMPs, irrespective of size. Of note, HCAs arising in patients with underlying liver diseases, such as GSD and hepatic vascular disorders seem to have a higher risk of malignant transformation, that should also be considered when manage these HCAs.

CONCLUSION

Recent molecular studies have significantly expanded our knowledge of HCAs. The newly developed phenotype-genotype classification system not only fulfils our curiosity academically, but more importantly, helps improving clinical management through identifying the "high risk" subtype of HCAs. However, further studies are needed, including how to incorporate the uncommon HCA subtypes into the classification system, how to effectively guide the HCA management by using the classification system and identifying the underlying molecular changes of the unclassified HCAs.

FOOTNOTES

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ORCID number: Xi Wang 0000-0001-8916-5132; Xuchen Zhang 0000-0002-1484-4672.

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OPINION REVIEW

Endoluminal vacuum-assisted therapy to treat rectal anastomotic leakage: A critical analysis

Andrea Vignali, Paola De Nardi

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Andrea Vignali, Paola De Nardi, Department of Gastrointestinal Surgery, IRCCS San Raffaele Scientific Institute, Milano 20132, Italy

Andrea Vignali, Department of Surgery, Vita-Salute University, Milano 20132, Italy

Corresponding author: Paola De Nardi, FASCRS, MD, Staff Physician, Surgeon, Department of Gastrointestinal Surgery, IRCCS San Raffaele Scientific Institute, *via* Olgettina 60, Milano 20132, Italy. denardi.paola@hsr.it

Abstract

Endoluminal vacuum-assisted therapy (EVT) has been introduced recently to treat colorectal anastomotic leaks in clinically stable non-peritonitic patients. Its application has been mainly reserved to low colorectal and colo-anal anastomoses. The main advantage of this new procedure is to ensure continuous drainage of the abscess cavity, to promote and to accelerate the formation of granulation tissue resulting in a reduction of the abscess cavity. The reported results are promising allowing a higher preservation of the anastomosis when compared to conventional treatments that include trans-anastomotic tube placement, percutaneous drainage, endoscopic clipping of the anastomotic defect or stent placement. Nevertheless, despite this procedure is gaining acceptance among the surgical community, indications, inclusion criteria and definitions of success are not yet standardized and extremely heterogeneous, making it difficult to reach definitive conclusions and to ascertain which are the real benefits of this new procedure. Moreover, long-term and functional results are poorly reported. The present review is focused on critically analyzing the theoretical benefits and risks of the procedure, short- and long-term functional results and future direction in the application of EVT.

Key Words: Anastomotic leakage; Rectal surgery; Endoluminal vacuum therapy; Endosponge; Complications

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Core Tip: Endoluminal vacuum therapy for the treatment of rectal anastomotic leakage, in clinically stable patients, has been reported to be promising, in term of high rate of anastomotic salvage and length of hospital stay. Nevertheless, inclusion criteria, definition of success and complications, are heterogeneous. Moreover, long-term anorectal function is poorly reported. This opinion review aims at clarify, through a critical analysis, all the raised points to stimulate the surgical community to a more standardized approach and algorithm of treatment, and to further study the long-term consequences of this technique.

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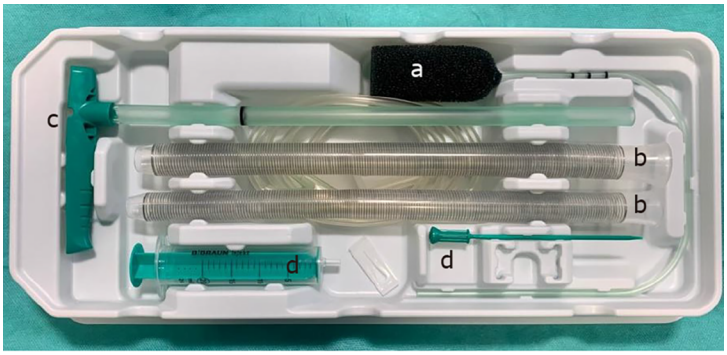
INTRODUCTION

Anastomotic leak (AL) still represents the most dreaded complication following colorectal resection due to its consequences that could severely affect functional and oncological outcome[1,2]. The gravity of the phenomenon is amplified when rectal cancer only is considered, with reported leakage rate ranging from 3% to 19%[3] and a mortality rate varying from to 1.7% to 16.4%[4]. The choice of the treatment is strictly influenced by the patient general condition and by the dimension of the anastomotic defect[5]. In 2007, the international rectal cancer study group, which includes expert colorectal surgeons and interventional radiologists from several regions in the world, proposed a standardized algorithm for treating ALs. Treatment options varies according to the location of the anastomosis, dimension of the abscess, and patient's clinical conditions[6]. In patients in whom a diffuse peritonitis has occurred, a laparotomy and takedown of the anastomosis is the suggested surgical treatment. More conservative treatments including the salvage of the anastomosis could be an option in patients who remain clinically stable or in presence of a small defect. Different options have been reported including trans-anastomotic tube drainage, percutaneous drainage of the peri-anastomotic abscess in association with fecal diversion, if not fashioned at primary operation, placement of stent or endoscopic clipping of the defect [4,6]. Nevertheless, in presence of a large defect, even in a clinically stable patient, the healing process is extremely long, resulting in a delay of diverting stoma and devastating future function of the neorectum, in particular when an extra-peritoneal anastomosis is taken into consideration. In the early 2000s, an endoluminal vacuum-assisted therapy (EVT) was introduced to treat presacral anastomotic abscesses in stable, non-peritonitic patients[7]. The principle is based on the application of topic negative pressure in order to drain, to clean, to induce the collapse of the cavity, and to prevent the development of chronic sinus. Several case reports papers, reviews, and meta-analyses[7-20] have been published so far with promising results. Nevertheless, the majority of papers are heterogeneous both in term of success rate definition, salvage and long-term results, paucity of comparative studies and thus definitive conclusions are not warranted at present time. In particular, the majority of the studies were focused on success rate, healing time and stoma closure rates, while only few papers dealt with long-term anastomotic function and complications that play a pivotal role when the issue of the efficacy of a novel treatment is taken into consideration. In this narrative review, we aim to critically appraise the literature with regard to the results of the EVT in term of success rate and complications and to evaluate the long-term functional results of this novel treatment.

EVT DEVICE DESCRIPTION

The endoscopic vacuum device consists of an open-cell polyurethane sponge measuring 7 cm × 3 cm, which can be cut down until minimum size, depending on the size of the cavity. The sponge dressing is placed into the abscess cavity using a specially developed introducer system. The end of the evacuation probe is connected to a vacuum wound drainage system *via* a variable drain connector (Figure 1).

In the majority of available studies the evacuation probe was connected to a low vacuum suction bottle (Redyrob® Trans Plus bottle with variable vacuum) (Figure 2), creating a constant negative vacuum pressure of 125-150 mmHg[20]. Higher values were reported by Arezzo *et al*[15] who connected the tube to a vacuum system producing continuum negative pressure up to 700 mmHg when in hospital, and a portable system producing continuum negative pressure up to 200 mmHg when discharged.



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Figure 1 Endo-SPONGE® kit including open-pore sponge drain (a), two silicon overtubes (b), the sponge pusher (c), and the irrigation set (d).



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Figure 2 Redyrob® Trans Plus vacuum bottle: this device is meant to be used connected to the Endo-SPONGE drain.

INCLUSION CRITERIA

The main indication to the use of EVT was represented by the presence of an extraperitoneal leak confirmed by flexible endoscopy ± computed tomography scan (CT) and on clinical and/or laboratory deterioration and drain secretion. Only patients in stable condition with no sign or only localized peritonitis were included. In the majority of patients, a leak following colorectal resection for cancer was considered. Table 1 reports the inclusion criteria of the available studies. The table shows up the lack of standardization in the inclusion criteria, some including both benign and malignant, some purely malignant or pure benign disease, to the different anastomotic heights, surgical approach, including both PME and TME, or in the different indications to EVT therapy, including Hartmann "stump insufficiency" or rectal perforation, as well as leaks following a proctectomy with a J-pouch ileoanal anastomosis. These data lead us to extreme caution in their interpretation, in the effort to make a critical analysis of the reported results. Moreover, a strong clinical heterogeneity has been reported with respect to initial cavity size ranging from 4.9 cm to 10 cm among the reported series[8-22]. Limitations to inclusion with respect to the initial cavity dimension have been reported only in one study[23].

Recently, it has been reported on the intraoperative use of indocyanine green (ICG) fluorescence from the luminal side *via* a trans-anal approach which enables to evaluate the whole circumference of anastomosis in the proximal and distal intestines[24]. Results showed that in patients in whom the vessels were not depicted by the ICG, a higher incidence of AL was observed. This finding is of relevance and it will be probably useful in selecting patients who will beneficiate of EVT *vs* patients in whom a more aggressive operative strategy is recommended. Possible future applications of this technique combined with endoscopy, will be the evaluation in patients with suspected leak, in order to confirm the presence of a leak and to evaluate local perfusion over the entire circumference of anastomosis in real time.

Table 1 Inclusion criteria among different studies

Ref.	# Patients	Anastomosis location	Inclusion criteria	Type of disease (*n*)	Neoadjuvant therapy (%)
Weidenhagen *et al* [7], 2008	29	Lower rectum; Middle rectum	Local peritonitis (20); General peritonitis (9)	Cancer of the rectum (22); Rectosigmoid cancer (3); Benign disease (4)	9 (40.9)
von Bernstorff *et al* [27], 2009	26	Lower rectum; Ileorectal	Local peritonitis	Cancer of the rectum + rectosigmoid	14 (54)
Riss *et al*[30], 2010	20	Lower rectum; Middle rectum	Not reported	Cancer of the rectum	6 (30)
van Koperen *et al* [8], 2009	16	Lower rectum; Ileo-anal	Not reported	Cancer (13); Ulcerative colitis (3)	9 (56)
Nerup *et al*[25], 2013	13	Lower rectum	Local peritonitis	Cancer	6 (46.1)
Mees *et al*[23], 2008	5	Lower rectum	Local peritonitis Abscess (> 3 cm × 3 cm, or < 10 cm × 10 cm)	Cancer of the rectum	No
Arezzo *et al*[15], 2015	14	Lower rectum	Local peritonitis	Cancer of the rectum (7); Other (1)	7 (50)
Strangio *et al*[11], 2015	25	Lower rectum (19); Middle rectum (5); Ileoanal (1)	Local peritonitis Anastomotic leak less than 270	Rectal cancer (18); Endometriosis (1); Left sided colon cancer (4); Diverticulitis (1); Ulcerative colitis (1)	18 (84)
Mussetto *et al*[31], 2017	11	Lower rectum(8); Middle (3)	Local peritonitis	Rectal cancer	5 (45)
Keskin *et al*[13], 2015	15	3 (20)	Local peritonitis	Rectal cancer (12); Other (3)	NR
Milito *et al*[16], 2017	14	Lower rectum	Local peritonitis	Cancer of the rectum	14 (100)
Srinivasamurthy *et al*[14], 2013	8	Lower rectum; Ileoanal	Not reported	Ulcerative colitis (1); Cancer of the rectum (8)	8 (100)
Abdalla *et al*[24], 2020	47	Middle (5); Lower (42)	Local peritonitis + asymptomatic leak	Cancer of the rectum (44); Other (3)	27 (57.4)
Kühn *et al*[28], 2021	281	Lower rectum; Ileoanal; Middle rectum	Local peritonitis extraperitoneal anastomotic leak; Rectal defect	Sigmoid or rectal cancer 183 (65); Other malignancies 50 (18); Diverticular disease 17 (6); Inflammatory bowel disease 12 (4); Perforation 8 (3); Benign/malignant diseases 11 (4)	84 (30)

EVT THERAPY RESULTS

EVT has been identified as a successful method in order to treat AL in clinically stable and non-peritonitic patients with reported figures ranging from 60% to 100% and a rate of diverting stoma closure ranging between 31% to 100%, as emerged by recently published systematic reviews on this issue[20,21]. The rate of success was significantly influenced by early therapy start (within 6 wk from onset) with summarized odds ratio of 3.48 as reported by Mahendran *et al*[18] in their review paper including 266 patients from 16 studies. Nevertheless, data extracted from the same paper underlined that an additional treatment was needed in 12.8% of patients, due to the persistence of the abscess cavity, including fibrin glue application, sutures under general anesthesia, clips placing over the scope or a combination of different techniques. Treatment duration, in current literature, varies between 11 and 244 d. Data are encouraging and promising showing that approximatively 67% of the patients had their anastomosis saved with no need of abdominal surgery[9]. Moreover, in selected cases, the EVT treatment could be performed without the need of a diverting stoma[7]. These percentages favorably compares with results reported by Kühn *et al*[21] in the largest comparative study recently published including 21 patients treated with EVT *vs* 41 historical controls treated with conventional management. The authors reported a significant higher preservation of the intestinal continuity in the EVT *vs* conventional group (86.7% *vs* 37.5%; $P = 0.001$) and shorter duration of hospital stay. Similarly, Nagell and Holte[22], who compared 4 patients treated with EVT with 10 historical controls, reported favorable results in healing time and length of stay in the EVT group when compared to conventional treatment. Mees *et al*[23] also reported a significantly shorter time for closure and reduced length of stay in the EVT group.

DEFINTION OF SUCCESS

Another point of discussion is represented by a lack of a standardization in the definition of success of the EVT treatment. In particular, data from the two large series (CLEAN study and GRECCAR study) including 39 and 47 patients respectively, defined the success rate as the absence of extravasation of contrast during abdominal CT or the presence of an intact anastomosis on endoscopy[17,25]. Other authors identified the cavity size, associated or not with the presence of granulating healthy tissue, to define the success rate, with figures ranging from 0.5 cm to 3 cm with no consensus among authors[7,9,26-28]. Kühn *et al*[28], in the largest monocentric series recently published, including both AL and rectal stump leak, defined success as granulating closure of the cavity, more than 90% clean and granulating tissue, decreasing wound secretion, reduction of fibrinous tissue, and no interventional or surgical procedure required in the further course.

FACTORS INFLUENCING SUCCESS

Different variables could potentially influence the success rate such as neoadjuvant therapy, presence of diverting stoma during the treatment, and length of follow-up[7-24]. von Bernstorff *et al*[27] first evaluated the effect of radiotherapy on the healing process following EVT therapy. The aforementioned author, and others later, reported a longer duration of therapy, more endoscopies, more sponge exchanges, and longer time to close the abscess cavity in patients who underwent neo-adjuvant treatment[7,15,27,29]. In contrast, others did not find any correlation of neo-adjuvant treatment on healing time and success rate[26,30]. A definitive answer to this topic has come from a subgroup analysis on the radiotherapy subject including eight studies, extrapolated from a meta-analysis, which reported a negative effect of radiotherapy on healing and success rate with a odds ratio of 0.56[20]. Similarly, Shalaby *et al*[9] in a cumulative analysis, encompassing more than 300 patients, identified preoperative radiotherapy (P = 0.018), development of complications (P = 0.002), male sex (P = 0.014) and absence of diverting stoma before treatment, as predictive factors for failure. This latter point deserves some consideration since, intuitively, the presence of a stoma plays a main role in the healing process, nevertheless, to the best of our knowledge, there is no paper or meta-analysis specifically addressing its role and future subgroup analyses will be advisable. In particular, to further clarify this issue, a detailed report of the stoma formation differentiating patients receiving a stoma at primary operation or after the leak was diagnosed will be mandatory, which actually is not fully available from the reported studies as recently stressed by Sharp *et al*[19] in their meta-analysis. Despite stoma reversal was considered by the majority of authors as a marker of success, concern has been expressed by some authors due to the complexity of this variable and to the fact that its closure could be influenced by different factors such as severe co-morbidity, insufficiency of the anal sphincter, chronic pre-sacral sinus, or local recurrence and malignancy[18,20].

Finally, with respect to the duration of follow-up, the latter is of pivotal importance since, as emerged by the multicenter study of Riss *et al*[30], more than 25% of the patients developed a recurrent abscess after a median follow-up of 17 mo. However, data extrapolated from the three recent meta-analyses published in the international literature, show that some of the included studies do not comprise follow-up data, or reported figures ranging within 1 and 4 mo, while less than 50% of the studies report a follow-up time longer than 12 mo, ranging between 14 and 41[18-20].

COMPLICATIONS

The overall EVT-related complication rate among the published series ranges from to 0% to 34.5%, with a mean of 11.1% (96 per cent confidence interval 6.0 to 16.2) as recently reported by Shalaby *et al*[9]. However not all the available studies report on this variable. The complication issue has been recently analyzed and discussed in two systematic reviews on EVT for rectal anastomotic leakage, including 295 and 335 patients respectively[20,21]. According to the review of Nagell and Holte[22], the most common complication is represented by pelvic abscess accounting for 11.5% of cases. Shalaby *et al*[9] reached similar conclusions in another review paper. The majority of abscesses were managed with a conservative treatment or *via* repeated EVT with success rate of 71%-75%, while in case of failure Hartmann's or Miles operation were performed (Table 2). From a more accurate analysis it emerged that in 1% of cases, the abscess occurred early and it should be considered as a treatment failures, while in 10% they were recurrent and thus they should be considered as a relapse after a primary healing has occurred [20]. According to these findings, could we still consider them as a complication or it would be more appropriate to classify them as a treatment failure? The same question spontaneously arises for the fistula issue. According to data extracted from studies reporting complications, overall 13 cases of fistulas were reported, of which 7 in a single series (Table 3). The majority of them were managed with Hartmann's procedure. Of note, the high incidence of fistulas recently reported by Kühn *et al*[28] in their series including 281 patients. In the aforementioned paper, all the fistulas were recto-vaginal in their

Table 2 Reported complications and treatment among studies reporting complications

Ref.	# Patients	Overall complications *n* (%)	Type of complication	Treatment	Ileostomy closure %
Weidenhagen *et al* [7], 2008	29	10 (34.5)	10 anastomotic stenosis; 2 fistulas	Bougienage/balloon dilatation (*n* = 10); Hartmann procedure for persistent fistula (*n* = 1)	88
von Bernstorff *et al* [27], 2009	26	2 (7.7)	2 intra-abdominal fistulas	Hartmann procedures (*n* = 2)	NR
Riss *et al*[30], 2010	23	6 (23)	1 stenosis; 5 recurrent abscess	Dilatation for stenosis (*n* = 1); Hartmann's procedure (*n* = 3); CT-guided drainage (*n* = 1); No further action (*n* = 1)	76.5
van Koperen *et al* [8], 2009	16	4 (25)	2 abscesses; 1 bleeding; 1 severe pain; 1 anastomotic stenosis	Hartmann procedure for recurrent abscess (*n* = 2); 1 stopped treatment for pain; 1 dilatation for stenosis	55.6
Nerup *et al*[25], 2013	13	1 (7.7)	1 anastomotic stenosis	Permanent colostomy (*n* = 1)	91
Mees *et al*[23], 2008	5	1 (20)	1 anastomotic stenosis	Dilatation (*n* = 1)	20
Arezzo *et al*[15], 2015	14	1 (14)	1 peritonitis; 2 poor compliance	Fibrin glue injection	NR
Strangio *et al*[11], 2015	25	3 (12)	2 fistulas (1 ureteric, 1 ileal); 1 recurrent abscess	Surgery (*n* = 3)	84.6
Mussetto *et al*[31], 2017	11	2 (18)	2 anastomotic stricture	1 endoscopic dilatation; 1 stent placement	91
Keskin *et al*[13], 2015	15	3 (20)	2 pelvic sepsis; 1 bleeding	Treatment discontinued	71
Milito *et al*[16], 2017	14	5 (36)	Moderate pain	None	NR
Srinivasamurthy *et al*[14], 2013	8	1 (12)	Iatrogenic injury during sponge placement	End stoma	64
Abdalla *et al*[24], 2020	47	4 (8.5)	1 intractable pelvic pain; 3 anastomotic stenosis	Treatment discontinued (pain); Endoscopic dilatation	NR
Kühn *et al*[28], 2021	281	27 (10)	10 anastomotic stenosis; 7 rectovaginal fistulas; 4 bleeding	Endoscopic dilatation (*n* = 10); Surgery (*n* = 7); Endoscopic haemostasis (*n* = 3); Surgery for intractable beelding (*n* = 1)	62

NR: Not reported.

nature, occurred in the early phase of EVT treatment, and the majority of the patients had initial surgery involving partial resection of the vagina or the uterus, suggesting that EVT might have either prompted or revealed a vaginal leak. In other series, fistulas were classified as abdominal, colovescical, ileal or ureteric, thus suggesting a progression of the leakage process and thus a failure of the treatment more than a complication[7,11,28].

Other complications of endoscopic vacuum treatment include anastomotic stenosis, with an estimated incidence of 4.4% in the cumulative analysis by Popivanov *et al*[20]. Widenhagen reported the occurrence of anastomotic stenosis in 33% of patients in a retrospective analysis of 29 cases. Stenoses were managed with bougienage or balloon dilatation[7]. In the series of Mussetto *et al*[31], including 11 patients, anastomotic stenosis accounted for 16%, while figures ranging from 6% to 11% were reported by others[26,32,33]. Nevertheless, the real incidence of the phenomenon is difficult to establish due to the limited follow-up period in a large percentage of the published studies. Moreover, anastomotic stenosis can also normally occur because of chronic inflammation related to the anastomotic leakage itself not as a direct consequence of EVT treatment, and thus considering purely as an EVT complication is questionable. Under this view, a comparative study with adequate follow-up period with patients who had received conventional treatment should be advisable to reach definitive conclusions.

The occurrence of moderate pain was a common complication in the series by Milito *et al*[16], accounting for 36% (5/14 patients), while intractable pain leading to a discontinuation of the EVT treatment been reported in two cases only[8,16,25]. The phenomenon is rare, 2%, for moderate pain and 0.4% for severe pain as emerged by data derived from the systematic review of Popivanov *et al*[20]. Other reported complications were bleeding from the cavity, which generally occurs in the act of changing the sponge. In the majority of cases, an endoscopic management has been successful, with only one case requiring conventional surgery. Migration of the sponge into the abdominal cavity has been also reported with an estimated overall incidence of 1%[20].

Table 3 Assessment of ano-rectal function after treatment of anastomotic leak with endoluminal vacuum-assisted therapy

Ref.	# Patients	# Patients with functional assessment	Follow-up time	Instrument to evaluate ano-rectal function	Results
Borstlap *et al*[17], 2018	30	15	6, 9, 12 mo	LARS score; COREFO	81% major LARS; 13% minor LARS
Huisman *et al*[36], 2019	20	13	2.6 (0.8-3.5) yr	LARS score	77% major LARS; 23% minor LARS
Katz *et al*[42], 2018	6	4	Not reported	None	Reasonable function
Srinivasamurthy *et al*[14], 2013	8	6	41 (10-45) mo	None	Good or reasonable function
Abdalla *et al*[24], 2020	47	17	14.8 ± 8.9 mo	LARS score	47.1% major LARS; 52.9% no or minor LARS
Rottoli *et al*[12], 2018	8 (pouch)	7	11.6 (6-18) mo	None	No feces or gas incontinence; BM: Daytime: 5 (3-8); Nighttime: 1.7 (1-4)
Weréen *et al*[43], 2020	14	6	5.9 (0.53-13) yr	LARS score	67% major LARS

LARS: Low anterior resection syndrome score; COREFO: Colorectal functional outcome; BM: Bowel movements.

CHRONIC SINUS AND ABSCESS RECURRENCE

One of the long-term sequelae of anastomotic failure is the development of chronic sinus. The true incidence is unknown, however, a large multicenter retrospective study on 1063 patients, reporting an incidence of 6.4% of anastomotic leakage after colorectal anastomosis or restorative proctocolectomy, 36% of whom developed a chronic sinus[34]. The occurrence of chronic sinus involves multiple interventions and a high risk of permanent stoma[35]. A proportion of chronic sinuses may heal spontaneously over time, nevertheless when sinus eventually resolves, it is associated with poor functional outcome[36]. The occurrence of chronic sinuses after AL treatment with EVT is poorly studied. Borstlap *et al*[17] reported a 34% rate after a 6 mo follow-up. Comparing patients who underwent early treatment (before 3 wk) *vs* late treatment (after 3 wk), they observed a higher rate (47%) in the latter group *vs* 21% in the former. Accordingly, the diverting ileostomy could be reversed in 60% of the patients in the late group as compared to 73% in the early group[37]. The authors argued that a late starting could lead to excessive fibrosis of the bowel thus hampering fistula closure.

Time for beginning EVT treatment was evaluated as prognostic factors by other authors with conflicting results. Huisman *et al*[36] reported that 3/20 patients (15%) experienced a chronic sinus, after a median follow-up of 10 mo, and all three received a permanent stoma because of the sinus. They grouped the patients according to start of EVT, before or after 20 d from surgery, but no significant difference between the early and late treatment groups were found.

Another important issue is recurrent leak after the anastomosis is healed. Riss *et al*[30] studied 20 patients treated by rectal resection for rectal cancer and by successful endoscopic EVT of AL (17 patients) or insufficiency of the rectal stump after Hartmann's procedure (3 patients). The patients received annual routine visits and colonoscopy. Five patients (25%) developed a recurrent pelvic abscess, three of them underwent surgery (Hartmann's procedure) and one to CT guided drainage; the treatment for the last patient was still under discussion at the time of publication. Interestingly, the authors did not identify any demographic, therapeutic, or temporal related significant factors that could predict the occurrence of late leak recurrence. The authors concluded that a surveillance of at least 2 years would be recommended for early identification and treatment of this problem. Actually, treatment of chronic sinus is challenging, since its presence precludes the closure of the ileostomy or dictates other surgical treatments such as Hartmann's procedure with closure of the ileostomy, and creation of a permanent colostomy, in the majority of the patients[7,28]. Jagielski *et al*[37] reported a different experience. They treated two patients, with recurrent abscess and anastomotic fistula, once more with EVT. The treatment duration was 15 d with four endosponge changes; they obtained fistula healing without need of major surgery. The authors also underlined that the endoscopic treatment could be interrupted as far as the cavity reached 30 mm with granulation tissue on the wall, without waiting for complete healing; this approach allowed faster endoluminal treatment with no impact on recovery.

In conclusion, despite endoscopic vacuum-assisted treatment allows successful treatment of AL, persistent sinus or recurrent fistula and abscess may occur. A constant follow-up is advisable for early diagnosis and care. Therapy often entails surgical treatment; however, a conservative treatment might also be attempted in selected cases.

FUNCTIONAL RESULTS

After restorative anterior rectal resection, bowel function could be impaired, which adversely affects quality of life. Low anterior resection syndrome (LARS), including incontinence, urgency, diarrhea, frequency and clustering of bowel motions, is being increasingly recognized[38]. The scores are categorized into three groups: 1- no LARS (0-20 points), 2- minor LARS (21-30 points) or 3- major LARS (31-42 points). Although an altered function could be present, in patients with rectal cancer, before any treatment, radiotherapy and surgery represent detrimental factors since they can affect both internal and external anal sphincter function as well as rectal compliance and sensory thresholds[39]. Recently a patient-reported outcome measure on bowel related quality of life showed that 85% of patients had some degree of impairment up to 5 years after surgery. Moreover the degree of quality of life impairment increased as the LARS and Wexner fecal incontinence scores increased, indicating that severity of quality of life impairment reflects severity of bowel dysfunction[40]. Bowel function can improve up to 18 mo after surgery or stoma reversal, however, after that time, further recovery is unlikely. A recent systematic review on 11 studies on rectal cancer patients who underwent low anterior rectal resection and completed a LARS score, with a mean or median follow-up of at least 18 mo, found a prevalence of major LARS of 41%, ranging between 18% to 56%. Radiotherapy and tumor height were the most significant factors for LARS development; AL, diverting ileostomy, and having a stoma for prolonged period, were also associated with increased risk of developing major LARS[41].

Articles on the EVT treatment of rectal AL mainly focus on leak resolution and preservation/restoration of the intestinal continuity. Very few assess long-term functional results, and the majority of them did not employ validated score systems or systematic search of the functional outcomes. Two small series observed "reasonable function", both in 5 patients who underwent stoma closure, after a mean follow-up of 28 and 41 mo respectively[14,42]. However, no score was used to grade bowel function. In another study, of the seven patients who had the ileostomy reversed, none reported incontinence to feces or gas after 11.6 mean follow-up, though it is not described if these symptoms were systematically explored[12].

Intestinal function was evaluated with the LARS questionnaires by few authors. Abdalla *et al*[24] studied 17 patients (out of 26 who had successful EVT treatment) after 14 mo on average following stoma reversal or AL healing, and reported 47.1% with major LARS.

The more complete assessment of postoperative function and related quality of life was performed by Borstlap *et al*[17]. The authors evaluated the LARS score, the colorectal functional outcome (COREFO) scale, the short form 36 (SF-36), the gastrointestinal quality of life index (GIQLI) questionnaire and the EQ-5D-5L at fixed time points. Eighty-one per cent of the patients experienced major LARS, 13% minor-LARS and only 6% did not report any functional problem. Analysis of the COREFO showed that function did not improve from 6 mo to 12 mo postoperatively. On the other hand improvement of the EQ-5D-5 L, GIQLI and SF-36 scores was noted during follow-up.

Only one study compared functional outcome in patients with AL treated with EVT with a group of patients without AL, after rectal resection[37]. A worse LARS score was found in the EVT group (37, range 23-42 points) with respect to the control group (30, range 4-41) points (P = 0.009), with 77% of patients reporting major LARS in the EVT group as compared with 48% in the control group. This study supports the impairment of long-term anorectal function caused by anastomotic dehiscence, possibly due to fibrosis and reduced rectal compliance. However, the question whether EVT treatment could improve functional results in patients with AL remain unanswered.

CONCLUSION

Our narrative review shows, with all the limitations related to the nature of the available studies from international literature, that EVT represents, in clinically stable non-peritonitic patients, a valid alternative to conservative approach (diverting stoma, drain) with relatively low percentage of complications, higher rate of stoma closure, and shorter length of hospital stay. Some questions currently remain unanswered, in particular with respect to quality of life and functional results following EVT treatment. Moreover, it seems to us extremely difficult to identify patients who will beneficiate most of these new treatment, due to strong heterogeneous inclusion criteria, different materials and treatment algorithms.

Objectively a randomized trial would be advisable to assess the real efficacy of a new therapy, nevertheless due to ethical considerations and to the “fragile” status of these patients, may prove difficult to perform. A possible alternative could be represented by a well-designed multicenter control study with standardized inclusion criteria, standardized definition of success, and adequate follow-up period and control group. These latter two points, in our opinion, are of pivotal importance in order to give a definitive answer on the diverting stoma closure issue, which identification as a marker of success, due to its complexity, should be re-considered.

FOOTNOTES

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Country/Territory of origin: Italy

ORCID number: Andrea Vignali 0000-0002-7887-4088; Paola De Nardi 0000-0002-9337-6765.

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REVIEW

Viral hepatitis: Past, present, and future

Matthew August Odenwald, Sonali Paul

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Matthew August Odenwald, Sonali Paul, Department of Medicine, Section of Gastroenterology, Hepatology, and Nutrition, Center for Liver Diseases, University of Chicago, Chicago, IL 60637, United States

Corresponding author: Sonali Paul, MD, MS, Assistant Professor, Department of Medicine, Section of Gastroenterology, Hepatology, and Nutrition, Center for Liver Diseases, University of Chicago, 5841 S. Maryland Ave. Rm J-517, MC7120, Chicago, IL 60637, United States. spaul@medicine.bsd.uchicago.edu

Abstract

Each hepatitis virus—Hepatitis A, B, C, D, E, and G—poses a distinct scenario to the patient and clinician alike. Since the discovery of each virus, extensive knowledge regarding epidemiology, virologic properties, and the natural clinical and immunologic history of acute and chronic infections has been generated. Basic discoveries about host immunologic responses to acute and chronic viral infections, combined with virologic data, has led to vaccines to prevent Hepatitis A, B, and E and highly efficacious antivirals for Hepatitis B and C. These therapeutic breakthroughs are transforming the fields of hepatology, transplant medicine in general, and public and global health. Most notably, there is even an ambitious global effort to eliminate chronic viral hepatitis within the next decade. While attainable, there are many barriers to this goal that are being actively investigated in basic and clinical labs on the local, national, and international scales. Herein, we discuss pertinent clinical information and recent organizational guidelines for each of the individual hepatitis viruses while also synthesizing this information with the latest research to focus on exciting future directions for each virus.

Key Words: Viral Hepatitis; Hepatitis A; Hepatitis B; Hepatitis C; Hepatitis D; Hepatitis E; Hepatitis G

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Core Tip: Viral hepatitis encompasses a wide array of clinical diseases—from asymptomatic and self-limited to chronic liver disease to acute liver failure. Extensive historical research has resulted in vaccines to prevent Hepatitis A, B, and E and highly efficacious antivirals for Hepatitis B and C, and these therapeutic breakthroughs are transforming the fields of hepatology, transplant medicine in general, and public and global health. While these breakthroughs are highly promising, there are many barriers to eventually elimination of chronic viral hepatitis. These barriers are being actively investigated, and we discuss ongoing research in the historical context of viral hepatitis research.

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INTRODUCTION

In this review of viral hepatitis infections, we discuss the pertinent clinical information and recent organizational guidelines for each of the individual hepatitis viruses while also synthesizing this information with the latest research to focus on exciting future directions for each virus.

HEPATITIS A

Hepatitis A

The Hepatitis A Virus (HAV) is a single stranded, non-enveloped ribonucleic acid (RNA) molecule. HAV is a member of the Picornaviridae family and the *Hepatovirus* genus that is transmitted primarily through a fecal-oral route *via* person-to-person contact or ingestion of contaminated food or water[1,2]. Globally, serologic evidence of prior infection is quite high, but prevalence has high geographic and demographic variability[3]. Acute HAV infection characteristically causes a self-limited illness. However, cases of fulminant liver failure have been reported with advanced age being the greatest risk factor for symptomatic disease[4]. Treatment is primarily preventative with vaccination prior to possible exposures, and both vaccination and HAV immunoglobulin to confer both active and passive immunity after exposure[5].

Epidemiology

An estimated 1.4 million cases of hepatitis A occur globally each year[6]. Estimates of disease prevalence vary regionally and are highly dependent on socioeconomic status and access to clean water. In developing countries with poor sanitation, there is nearly 100% seropositivity for HAV immunoglobulin G (IgG). In these countries, it is presumed that most children are infected at very early ages, when minimal symptoms develop and therefore clinical presentation of HAV infection is very rare[3]. In wealthy nations, including the United States, HAV IgG seropositivity rates are much lower[7]. Seropositivity increased with age and was lower among United States-born residents compared to immigrants [8]. Since the introduction of the HAV vaccine in 1996, new cases of HAV have declined by over 90% in the United States despite relatively low rates of HAV vaccination[9]. International travel to endemic areas or person-to-person contact with an infected person are the main risk factors for HAV.

Natural History of Infection and Clinical Course

The primary route of HAV infection is the fecal-oral route from contaminated food and water. This is evidenced by detection of HAV RNA in stool during the incubation period and for up to 4-5 mo after the onset of symptoms[10]. In small children, the infection is largely asymptomatic with fewer than 10% of children < 6 years old developing jaundice with HAV infection, and the only evidence of infection is serologic presence of anti-HAV antibodies. In older children and adults, HAV infection follows a characteristic pattern[5]. Infection is followed by an average 28-d incubation period where the virus actively replicates in hepatocytes. HAV itself is not thought to be directly hepatotoxic as there is no laboratory or clinical evidence of liver damage during the incubation period, and HAV can be propagated *in vitro* without any evidence of cytopathology[11]. After the incubation period, however, there is immune-mediated damage to the hepatocytes that results in non-specific symptoms of fever, malaise, fatigue, and loss of appetite followed by jaundice in approximately 70% of patients[12]. The majority of patients (approximately 60%) have a full recovery within 2 mo. Of the patients who do not fully recover within 2 mo, some develop prolonged cholestasis and others have relapsing disease with 2 or more bouts within a 6-10 wk period driven primarily by viral shedding within the stool and

reinfection. The overwhelming majority (nearly 100%) of these patients fully recover within 6 mo of disease onset, and there is no increase in mortality with any of these disease presentations[9].

Fulminant HAV is associated with low HAV RNA titers and high bilirubin levels, likely related to a robust host immune response reducing HAV viral load and resulting in significant hepatocyte damage [4]. Acute liver failure occurs in less than 1% of cases of HAV infection[4]. It is more common among patients with advance age (> 75 years old), underlying liver disease, or chronic kidney disease. The incidence of fulminant HAV in the United States has decreased dramatically from 1990 to 2005[13]. Data from the United States Acute Liver Failure Study Group (ALFSG) showed that the proportion of ALFSG cases due to HAV was low (29 of 925 patients, 3.1%), and of these patients, 55% recovered, 31% received transplant, and 14% died[13]. In 2006, the ALFSG study group designed a prognostic model based on clinical features at presentation [alanine transaminase (ALT) < 2600 IU/L, creatinine > 2.0 mg/dL, intubation, and vasopressors] that predict the likelihood of death and need for transplant with high accuracy[13]. Subsequently, a refined scoring system was derived from a cohort of 294 Korean patients with fulminant hepatitis A to predict the likelihood of death or need for liver transplant[14]. This scoring system takes multiple objective values (age, international normalized ratio, bilirubin, ammonia, creatinine, and hemoglobin) at the time of HAV-associated ALF into account, and compared to the ALFSG study group, this new model better predicted the likelihood of death or need for transplantation in both the Korean discovery cohort and international validation cohorts[14]. These scoring systems are useful in determining the level of care that a patient with acute HAV infection should receive. Nevertheless, there is an unusually high rate of recovery for HAV-related acute liver failure, and given this, auxiliary transplantation and artificial liver devices have been proposed as therapeutic bridges to native liver recovery and regeneration[15]. However, these are not commonly used in clinical practice.

Prevention, Diagnosis, and Treatment

There are currently 4 inactivated HAV vaccines available, all with similar efficacy and side effect profiles. However, widespread vaccination programs are not currently universal[16]. In fact, the World Health Organization (WHO) recommends that large-scale efforts should not be undertaken in highly endemic areas where nearly 100% of children contract HAV early in life and are asymptomatic[16]. On the other hand, in regions with lower rates of disease and higher acute infection rates later in life (when it is more likely to be symptomatic and result in increased healthcare costs) the WHO recommends either targeted vaccination of high-risk groups (in very low prevalence areas) or universal vaccination programs (in intermediate endemic areas)[16]. Given recent outbreaks and increases in the number of cases reported in the United States each year, the United States Centers for Disease Control (CDC) now recommends vaccination of all children > 1 year old in addition to the traditional at-risk groups[17].

Clinically, HAV infection is indistinguishable from other forms of acute viral hepatitis and diagnosis relies on serologies. An acute infection is defined by the presence of anti-HAV-immunoglobulin M (IgM) antibodies, which are present within a couple of weeks of exposure and at the onset of symptoms. Anti-HAV-IgG antibodies are also present at the onset of symptoms. While the anti-HAV-IgM titer decreases over time and is generally undetectable after 1 year of exposure, the IgG antibody is present for life and confers lifelong immunity. HAV RNA can be found in various bodily secretions and excretions, which can determine infectivity but levels are not currently used clinically.

Treatment for HAV is largely supportive with spontaneous recovery in the overwhelming majority of patients. While there is no anti-viral treatment for HAV, some studies have investigated post-exposure prophylaxis with both active immunity with HAV vaccination and conferring passive immunity through HAV immunoglobulin infusions. The most comprehensive study came in 2007 when Victor *et al* [18] performed a randomized control trial comparing HAV vaccination to HAV immunoglobulin in 1090 household contacts aged (2 to 40) of HAV patients. Both groups had low rates of hepatitis A, and the study's noninferiority criteria were met[18]. As such, the United States CDC recommends either HAV vaccine or HAV immunoglobulin for post-exposure prophylaxis within 2 wk of exposure[19]; however, the HAV vaccine does have an advantage over immunoglobulin, including active immunity and longer duration of action.

In summary, while effective HAV vaccines are available, the available data support the current practice of targeted vaccination in areas where patients who are more prone to more severe symptoms from HAV are more likely to be exposed rather than vaccinating all individuals in endemic areas. For those who are exposed, it will be interesting to see if further improvements can be made to already good predictive models to determine the clinical trajectory of patients with acute, fulminant HAV to determine whether liver transplant will be needed. Finally, we will be eagerly watching for further data on currently investigational liver support devices, which hold promise to provide supportive care through fulminant HAV and obviate the need for liver transplantation.

HEPATITIS B

Hepatitis B infection is caused by the Hepatitis B Virus (HBV), a deoxyribonucleic acid (DNA) virus belonging to the Hepadnaviridae family and the Orthohepadnavirus genus[20]. It is transmitted *via*

exposure to infected blood or bodily fluids, most commonly from intravenous drug use, sexual contact, or vertical transmission from mother to child[20]. The burden of HBV is declining in the developed world due to vaccination[21], but HBV prevalence is still quite high in endemic areas primarily due to vertical transmission between mother and child and early life exposures[22]. The age of HBV infection is the principal factor determining the course of disease; the overwhelming majority of perinatally infected patients develop chronic hepatitis B whereas the majority of adults who are infected readily clear the virus[23]. Antiviral medications can stop viral replication and subsequent liver damage. While no available treatments can clear HBV infection, there are exciting investigational agents that may provide therapeutic benefit in the future[24]. Moreover, there is a broad global health effort to eliminate HBV *via* a combination of aggressive vaccination, diagnostic, and treatment programs[25].

Epidemiology

In 2006, it was estimated that 2 billion people had been infected with HBV and that 360 million people were living with chronic hepatitis B worldwide. There is geographic variation in hepatitis B prevalence. Endemic regions like Southeast Asia, Sub-Saharan Africa, and parts of South America have prevalence rates greater than 8% compared to 2% in non-endemic areas, including the majority of North America [26]. Routes of transmission differ between endemic and non-endemic areas and determine the course of HBV infection. In endemic areas, vertical transmission between mother and child and horizontal transmission among young children are the most common routes of HBV infection, but in non-endemic areas, intravenous drug use and sexual transmission in adults are the predominant modes of infection [27]. Exposure to HBV within the first six months of life confers a nearly 90% risk of developing a chronic infection due to immunologic tolerance, which decreases to approximately 50% risk if exposed before the age of 6[27]. Acutely infected adults with intact immune systems, however, spontaneously clear HBV infection in a remarkable 95% of cases. Taken together, these data indicate that the majority of chronic HBV in the world is within endemic areas. In line with this, recent studies have shown that more than 90% of cases of chronic HBV in the United States are in immigrants from endemic areas[28]. While the incidence of acute HBV is declining in the United States due to vaccination, blood product screening, and perinatal screening, the incidence of chronic HBV is increasing due to changing immigration patterns and increasing immigration from endemic areas[28]. A recent meta-analysis assessed the prevalence of hepatitis B surface antigen (HBsAg) in hemodialysis patients in the Middle East found a 4.4% positivity rate, which is decreasing over time[29].

Natural History of Infection and Clinical Course

Hepatitis B is a small DNA virus with 10 known genotypes. The enveloped hepatitis B virus is recognized *via* the HBsAg and enters the cell *via* receptor-mediated endocytosis[20]. Upon entry into the cell, HBV is uncoated and undergoes repair of the single-stranded DNA to either integrate into the host genome or form covalently closed circular DNA (cccDNA), both of which serve as templates for transcription and translation[20]. The cccDNA persists in hepatocytes even after other signs of declining virus activity, including HBsAg loss, and is the main cause of HBV persistence despite antiviral treatment. The 3.2kb genome has 4 open reading frames that encode for the (1) Core gene (important for viral packaging and production of e-antigen (eAg); (2) surface gene (encodes surface proteins); (3) X-gene (which maintains expression of cccDNA); and (4) polymerase gene (encodes multiple proteins important for DNA replication, including a reverse transcriptase and polymerase)[24]. Once transcribed from cccDNA, immature RNA molecules are packaged into nucleocapsids that can either be recycled to the nucleus or further packaged and trafficked to budding sites in a HBsAg-dependent manner.

HBV is not directly cytotoxic but instead the clinical course of HBV is determined by the intensity of the immune response[30,31]. Acute HBV infection is a subclinical illness in approximately 2/3 of the cases while the other 1/3 develop symptomatic hepatitis and 1% develop acute liver failure with survival rates of only 20%. Regardless of the initial clinical manifestation, with an intact immune system, the majority of HBV is rapidly cleared, and understanding the modes of natural clearance are important for developing new treatment strategies.

The innate immune system is classically thought of as the initial line of defense against pathogens: It uses non-specific defenses including proinflammatory cytokines, interferons, and natural killer (NK) cells to keep the virus under reasonable control[31]. The adaptive immune system is fine-tuned to more specifically fight the pathogen. In hepatitis B, however, the line between these two arms of the immune system are blurred. In acute HBV infection, the innate immune system has a relatively weak release of the prototypical cytokines and weak induction of interferon and interferon-stimulated genes[32]. In fact, HBV has been shown to actively suppress interferon release and inhibit with interferon functions[33]. In one study of 21 patients with acute HBV infection, these patients had an increase in the anti-inflammatory cytokine interleukin (IL)-10 while there was no change in interferon levels from baseline[34]. Increases in IL-10 were accompanied by a decrease in NK cell activation and attenuated HBV-specific $CD4^+$ and $CD8^+$ T-cell responses[34]. Moreover, there is a decrease in NK cell function in clearing the initial infection. However, non-traditional roles for NK cells, including regulating the adaptive T-cell response, and the non-classical NK T-cells (NKT cells) can clear HBV without the help of $CD4^+$ or $CD8^+$ T-cells. These defects in the innate immune response to HBV infection are likely the culprit for the relatively low rate of symptomatic hepatitis in the acute phase and have led to HBV being considered a

"stealth virus"[35].

Adaptive immunity plays a crucial role in clearance of acute HBV infection. Classically, $CD8^+$ T-cells selectively eliminate virus-infected cells by recognizing short viral epitopes on infected cells, and HBV-specific $CD8^+$ T-cells are an integral component of natural HBV control[31]. This was suggested by a study of 23 patients with acute, self-limited HBV[36]. Interestingly, the highest frequency of HBV-specific $CD8^+$ T-cells correlated with the clinically acute phase of infection[36]. Causation was more convincingly demonstrated with an experimental chimpanzee model with selective depletion of either $CD4^+$ T-cells or $CD8^+$ T-cells prior to acute HBV infection[37]. Chimpanzees with depletion of $CD8^+$ T-cells had delayed viral clearance, and viral clearance did not occur until the reappearance of $CD8^+$ T-cells[37]. Repopulation of $CD8^+$ T-cells and viral clearance also coincided with interferon expression. Depletion of $CD4^+$ T-cells did not have any appreciable effect on viral clearance compared to controls [37].

Patients who are unable to mount this initial immune response fail to clear the virus and progress to chronic HBV. Immunologic hallmarks of chronic hepatitis B infection include numerical and functional deficiency of HBV-specific $CD8^+$ T-cells as well as decreased B-cell function[38-40]. Given their crucial role in clearing acute HBV infection, augmenting the number and function of $CD8^+$ T-cells is of great interest in therapeutic development.

Chronic hepatitis B has four distinct phases: the immune tolerant phase, immune active phase, inactive carrier phase, and reactivation. In the immune tolerant phase, there are high HBV viral loads without lab evidence of liver inflammation. Immune active phase is evidenced by lower viral loads with elevated transaminases. If untreated, patients in the immune active phase have a very high chance (approximately 20%) of progressing to chronic liver disease with cirrhosis and hepatocellular carcinoma (HCC) in approximately 25%-30% of patients with the presence of active viral replication and necroinflammatory liver disease being predictors of disease progression. In the absence of antiviral therapy, patients with HBsAg-positive cirrhosis have an 84% 5-year survival when compensated but a bleak 14% 5-year survival rate after the initial decompensation event[41]. While current antivirals can help improve liver histology, decrease hepatic decompensation, and improve long-term survival, achieving a functional cure (*i.e.*, HBsAg loss) is an uncommon event with unknown predictive factors[42,43]. In the inactive carrier state, patients have normalization of transaminases, undetectable HBV virus levels, and in some patients, fibrosis improvement[44]. However, these patients can reactivate either due to loss of immune control whether spontaneously or induced by immunosuppressive therapies[45]. Despite available treatments, the burden of chronic HBV is still very high and is estimated to account for 700000 deaths each year from decompensated cirrhosis and HCC[46].

Prevention, Diagnosis, and Treatment

As discussed above, vertical and horizontal transmission in childhood are responsible for the majority of chronic HBV infections. HBV can be prevented with administration of an effective HBV vaccine, which has been available since the 1980s. This was demonstrated with mass childhood vaccination programs in the endemic area of Taiwan that started in 1984[47]. Seroprevalence of HBV was tested 10 years after the introduction of mass vaccination programs and showed marked declines in the childhood presence of HBsAg (from 9.8% to 1.3%) and rising rates of immunity as marked by hepatitis B surface antibody (HBsAb) (from 23% to 79%)[47]. Importantly, a subsequent study showed that universal childhood HBV vaccination was also linked to reduced incidence of childhood HCC and HCC-associated mortality[48]. A similar aggressive vaccination program for Native Alaskans was conducted in newborns and young-children and was linked to elimination of acute, symptomatic HBV infections and HCC[49]. Additionally, despite a growing population, the number of children identified with HBsAg fell from 697 to 2 after initiation of this vaccination program[49]. The effect of vaccination has also been studied in infants at highest risk of vertical transmission—those born to HBeAg positive, HBsAg carrier mothers[50]. In combination with hepatitis B immunoglobulin (HBIG), neonatal vaccination for HBV was extremely efficacious. Persistent HBsAg was found in only 6% of the infants receiving both vaccination and HBIG compared to 88% of those receiving placebo[50]. With this marked efficacy in mind, the World Health Organization has recommended universal birth dose vaccination against HBV. However, despite the demonstrated efficacy more than 35 years ago, this has still not occurred in the majority of endemic countries. Expanding HBV vaccination is therefore the focus of many ongoing global health efforts to eliminate HBV[25].

Available antiviral medications against HBV are interferon-based regimens or nucleotide/nucleoside reverse transcriptase inhibitors (NRTIs), including entecavir, tenofovir, lamivudine, adefovir, and telbivudine[42]. Interferon-based therapies have both immune stimulating and antiviral effects and have higher potency than NRTIs; however, it is used much less frequently because of its adverse effects. The first generation NRTIs (lamivudine, adefovir, and telbivudine) suffered from low-barriers to viral resistance and have been replaced by second generation NRTIs (entecavir and tenofovir) in clinical practice[42]. When used alone, NRTIs can achieve HBV DNA negativity in 70%-85% of patients and e-antigen seroconversion in 20%-25% of patients after 1 year of treatment with even further benefit after 3 years[51]. However, even patients with seroconversion have high rates of HBV relapse upon withdrawal of medication (approximately 30% by 5 years), which has led to prolonged therapy even after e-antigen seroconversion to decrease the risk of reactivation[52]. While many patients are treated

with lifelong NRTIs with minimal side effects, even the simplest treatment algorithms require physician visits, lab draws, and HCC screening every 6 mo. This is costly and inconvenient, which can lead to non-adherence to care for a variety of reasons, especially in many resource-limited endemic countries. Indeed, worldwide, many patients that guidelines would suggest to be on NRTI therapy do not actually receive treatment[25]. Multiple approaches to remove this barrier to receiving care are being investigated and include determining if and when therapy can safely be withdrawn and combining NRTI therapy with other therapies to improve durable, off-treatment responses. In retrospective analyses of patients who have discontinued NRTI, it appears safe to stop therapy if patients do not have HBeAg, have very low levels of HBsAg, and have minimal fibrosis. However, flares can still occur and HCC screening is still required, and therefore close follow up is still warranted, suggesting that this approach may not be feasible on a global scale[53]. NRTI withdrawal appears to be more feasible in non-cirrhotic, HBeAg negative patients with low HBV DNA titers if NRTI is combined with interferon therapy; however, this too was done in a highly regimented clinical trial setting with relatively short-term follow up[54]. At the moment, this approach is only experimental, and real-world experience with longer term data to determine HBV relapse rates and HCC occurrence has yet to be seen.

In sum, the most promising method for prevention of primary HBV infections is early, universal vaccination, and we are hopeful that aggressive vaccination campaigns already underway will substantially reduce the burden of HBV in the coming decades. Fortunately, highly active antivirals are capable of controlling the virus and reducing the burden of advanced liver disease from HBV; however, there remains no cure for HBV. In the next section, we discuss many exciting experimental approaches aimed at curing HBV *via* multiple different mechanisms.

Future Therapies Under Investigation

The high rate of HBV relapse after NRTI withdrawal reflects the persistence of cccDNA and integrated HBV DNA. Multiple drugs in development target components of the HBV lifecycle as well as the immune response to HBV infection. Future approaches to achieving a cure for hepatitis B will likely exploit all three pathways. That is, (1) Use of existing potent antivirals in combination with; (2) novel, direct acting antivirals; and (3) immunomodulators that enhance clearance of cells that harbor HBV DNA[24]. Novel direct-acting antiviral therapies under investigation include gene editing and suppression *via* clustered regularly interspaced short palindromic repeats (CRISPR-CAS-9) technology and gene suppression *via* silencing RNA (siRNA) and antisense oligonucleotides (ASOs)[24].

The CRISPR approach has been used with guide RNAs designed to target cccDNA components (core, polymerase, or X open reading frames)[55]. This study demonstrated the ability of CRISPR technology to directly cleave cccDNA and significantly reduce both HBV RNA titers and HBsAg concentrations *in vitro* with both transient transfection and sustained expression. In a mouse model of HBV infection, simultaneous delivery of HBV and guide RNA was also shown to result in decreased HBV viral titers and HBsAg levels[55]. Given that both integrated HBV DNA and cccDNA can contribute to HBV persistence, another group successfully used CRISPR to excise a full-length integrated DNA fragment while simultaneously disrupting cccDNA in a cell line that stably expressed HBV DNA[56]. All measures of HBV chronicity were undetectable for 12 mo after this therapy[56]. While this is promising, CRISPR-Cas9 targets the integrated DNA by inducing double stranded DNA breaks within the host genome, which has the potential to have detrimental off-target effects including host genome rearrangement. More targeted approaches with CRISPR-Cas-9 "nickases" to mediate only single strand nicks and base-editing to introduce mutations have also been employed[57]. A more targeted approach with CRISPR-Cas9-mediated base-editing to introduce mutations has also been attempted *in vitro*. The introduction of either missense or nonsense mutations resulted in inactivation of both integrated and cccDNA with associated reductions in HBV viral titers, HBsAg, and reductions in both surface and polymerase proteins[57]. Moreover, CRISPR-Cas-9-mediated knockout of HBsAg in an HCC cell line reduced proliferation *in vitro* and the ability of these cells to form tumors in mice, suggesting therapeutic potential for HBV-associated HCC as well[58]. Together, these data suggest that CRISPR-Cas9 technology has the potential to promote a true HBV cure.

Alternative approaches that directly target the hepatitis B virus include targeting the RNA products of both cccDNA and integrated HBV transcription *via* specific siRNA or ASOs. Both siRNA and ASOs are short RNA sequences that are complementary to the viral mRNA and therefore generate double stranded RNA that is targeted for degradation *via* dicer or RNase-H, respectively[59]. With multiple siRNA or ASOs administered in each dose, this approach has the potential to target multiple mRNA products simultaneously[59].

One siRNA molecule, ARC-520, is designed to target the open reading frame of HBV X but overlaps with all cccDNA transcripts and therefore has the potential to target all cccDNA transcripts for degradation. ARC-520 was shown to be safe in healthy volunteers[60], and in a phase II study, ARC-520 resulted in significant reduction in HBsAg production in patients that were NRTI-naïve or had HBeAg [61]. The difference in response to RNA interference (RNAi) between HBeAg+ and HBeAg- patients was investigated using chimpanzees and determined to be due to the presence of integrated DNA in addition to cccDNA[61]. In follow up studies of ARC-520 activity against chronic HBV in NRTI-experienced patients, four monthly doses of the siRNA resulted in dose-dependent reduction in HBsAg concentrations regardless of HBeAg status. The reductions were only modest (approximately 0.4 log

reduction from baseline), possibly resulting from the presence of integrated DNA that is not targeted by this particular siRNA[62]. Additional siRNAs are currently under development and preliminary data from one siRNA that uses a N-Acetylgalactosamine ligand to target the siRNA to the liver suggests improved HBsAg reduction (approximately 1.75 log reduction) with dosing every 4 wk[63].

The potential of ASO in treating chronic HBV was recently demonstrated in preclinical *in vitro* and *in vivo* mouse models[64]. A target second-generation ASO that was complementary to reference sequences in HBV genotypes A-H was identified as an effective ASO using *in vitro* models. This ASO reduced HBV DNA expression, replication, viremia, and HBsAg and HBeAg production in multiple HBV genotypes[64]. Importantly, the ASO used had no interference with the anti-viral activity of NRTIs when given simultaneously, suggesting that combination with existing therapy is feasible[64]. An HBV-targeted ASO was recently shown to be safe in escalating doses in healthy human subjects[65], and subsequently shown to have excellent antiviral activity in patients with chronic hepatitis B with reduction in HBsAg regardless of concurrent therapy after 29 d[66]. In addition to the longer-term follow up that is underway, it will be important to determine if there can be a further decline in measures of HBV infection if combined with additional ASOs or if sequence variations between individuals can be accounted for with use of multiple ASOs simultaneously[67]. Moreover, further modifications to ASOs are currently in development to improve delivery to hepatocytes[67].

Immunomodulation aims to rectify the relative functional and numerical deficiency of $CD8^+$ T-cells that is present in patients with chronic hepatitis B infections. This can be achieved either by augmenting the function of existing $CD8^+$ T-cells, creating a new source of $CD8^+$ T-cells, or immune mobilization[24,31,68,69]. One hallmark of $CD8^+$ T-cells in chronic HBV is overexpression of inhibitory molecules, among them programmed cell death protein-1 (PD-1). This has led to the hypothesis that PD-1 inhibition may increase recruitment of T-cells to help clear chronic hepatitis B. Efficacy of a single dose of the PD-1 inhibitor nivolumab was tested in patients who already had viral suppression and were HBeAg negative and demonstrated a modest further reduction in HBsAg production at 12 wk (average reduction was 0.3 log reduction) with only 1 patient achieving loss of HBsAg production in the study period[70]. While these data demonstrate some additive benefit of immunotherapy with currently available antivirals, it is notable that this study specifically excluded patients with advanced fibrosis leaving open the possibility that patients with cirrhosis may not be ideal candidates for this approach. Moreover, given extremely low number of HBV-specific $CD8^+$ T-cells in chronic hepatitis B, it is unlikely that recruitment of autologous T-cells alone will provide a durable cure.

This realization has led to efforts to generate new functional pools of effector T-cells by engineering large numbers of HBV-specific T-cells using chimeric antigen receptor (CAR) and T-cell receptor (TCR) technology. This approach has been successfully employed in mouse models of chronic HBV. One group isolated $CD8^+$ T-cells from mice and engineered them to express CAR that bind to HBV envelope proteins prior to transferring them into HBV transgenic mice[71]. These adoptively transferred HBV-specific T-cells engrafted, expanded, honed to the liver, reduced HBV replication, and caused only transient liver damage[71]. Similar success was achieved in HBV-infected humanized mice using human T-cells that were engineered to express an HBV-specific T-cell receptor[72]. Further, simultaneous treatment with the TCR-engineered cells and the HBV entry inhibitor myrcludex led to long-term control of HBV infection with limited liver injury[72].

A third approach has recently been described and acts as a hybrid between the above described methods of recruitment autologous T-cells and engineering larger pools of T-cells[69]. This approach uses immune-mobilizing monoclonal T-cell receptors against the virus (immTAV), which are soluble bispecific proteins that bind to both the TCR, which specifically recognizes the HBV viral peptide-human leukocyte antigen complex, and to CD3, which recognizes non-specific T-cells. Using an HBV envelope protein-specific immTAV, one group demonstrated that this approach can redirect polyclonal T-cells to destroy hepatocytes that are either infected with HBV or have integrated HBV DNA[69].

Despite the success of these immunotherapy approaches in cell culture and animal models, these have not yet been translated into human trials. Investigators are likely to proceed with caution given that these cytotoxic T-cells are targeting infected tissue within an essential organ and any exaggerated or off-target effect has the potential to induce irreparable liver damage and prove fatal. One possible way to minimize this chance is to minimize the portion of hepatocytes harboring HBV infection with existing antivirals. It is therefore likely that this approach will be most useful after existing or novel antiviral medications deplete significant portions of HBV DNA. Enhancing $CD8^+$ T-cell function will therefore result in destruction of the few remaining infected hepatocytes to allow for a cure.

HEPATITIS C

Hepatitis C infection is caused by the Hepatitis C virus (HCV), a single stranded RNA virus. HCV is a member of the Flaviviridae family and *Hepacivirus* genus that is transmitted primarily through direct bloodstream inoculation[73,74]. HCV successfully evades the immune system to cause a chronic hepatitis in the majority of cases, which often leads to advanced fibrosis and cirrhosis if untreated[73]. While there are no effective vaccines for HCV prevention, with the advent of direct-acting antivirals

(DAA) for the treatment of HCV, HCV can be easily cured in the overwhelming majority of cases. This has now led to ambitious global efforts to eliminate HCV[75]. Additionally, DAAs have led to the exciting possibility of transplanting organs from HCV-positive donors, which has the potential to greatly expand the organ donor pool and increase the availability of scarce resources.

Epidemiology

HCV is estimated to effect approximately 3% of the population worldwide, which translates into nearly 200 million cases worldwide. There is a wide geographic variation in disease prevalence with rates of approximately 1.5% in the United States to nearly 15% in Egypt and up to 50% in certain age groups in Egypt[74,76,77]. The unusually high rate of HCV positivity in Egypt has been traced to campaigns to administer parenteral anti-schistosomiasis treatment with inadequate needle sterilization in the 1950-1980s and subsequent spread with blood transfusions and medical and dental procedures[78]. More recently, a large effort to screen and treat Egyptians for HCV was undertaken and successfully screened 49.6 million people for HCV and showed a lower seroprevalence than previous estimates after large treatment efforts, now at 4.61%[79]. Remarkably, after DAA treatment 98.8% of patients with a known treatment outcome had achieved sustained viral response, suggesting an even lower disease prevalence in Egypt now[79].

The estimates of prevalence in the United States are based on the National Health and Nutrition Examination Survey (NHANES) database, which surveys a representative sample of 5000 adults annually; however, it is notable that this survey does not include homeless or incarcerated individuals, where HCV prevalence is estimated to be significantly higher, with weighted prevalence of 23.1% in the incarcerated population and 32.1% in the homeless[80]. The estimates in the United States are therefore likely to underestimate the true disease burden with one study calculating that NHANES underestimates the number of HCV infections in the US by approximately 1 million[80]. Moreover, in recent years, the opioid epidemic has led to increasing rates of HCV cases and a shifting demographic, now with rates of infection rising fastest in young people while rates in the baby boomer generation fall due to screening efforts and treatment[81].

Hepatitis C transmission occurs with parenteral exposures, most commonly from intravenous drug use and contaminated blood transfusions with a notable exception in Egypt, as discussed above. A case control series from 1997 surveyed both 2316 HCV-positive and 2316 HCV-negative blood donors from the United States for lifestyle and socioeconomic factors to elucidate possible mechanisms of HCV exposure and transmission and to assist with finding populations that may benefit from closer screening [82]. They found that the strongest risk factors for HCV-positive individuals were intravenous (IV) drug use (OR 49.6), blood transfusion, (OR 10.9), and sex with an IV drug user (OR 6.3)[82]. Since 1992, the United States has enacted universal HCV screening of donated blood, which has dramatically reduced the risk of HCV transmission *via* blood transfusions[83]. HCV can survive on an unsterilized needle for many days depending on the temperature and inoculum[84]. There are an estimated 11-21 million IV drug users worldwide[85], with rates rising in many countries, most notably with the opioid epidemic in the United States[81]. This has already led to rates of HCV infection rising sharply since 2010[86] and is likely to continue to do so until the opioid epidemic is under better control.

Natural History of Infection and Clinical Course

Nearly all cases of acute hepatitis C are asymptomatic, and fulminant hepatitis C has been reported only in case reports[87]. The lack of symptoms in early infections has precluded identification of patients for large studies to fully characterize this phase of the disease. Studies of transfusion-associated infections have been able to identify some patients with acute, transfusion-related HCV infections[88]. These studies have identified that HCV RNA rises rapidly after infection followed by a rise in ALT in 1-3 mo, indicating hepatocyte damage. The latent period (defined as the period prior to ALT elevations) is inversely proportional to the donor HCV viral load and is shorter in patients with clinically overt disease[88]. Importantly, these studies have helped identify host factors that assist with HCV clearance and those that make hosts susceptible to developing chronic disease. More recently, the factors that mediate this rapid immune response are being used in the hopes of developing a hepatitis C vaccine.

One such study analyzed women who were infected with HCV when prophylactically treated with Rh_0 (D) immunoglobulin and found that spontaneous viral clearance is strongly associated with genetic polymorphisms near the interleukin 28B (*IL28B*) gene, which encodes interferon (IFN)-λ-3[89]. Moreover, women who did not have the favorable *IL28B* polymorphism had increased chance of viral clearance if they developed jaundice in the acute phase of infection[89]. This finding has been confirmed in additional studies, and one study followed 632 patients with acute HCV and found that 25% of patients cleared acute HCV with clearance being more likely if patients were female, had the favorable *IL28B* genotype (C/C), or were infected with HCV genotype 1[90]. This large study suggests that HCV becomes a chronic infection in approximately 75% of acutely infected individuals, which is in line with widely quoted estimates of 50%-85% chronicity rates[90].

Chronic HCV is also largely asymptomatic prior to the development of advanced fibrosis, and cirrhosis is estimated to occur in 16% of patients within 20 years of HCV infection[91]. Factors that contribute to chronic HCV progressing to cirrhosis include advanced age, concurrent HBV, ongoing alcohol use, immunocompromised states, and risk factors for non-alcoholic steatosis including obesity

and insulin resistance[92]. Once a patient has cirrhosis, they are at higher risk for hepatic decompensation and the development of HCC. While HCV-associated HCC can develop in non-cirrhotic livers[93], the risk is much higher in patients with cirrhosis and additional risk is conferred by many of the factors associated with progression to cirrhosis in the first place, including age, alcohol use, and male sex[92].

Diagnosis and Treatment

As briefly discussed above, Hepatitis C is almost universally a chronic, asymptomatic disease until it ultimately causes advanced fibrosis and cirrhosis, when it has symptoms that overlap with a variety of advanced liver diseases. As such, diagnosis relies entirely on serologies. Given the frequency of HCV in the general population, the asymptomatic nature of early HCV, and the ease of treatment (discussed more below), it is recommended that all adults in the United States be screened for HCV at least once and that high-risk individuals be screened more frequently[94]. In most patients, diagnostic testing consists of a hepatitis C antibody test with a reflex to HCV RNA viral load if the antibody test is positive. Alternatively, in high-risk patients, some physicians may choose to send an HCV RNA level regardless of antibody result. If any test yields a positive result, further characterization of liver function—including a fibrosis assessment—will help direct further treatment and screening procedures.

The introduction of direct acting antivirals (DAA) has revolutionized the care of patients with HCV [95-98]. With pan-genotypic treatments now available, insurance coverage, falling costs of available agents, and widely available algorithms for simplifying treatment of patients with HCV (*e.g.*, https://www.hcvguidelines.org), treatment of HCV is readily available to the majority of patients in developed countries such as the United States. Treatment goals have therefore shifted towards reaching as many patients as possible and have led to the aggressive goals of eliminating HCV globally[25,75]. As briefly discussed above, an impressive voluntary global health effort in Egypt was able to screen 49.6 million citizens, which identified over 1 million untreated HCV RNA patients and led to successful treatment in 98.8% of patients with long-term follow up[79]. Remarkably, the cost of identifying and curing each case of Hepatitis C was $130.62 in contrast to the cost of chronic medical care and disability in patients with untreated HCV, which is estimated to be in excess of $100000 per patient[79]. This study highlights the feasibility of large-scale screening and treatment efforts in resource-limited settings. With the recent changes to the United States Preventative Services Task Force recommendations to expand screening from all adults born between 1945 and 1965 to all adults between age 18 to 79[94], it will be interesting to see if similar massive-scale screening and treatment can be successfully completed in the United States.

Despite this impressive effort, several factors remain barriers to global elimination of HCV[25]. Notably, in the Egyptian effort, 20.6% of the population did not participate in voluntary screening with men and young people (< 25 years old) having the lowest participation rates[79]. While these efforts will likely be successful in patients without ongoing risk factors for HCV infection, IV drug users who are at particularly high risk for HCV infection are among the patients least likely to seek out regular medical care and to adhere to a course of antiviral therapy. With this in mind, multiple studies in the United States have experimented with modified treatment protocols to decrease the burden of treatment and improve access to care. For example, the MINMON study (Clinical Trial Number: NCT03512210) aims to test whether a minimal monitoring approach is safe and effective when using the pan-genotypic agent sofosbuvir/velpatasvir in treatment-naïve HCV patients. To do this, they require no pretreatment genotyping, provided patients all of the necessary medication up front, and do not schedule any clinic or lab visits while patients were undergoing treatment but did remotely contact patients at 4 and 22 wk. Promising results for this study were presented at the American Association for the Study of Liver Diseases Liver Meeting in 2020 and showed that sustained virologic response (SVR) was near 95%[99]. If adopted on a broader scale, this approach has the potential to further simplify HCV treatment and remove some of the treatment burden. Additional studies have attempted to identify the most effective way to treat patients who inject drugs in the multi-center HERO study[100]. In this study, patients are randomly assigned to receive HCV treatment in one of two ways: (1) Directly observed treatment where patients take medication in front of a staff member; or (2) With the help of patient navigators who attempt to help patients overcome barriers to taking medication. Final results are forthcoming but will hopefully help provide guidance for HCV treatment in this difficult to treat population.

Prevention Efforts

Expansion of HCV prevention strategies are also vital to elimination efforts. Given HCV is most commonly transmitted from unsafe injection practices, especially among injection drug users, programs to increase safe injections are critically important to efforts to prevent HCV transmission and are gaining acceptance[101].

As with other hepatitis viruses, primary prevention of HCV with vaccination would be extremely beneficial. Moreover, reinfection is a significant risk for patients who have successfully completed DAA therapy but continue to have risk factors for HCV infection. Development of an effective HCV vaccine has proven difficult due to the extreme genetic diversity of HCV—7 known genotypes with over 80 known subtypes—and an error-prone viral polymerase that confers HCV with rapid mutability[102]. Hope for a vaccine comes from the observation that approximately 25% of acutely infected individuals

spontaneously clear HCV[103]; however, re-infection can occur despite the appearance of broadly neutralizing antibodies in patients who clear their initial HCV infection. Nevertheless, a study that followed 22 active IV drug users who had previously cleared HCV demonstrated that upon reinfection with HCV, virus clearance occurs 83% of the time[104]. Moreover, reinfection is characterized by reduced maximal viral titers, shorter duration of viremia, and augmented T-cell responses[104]. Benefit of humoral immunity in preventing HCV infection has been demonstrated in chimpanzees treated with immunoglobulin derived from a human patient in the acute phase of post-transfusion hepatitis C infection[105]. In this case, human immunoglobulin directed against the hypervariable region 1 of the envelope 2 protein prevented infection with homologous HCV strains[105]. In another study, HCV neutralizing antibodies derived from a patient infected with HCV genotype 1a protected chimpanzees from infection with genotype 1a and 6a but failed to protect them from infection with HCV genotypes 4a or 5a[106]. Pools of broadly neutralizing antibodies can prevent infections with multiple HCV genotypes in humanized mice[107,108], and together with the extreme genetic variability of HCV, this suggests that in order for a vaccine to be widely effective, it should be able to induce generation of broadly neutralizing HCV antibodies. Multiple experimental vaccines have used this approach; however, to date, the majority of recipients of these vaccines—either chimpanzee or human—have failed to produce sufficient titers of broadly neutralizing antibodies in most subjects[102,109]. Nevertheless, new culture strategies may enable use of whole inactivated HCV rather than only envelope protein epitopes to allow for additional vaccine epitopes and promote generation of more broadly neutralizing antibodies.

At the moment, prevention of HCV infection is dependent upon behavioral risk reduction (*e.g.*, clean needle programs), which is unfortunately being overpowered by a surge of new cases with the ongoing opioid epidemic in the United States. While a vaccine would be ideal, there are various obstacles to overcome, as detailed above. Fortunately, widespread screening and treatment programs are underway on a global scale, and we are hopeful that these will achieve the goal of elimination of chronic hepatitis.

Hepatitis C and Organ Transplantation

DAAs have also made it possible to transplant solid organs from hepatitis C positive donors, which has the potential to greatly expand the donor pool, shorten time on the waitlist, and improve mortality in patients in need of organ transplantation[110]. Solid organ transplantation can transmit HCV to the organ recipient and was a major concern in the pre-DAA era. This was demonstrated by a retrospective review of all cadaveric donors to the New England Organ Bank between 1986 and 1990, and of 716 organ donors, 1.8% (13) were determined to be HCV positive[111]. Of these 13 HCV positive donors, their organs—19 kidneys, 6 hearts, and 4 livers—were transplanted into 29 different recipients, and 14 of the recipients (48%) developed HCV, which caused chronic liver disease in the majority of infected patients[111]. This study raised serious concerns about whether HCV-positive organs should be offered for transplant given the lack of effective treatment for HCV at the time of the study. Subsequent studies from the pre-DAA era showed that transplant patients tend to have worse outcomes if they receive HCV positive organs. HCV-positive liver transplant recipients have decreased patient and graft survival, primarily attributed to HCV recurrence after transplantation[112]. Additionally, in immunosuppressed patients, including recipients of both liver transplants and other solid organs, HCV positivity is associated with exceptionally high HCV replication and the development of fibrosing cholestatic hepatitis C, an aggressive presentation of HCV that has a high risk of liver failure and mortality[113]. For heart transplant patients who receive hearts from HCV-positive donors, there is significantly increased mortality at 1, 5, and 10 years that may be attributable to both the development of liver disease and accelerated and more severe coronary artery vasculopathy[114,115]. In kidney transplant recipients, HCV, whether from the donor organ or already present in the recipient, is associated with both increased mortality and graft failure[116,117].

Prior to the introduction of highly effective treatment for HCV, these findings understandably led to reservations about using organs from HCV-positive donors and historically resulted in discarding high-quality organs from HCV-positive donors at much higher rates. Compared to high-quality HCV-negative livers, high-quality HCV-positive livers were 3 times more likely to be discarded from 2005 to 2010, a rate which has decreased after DAAs but is still approximately 1.7 times more likely[118]. Similarly, high-quality kidneys from HCV-positive donors were 2.6 times more likely to be discarded between 1995 and 2009, and a more recent analysis (from 2005-2014) concluded that only 37% of kidneys from HCV-positive donors were transplanted (*i.e.* 63% were discarded) despite most being of good quality and many available recipients[119,120]. Hearts from HCV-positive donors are also significantly less likely to be used. In 2015, hearts from HCV-naïve donors were used approximately 30% of the time compared to 0.7% and 1.4% for hearts from HCV-viremic and non-viremic HCV-Ab positive donors, respectively[121].

If attitudes towards transplanting solid organs from HCV-positive patients do not change, there is likely to be an even greater mismatch between available high-quality donor organs and patients on transplant waitlists, as the opioid epidemic is resulting in an alarming rise in overdose deaths[122]. A recent National Registry Study characterized organ donations form deceased donors who died of drug overdose (ODD, overdose death donor) between 2000 and 2017 found that ODDs have risen significantly in that time from 1.1% of donors in 2000 to 13.4% of donors in 2017[122]. Compared to non-

ODDs, ODDs were more likely to be young, white, have little comorbidity, and have HCV infection (18.3% were HCV-positive)[122]. Despite the higher rates of HCV positivity, the authors found similar 5-year patient and graft survival rates between non-ODDs and ODDs[122].

Along with the organ shortage and rise in availability of high-quality organs from HCV-positive donors, the success of DAAs in curing HCV has led to several clinical trials to determine the outcomes of transplanting organs from HCV-positive donors. In general, these trials have been an overwhelming success. Two separate studies of 10 HCV-mismatched kidney transplant recipients (Donor positive, recipient negative) showed 100% success rate of achieving SVR in recipients[123,124]. Moreover, this mismatched strategy reduced wait times, and recipients had excellent graft function at 6 mo post-transplant[124]. Similar results have been shown in both mismatched heart and lung transplant patients. An early study performed HCV-mismatched heart transplants on 13 patients, and 9 of the 13 developed HCV viremia shortly after transplant. Eight of these patients completed DAA treatment and demonstrated SVR (the 9th patient died of a pulmonary embolus)[125]. Another study enrolled 44 patients awaiting heart or lung transplant and performed 36 HCV-mismatched lung transplants and 9 heart transplants regardless of HCV genotype[126]. Ninety-five percent of patients had detectable HCV RNA immediately after transplant, and 100% achieved SVR with the pan-genotypic DAA sofosbuvir–velpatasvir[126]. Importantly, at 6 mo, the patients continued to have excellent graft function and no detectable HCV RNA[126].

Finally, a larger, more recent study that performed 80 HCV-mismatched heart transplants and followed patients for 1 year showed comparable 1-year survival and similarly, low rates of allograft rejection between patients who received HCV-positive organs and those who received HCV-negative organs[127]. However, there was significantly more primary graft dysfunction and a trend towards increased early coronary allograft vasculopathy in patients who received organs from HCV-positive donors, although this was not statistically significant[127]. A large retrospective registry study of patients who underwent a single organ liver transplant from 2008 through 218 demonstrated similar 2-year graft survival rates (approximately 88%) among all combinations of donor-recipient HCV RNA status[128]. Importantly, graft survival from HCV-positive donors has improved in the DAA era, with 3-year graft survival now approximately 88% regardless of recipient HCV status[128]. Moreover, this study demonstrated a trend towards increasing use of organs from HCV-positive donors for HCV-negative recipients from 7 in 2008 to 107 in 2018, highlighting increased acceptance of this novel strategy of expanding the pool of available organs[128].

Together, these data are extremely promising in demonstrating early, post-transplant HCV cure and similar graft and recipient survival in short- and mid-term follow-up. They also provide justification for the use of organs from HCV-positive donors, regardless of recipient HCV status. This strategy is gaining popularity: The Scientific Registry Transplant Recipients indicates increasing numbers of mismatched transplants, and there are now similar utilization rates of available HCV-positive and HCV-negative donor hearts[128]. Nevertheless, there still exists some skepticism of this strategy, as a recent survey of 99 transplant nephrology providers demonstrated that fewer than half support offering HCV-positive kidneys as part of routine care outside of a research setting[129]. These attitudes will likely shift to usher in more widespread use of this practice if long-term outcomes are as promising as the available clinical trial results.

HEPATITIS D

Hepatitis D infection is caused by the Hepatitis D virus (HDV), a single stranded, enveloped RNA molecule. HDV is the smallest virus known to infect humans, and it is often classified as a subvirus given that the HDV lifecycle is entirely dependent on HBV[130,131]. Transmission occurs through similar means as HBV and can occur either at the same time as an HBV infection (*i.e.*, coinfection) or in patients with chronic HBV infections (*i.e.*, superinfection). This relationship to HBV infection timing determines the natural history of HDV infection, with superinfection more often leading to a rapid clinical deterioration with progressive hepatitis, cirrhosis and development of complications of cirrhosis, including HCC. Current treatment strategies for HDV are centered around prevention and the management of HBV, as HDV is entirely dependent on the HBV lifecycle; however, directed therapies against HDV are under investigation[132].

Epidemiology

There are multiple estimates of HDV prevalence, as high as 13% of all HBV carriers; however, a recent meta-analysis estimated that approximately 4.5% of HBsAg-positive people are coinfected with HDV, which corresponds to approximately 12 million HDV infections worldwide[133]. HDV is present worldwide with geographic variation that does not align with HBV prevalence. There is a very high prevalence of HDV in Mongolia (36.9% of patients with HBsAg) and in central African countries (with estimates of > 10% prevalence in the HBsAg-positive population). Despite high rates of HBV infection, there are very low rates of HDV co-infection in other Asian countries, including China[133]. Regardless of geography, the populations at highest risk for HDV include people who inject drugs and those with

human immunodeficiency virus (HIV) or HCV[133]. HDV is even more prevalent in patients with HBV-associated cirrhosis and HCC, highlighting the pathogenic importance of HDV.

Natural History of Infection and Clinical Course

The primary modes of transmission for HDV are similar to those for HBV with the highest infection rates in people who inject drugs. HDV is a 1.7kb single stranded RNA molecule that encodes for the hepatitis D antigen (HDAg)[134]. Depending on RNA processing, the HDAg has one of two forms—the short form (HDAg-S) or long form (HDAg-L)—each with distinct functional roles. HDAg-S activates further HDV RNA synthesis, while HDAg-L inhibits HDV RNA synthesis and promotes HDV assembly with HBsAg to allow for packaging and transport[134]. It has been proposed that HDV mediates liver toxicity *via* both direct hepatotoxicity and indirect immune-mediated hepatocellular damage, although immune-mediated damage is the current prevailing theory.

Evidence suggesting direct hepatotoxicity is based on (1) Histopathology with limited immune infiltrate in acute HDV infection[135]; (2) ultrastructural analysis of hepatocytes infected with HBV and with HDV that showed a strong correlation between the appearance of cytoplasmic structures and hepatocellular damage as assessed by ALT levels[136]; and (3) *in vitro* cell culture data in which HDAg expression is associated with cell death in the absence of immune cells[137]. However, the conclusion that HDV causes direct cytotoxicity has been questioned. Transgenic mice with hepatocyte-specific expression of both HDAg-S and HDAg-L demonstrated no direct cytotoxic effect regardless of the level of HBsAg coexpression[138]. This finding was mirrored with histologic assessment of HBsAg carriers with chronic HDV infection, which demonstrated that HDAg expression was low during acute hepatitis and increased with the development of chronic disease[139]. However, these studies do not test the possibility of other stages of the HDV lifecycle causing direct cytotoxicity and some have suggested that HDV viral replication itself leads to cytotoxicity. More convincing evidence is present for HDV infection causing immune-mediated hepatotoxicity. Nevertheless, long-term follow up of 76 patients who underwent liver transplant for HDV-related cirrhosis showed that either HDV RNA (serum) or liver HDAg were present in 88% of patients within the first year[140]. However, these patients did not develop hepatitis unless active HBV infection recurs, suggesting that HDV is not hepatotoxic when expressed alone but instead requires HBV expression[140].

Regardless of the mode of hepatotoxicity, HDV has different clinical courses that depend on the timing of HDV infection in relationship to HBV-mediated liver disease. For coinfection, HBV and HDV are acquired simultaneously, whereas a superinfection occurs when HDV is acquired in a patient with an established HBV infection. Coinfection is most common in patients who use IV drugs. Since HDV is dependent on HBV and HBV infection is cleared in the majority of cases acquired in adulthood, HDV acquired in this manner also spontaneously clears. When the coinfection does not clear, some evidence suggests that HDV may cause a more intense hepatitis[141]. This was suggested by the high seroprevalence of HDV in patients with fulminant hepatitis B (52% in 1 study), but in this same study of fulminant hepatitis B, HDV coinfection correlated with better survival than with those infected with HBV alone (57.8% survival in coinfection vs. 16.7% survival with HBV alone)[141].

HDV superinfection occurs when a patient with a preexisting liver disease from chronic HBV infection contracts HDV. This is often accompanied by very high levels of HDV RNA expression with a subsequent severe hepatitis and decompensation of preexisting liver disease, as the HDV replication is protected by high levels of HBsAg[142]. Despite HDV inhibition of HBV replication, HBV infection is already established and allows development of chronic HDV infection in the majority of cases. Chronic HDV infection often leads to hepatitis with rapidly developing cirrhosis and increased risk of decompensation and HCC[143]. The increased risk of HCC in patients chronically infected with HDV is evidenced by multiple long-term cohort studies. A 28-year study of 299 patients with HDV infection with a median follow up of 233 mo demonstrated that active HDV replication portends an increased risk of cirrhosis and HCC with annual rates of 4% and 2.8%, respectively[144]. Moreover, this study also reported that HDV replication was an independent predictor of mortality in these patients[144]. Another cohort demonstrated that HCC developed in 42% of patients with chronic HDV within 12 years of diagnosis[131].

Prevention, Diagnosis, and Treatment

As discussed above, HDV infection and replication is entirely dependent upon HBV infection, and therefore HDV prevention efforts are largely centered around HBV prevention with vaccination programs. HBV vaccination programs have therefore led to a significant reduction in HDV infections in developed countries[145]. The impressive ongoing global efforts to eliminate chronic viral hepatitis—including HBV—should also significantly reduce the burden of HDV.

The only currently available treatment for HDV is interferon, which may exert its effect on HDV either *via* direct inhibition of HDV or inhibition of HBV[146]. Despite initial control of HDV viral load and improved histology during the treatment phases, multiple trials have shown exceptionally high rates of HDV relapse after completing interferon therapy[147]. The largest of these studies randomly assigned 42 patients with chronic HDV to receive either placebo or 3 million or 9 million units of interferon-alpha-2a[147]. This study demonstrated a dose-dependent response with regards to ALT levels, HDV RNA levels, and histology scores[147]. While the decreases in ALT levels were maintained

for 4 years of follow up, even patients treated with high dose interferon demonstrated relapses in HDV replication during post-treatment follow up[147]. Nevertheless, when these patients were followed for an additional 2-14 years, this group discovered that patients who received high dose interferon therapy had a dramatic long-term survival benefit[148]. Further, non-randomized, studies assessed the response of chronic HDV to peginterferon-alpha-2b[149,150]. These studies demonstrated variable responses to peginterferon-alpha-2b with a 57% sustained response in one study and only 17% sustained response in the other study with sustained response defined as undetectable HDV-RNA 6-mo post-treatment[149,150]. Those most likely to have a sustained response are likely to also have early reductions in HDV-RNA levels[149,150]. Conversely, no patients with cirrhosis demonstrated a sustained response[149].

Given the dependence of HDV on HBV, it is logical that therapies directed against HBV may also treat HDV. However, HDV replication is independent of HBV replication and only requires HBsAg. Therefore, any therapeutic benefit of HBV therapy on HDV infection is an indirect result of decreasing HBsAg production, which is not significantly reduced by nucleos(t)ide analogs. Unfortunately, trials of nucleos(t)ide analogs have not proven any sustained benefit in chronic hepatitis D despite excellent suppression of HBV DNA[151]. While there have been reports of Tenofovir resulting in HDV virus suppression in patients with either HBV-HDV co-infection or triple infection with HIV, HBV, and HDV [152,153], but it is questioned whether this results in a sustained benefit if tenofovir is withdrawn. Moreover, when combined with peginterferon alpha-2a in a placebo-controlled randomized control trial, tenofovir had no additional benefit with respect to HDV RNA level[154]. Similarly, the nucleoside analog adefovir also had no benefit on HDV levels when used alone and had no added benefit to interferon therapy[155]. Taken together, these data highlight the need for more directed HDV therapies. Directed treatments against HDV are currently under investigation and included inhibitors of cell entry, prenylation inhibitors, and subviral particle release inhibitors[132,146]. However, none of these are currently approved for use outside of clinical trials.

HEPATITIS E

Hepatitis E infection is caused by the Hepatitis E virus (HEV), a single stranded, quasi-enveloped RNA molecule in the Hepeviridae family[156]. There are 8 known genotypes, with genotypes 1-4 being the most studied[157]. Transmission occurs either *via* fecal-oral route (genotypes 1 and 2) or zoonotic transmission with ingestion of raw or undercooked meat (genotypes 3 and 4). HEV typically causes an acute, self-limited hepatitis that is particularly severe in pregnant women; however, cases of chronic hepatitis E have been reported in immunocompromised hosts. Prevention is centered around improving hygiene and vaccination, although a vaccine is available for use only in China[158]. Currently, treatment of acute HEV is supportive, and chronic HEV is treated with a multipronged approach with decreasing the underlying immunosuppression and occasionally ribavirin.

Epidemiology

There are two distinct, genotype-dependent epidemiologic patterns of Hepatitis E. Hepatitis E virus genotypes 1 and 2 are only known to infect humans and are highly endemic in developing countries throughout the world[157]. In these regions, HEV genotypes 1 and 2 result in large outbreaks in areas of poor sanitation, primarily in developing countries and related to drinking water contaminated with feces[159]. This was demonstrated by a naïve volunteer who developed a hepatitis-like illness after ingesting pooled feces extracts from patients with non-A, non-B hepatitis, which led to the discovery of hepatitis E[160]. Genotypes 1 and 2 are estimated to cause at least 20 million acute infections that result in approximately 3.4 million symptomatic cases, 70000 deaths, and 3000 stillbirths each year[161].

In addition to humans, HEV genotypes 3 and 4 are known to infect multiple species including swine, deer, and rabbits and are more commonly reported in the developed world (Europe, North America, Australia, and Japan)[156]. HEV genotypes 3 and 4 account for very few (less than 1%) of the cases of viral hepatitis in the developed world, and transmission is thought to be from consumption of undercooked meat, primarily pork. This is evidenced by the exceptionally high rate of HEV infection in both wild and domesticated pigs in multiple European countries[162,163]. A study of the Swedish swine population also sequenced partial genomes of HEV strains from humans, pigs, and wild boars and found a high degree of relatedness, suggesting zoonotic transmission[163]. The ability of swine HEV genotype 3 to cross species was confirmed with inoculation of rhesus monkeys and chimpanzees[164]; however, pigs have varying degrees of susceptibility to experimental infection with HEV from humans [165,166]. Nevertheless, HEV has been detected in multiple stages of consumer pork product processing across Europe[167-169], but HEV is inactivated by adequate food preparation, as pigs cannot be inoculated with HEV-infected pureed pig livers subjected to heating to 71 °C for 20 min or longer[170].

While very few cases of HEV are reported in the developed world each year, this likely owes to undertesting and the self-limited nature of the disease. Screening blood donations in Western Europe has shown HEV RNA in 0.02%-0.14% of all screened blood products. In England, one study that screened 225000 blood donations found that 79 donors (0.04%) were infected with HEV RNA[171]. These infected donors generated in 129 blood components and resulted in an estimated 42% infectivity

Table 1 Overview of epidemiology, symptoms, natural history and clinical management of viral hepatitis infections

Virus	Estimated number of infections worldwide	Mode of transmission	Typical clinical signs/symptoms	Natural history	Diagnosis	Treatment	Prevention
Hepatitis A	1.4 million annually	Fecal-oral route	Many asymptomatic. Most with non-specific symptoms of fatigue, nausea, vomiting, anorexia, jaundice	Asymptomatic, self-limited illness, prolonged cholestasis, relapsing, fulminant hepatitis (very rare)	Hepatitis A IgM	Supportive care, post-exposure vaccination and HAV immuno-globulin	Sanitation efforts, vaccination
Hepatitis B	257million chronic HBV infections (WHO 2017 Global Hepatitis Report)	Vertical transmission (common for chonic HBV); IVDU, blood product transfusions, sexual contact (common for acute HBV)	Acute: non-specific symptoms (fatigue, nausea, vomiting, anorexia, jaundice); chronic: often asymptomatic, can progress to cirrhosis and HCC	Infection at birth: chronic HBV infection (immune tolerance, immune clearance, inactive carrier, reactivation). Eventual progression to cirrhosis and HCC; infection in adulthood: > 95% clearance	Past infection-HBsAg negative, HBsAb positive, HBcAb positive, HBeAb +/-; current infection-HBsAg positive, HBsAb negative, HBcAb positive, HBeAb +/-	Nucleot(s)ide reverse transcriptase inhibitors (entecavir, tenofovir); interferon	HBV vaccine (universal vaccination recommended at birth); HBIG in select cases
Hepatitis C	71 million (WHO 2017 Global Hepatitis Report)	Direct blood stream inoculation (IVDU, unregulated tattoos/piercings, blood transfusion and organ transplants)	Typically asymptomatic until cirrhosis develops	Spontaneous clearance: 10%-25%; chronic Infection: 75%-90%, can progress to cirrhosis and HCC	HCV antibody, HCV RNA viral load	Direct acting antivirals	Widespread screening efforts
Hepatitis D	12 million cases annually, 4.5% of HBV-infected individuals	Similar to Hepatitis B (IVDU, blood product transfusions, sexual contact)	Non-specific symptoms of fatigue, nausea, vomiting, anorexia, jaundice	Simultaneous coinfection of HDV and HBV: rare fulminant hepatitis, usually complete recovery; superinfection on chronic HBV: accellerated progression of chronic HBV	HDV IgM (acute), HDV IgG (chronic)	Hepatitis B treatment	Hepatitis B vaccination
Hepatitis E	20 million acute infections (The Global Burden of Hepatitis E Virus Genotypes 1 and 2 in 2005)	Genotypes 1 and 2: Fecal-oral route; genotypes 3 and 4: Zoonotic, contam-inated meat	Commonly asymptomatic; prodromal flu-like symptoms, nausea, vomiting, anorexia, fatigue followed by jaundice	Acute self-limited in majority of cases, severe in pregnant women; chronic hepatitis in immunocompromised hosts	HEV IgM (acute), HEV IgG (chronic)	Chronic infection: decrease immunosup-pression, ribavirin	Genotypes 1 and 2: Sanitation efforts, vaccine available in China
Hepatitis G	4.8% worldwide	Direct blood stream inoculation (IVDU, unregulated tattoos/piercings, blood transfusion and organ transplants)	Not well-described, likely asymptomatic	Not well-described. Unlikely to cause clinically significant hepatitis in humans.	Hepatitis G RNA; not currently used clinically	None	None

HAV: Hepatitis A virus; HBV: Hepatitis B virus; HCV: Hepatitis C virus; HDV: Hepatitis D virus; HEV: Hepatitis E virus; HGV: Hepatitis G virus; WHO: World Health Organization; IVDU: Intravenous drug use; HCC: Hepatocellular carcinoma; HBIG Hepatitis B immunoglobulin; HBsAb: Hepatitis B surface antibody; HBsAg: Hepatitis B surface antigen; IgG: Immunoglobulin G; IgM: Immunoglobulin M.

rate (18 of 43 followed up), which has led some to call for universal screening of blood donations for HEV[171,172]. Seropositivity in the developed world ranges from 6% in the United States to 39.1% in Southern France, although assays for HEV IgG have reportedly wide ranges of sensitivity[156]. A 2017 systematic review that analyzed the prevalence of HEV in Iran found an overall positivity rate of 9.7%

with varying rates between cities (1.1% in Tehran *vs* 46.1% in Ahvaz)[173]. Finally, a recent systematic review and meta-analysis of 56 studies on the seroprevalence of HEV in patients on maintenance hemodialysis (HD) found that the pooled seroprevalence of HEV was 11.13% between 2016 and 2020, which had increased from 6.6% from 1994 to 2000[174]. Moreover, the length of time on HD was associated with higher seroprevalence as those on HD for more than 60 mo had a significantly higher chance of being seropositive (27.69% more than 60 mo *vs* 15.78% less than 60 mo)[174].

Natural History of Infection and Clinical Course

Hepatitis E enters into the hepatocyte in 1 of 2 distinct mechanisms depending on whether the virus is enveloped or not, and upon entry, the virus is uncoated, transcribed and translated into 3 different proteins that facilitate viral replication, repackaging, sorting and release into both the blood stream and bile[156]. Upon HEV release into the bile, the envelope is degraded by bile acids and detergents prior to being released into the intestinal lumen and excreted with stool. The unenveloped version of the virus released into the feces has a much more efficient mechanism of hepatocyte entry and helps explain the most common form of HEV transmission. HEV is not thought to be directly cytotoxic. Instead, HEV causes hepatotoxicity *via* immune mediated mechanisms as patients with acute hepatitis E infection show both $CD4^+$ and $CD8^+$ T-cell responses[175].

HEV infection is most commonly asymptomatic as evidenced by the high seroprevalence of anti-HEV IgG and lack of recollection of acute symptoms[176]. When symptomatic, HEV has a 2-10 d incubation period followed by non-specific prodromal symptoms of fatigue, nausea, vomiting, and anorexia, similar to other acute hepatitis infections. Ultimately, HEV causes an acute, self-limited hepatitis characterized by jaundice and marked AST and ALT elevations. These symptoms typically self-resolve within 1 mo, although some cases of prolonged cholestasis (up to 6 mo) and even cases of fulminant hepatic failure with resultant death have been reported[177].

Pregnant women are particularly prone to severe hepatitis E infections that can result in fulminant hepatic failure in approximately 20% of infected patients with an associated high mortality rate and high rate of fetus and neonatal complications, including spontaneous abortions, stillbirths, and neonatal deaths. Infections within the third trimester are the most problematic and are thought to be so detrimental because of an altered immune system and hormonal changes[178].

All reported cases of chronic hepatitis E infection have been linked to genotype 3 and have been reported in immunocompromised patients including those with HIV[179], undergoing chemotherapy [180], or solid organ transplant recipients[181]. This has been linked to the development of cirrhosis and HCC. However, the limited number of cases reported precludes detailed assessment of risk of these complications of chronic HEV infection.

Prevention, Diagnosis, and Treatment

Given that the primary mode of transmission for the most common HEV variants (genotypes 1 and 2) is through contaminated water, the main mode of prevention is through increased sanitation efforts to provide citizens of the developing world with clean drinking water. A vaccine to protect against HEV is now approved for human use in China but not elsewhere; however, this is likely to benefit those infected in the rest of the world[158]. Efforts to bring clean drinking water to all parts of the world should reduce the burden of HEV, and we are hopeful that the vaccine for HEV may be approved outside of China for further HEV prevention.

There is currently no direct acting antiviral to treat HEV. In immunocompromised patients, the mainstay of treatment is to reduce immunosuppression if possible[181,182]. This results in a sustained viral response (*i.e.*, no detectable HEV RNA) in approximately 30% of patients. In patients where decreasing immunosuppression is either not possible or does not result in SVR, ribavirin is currently the main therapeutic option[183]. Unfortunately, ribavirin is a category X drug, meaning that is should not be given to pregnant women. Some cases of ribavirin exposure do occur during pregnancy, which is the subject of study in the ribavirin pregnancy registry[184]. Interim analysis has failed to show a direct link between ribavirin and teratogenicity, but full results of the study are forthcoming and for the moment, ribavirin remains a category X drug[184]. Additional therapies, including pegylated interferon and sofosbuvir have been evaluated only in limited clinical settings[185,186].

HEPATITIS G

What is now regarded as the hepatitis G virus (HGV) was initially described in 1966 and named the GB virus (GBV) after the surgeon G. Barker, who fell ill with hepatitis. Subsequent studies isolated genetically similar agents, named GBV-A, GBV-B, and GBV-C, and GBV-C was genetically similar to a separate isolate already named HGV[187].

Hepatitis G virus is a less well-described entity with a global distribution but varying reports of HGV prevalence in the general population. A systematic review evaluated studies reporting the prevalence of HGV in healthy voluntary blood donors and found 649 infections in 13610 voluntary blood donors (4.8% positivity rate) [188]. The studies included in the review reported HGV positivity rates ranging

from 0.5% prevalence in a Japanese study to 18.9% in a South African study[188]. Currently, HGV is not widely tested for clinically, so the true incidence is not well-known.

The natural history of HGV is not well-characterized, and it is questioned whether HGV causes a clinically significant liver disease. While HGV has been detected in many different clinical scenarios, including acute presumed viral hepatitis, cirrhosis of unknown cause, and HCC, the clinical relevance is still questioned given the high co-infection rate with other causes of viral hepatitis, including HCV and that HGV co-infection does not exacerbate the course of HCV[189]. Moreover, greater than 75% of patients who are positive for HGV have normal liver function tests[190]. This group also reported that among HGV-positive individuals with elevated liver function tests, aminotransferase elevations are negligible and that there is no correlation between HGV viral load and aminotransferase elevations in patients on chronic dialysis[190]. In another study, 0 of 16 HGV-positive patients on chronic hemodialysis had elevated aminotransferase levels despite prolonged viral persistence[191].

Additionally, individuals who undergo liver transplantation are often incidentally HGV-positive, and both HGV-positive and HGV-negative liver recipients have similar outcomes (including 40-mo survival), suggesting that HGV positivity does not affect engraftment or graft function[192]. Additional studies have suggested that the liver is not the site of viral replication as the liver-to-serum ratio of HGV RNA is less than 1, which is consistent with blood contamination of liver tissue[193]. Finally, despite initial reports of HGV-associated fulminant liver failure, it is now thought that these patients were positive for HGV RNA due to blood product administration during the hospitalization rather than HGV being present at the time of admission[194]. Experimental models also argue against HGV causing a clinically significant disease as studies in chimpanzees demonstrate that HGV can reach high levels of viremia in chimpanzees without causing a concurrent rise in liver enzymes[195]. Taken together, these data provide evidence that HGV does not cause clinically significant liver disease. As such, HGV is not a common test ordered on the clinical wards.

CONCLUSION

As a group, viral hepatitis represents an ongoing global health concern (Table 1). Acute viral hepatitis infections—HAV and HEV—tend to be self-limited infections with little-to-no long-lasting effect, and both vaccines and improved sanitation conditions will decrease the burden of disease over time. Moreover, ever-improving understanding of the risk factors for acute liver failure from acute hepatitis and experimental supportive care options will aid in further reducing the impact of acute viral hepatitis. Chronic viral hepatitis infections—HBV and HCV—on the other hand, often result in cirrhosis and death if left untreated. While vaccination for HBV is highly effective in reducing transmission, and treatments for HBV are effective in reducing HBV viral load and progression of liver disease, an effective cure for HBV remains elusive. Conversely, an effective vaccine for HCV has not been achieved, but highly effective treatments cure HCV in nearly 100% of cases and are now revolutionizing the fields of transplant medicine. Despite unique barriers, there are ongoing global efforts to eliminate chronic viral hepatitis, and given substantial progress, we are hopeful that this ambitious goal will be realized.

FOOTNOTES

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Country/Territory of origin: United States

ORCID number: Matthew August Odenwald 0000-0003-1740-6472; Sonali Paul 0000-0002-0136-5496.

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REVIEW

Osteosarcopenia in autoimmune cholestatic liver diseases: Causes, management, and challenges

Nicola Pugliese, Ivan Arcari, Alessio Aghemo, Andrea G Lania, Ana Lleo, Gherardo Mazziotti

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Nicola Pugliese, Ivan Arcari, Alessio Aghemo, Andrea G Lania, Ana Lleo, Gherardo Mazziotti, Department of Biomedical Sciences, Humanitas University, Pieve Emanuele 20090, Milan, Italy

Nicola Pugliese, Ivan Arcari, Alessio Aghemo, Ana Lleo, Division of Internal Medicine and Hepatology, Department of Gastroenterology, IRCCS Humanitas Research Hospital, Rozzano 20089, MI, Italy

Andrea G Lania, Gherardo Mazziotti, Department of Endocrinology, Diabetology and Medical Andrology Unit, IRCCS Humanitas Research Hospital, Rozzano 20089, MI, Italy

Corresponding author: Ana Lleo, MD, PhD, Professor, Department of Biomedical Sciences, Humanitas University, Via Rita Levi Montalcini 4, Pieve Emanuele 20090, Milan, Italy.
ana.lleo@humanitas.it

Abstract

Primary biliary cholangitis and primary sclerosing cholangitis (PSC) are the most common cholestatic liver diseases (CLD) in adults and are both characterized by an immune pathogenesis. While primary biliary cholangitis is a model autoimmune disease, with over 90% of patients presenting very specific autoantibodies against mitochondrial antigens, PSC is considered an immune mediated disease. Osteoporosis is the most common bone disease in CLD, resulting in frequent fractures and leading to significant morbidity. Further, sarcopenia is emerging as a frequent complication of chronic liver diseases with a significant prognostic impact and severe implications on the quality of life of patients. The mechanisms underlying osteoporosis and sarcopenia in CLD are still largely unknown and the association between these clinical conditions remains to be dissected. Although timely diagnosis, prevention, and management of osteosarcopenia are crucial to limit the consequences, there are no specific guidelines for management of osteoporosis and sarcopenia in patients with CLD. International guidelines recommend screening for bone disease at the time of diagnosis of CLD. However, the optimal monitoring strategies and treatments have not been defined yet and vary among centers. We herein aim to comprehensively outline the pathogenic mechanisms and clinical implications of osteosarcopenia in CLD, and to summarize expert recommendations for appropriate diagnostic and therapeutic approaches.

Key Words: Cholestatic liver diseases; Primary biliary cholangitis; Primary sclerosing

cholangitis; Osteoporosis; Sarcopenia

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Core Tip: Cholestatic liver diseases (CLD) in adults are characterized by an immune pathogenesis. Osteoporosis is the most common bone disease in CLD, resulting in frequent fractures and leading to significant morbidity. Sarcopenia is emerging as a frequent complication with a significant prognostic impact and severe implications on the quality of life of patients. The lack of useful preventive measures and efficacious treatment strategies remains one of the largest challenges in the management of patients with CLD.

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INTRODUCTION

Cholestatic liver diseases (CLD) are characterized by progressive inflammation, damage, and destruction of bile ducts that lead to liver damage and, eventually, liver cirrhosis and systemic alterations. Accumulation of bile acids within liver cells cause detergent-induced damage of cellular membranes, which ultimately determines the development of apoptosis, inflammation, necrosis, fibrosis, and carcinogenesis[1,2]. Primary biliary cholangitis (PBC) and primary sclerosing cholangitis (PSC) are the most common CLD in adults. Although PBC is considered a classic autoimmune disease with nearly 95% of patients presenting very specific autoantibodies against mitochondrial antigens, PSC is recognized as an immune-mediated disease, with immunogenetic features and a strong association with inflammatory bowel disease (IBD). Both PBC and PSC are associated with a vast group of extrahepatic manifestations, including, but not limited to, fatigue, low bone mass, and other autoimmune diseases such as IBD, systemic sclerosis, and Sjogren syndrome[2,3].

Bone disease, including osteopenia and osteoporosis, is a common complication of CLD. Osteoporosis is characterized by a decreased bone density that leads to an increased risk of fractures. It increases morbidity and mortality in patients, and it is four times more common in patients with PBC compared to gender and age-matched controls. Moreover, sarcopenia and skeletal frailty have recently emerged as frequent complications of CLD, leading to severe morbidity, worse clinical outcome of disease, and lower quality of life of patients. The burden of osteosarcopenia in patients with CLD remains significant and therefore prevention is essential. In recent years, there have been advances in elucidating the risk factors and pathogenetic mechanisms underlying osteoporosis and sarcopenia in CLD but, unfortunately, validated diagnostic and therapeutic guidelines are not yet available. This review focuses on the pathogenic mechanisms and clinical implications of osteosarcopenia in CLD and summarizes expert recommendations for appropriate diagnostic and therapeutic approaches.

OSTEOPENIA AND OSTEOPOROSIS

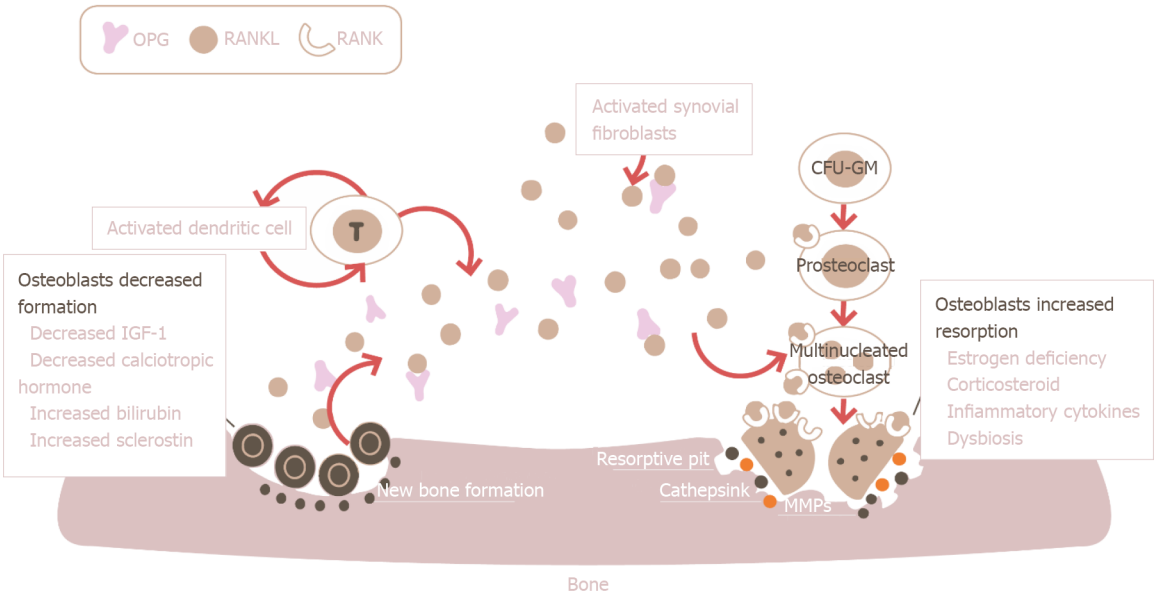
The World Health Organization (WHO) defines osteoporosis as a skeletal disorder characterized by compromised bone strength predisposing to an increased risk of fracture. In clinical practice, this condition is diagnosed using dual X-ray absorptiometry (DXA) for measurement of bone mineral density (BMD) at the lumbar spine, femoral neck, and total hip. In individuals older than 50 years of age and post-menopausal women, skeletal demineralization is graded based on comparisons of patient's BMD with the average for young adults, after adjusting for race and gender. A T-score less than or equal to −2.5 standard deviations (SD) at the hip or spine is defined as osteoporosis, whereas osteopenia is defined as a T-score between −1 and −2.5 SD. These densitometric definitions cannot be applicable for younger subjects in whom the Z-score (*i.e.*, the number of SD from age-matched controls) of 2.0 or lower is used to define a BMD "below the expected range for age"[4]. Although low BMD is consistently correlated with an increased fracture risk in the general population, fragility fractures may develop even in the context of normal BMD especially in subjects with secondary osteoporosis, in whom bone quality is affected more than bone quantity[5]. In these cases, evaluation of trabecular and cortical bone microstructure by high-resolution peripheral computed tomography can provide more reliable information on risk of fractures[6]. In the United States and Europe, the estimated number of

osteoporosis-related hip fractures is about 0.3 and 1.7 million *per* year, respectively. More relevant is the impact of vertebral fractures (VFs) that have been consistently reported as an earlier and frequent complication of osteoporosis in the general population potentially associated with decreased survival and impaired quality of life[7-9].

Osteomalacia, defined by a reduction in bone mineralization with a preserved bone mass, was once thought to play a primary role in increasing fracture risk in patients with CLD[10], but more recent studies have proven that it is rarely associated with liver diseases, as it has been only reported in patients with advanced PBC and severe intestinal malabsorption associated with limited exposure to sunlight[11]. However, skeletal fragility can be associated with a worse outcome in patients with liver disease, as it entails an increased risk of fractures and consequently an overall increased disability prevalence and reduced quality of life[12].

Although osteoporosis is associated with liver diseases including cirrhosis, it is most prevalent in cholestatic disorders[13]. Most of the studies that try to understand the relationship between the bone and liver have focused on PBC, though the pathophysiological mechanisms likely overlap in the case of end-stage liver disease from other etiologies. These mechanisms are numerous and not fully elucidated (Figure 1). The predominant process determining the reduction in bone mass appears to be a reduction in bone formation[11,13], although it seems that in some cases an increase in bone resorption is also involved, such as in post-menopausal women and patients with hypogonadism[14-16]. Bone formation, mediated by osteoblasts, and bone resorption, dependent on osteoclasts, are the two opposite processes that influence bone mass: When resorption exceeds formation, bone mass inevitably decreases and this negative balance leads to bone loss and osteoporosis. Osteoblast dysfunction may be directly linked to elevated serum levels of bile acids and bilirubin[17]. Some *in vitro* studies showed that lithocholic acid (LCA), a monohydroxylated secondary bile acid, can negatively influence osteoblasts' activity, both directly and indirectly through the ligation to vitamin D receptor (VDR) and the successive modification of expression of VDR-mediated genes, such as receptor activator for nuclear factor kappa B (NF-kB) ligand (RANKL) and bone gamma-carboxyglutamate protein, which serve as a regulator of osteoclast and osteoblast maturation, respectively[18]. Curiously, it appears that albumin, when added to cultures of osteoblasts exposed to LCA, can reduce the toxic effects of the molecule on the osteoblasts. It could be hypothesized that the amount of circulating albumin is one of the critical factors linked to the harmful effects on bone of circulating bile acids[11], but data regarding this association are still lacking. Osteoblast dysfunction can also depend on the reduced circulating levels of osteoblast stimulating factors such as insulin-like growth factor 1 (IGF1) secondary to the lack of hepatic synthesis seen in advanced chronic liver disease (ACLD) and reduced absorption[19,20], respectively. IGF1 acts directly on bone to promote longitudinal growth during the development phase and maintain adequate levels of bone mass once peak bone mass is reached[21]. Liver diseases impair the somatotropic axis and associate with liver growth hormone (GH) resistance; the consequent reductions in serum IGF1 can contribute to impair osteoblast function and cause skeletal fragility in individuals with CLD[21,22]. In this context, the role of paracrine and autocrine actions of locally produced IGF1 in the skeleton under control of parathyroid hormone (PTH) is unknown and in need of future studies[21].

In patients with PBC, low liver tissue and serum levels of RANKL and high levels of osteoprotegerin (OPG) have been previously reported[23-25]. This is at first glance unexpected, since OPG has a bone preserving function while RANKL activates osteoclastogenesis and tends to increase bone loss. However, RANKL can be interpreted both as a marker of bone resorption and as a marker of osteoblasts activity: Low RANKL would therefore indicate low osteoblast activity and a reduced bone turnover, which inevitably leads to increased bone fragility and risk of fractures[26]. High OPG levels on the other hand could indicate the homeostatic response attempting to prevent bone loss. Finally, the importance of osteoblast dysfunction has been proved by a series of studies that evaluated bone histomorphometry in patients with advanced CLD undergoing orthotopic liver transplant (OLT). In these studies, osteoblast numbers and bone formation rates appear to be decreased when compared to controls[27,28]. This data correlates with the low levels of osteocalcin, a non-collagenous marker of bone formation, seen in patients with cholestasis[29]: Two different studies observed that osteocalcin levels were decreased in up to 74% of PBC patients[30,31]. However, a previous study has revealed that increased bone resorption and turnover showed by bone histomorphometry are early characteristics of PBC-related bone disease[32]. More studies are needed to evaluate the variation of bone formation and bone resorption markers and to link them to cholestasis. An exemplary study on PBC patients showed no significant decrease in the levels of bone-specific alkaline phosphatase, a bone formation marker, but up to 95% of patients showed above-normal values; also, no significant variation was observed in the urine levels of type I collagen-cross-linked N-telopeptide, a marker of bone resorption[33]. Although it seems reasonable to think that a defect in the secretion of bile acids leads to reduced intestinal absorption of vitamin D, thus leading to hypocalcemia and secondary hyperparathyroidism, data in this regard are conflicting and not conclusive. Old studies have found decreased calcium absorption and serum vitamin D levels in PBC patients[34], but others have found normal vitamin D, calcium, and PTH levels even among osteoporotic patients with PBC[27,35]. It also appears that vitamin D supplementation in patients with cholestasis and malabsorption is unable to significantly improve BMD[36,37], although some old studies proved otherwise[34].



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Figure 1 Possible pathophysiological mechanisms of development of osteoporosis in cholestatic diseases. The figure describes the role of multiple factors linked to cholestatic diseases in the development of osteoporosis. Emphasis is placed on the difference between factors that cause osteoblasts dysfunction and factors that cause increased osteoclasts activity. OPG: Osteoprotegerin; RANK: Receptor activator for nuclear factor kappa B; RANKL: Receptor activator for nuclear factor kappa B ligand; IGF-1: Insulin-like growth factor 1; CFU-GM: Granulocyte-macrophage-colony forming unit; MMPs: Matrix Metalloproteinases.

Since the main risk factors for the development of osteoporosis in OLT patients are pre-transplant bone mass state and pre-transplant fragility fractures, it is not surprising that cirrhotic patients who undergo liver transplant for PBC and PSC are at an extremely high risk of developing osteoporosis and suffering pathological fractures. Osteoporosis is a primary co-morbidity in post-transplant patients and is becoming more and more relevant, as their longtime survival has significantly increased in the last few years. In these patients, there is a rapid bone loss within the first 3 to 6 mo after transplantation and a frequency of fragility fractures of about 21%, most of which happen in the first period after transplant [38]. A study reported that the severity of bone loss was more frequently seen in patients of younger age with PSC, higher pretransplant BMD, no IBD, shorter duration of disease, current smoking, and ongoing cholestasis at 4 mo since OLT[39]. An important contributing cause of rapid bone loss in the immediate postoperative period of these patients is probably the use of high doses of corticosteroids and other immunosuppressive agents, such as tacrolimus and cyclospirn A, as well as immobilization during hospitalization after OLT[40]. After the first 3 to 6 postoperative months, bone gain occurs during the first 2 years with favoring factors for improvement of lower baseline and/or 4-mo BMD, premenopausal status for females, lesser glucocorticoids, no ongoing cholestasis, and higher levels of vitamin D and parathyroid function[39].

SARCOPENIA

Sarcopenia, a progressive and generalized loss of skeletal muscle mass, strength, and function, is the other side of the coin of the metabolic abnormality in patients with liver disease[41]. It can be assessed in numerous ways, such as by bioelectrical impedance analysis or DXA, but the recommended method is by measuring anthropometric parameters, such as skeletal muscle area (SMA) and the Skeletal Muscle Index (SMI), by computed tomography and magnetic resonance imaging[42,43]. SMI is the metric recommended by the International Consensus panel on cachexia. It is the result of SMA (cm^2) depicted on a single image slice [usually at the level of the third lumbar vertebra (L3)], adjusted by the height of the patient (m^2) (this is often referred to as L3SMI method). This measurement can be easily compared to specific cut-off values based on healthy European young adults[44]. Importantly, sarcopenia is significantly associated with mortality and reduced quality of life in patients with liver cirrhosis[45,46].

Although recent data demonstrate that sarcopenia can be identified in up to 70% of cirrhotic patients [47], no data are available on the prevalence of sarcopenia in non-advanced cholestatic diseases. An outstanding study has recently been published analyzing the relationship between PBC, bone diseases, and sarcopenia[48]. Saeki *et al*[48] demonstrated that the association between osteoporosis and sarcopenia was stronger than the association among osteoporosis, female gender, and menopause in PBC patients, and *vice versa* osteoporosis and VFs were important risk factors for sarcopenia,

independently of sex and menopause[48]. The increasing evidence of the existing association between bone mass and muscle loss, defined all together as osteosarcopenia, is of incredible relevance in liver diseases, given that it impacts the prognosis and health-related quality of life. The association of the two conditions is particularly hazardous, since it causes both ease of falling (due to sarcopenia) and bone vulnerability (due to osteoporosis)[49]. More data are, however, needed to fully comprehend the real impact of sarcopenia on non-advanced CLD. At the moment, the only considerations that can be made are based on studies on cirrhotic patients, in which, however, it appears that the main mechanism is linked to hyperammonemia, a phenomenon not present in the early stages of cholestatic diseases[50]. The role of cholestasis in sarcopenia in cirrhotic patients is only hinted at and remains anecdotal. Protein synthesis, protein breakdown, and muscle regenerative capacity mediated by satellite cells are the main processes that influence skeletal muscle mass: When their balance is disrupted, there is a loss in lean mass leading to sarcopenia. Skeletal muscle expression and serum levels of myostatin, a member of the TGFβ superfamily, are increased in patients with ACLD[51,52] and this hormone increases autophagy and proteolysis and prevents protein synthesis by inhibiting the mTORC1 pathway. Serum ammonia has been recognized as a stimulus in the synthesis of myostatin, by acting *via* a NF-kB mediated mechanism[53-55]. Hyperammonemia also impairs the formation of α-ketoglutarate (αKG), a molecule involved in the cycle of tricarboxylic acids (TCA)[53]. This results in several potential consequences including lower flux of the TCA cycle, impaired mitochondrial function, and decreased ATP synthesis. Since protein synthesis is an energy intense process, low ATP concentrations may also cause reduced protein synthesis. Similarly to hyperammonemia, hypoglycemia and low glucose levels in skeletal muscle cells are linked to the lack of glycogenolysis secondary to the reduction of hepatic glycogen stores, and can lead to the consumption of muscle amino acids for the production of energy[56]. Although hyperammonemia does not manifest itself in non-advanced CLD, the mechanisms leading to sarcopenia may be in some way analogous. Liver damage caused by the underlying pathology, malabsorption, and increased energy demands may lead to an energy deficit that would push the skeletal muscles to use amino acids as an alternative source of energy, in a similar way to how it occurs in case of hypoglycemia due to reduced hepatic glycogen reserve[57].

PREVALENCE AND DETERMINANTS OF OSTEOSARCOPENIA IN CLD

PBC

PBC is a chronic inflammatory autoimmune cholestatic liver disease which, if left untreated, could culminate in end-stage biliary cirrhosis[58]. It is the most studied condition as regards metabolic bone disease, both for the high prevalence of osteoporosis in PBC patients and for the possibility of directly studying the pathophysiological mechanisms underlying the interaction between cholestasis and reduction in bone mass.

Osteoporosis is a common complication of PBC, with the most recent studies reporting a prevalence ranging from 20% to 45%, four-fold higher than that in the general population[59,60], with the highest prevalence in patients with cirrhosis on the liver transplant list[39]. Accordingly, the incidence and prevalence of fractures are also increased in PBC patients, ranging from 0% to 14% over a 2-year period and from 9% to 22%, respectively[61]. In a prospective study, Guañabens *et al*[62] observed a prevalence of vertebral, non-vertebral, and overall fractures of 11.2%, 12.2%, and 20.8%, respectively (Table 1). In that study, more than 20% of fractures occurred without a densitometric diagnosis of osteoporosis consistent with the pathophysiological concept that alteration of osteoblastogenesis and bone formation in CLD may induce impairment of bone quality more than bone quantity as in other forms of secondary osteoporosis. In addition to the common risk factors associated with skeletal fragility, such as age and post-menopausal state, additional independent risk factors have been identified in patients with PBC. The most prominent risk factor for developing osteoporosis is the stage of the disease. A study based on histologic staging observed that patients with more advanced histologic stages (such as stage 3 or 4) had more than a 5-fold increased risk of developing osteoporosis than patients with an earlier stage of the disease and the rate of bone loss over time was significantly greater in the former as compared with the latter[63]. A more recent study observed that liver stiffness measured by FibroScan was directly related with the reduction of both cortical and trabecular bone parameters in the tibia and distal radius[64]. The stage of disease appears to have an effect far stronger than the post-menopausal state in PBC patients [65]. Other less important risk factors that seem to have been identified include female gender, the necessity of transplant and, to some extent, genetic predisposition[58,66]. Indeed, some studies identified some gene loci that positively correlate with osteoporosis[67-69]. Although vitamin D does not seem to play an important role in determining skeletal fragility in CLD, polymorphisms of VDR have been associated with osteoporosis in individuals with PBC. One study in women affected by PBC concluded that VDR genotype is an independent genetic predictor of osteoporosis[67]. In addition, a polymorphism of the gene encoding collagen type I alpha1 (COLIA1), Sp1, is associated with reduced baseline BMD in patients with PBC[69]. Lastly, some polymorphisms of a tight junction membrane gene, the claudin-14 (CLDN-14), suspected to be involved in the pathogenesis of CLD, has been proved to be associated with low BMD in PBC patients[70].

Table 1 Prevalence of osteoporosis and fractures according to the severity and stage of primary biliary cholangitis

Ref.	N of patients	Age (yr) mean	Female (%)	Diagnosis	Osteoporosis (%)	Vertebral fractures (%)	Peripheral fractures (%)	Overall fractures (%)	Cirrhosis (%)
Guañabens *et al* [114]	38	51	100	DPA	45	13	NR	13	94
Parés *et al*[68]	61	54	100	DXA	21	10	10	13	26
Guañabens *et al* [65]	142	54	100	DXA	31	14	11	14	26
Guichelaar *et al* [39]	156	53	86	DXA	44	22	NR	22	100
Guañabens *et al* [62]	185	56	100	DXA	32	11	12	21	23
Solaymani-Dodaran *et al* [115]	930	NR	88	NR	NR	NR	7.4	14.7	31

NR: Not reported; DXA: Dual-energy X-ray absorptiometry; DPA: Dual-photon absorptiometry.

Another important independent risk factor is low body mass index (BMI). Beyond the direct association of reduced muscle mass and low bone mass, it seems that in PBC patients, preponderant hormonal mechanisms associated with BMI come into play: Leptin, an adipocyte derived hormone, seems to indirectly regulate bone metabolism since it increases osteoblast proliferation and bone matrix synthesis, resulting in increased bone formation, and inhibits RANKL production, decreasing bone resorption. Szalay *et al*[71] observed decreased leptin levels in PBC patients and also demonstrated a positive correlation between leptin, BMI, and BMD[71].

Although sarcopenia is a fascinating new topic in hepatology and has been recently studied in ACLD patients, very few data are available on its relationship with PBC in non-cirrhotic patients. The only article available in the literature at the time of writing this review has been previously cited and dates back to May 2020[48]. Saeki *et al*[48] reported the prevalence of sarcopenia, diagnosed according to the Japan Society of Hepatology guidelines[48,72], between PBC patients: 23.1% for all patients and 25% for female patients, greater than in other non-cirrhotic liver conditions, where the prevalence was reported to be approximately 15%[73,74]. These findings suggest that patients with PBC are more susceptible to sarcopenia, compared to those with other chronic liver diseases. This study also proved that sarcopenia is strongly correlated with osteoporosis and increased fracture risk (especially VFs) and *vice versa*, proving that osteosarcopenia as a unified clinical entity is an important complication of PBC, occurring in up to 15.4% of patients, requiring careful monitoring in all patients, especially post-menopausal women, who represent the majority of PBC patients. The clinical relevance of osteosarcopenia has been proven in studies focused on geriatric patients, in which the osteosarcopenic group had greater impairment of physical performance and balance than the non-osteosarcopenic and sarcopenia/osteoporosis alone groups. Consequently, osteosarcopenia conferred an increased rate of falls and fractures and a consequent higher mortality rate[75-77]. Despite the clinical relevance, recommendations on follow-up and treatment cannot yet be made, as these aspects are still in the early stages of definition [78].

PSC

PSC is a chronic, cholestatic liver disease characterized by immune-mediated inflammation and fibrosis of both intrahepatic and extrahepatic bile ducts, leading to the formation of multifocal bile duct strictures and to the development of biliary cirrhosis[79]. Although it is a clinical entity of considerable interest, both for its hepatological implications and for the set of clinical conditions with which it is associated, there are still very few studies that focus on the link between PSC and bone disease, and almost none that investigate a possible relationship of PSC with sarcopenia.

As with other chronic liver diseases, the prevalence of osteoporosis in PSC is higher than that of the general population, accounting for 15%–30% of patients with PSC[80,81]. General risk factors include female gender, age, and low BMI. Studies are still needed to evaluate if duration of the disease is a risk factor and, curiously, osteoporosis does not appear to be related to the severity of the underlying PSC [27]. Although previous studies reported a possible association between osteoporosis and the stage of disease[82,83], more recent studies observed otherwise and failed to prove a significance correlation between osteoporosis and the severity of liver disease. It is possible that this difference is based on population cohorts analyzed, since in previous studies the patients were less heterogeneous and predominantly post-menopausal women[84]. Moreover, the close association between PSC and IBD [85], the consequent malabsorption, and the possible use of steroid therapy at high doses for long

periods of time can certainly influence bone metabolism and represent an important risk factor for the development of osteoporosis. In addition, patients with IBD presents themselves with a lower bone mass at the diagnosis[86], likely due to the systemic inflammatory state of IBD.

No conclusive data are yet available regarding the possible relationship between PSC and sarcopenia but, considering the cholestasis-associated malabsorption, which may be worsened by the eventual concomitant IBD related to PSC, and the increased prevalence of sarcopenia in patients with other liver diseases including PBC, it is reasonable to assume that these patients also have an increased prevalence of sarcopenia compared to the general population that should be investigated at the time of diagnosis and during the follow-up. Interestingly, Shteyer *et al*[87] failed to prove a significant difference between pediatric patients with PSC and the control group; also, children and young adults with concomitant PSC and IBD appeared to have lesser degree of sarcopenia in comparison to patients with PSC alone [87]. Although interesting, larger studies are required to confirm these curious findings.

MANAGEMENT OF OSTEOSARCOPENIA IN CLD

Osteoporosis

Management of osteoporosis in cholestatic diseases cannot be evidence-based and no guidelines have been developed for diagnosis and treatment of osteoporosis in this specific clinical setting. However, based on our personal experience and the few studies so far published on the topic, some recommendations could be provided (Table 2). DXA measurement of BMD should be performed at the initial diagnosis of PBC to identify subjects with low BMD at higher risk of fractures.

The optimal timing of monitoring PBC patients is yet to be defined, but in clinical practice bone densitometry with DXA should be performed, depending on the presence of risk factors for osteoporosis and fractures, in 1 to 3 years if initial results are normal. A more stringent follow-up is indicated in the presence of altered BMD or risk factors such as severe cholestasis, menopause before the age of 45 years old, family history of osteoporosis or fragility fractures, BMI less than 19 kg/m^2, tobacco use, heavy alcohol abuse, and glucocorticoid use greater than 3 mo and or > 5 mg daily. In patients who are already on osteoporosis treatment, DXA should be performed annually to assess treatment response. In addition to imaging, routine monitoring of vitamin D, calcium, phosphorus, and PTH should be performed every 1 to 2 years based on the current risk of developing bone disease[58,61].

Over the last decade, several algorithms (*e.g.*, FRAX) have been proposed to improve the value of DXA results in predicting fracture risk[88] but their use in secondary osteoporosis in general and in CLD in particular has not been validated. Noteworthy, the traditional risk factors of osteoporosis and fractures included in the algorithm FRAX seem to have a role also in influencing the occurrence of fragility fractures in subjects with CLD.

The new generation DXA machines can also provide information on bone quality. For instance, the trabecular bone score (TBS) is a texture parameter obtained directly from DXA images through the evaluation of the average pixel gray-scale variation. A low TBS value correlates with a weaker microarchitecture with reduced and scarcely interconnected trabeculae, resulting in lower bone strength and mechanical resistance[89]. Measuring this parameter, the clinicians may have another reliable information on risk of fractures even in individuals with either normal or only slightly decreased BMD [90].

As in other forms of secondary osteoporosis, the search of VFs is indicated in all subjects at diagnosis of CLD since they may occur even in the context of normal BMD. In more than 55% of the cases, VFs occur without specific clinical symptoms and the radiological and morphometric approach has emerged as the method of choice for evaluating the true prevalence and incidence of these fractures in the clinical practice. VFs are identified by marking the vertebral body with six points to describe the vertebral shape and heights. According to the quantitative morphometric approach, VFs are defined mild, moderate, and severe based on a height ratio decrease of 20%-25%, 25%-40%, and more than 40%, respectively[91]. VFs are routinely assessed by examining lateral projection images of conventional spine X-ray radiographs, although other approaches using DXA and the low-dose biplane X-ray imaging system (EOS imaging, Paris, France) have been proposed as alternative tools to limit radiation exposure in clinical practice[92,93]. The current guidelines indicate that finding of non-traumatic VFs, regardless of underlying disease and BMD values, is sufficient to establish the diagnosis of osteoporosis and to consider pharmacologic treatment as secondary prophylaxis[94].

Bone active agents used to treat osteoporosis are classified as anti-resorptive and anabolic drug[95]. Bisphosphonates inhibit bone resorption and are the most prescribed drugs for the treatment of osteoporosis. Denosumab is a human monoclonal antibody (IgG2 immunoglobulin isotype) binding RANKL with high affinity and specificity and inducing a reversible inhibition of osteoclastogenesis and bone resorption. Teriparatide is the 1-34 active fragment of PTH with stimulating effects on osteoblastogenesis and bone formation when intermittently administered once daily. Teriparatide is currently the only anabolic drug approved for treatment of osteoporosis at high risk of fractures and for glucocorticoid-induced osteoporosis.

Table 2 General expert recommendations for management of osteosarcopenia in patients with cholestatic liver diseases

Risk for osteoporosis should be considered in all patients with cholestatic liver diseases	
DXA should be considered to assess BMD at presentation and at follow-up where indicated	T-score > -1.5 - > repeat in 2-3 yr Osteopenia, T-score ≤ -1.5 but > -2.5, or presence of risk factors - > repeat in 1-2 yr Osteoporosis, T score ≤ -2.5, or pathological fractures with normal BMD - > repeat in one year
VFs should be investigated at presentation with lateral spine X-rays radiograph in all patients with cholestatic liver diseases	
Alcohol and smoking cessation in addition to increasing aerobic exercise and practicing routine weight-bearing exercises are highly recommended in all patients with cholestatic liver diseases	
Consider including supplements of 25-(OH)-vitamin D (800 IU daily) and calcium (1000–1500 mg daily) in patients with cholestatic liver disease and osteopenia or osteoporosis	
Consider utilizing bisphosphonates in patients with osteoporosis and patients with VFs, regardless of underlying disease and BMD values	
For patients with PBC, denosumab might have a beneficial role both for osteoporosis treatment and for PBC but data are scarce, and recommendation cannot be made yet	
Consider evaluating sarcopenia by cross-sectional imaging when strong clinical suspicion is present in all patients with cholestatic liver diseases	
Consider exercise programs and adequate nutritional and caloric intake in all patients with sarcopenia and cholestatic liver diseases	

DXA: Dual-energy X-ray absorptiometry; BMD: Bone mineral density; VFs: Vertebral fractures; PBC: Primary biliary cholangitis.

Data on the efficacy and safety of bone-active drugs in CLD are scant and their use in this clinical setting can be guided by evidence extrapolated from the literature on the treatment of postmenopausal osteoporosis[96]. A comprehensive Cochrane systematic review published in 2011 concluded that there are no conclusive data showing the benefits of bisphosphonate use on BMD, mortality, or reduced fracture risk in this specific clinical context[97]. There is only one randomized clinical trial that compares newer generation bisphosphonates (in this case alendronate) to placebo in PBC[98]: It proved that after 1 year, there was a significant improvement in lumbar spine BMD in patients treated with bisphosphonates (10.4% *vs* –0.12% in patients in the placebo arm, $P < 0.005$) but failed to prove a significant reduction in fractures (0% *vs* 7.1% in patients in the placebo arm, $P = 0.3$)[98]. Particular attention must be given to patients with ACLD with a high risk of oesophageal varices since esophagitis and oesophageal ulcers are side-effects of oral bisphosphonates. In these patients, parenteral bisphosphonates can be proposed[99,100].

Preliminary data on denosumab in patients with PBC indicate that lumbar spine T-score significantly improved after 1 and 3 years of treatment, along with levels of markers of bone formation, despite that the prevalent mechanism determining osteoporosis is osteoblast dysfunction[101,102]. As the studies are extremely small, no recommendations can be made yet, but certainly the use of denosumab in these patients is promising. Importantly, recent evidence strengthens a critical role of RANK/RANKL signaling in autoimmunity besides bone density, with the immune and skeletal systems being closely interconnected. It has been demonstrated that cholangiocytes from PBC patients express high levels of RANK; most importantly, the immune infiltrates within the portal areas around bile ducts in PBC are highly RANKL positive and the hepatic level of RANKL was associated with disease severity[25]. Lleo *et al*[25] hypothesized that damaged cholangiocytes in PBC, which show high levels of RANK, determine the recruitment of RANKL positive cells and consequently portal tract infiltrates. Taken all together, a number of recent and old evidence point out that denosumab might have a beneficial role in PBC therapy, besides osteoporosis, but data are scarce and more studies are needed to make recommendation[25].

No data are available on the efficacy and safety of teriparatide and PTH-analogues but, whereas the main pathogenetic mechanism that determines the development of osteoporosis in patients with PBC is reduced osteoblasts activity, osteoanabolic agents could play a crucial role in the treatment of the condition in this setting.

Inactivity, alcohol consumption, tobacco use, and reduced dietary calcium intake can all lead to reduced bone density. For this reason, alcohol and smoking cessation in addition to increasing aerobic exercise and practicing routine weight-bearing exercises is highly recommended. Dietary supplementation of calcium (1000-1500 mg daily) and vitamin D [800 international units (IU) daily] is also recommended in patients who are at particularly high risk of developing osteoporosis, especially in patients with ACLD[58,103]. Patients receiving cholestyramine should be monitored closely since its

administration may reduce intestinal absorption of vitamin D[104,105]. Although this is standard clinical practice, data on the effect of calcium and vitamin D supplementation are controversial: As previously stated, some studies prove a significant improvement in BMD[34] while other studies failed to do so[36,37].

It is important to assess the presence of osteoporosis by DXA at the moment of diagnosis of PSC, as well as considering supplementation with calcium and vitamin D in all patients, though this has no clear benefit based on the literature[106,107]. No protocols exist on how to handle the specific follow-up and treatment of these patients, so WHO or ISCD guidelines for the management of osteoporosis are usually used[4] and the recommended diagnostic-therapeutic approach is the same as in PBC, even if the two diseases differ both in pathogenesis and in the phenotype of the population affected.

Sarcopenia

The diagnosis of sarcopenia should be made by cross-sectional imaging when strong clinical suspicion is present. Treatment can be derived by studies based on ACLD, but even in this setting clear guidelines are not currently available, both because the studies existing so far showed great heterogeneity and were based on a small number of patients, thus limiting the possibility of defining specific and reliable guidelines for the pathology, and no treatment had proven to be particularly effective[57]. Exercise programs finalized on avoiding natural deterioration of muscle mass[108,109] and nutritional supplementation with BCAA[110-112] are no established treatment and the data on these approaches are controversial. It should also be noted that physical activity may be difficult as one of the salient clinical features of this disease is chronic asthenia and fatigue. Considering the molecular mechanisms that lead to sarcopenia, improving protein synthesis and reducing autophagy with molecules like myostatin antagonists, direct mTORC1 activators, antioxidants, and mitochondrial protective agents could have the potential to benefit skeletal muscle protein turnover but have not been adequately evaluated[113]. More studies are needed to define the correct timing of follow-up.

CONCLUSION

Osteosarcopenia is a common complication of CLD that strongly influences quality of life and leads to severe morbidity. Indeed, cholestasis is directly associated with both bone and lean mass loss and the prevalence of bone damage is demonstrated to be higher in CLD than in the general population. Risk factors and etiopathogenesis of osteoporosis in PBC have been widely investigated; however, evaluation of the efficacy of osteoporosis drugs and preventive measures remain poorly known and data on PSC are scarce. On the other hand, studies on risk factors, etiologic mechanisms, and management of sarcopenia in CLD are lacking.

It is widely accepted that preventing the reduction of bone density is important to decrease the risk of fractures and improve morbidity and mortality. Further, PSC and PBC mostly affect young patients, and therefore prevention and screening are widely recommended. However, the timing is not yet defined, and no clinical guidelines are available for management of osteosarcopenia in CLD. Unfortunately, the overall quality of evidence is low and data on the treatment of CLD-related osteosarcopenia are inadequate.

Table 2 provides a proposed algorithm for the management of osteosarcopenia in CLD. Not pharmacological measures, including alcohol and smoking cessation, and aerobic and weight-bearing exercises, are highly recommended in all patients. The primary medical intervention for the treatment of osteoporosis in CLD is bisphosphonates in association with calcium and vitamin D supplements, though a benefit in terms of fracture reduction has never been shown. The use of further therapies for osteoporosis in CLD are based on the postmenopausal osteoporosis literature and new studies are desperately needed to define the best therapeutic approach to osteosarcopenia in a group of patients with high prevalence.

FOOTNOTES

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Country/Territory of origin: Italy

ORCID number: Nicola Pugliese 0000-0001-6466-1412; Ivan Arcari 0000-0002-3601-1612; Alessio Aghemo 0000-0003-0941-3226; Andrea G Lania 0000-0002-5380-2141; Ana Lleo 0000-0002-0561-7902; Gherardo Mazziotti 0000-0001-5458-701X.

Corresponding Author's Membership in Professional Societies: American Association for the Study of Liver Diseases; European Association for the Study of the Liver (EASL).

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ORIGINAL ARTICLE

Basic Study

Syngeneic implantation of mouse hepatic progenitor cell-derived three-dimensional liver tissue with dense collagen fibrils

Miho Tamai, Eijiro Adachi, Masaya Kawase, Yoh-ichi Tagawa

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Miho Tamai, Yoh-ichi Tagawa, School of Life Science and Technology, Tokyo Institute of Technology, Yokohama-shi 226-8501, Japan

Miho Tamai, Faculty of Dental Medicine, Hokkaido University, Sapporo 060-8586, Japan

Eijiro Adachi, Department of Molecular Morphology, Kitasato University, Yokohama-shi 319-3526, Japan

Eijiro Adachi, Long-Term Care Health Facility Yasuragi, Ibaraki Zip or Postal Code, Japan

Masaya Kawase, Nagahama Institute of Bio-Science and Technology, Shiga 526-0829, Japan

Corresponding author: Yoh-ichi Tagawa, DSc, Associate Professor, School of Life Science and Technology, Tokyo Institute of Technology, 4259 B51, Nagatsuta-cho, Midori-ku, Yokohama-shi 226-8501, Japan. ytagawa@bio.titech.ac.jp

Abstract

BACKGROUND

Liver transplantation is a therapy for irreversible liver failure; however, at present, donor organs are in short supply. Cell transplantation therapy for liver failure is still at the developmental stage and is critically limited by a shortage of human primary hepatocytes.

AIM

To investigate the possibility that hepatic progenitor cells (HPCs) prepared from the portal branch-ligated hepatic lobe may be used in regenerative medicine, we attempted to enable the implantation of extracellular matrices containing organoids consisting of HPC-derived hepatocytes and non-parenchymal cells.

METHODS

In vitro liver organoid tissue has been generated by accumulating collagen fibrils, fibroblasts, and HPCs on a mesh of polylactic acid fabric using a bioreactor; this was subsequently implanted into syngeneic wild-type mice.

RESULTS

The *in vitro* liver organoid tissues generated transplantable tissues in the condensed collagen fibril matrix and were obtained from the mouse through partial hepatectomy.

CONCLUSION
Liver organoid tissue was produced from expanded HPCs using an originally designed bioreactor system. This tissue was comparable to liver lobules, and with fibroblasts embedded in the network collagen fibrils of this artificial tissue, it is useful for reconstructing the hepatic interstitial structure.

Key Words: Liver; Three-dimensional tissue culture; Hepatic progenitor cells; Angiogenesis; Biomimetic extracellular matrix

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Core Tip: Liver transplantation is a therapeutic procedure used to recover liver function in patients with irreversible liver failure; however, there is presently a shortage of transplant organs available. Hepatic stem and progenitor cells are expected to allow regenerative medicine to produce a cell source as an alternative to whole organs. The portal branch-ligated, hepatic lobe-derived HPCs multiplied in a bioreactor chamber to form liver organoid tissues comparable to liver lobules. These organoid tissues were implanted into syngeneic mice. This portal branch-ligated-derived HPC line has the potential to proliferate, mature, and form implantable hepatic tissue.

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INTRODUCTION

Liver transplantation is a therapy for irreversible liver failure; however, donor organs are currently in short supply[1,2]. Cell transplantation therapy for liver failure is still at the developmental stage and has a critical problem in terms of a shortage of human primary hepatocytes[3]. Human embryonic stem/induced pluripotent stem (ES/iPS) cell-derived hepatocytes are thought to be an alternative to human primary hepatocytes, but ES/iPS cells are difficult to differentiate into mature hepatocytes in culture[4,5]. ES/iPS cell-derived immature hepatocytes successfully developed into mature liver tissue in animals after implantation[6,7]. However, this process requires a great deal of time, effort, and expense in order to obtain a sufficient number of ES/iPS cell-derived hepatocytes in culture to achieve the amount needed for them to continue to proliferate. There have been several reports of rat HPCs, such as small hepatocytes[8] and Lgr5+ rat and mouse liver stem cells[9,10], becoming established in culture. These hepatic stem/progenitor cells have the ability to proliferate and differentiate into hepatocytes and cholangiocytes[11,12]. Recently, we also succeeded in establishing HPC lines prepared from the portal branch-ligated hepatic lobe in mice (PBL-HPCs)[13]. These cells could differentiate into mature hepatocytes in the presence of oncostatin M, or to cholangiocytes in EHS gel.

Besides dissociated hepatocyte implantation, regenerative medicine is also expected to enable the implantation of extracellular matrices containing aggregate, or organoids consisting of hepatocytes and non-parenchymal cells[14-16]. *In vitro* liver organoid tissue has previously been generated by accumulating collagen fibrils, human fibroblast cell line (HFO cell), and human hepatocarcinoma cell line (Hep G2) on a mesh of polylactic acid (PLA) fabric using a bioreactor[14]. Also, instead of HFO and Hep G2, mouse embryonic fibroblasts and primary hepatocytes were used for this *in vitro* liver organoid tissue. These *in vitro* liver organoid tissues generated transplantable liver organoid tissues in the right portal vein branch-ligated *nu/nu* mouse with a condensed collagen fibril matrix[14]. The fibroblasts are embedded in the network collagen fibrils of this artificial tissue, and it is therefore useful for reconstructing the hepatic interstitial structure.

In this study, the PBL-HPCs were expanded and formed liver organoid tissue, which was comparable to liver lobules using an originally designed bioreactor system, and was also implanted into its syngeneic wild-type mouse.

MATERIALS AND METHODS

Animals

Pregnant BALB/cA mice at 13.5 d post-coitus (CLEA Japan, Tokyo, Japan) were used for embryonic fibroblast isolation. Six-week-old female and male BALB/cA Jcl and BALB/cA Jcl-nu/nu 3 mice (CLEA Japan, Tokyo, Japan) were used as transplant recipients. The animal protocol was approved by the Animal Experimentation Committee of the Tokyo Institute of Technology.

Cells and cultures

The PBL-HPCs were established in a previous study with portal vein ligated methods[13]. The cells were cultured in Williams' E medium (GIBCO Laboratories, Grand Island, NY, United States) supplemented with 5% fetal bovine serum, 10 mmol/L nicotinamide (Sigma-Aldrich, St. Louis, MO, United States), 0.1 μmol/L dexamethasone (Sigma-Aldrich), 1×Insulin-Transferrin-Sodium Selenite Supplement (Roche Diagnostics, Mannheim, Germany), and 20 ng/mL Recombinant Mouse Epidermal Growth Factor (R&D Systems, Minneapolis, MN, United States) in 5% CO_2 at 37°C. These cells were passaged by treatment with 0.05% trypsin (Invitrogen) and 20 μmol/L ethylenediaminetetraacetic acid (EDTA; NACALAI TESQUE, Kyoto, Japan).

Preparation of murine embryonic fibroblasts

A pregnant female BALB/c mouse at 13.5 dpc (days post-coitus) was sacrificed by cervical dislocation, and embryos were removed. The limbs of the embryos were minced and treated with 0.25% trypsin (Invitrogen, Tokyo, Japan) + 1 mmol/L EDTA (about 2 mL per embryo) and incubated with gentle stirring at 37 °C for 10-15 min. The cells were subsequently cultured in DMEM containing 10% (v/v) FBS.

Generation of three-dimensional liver tissue culture model

As shown in Figure 1, the three-dimensional (3-D) liver tissue culture model was generated by accumulating collagen fibrils, primary murine embryonic fibroblasts, and PBL-HPCs using a closed-loop system with a bioreactor chamber (diameter 17 mm; thickness 20 mm) developed by our group as previously reported[14,17]. Briefly, primary embryonic fibroblasts (1.0×10^5 cells/mL) in 10% FBS and 7.5 mg/mL type I collagen prepared from calfskin (Koken Collagen, Tokyo, Japan) in Williams' E medium flowed through the closed-loop system at a predetermined flow rate (1-5 mL/min) for 6 h. Subsequently, the same medium was circulated through the closed-loop system, and PBL-HPCs (5.0×10^6 cells/mL) were injected using a syringe into the system upstream of the bioreactor chamber for 2 h. Finally, a suspension of fibroblasts (1.0×10^5 cells/mL) was circulated for 6 h.

Morphological analyses

The 3-D liver tissue culture models were fixed with Zamboni's fixative for light microscopy. The samples were dehydrated with an ethanol series and embedded in paraffin. The sections were stained with hematoxylin and eosin or AZAN and examined with a light microscope.

Hepatic function assay in liver tissue culture model

Urea production in the medium was quantified using a urea assay kit (Bioassay Systems, Hayward, CA, United States) 24 h after the addition of 2 mmol/L NH_4Cl. Albumin production was quantified in the medium using an albumin EIA (Albuwell M) mouse kit (Exocell, Philadelphia, PA, United States).

The metabolites of testosterone in the medium were quantified by HPLC analysis[18]. The 3-D liver tissue culture models were incubated with fresh medium containing 0.25 mmol/L testosterone and the medium was collected at 24 h. After sample treatment, HPLC analysis was performed using LC-10ADVP (Shimadzu, Kyoto, Japan) with Cadenza columns (Cadenza CD-C18) (Imtakt, Kyoto, Japan) and SPD-10A VP (Shimadzu, Kyoto, Japan).

Transplantation of liver tissue culture model

Under isoflurane anesthesia, mice were subjected to an upper-abdominal incision, followed by exposure and ligation of the left portal vein branch and subsequent hepatectomy of the left and middle lobes (70%). The 3-D liver tissue culture model was transplanted into the subcutaneous layer of a mouse. Two weeks later, the 3-D liver tissue culture model was removed for histological analysis of the vascular network.

Statistical analysis

Results of multiple experiments were reported as the mean ± SE. Statistical comparisons were made using a Tukey-Kramer method and a Welch *t*-test using the IBM SPSS Statistic 27.

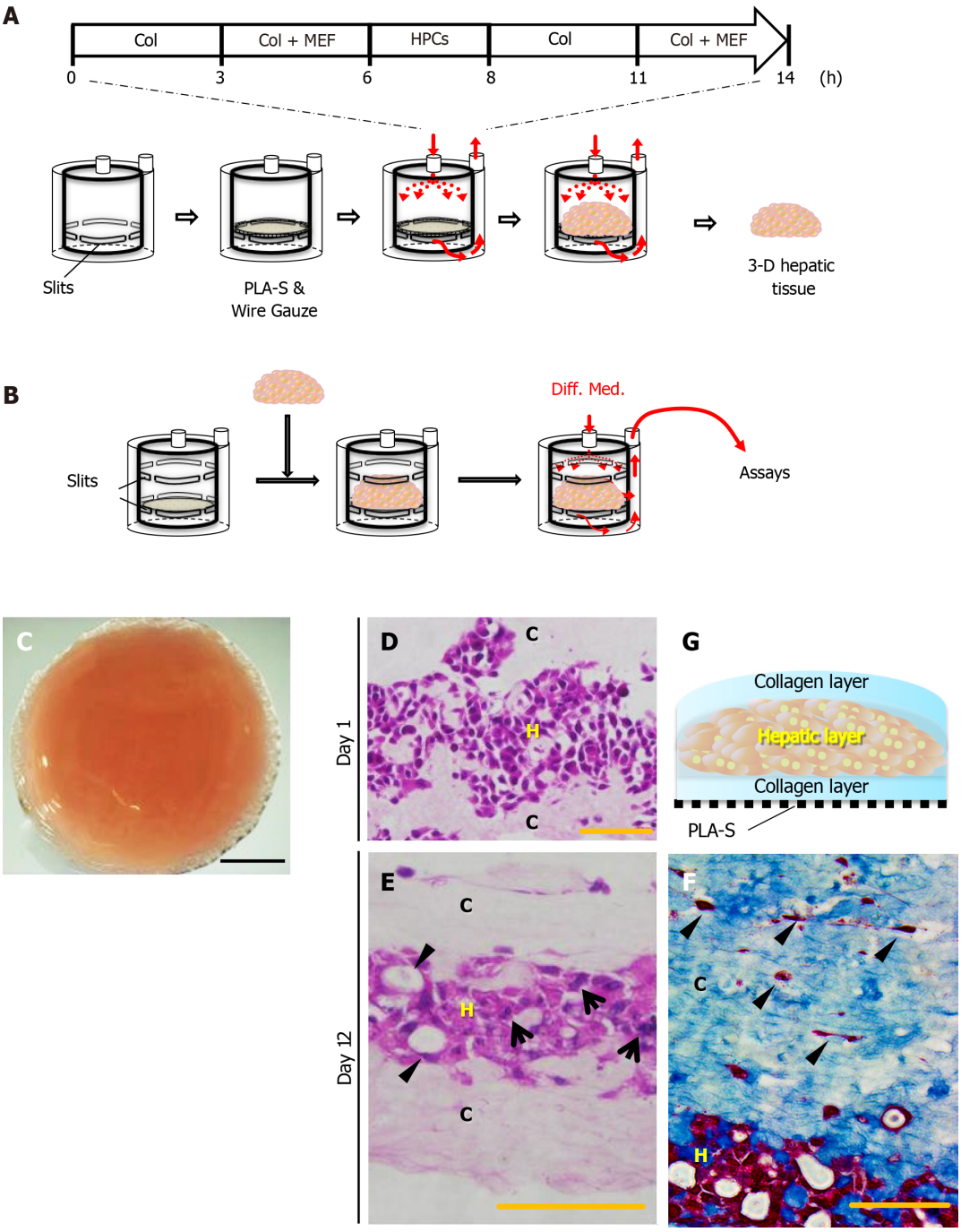


Figure 1 Reconstruction of the three-dimensional liver tissue culture model using hepatic progenitor cells. A and B: A schematic illustration showing reconstruction of the liver tissue culture model using a bioreactor; preparation steps (A), and culture steps (B); C: Micrographs of a reconstructed three-dimensional (3-D) liver tissue culture model with dense collagen fibrils; D and E: Histological analyses: hematoxylin-eosin staining of a section of the 3-D liver tissue culture model on day 1 (D) and day 12 (E). Arrows indicate binuclear populations, like hepatocytes. Arrowheads indicate a bile duct–like structure; F: Histological analyses: AZAN staining of a section of the 3-D liver tissue culture model on day 12. Arrowheads indicate fibroblasts at condensed collagen fibril matrices. Bar corresponds to (C) 5 mm, (D) 50 μm, and (E) and (F) 100 μm. Col: Collagen; HPCs: Portal branch-ligated-hepatic progenitor cells; PLA-S: Polylactic acid sheet; C: Collagen layer; H: Hepatic layer.

RESULTS

Preparation of 3-D hepatic progenitor cell-derived 3-D liver tissue culture model

Type I collagen solution was circulated through a sheet of PLA into a reverse radial flow-type bioreactor, followed by the suspension of 5.0 × 10^6 cells of mouse embryonic fibroblasts (MEFs). In the next step, the suspension of 1.0 × 10^7 PBL-HPCs was circulated without oncostatin M. After these steps, the type I collagen solution was circulated again followed by the suspension of 5.0 × 10^6 MEFs. Finally a 3-D aggregate was prepared (Figure 1A). The surface of this 3-D aggregate which included PBL-HPCs, primary fibroblasts, and type I collagen was glossy and measured 17 mm in diameter and 1.5 mm in

height. This glossy aggregate consisted of a layer of PBL-HPCs, sandwiched between two layers of collagen fibrils with MEFs, and was constructed on a sheet of PLA as shown in Figure 1G. Cross-sectional profiles of the 3-D liver tissue culture models were stained with hematoxylin-eosin and AZAN as shown in Figure 1D-F. The collagen layers were composed of densely packed collagen fibrils running parallel to the plane of the PLA sheet. A layer of PBL-HPCs 200-300 μm thick (Figure 1D), and two layers of collagen fibrils populated with embryonic fibroblasts, approximately 400 μm thick, were observed in the 3-D liver tissue culture model. To culture the 3-D liver tissue model, the cylinder inside the bioreactor was changed from construction to culture (Figure 1B). The 3-D liver tissue culture model was cultured using a differentiation medium containing 20 ng/mL oncostatin M in the bioreactor for 12 d. After this, PBL-HPCs differentiated into mature hepatocyte-like cells, in binuclear populations, and with a bile duct-like structure (Figure 1E). Collagen layers were maintained by the collagen fibers and fibroblasts on day 12.

Expression of liver-related genes in hepatic progenitor cell-derived 3-D liver tissue culture model

The expression of liver-specific genes was determined in the 3-D liver tissue culture model. To investigate whether the 3-D liver tissue culture models were able to show hepatic lineage differentiation (*i.e.*, to mature into hepatocytes and bile duct cells), the cells were cultured in the presence of oncostatin M. By day 12 of the culture in the hepatic lineage differentiation medium, quantitative real-time polymerase chain reaction analysis revealed that the cells expressed hepatocyte differentiation markers including: Afp (α-fetoprotein); Albumin; Tat (tyrosine aminotransferase); Tdo (tryptophan 2,3-dioxygenase); Cps1 (carbamoyl-phosphate synthetase 1); Cyp1a2 (Cytochrome P450, family 1, sub-family a2); Cyp2e1 (Cytochrome P450, family 2, sub-family e1); and Abcc2 [ATP-binding cassette, sub-family C (CFTR/MRP), member 2] (Figure 2A-H). On the other hand, the gene expression of *Bsep* (bile salt export pump) and *ABCB11* (ATP-binding cassette, sub-family B member 11) decreased in the 3-D liver tissue culture model (Figure 2I). The expression of *CK19*, a representative marker for HPCs, was confirmed in the PBL-HPCs. The gene expression of *CK19* decreased in the 3-D liver tissue culture models (Figure 2J). In addition, PBL-HPCs expressed the *CD44* gene, a progenitor cell marker, which decreased in the 3-D liver tissue culture models (Figure 2K). After that, *CD44* expression increased in the 3-D liver tissue culture models on day 12 of culture (Figure 2K).

The 3-D liver tissue culture model exhibits expression of multiple liver-specific functions

The expression of several liver-specific functions, such as the production of urea and albumin, and drug metabolism, were analyzed in the 3-D liver tissue culture models. Urea production was examined in cultured 3-D liver tissue culture models in a hepatic lineage differentiation medium containing 2 mmol/L NH_4^+. The level of urea production in the 3-D liver tissue culture model gradually increased and was significantly higher on days 6 and 12 than in the 2-D culture (Figure 3A). The amount of albumin released from the 3-D liver tissue culture model into the medium was measured in each medium on day 1, day 6, and day 12 by enzyme-linked immunosorbent assay. As seen in Figure 3B, the albumin level increased gradually from day 1 to day 12. These results suggest that the 3-D liver tissue culture model with PBL-HPCs had differentiated.

To quantify P450 activities, the hydroxylated pattern of testosterone by cultured 3-D liver tissue culture models in a hepatic lineage differentiation medium, containing 250 μmol/L testosterone, was examined using high-performance liquid chromatography. The concentration of each hydroxylated testosterone (6β-OHT, 7α-OHT, 16α-OHT, and 16β-OHT, respectively, corresponding to oxidation by Cyp3a, Cyp2a4/5 and 2d9, Cyp2d9 and 2b, and Cyp2c29 and 2e) was quantified. The concentrations of hydroxylated testosterones, such as 6β-OHT, 7α-OHT, 16α-OHT, and 16β-OHT, in the media of the 3-D liver tissue culture models after 12 days culture were measured. As compared with the hydroxylation levels of 6b-OHT, 7a-OHT, and 16a-OHT in the 2-D culture, the hydroxylation levels were significantly increased in the 3-D liver tissue models (Figure 3C).

The 3-D liver tissue was engrafted in the partially hepatectomized mouse

The 3-D liver tissue culture model of PBL-HPCs was syngeneically transplanted into the subcutaneous layer of a BALB/cA mouse which had received a 70% partial hepatectomy as shown in Figure 4A. The graft could be observed in the subcutaneous layer two weeks after transplantation. Microvascular networks were seen throughout this engrafted tissue. As shown in Figure 4B and C, the hematoxylin-eosin staining of this specimen in the graft area showed that collagen remained rich in the graft, fibroblasts existed in the collagen area, and vessel-like tube formation was observed. To investigate whether the cells in these areas were hepatocytes, an immunohistochemical examination using staining with anti-albumin antibodies was carried out (Figure 4D). This confirmed that the PBL-HPCs were accepted as albumin-positive cells after transplantation. These results indicate that the 3-D liver tissue culture model was successfully grafted with angiogenesis in the partially hepatectomized mouse.

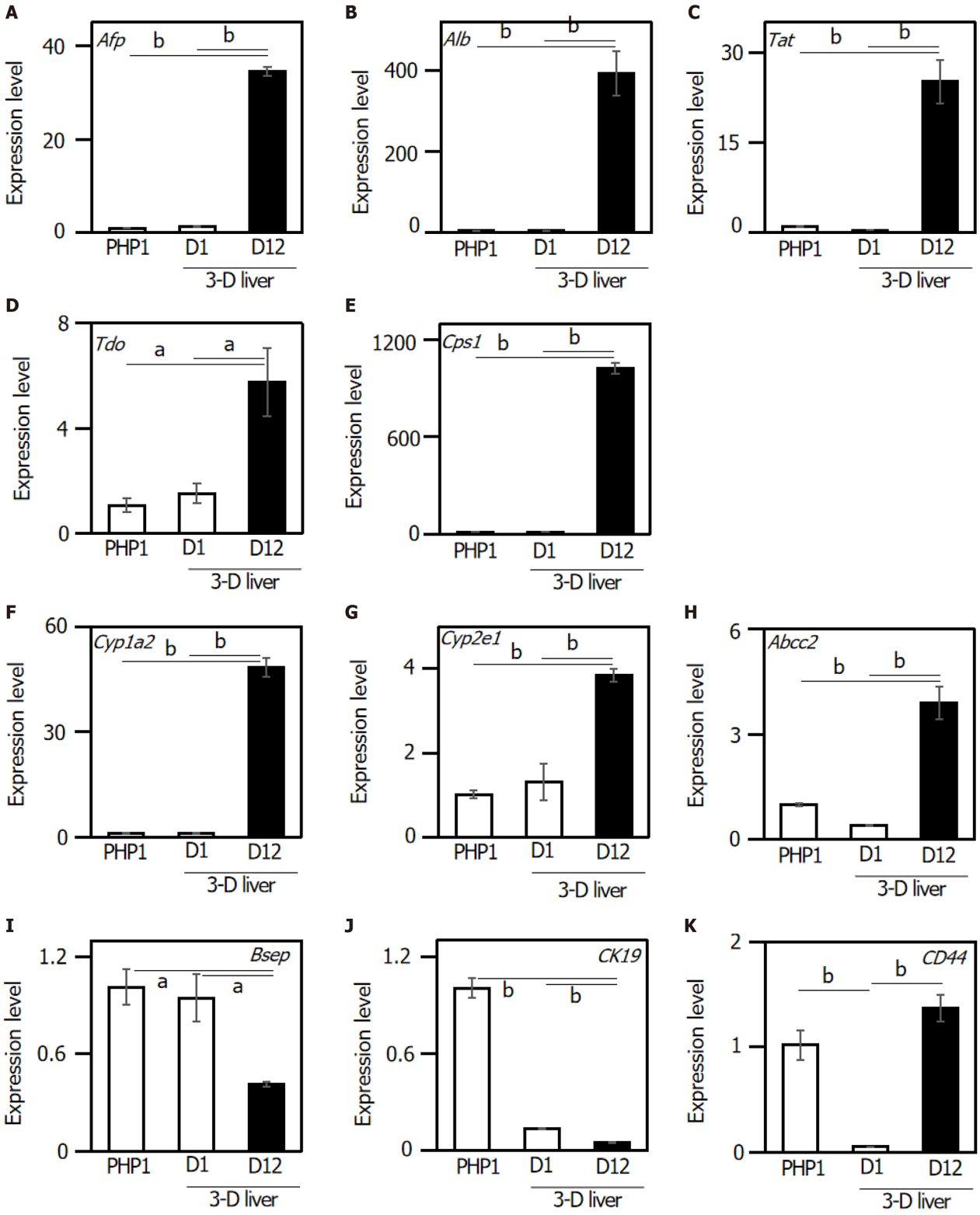


Figure 2 Relative gene expression levels in the three-dimensional liver tissue culture model cultured in a bioreactor on day 1 and day 12. Relative gene expression levels were analyzed in the three-dimensional liver tissue culture model. A: *Afp*; B: *Albumin*; C: *Tat*; D: *Tdo*; E: *Cps1*; F: *Cyp1a2*; G: *Cyp2e1*; H: *Abcc2*; I: *Bsep*; J: *CK19*; K: *CD44*. *Hprt* was used as an internal control. Statistical comparisons were made using the Tukey-Kramer method. Data are shown as means ± SE, $n = 3$, $^{a}P < 0.05$ and $^{b}P < 0.01$. Afp: Alpha-fetoprotein; Tat: Tyrosine aminotransferase; Tdo: Tryptophan 2,3-dioxygenase; Cps1: Carbamoyl-phosphate synthetase 1; Cyp1a2: Cytochrome P450, family 1, sub-family a2; Cyp2e1: Cytochrome P450, family 2, sub-family e1; Abcc2: ATP-binding cassette, sub-family C (CFTR/MRP), member 2; Bsep: Bile salt export pump; ABCB11: ATP-binding cassette, sub-family B member 11; CK19: Cytokeratin 19; CD44: Hyaluronic Acid Binding Protein.

DISCUSSION

It is crucial to develop a technology that enables transplantable engineered tissues to be functionally

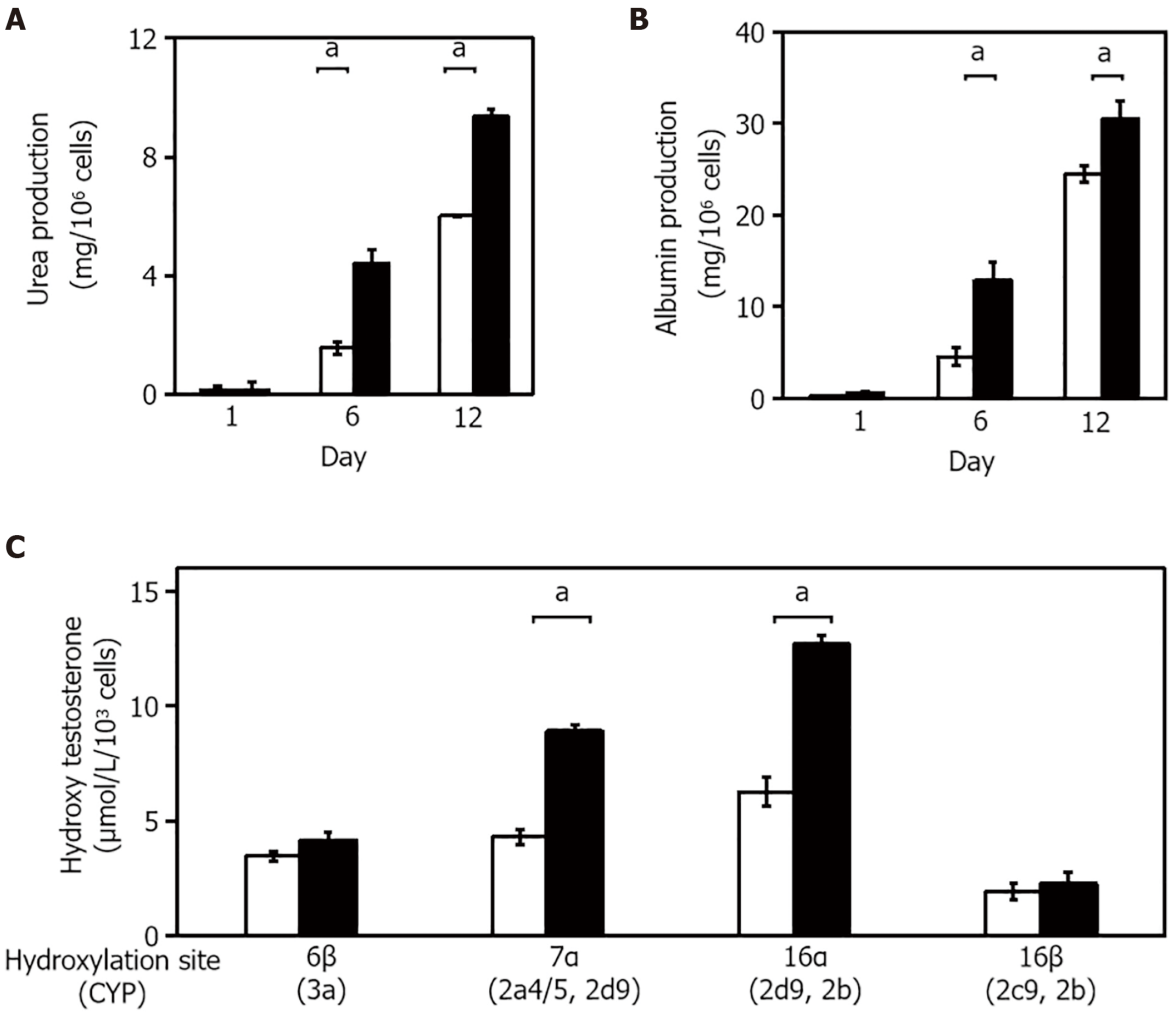


Figure 3 Validation of hepatic functions in the three-dimensional liver tissue culture model. A and B: Urea (A) and albumin (B) production levels in the culture medium in each culture condition; C: Hydroxylation activity of testosterone in the three-dimensional (3-D) liver tissue culture model. The amount of each hydroxylated testosterone in culture medium. Culture medium was changed every 2 d over the course of the measurement period. 2-D culture (open columns) and 3-D culture (closed columns) system. Statistical comparisons were made using the Welch *t*-test. Data are shown as means ± SE, $n = 3$, $^{a}P < 0.01$.

engrafted and long-lasting, in order to maximize the therapeutic effects of this procedure[19-21]. There are reports of transplanted hepatocytes at several different extrahepatic sites such as the small intestine [22,23]. Tissue engineering has been a promising procedure for providing transplantable tissues mimicking liver *ex vivo*[24,25]. The attachment of hepatocytes to extracellular matrix scaffolds can help in their engraftment in extrahepatic sites[26,27]. It is important to provide scaffold materials for hepatocytes to enable significantly greater hepatocyte survival in heterotypic transplantation[28]. The liver is encased mainly with collagen fibrils. By fabricating graded structures specific for target tissues and organs, one can obtain suitable scaffolds for tissue regeneration[29]. Taking into account the architecture of the liver, we have generated a 3-D liver tissue culture model of HPCs with a collagen fibril matrix using a bioreactor. Furthermore, defining and validating new sources is mandatory for ensuring functional hepatic cell supply[30]. Hepatic stem/progenitor cells have many advantages compared to adult hepatocytes as they are bipotent cells, so they can differentiate into hepatocytes and cholangiocytes[20]. Moreover, they have high proliferation ability.

In this study, HPCs — PBL-HPCs — were used in an original procedure to generate a 3-D liver tissue culture model. The histological structure of this model resembled that of the liver, with respect to its capillary network and surrounding cell clusters. As PBL-HPCs have the potential to reproduce themselves, it is easy to prepare the necessary numbers of cells (1×10^7 cells order). The PBL-HPC-derived 3-D liver tissue culture model reconstructed in a bioreactor produced cells that differentiated into hepatic-like cells, binuclear populations, and bile duct–like structures (Figure 1F). These cells expressed hepatocyte differentiation markers (Figure 2) after 12 days of culture. The PBL-HPCs differentiated not only into hepatic cells but also bile duct-like cells in a reconstituted collagen fibril matrix. In the 3-D liver tissue culture model derived from PBL-HPCs, the levels of urea and albumin production were gradually enhanced and were significantly higher than those cultured in dishes on days 6 and 12 (Figure 3A and B). Cyp3a, 2a4/5, 2d9, and 2b activities were significantly increased in the 3-D liver

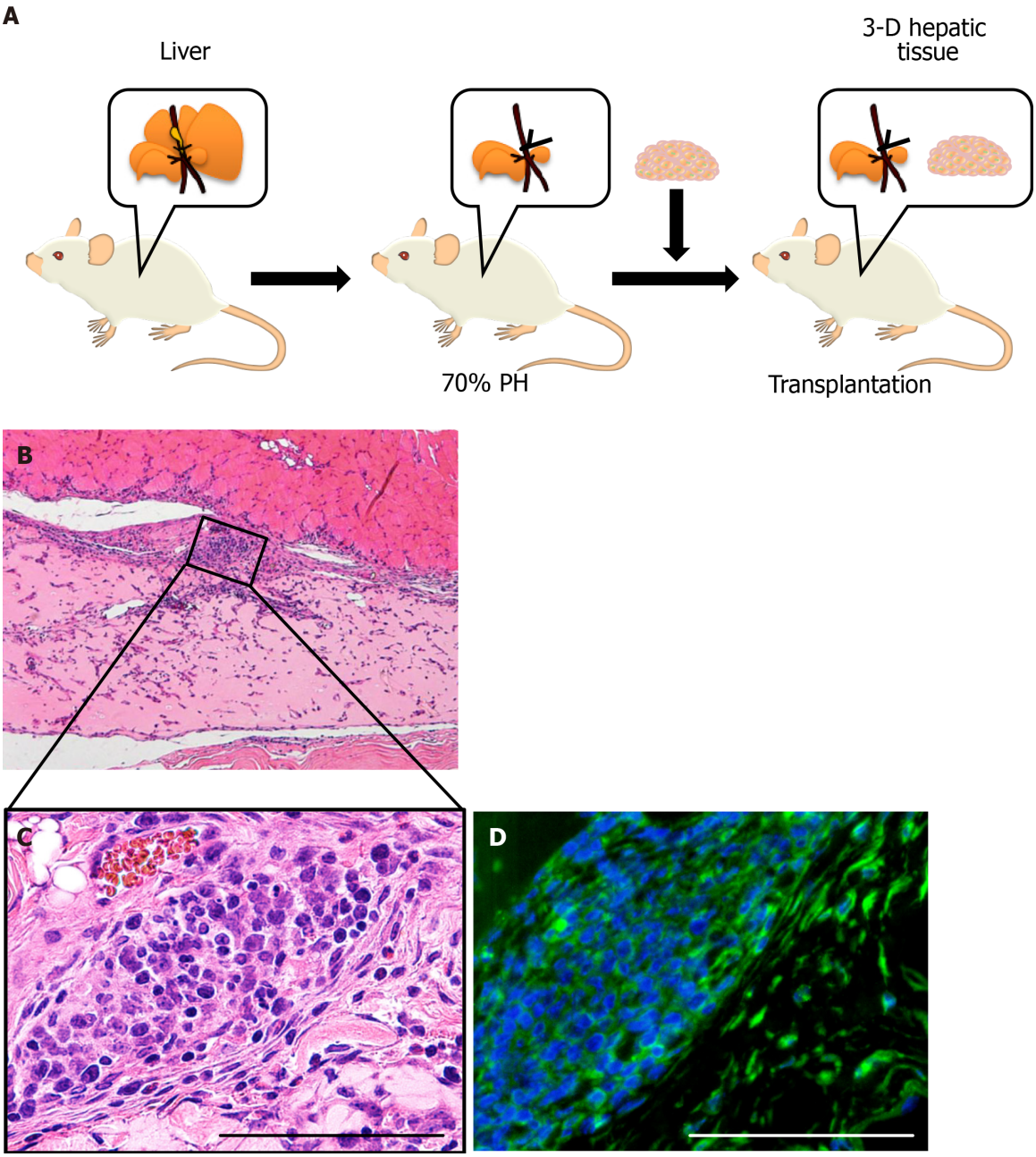


Figure 4 Transplanted three-dimensional liver tissue culture models in partially hepatectomized model mice. A: A schematic illustration showing the transplantation of the three-dimensional (3-D) liver tissue culture model; B: Histological analyses and hematoxylin-eosin staining of the section for the 3-D liver tissue culture model after transplantation; C: Higher magnification of the inscribed area in (B). The vascularization was observable at condensed collagen fibril matrices. Arrowheads indicate new blood vessels; D: Immunohistochemical analysis of the 3-D liver tissue culture model of albumin-positive hepatic cells after being transplanted using anti-albumin (green) antibodies. Bar corresponds to 100 μm.

tissue culture models (Figure 3C). These results suggest that the PBL-HPCs differentiated to mature hepatocytes in the 3-D liver tissue culture model.

Since PBL-HPCs differentiated into cells expressing hepatic functions in a 3-D liver tissue culture model, further investigation was carried out to determine whether PBL-HPCs also maintained their acquired functions after transplantation. Following implantation of the PBL-HPC-derived 3-D liver tissue culture model into mice, the 3-D liver tissue culture model was grafted, and vessel-like tube formation was observed (Figure 4B and C).

Also, the 3-D liver tissue culture model was grafted in the healthy mouse which did not undergo partial hepatectomy, and vascularization was observed (Supplementary Figure 1A and B). On the other hand, the Matrigel-embedded PBL-HPCs were transplanted into male healthy mice or nu/nu mice, and vascularization was not observed (Supplementary Figure 1C-F). PBL-HPCs differentiated into albumin positive cells (Figure 4D). This haptic tissue consisted of fibroblasts and HPCs which can be differentiated into only hepatocytes and bile duct cells, not immune cells. As some blood vessels were observed after implantation, lymphocytes and monocytes may have been circulating. However, we did not detect

Kupffer cells in the grafts. The 3-D liver tissue culture model was investigated for efficiency of transplantation in extrahepatic sites. These findings demonstrated that a reconstituted collagen fibril matrix can provide an extracellular microenvironment to promote the maturation of progenitor cells into hepatic cells. A local vascular network would allow nutrient and gas transport to the graft. These findings could make a significant contribution to the problem of liver graft shortage.

CONCLUSION

In conclusion, a 3-D liver tissue culture model was developed using HPCs. The advantage of our system is that it consists of proliferative HPCs. The 3-D liver tissue culture models can be generated in an originally designed bioreactor within 24 h. By mimicking the structure of the natural liver, our system was effective in constructing a functional liver tissue model.

ARTICLE HIGHLIGHTS

Research background

Liver transplantation is a therapeutic procedure to recover liver function in patients with irreversible liver failure; however, there is currently a shortage of available transplant organs, which limits the availability of this treatment.

Research motivation

Portal branch-ligated (PBL) HPCs are expected to allow regenerative medicine to produce a cell source to provide an alternate source for transplantation.

Research objectives

We aimed to development a liver model using HPCs.

Research methods

Hepatic stem/progenitor cells have the ability to multiply *ex vivo* and differentiate into hepatocytes and cholangiocytes. We have previously established HPC lines derived from the hepatic tissues of mice after ligation of venous drainage. In this study, the PBL hepatic lobe-derived HPCs multiplied in a bioreactor chamber to form liver organoid tissues comparable to liver lobules. These organoid tissues were implanted into syngeneic wild-type mice.

Research results

In the three-dimensional (3-D) liver tissue culture model, PBL-HPCs differentiated into mature hepatocyte-like cells, in binuclear populations, and with a bile duct–like structure. Quantitative real-time polymerase chain reaction analysis revealed that the cells expressed hepatocyte differentiation markers. In the 3-D liver tissue culture model derived from PBL-HPCs, the levels of urea and albumin production and activities of Cytochrome P450 enzymes were gradually enhanced.

Research conclusions

By mimicking the structure of the natural liver, our system was effective for the construction of a functional liver tissue model.

Research perspectives

This PBL-derived HPC line has the potential to proliferate, mature, and form implantable hepatic tissue.

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FOOTNOTES

Author contributions: Tagawa YI designed the study; Tamai M conducted all experiments; Kawase M performed statistical processing; Adachi E and Tagawa YI supervised the project; Tamai M and Tagawa YI wrote the manuscript; all authors read and approved the manuscript.

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Country/Territory of origin: Japan

ORCID number: Miho Tamai 0000-0001-8016-9663; Eijiro Adachi 0000-0002-0520-5211; Masaya Kawase 0000-0003-4003-4013; Yoh-ichi Tagawa 0000-0002-7673-6781.

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ORIGINAL ARTICLE

Retrospective Cohort Study

Clinical classification of symptomatic heterotopic pancreas of the stomach and duodenum: A case series and systematic literature review

Michael T LeCompte, Brandon Mason, Keenan J Robbins, Motoyo Yano, Deyali Chatterjee, Ryan C Fields, Steven M Strasberg, William G Hawkins

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Michael T LeCompte, Department of Surgical Oncology, University of North Carolina, Raleigh, NC 27608, United States

Brandon Mason, Department of Radiology, Stillwater Medical Center, Stillwater, OK 74074, United States

Keenan J Robbins, Department of General Surgery, Washington University St. Louis, St. Louis, MO 63110-8109, United States

Motoyo Yano, Department of Radiology, Mayo Clinic, Phoenix, AZ 8505, United States

Deyali Chatterjee, Department of Pathology and Immunology, MD Anderson Cancer Center, Houston, TX 77030, United States

Ryan C Fields, Department of Surgical Oncology, Washington University School of Medicine, St. Louis, MO 63110-8109, United States

Steven M Strasberg, William G Hawkins, Section of Hepatobiliary-Pancreatic and GI Surgery, Washington University St. Louis, St. Louis, MO 63110, United States

Corresponding author: Michael T LeCompte, MD, Assistant Professor, Department of Surgical Oncology, University of North Carolina, 2800 Blue Ridge Rd. Suite 300, Raleigh, NC 27608, United States. mtleco01@gmail.com

Abstract

BACKGROUND

Heterotopic pancreas (HP) is an aberrant anatomic malformation that occurs most commonly in the upper gastrointestinal tract. While the majority of heterotopic pancreatic lesions are asymptomatic, many manifest severe clinical symptoms which require surgical or endoscopic intervention. Understanding of the clinical manifestations and symptoms of HP is limited due to the lack of large volume studies in the literature. The purpose of this study is to review symptomatic cases at a single center and compare these to a systematic review of the literature in order to characterize common clinical manifestations and treatment of this disease.

AIM
To classify the common clinical manifestations of heterotopic pancreas.

METHODS
A retrospective review was conducted of pathologic samples containing heterotopic pancreas from 2000-2018. Review was limited to HP of the upper gastrointestinal tract due to the frequency of presentation in this location. Symptomatic patients were identified from review of the medical records and clinical symptoms were tabulated. These were compared to a systematic review of the literature utilizing PubMed and Embase searches for papers pertaining to heterotopic pancreas. Publications describing symptomatic presentation of HP were selected for review. Information including demographics, symptoms, presentation and treatment were compiled and analyzed.

RESULTS
Twenty-nine patient were identified with HP at a single center, with six of these identified has having clinical symptoms. Clinical manifestations included, gastrointestinal bleeding, gastric ulceration with/without perforation, pancreatitis, and gastric outlet obstruction. Systemic review of the literature yielded 232 publications detailing symptomatic cases with only 20 studies describing ten or more patients. Single and multi-patient studies were combined to form a cohort of 934 symptomatic patients. The majority of patients presented with abdominal pain (67%) combined with one of the following clinical categories: (1) Dyspepsia, (n = 445, 48%); (2) Pancreatitis (n = 260, 28%); (3) Gastrointestinal bleeding (n = 80, 9%); and (4) Gastric outlet obstruction (n = 80, 9%). The majority of cases (n = 832, 90%) underwent surgical or endoscopic resection with 85% reporting resolution or improvement in their symptoms.

CONCLUSION
Heterotopic pancreas can cause significant clinical symptoms in the upper gastrointestinal tract. Better understanding and classification of this disease may result in more accurate identification and treatment of this malformation.

Key Words: Heterotopic pancreas; Ectopic pancreas; Aberrant pancreas; Pancreatic rest; Groove pancreatitis

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Core Tip: This is a retrospective study that compares a single institution's experience with that of a systematic literature review to evaluate the common clinical symptoms and presentation of heterotopic pancreas. To date, there have been numerous small volume studies in the literature reporting heterotopic pancreas. However, these studies predominantly report asymptomatic or incidental cases and there are few studies characterizing the symptomatic presentation of this disease. The goal of this study is to classify symptomatic cases of heterotopic pancreas to better identify the presentation and management of this disease.

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INTRODUCTION

Heterotopic pancreas (HP) is an uncommon embryologic abnormality, defined as aberrant pancreatic tissue separated anatomically from the main body and blood supply of the pancreas. This tissue can be located throughout the gastrointestinal tract with a reported frequency of 1/500 in surgical specimens [1]. Most of these lesions are asymptomatic and are identified incidentally on pathologic specimens or at autopsy. However, some of these anatomic anomalies can manifest clinical symptoms. When this occurs, the clinical presentation can often be severe, requiring surgical intervention[2].

Knowledge of the clinical features of this disease process and the appropriate treatment is limited. While reports of clinically relevant cases are present in the literature dating back to 1727, the majority of the available literature consists of case reports, with few studies containing more than a handful of patients[3]. Thus, little is known about the "typical" clinical manifestations of HP due to small volume

studies. Classification of this aberrancy is further limited by the diversity of possible symptoms which are often dictated by the anatomic location, size and functionality of ectopic lesion. While HP is most commonly located in the foregut, it has been identified in tissues including the small bowel, colon, gallbladder, spleen, esophagus and mediastinum[4]. This leads to further complexity in appropriate diagnosis and treatment of HP as it is often mimics other disease processes.

To address the deficit in description and classification of symptomatic HP we evaluated our center's experience with symptomatic HP cases and compared it to a systematic review of the available literature. The scope of this study focuses on describing and characterizing the common clinical manifestations for HP within the stomach and duodenum as these locations represent the majority of cases. Cases identified in this study were compared with the results of a review of the available reported cases to identify the common symptoms, presentation, and treatment options for this disease process.

MATERIALS AND METHODS

A review of pathology specimens from 2000-2018 was conducted at a single academic institution. Approval was obtained from the institutional review board (#210811062). Specimens with pathologic confirmation of heterotopic pancreatic tissue were identified. Lesions found in the upper gastrointestinal tract were selected in adult patients over 18 for final review. Electronic medical records were analyzed and factors associated with the clinical presentation, symptoms, diagnosis and treatment were identified in each of the cases. Based upon the clinical history and presentation, patients were classified as symptomatic or asymptomatic. Symptomatic patients were defined as those who presented with upper gastrointestinal complaints which could be attributed to the identified pathology and ultimately resolved with treatment or removal of the ectopic tissue. Asymptomatic patients were classified as patients in whom the presence of heterotopic pancreas was identified in the workup or treatment of symptoms related to other clinically established pathology, or were found incidentally in a surgical pathologic specimen. Symptomatic patients were analyzed and categorized based upon their clinical features, presentation and treatment.

The cohort of patients with symptomatic manifestations from our institution was then compared that of published cases in the literature. A literature review of heterotopic pancreas using Embase and PubMED databases was conducted. The terms "heterotopic pancreas", "ectopic pancreas", and "aberrant pancreas" were searched. Publications printed in the English language were selected. Inclusion criteria were limited to publications reporting symptomatic cases of pathologically confirmed heterotopic pancreas located in stomach or duodenum. Publications which reported symptomatic and asymptomatic cases were excluded if they lacked characterization and description of clinical symptoms. Patients from the included publications were combined into a single cohort and categorized based on the presenting clinical symptoms, available demographic information and treatment. This sample was then compared with a series of patients identified at our institution for similarities. Descriptive statistics were employed to tabulate and report the results.

RESULTS

Institutional results

Twenty-nine pathology specimens were identified with pancreatic heterotopia in the upper gastrointestinal tract during the study period of January 2000 to December 2018. Of these, fourteen patients were female (48%). Six patients (20%) were identified as having symptoms related to an HP lesion. The remaining patients were identified as asymptomatic with incidentally noted lesions on pathology or discovered on endoscopy (Table 1). Five of the symptomatic patients underwent surgical treatment of their disease process and one declined surgery in favor of medical management.

Of the symptomatic patients, two (7%) presented with elevated lipase and evidence of pancreatitis within the heterotopic lesion, two patients presented with gastrointestinal bleeding or anemia, one patient (3.4%) presented with perforated gastric ulceration related to a heterotopic lesion, and one patient presented with gastric outlet obstruction from a HP lesion located at the pylorus. The six case presentations are briefly summarized below.

Case 1: 28-year-old male who presented as an outpatient with a history of mid-epigastric abdominal pain, nausea and daily vomiting for approximately 3 mo. He reported associated anorexia and a weight loss of approximately 40 Lbs. On exam he was noted to be cachectic with a BMI of 17 with moderate abdominal tenderness to palpation in the upper abdomen. Workup demonstrated an elevated lipase of 123 and an amylase of 141. Computed tomography revealed a 3.4cm hyper-enhancing mass near the pylorus on CT imaging. This was associated with a complex fluid collection within the wall of the stomach and evidence of inflammatory stranding, gastric thickening and edema. Endoscopy revealed a mass within the wall of the gastric antrum. Endoscopic ultrasound and biopsy demonstrated pancreatic acini. The patient was subsequently taken to the operating room for antrectomy and billroth II

Table 1 Pathologic specimens identified with heterotopic pancreas; specimens are divided into symptomatic classification

Clinical features	*n*	Percent
Total patients	29	100%
Symptomatic HP	6	20.0%
Pancreatitis	2	7.0%
Bleeding/anemia	2	7.0%
Peptic ulceration	1	3.4%
Gastric outlet obstruction	1	3.4%
Asymptomatic HP	23	80.0%
Incidental finding on pathology	5	17.2%
Noted on Endoscopy	17	58.6%
Identified at time of surgery	1	3.4%

HP: Heterotopic pancreas.

reconstruction. His pathology demonstrated heterotopic pancreatic tissue, 3.5 cm in size within the gastric antrum (Figure 1). In addition, there was an associated 4.3 cm pseudocyst within the gastric wall with surrounding inflammation. The patient was seen in follow up clinic one month later and was noted to be eating well with an increase in his weight of 14 Lbs since discharge.

Case 2: 37-year-old male who presented with a chief complaint of melena, epigastric abdominal pain, anorexia and weight loss of approximately 20 Lbs. An esophagogastroduodenoscopy was performed which demonstrated a 2 cm submucosal mass with overlying ulcerated mucosa. An endoscopic ultrasound and FNA were obtained. Results demonstrated atypical cells. Radiographic imaging was obtained, however computed tomography scan was unable to identify or localize the mass. Surgical resection was offered, and the patient underwent a laparoscopic assisted gastric wedge resection. Pathology from the specimen was identified as a 2.5 cm × 2.0 cm mass consistent with multilobulated pancreatic tissue located between the gastric mucosa and muscularis propria with evidence of inflammation and ulcerated mucosa. The patient recovered without complication and with resolution of his anemia.

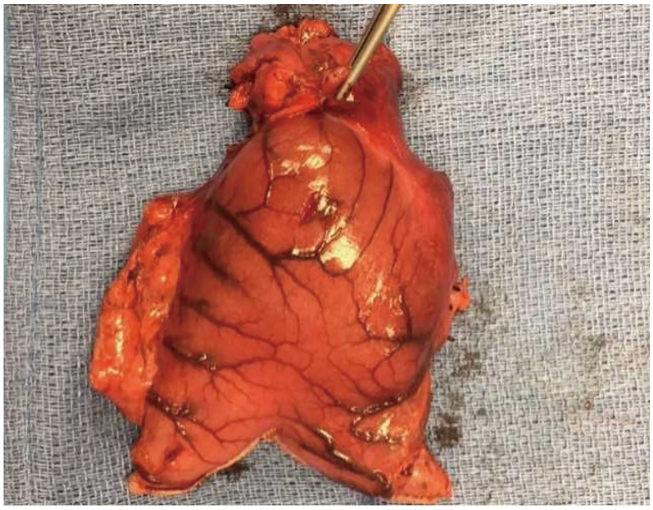
Case 3: 42-year-old male presented with acute abdominal pain and the presence of free air on upright abdominal film. He was noted to be tachycardic and with evidence of peritonitis on exam. A computed tomography scan confirmed the presence of free air and intraperitoneal fluid in the abdomen. His past medical history was notable only for a history of gastroesophageal reflux and indigestion that was refractory to years of proton pump inhibitor therapy. He was taken to the operating room for exploration where a large ulcer was identified upon opening the lesser sac in the posterior wall of the stomach approximately 6cm from the pylorus. A distal gastrectomy and reconstruction with a billroth II gastrojejunostomy was performed.

Pathology from the resected specimen demonstrated a transmural gastric perforation with evidence of associated pancreatic tissue within the gastric wall and along the edge of the ulcerated mucosa. The patient tolerated the procedure well and remained symptom free.

Case 4: 43-year-old male presented to the emergency department with chronic symptoms of non-bloody, non-billious vomiting and intolerance of oral intake. A detailed history revealed that the patient had suffered from intermittent vomiting and progressive oral intolerance over the prior 6 mo. A computed tomography scan was obtained which demonstrated a grossly distended stomach and a hyperenhancing mass as the level of the pylorus and first portion of the duodenum with an associated gastric outlet obstruction.

He was taken to surgery where an abnormal mass was palpated near the level of the pylorus. A distal gastrectomy was performed with a billroth II reconstruction. Final pathology demonstrated heterotopic pancreas at the level of the pylorus.

Case 5: 52-year-old female presented with anemia underwent upper endoscopy, which showed a submucosal mass along the lesser curve of the stomach near the incisura. EUS was performed, where a submucosal mas was identified with ulceration concerning for a gastrointestinal stromal tumor. Fine needle aspiration was non-diagnostic. She proceeded to surgery where a laparoscopic wedge resection was performed. Her pathology showed a 1.5 cm × 1.1 cm × 1.0 cm mass of pancreatic tissue. On post-operative follow up she was noted to have resolution of her anemia.



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Figure 1 Image demonstrating the gastric antrum with an intramural mass identified within the wall of the stomach.

Case 6: 29-year-old male presented with abdominal pain and anorexia over the course of on year with a weight loss of 75 Lbs. Imaging with computed tomography showed 2.6 cm × 1.6 cm mass along the lesser curve of stomach and the patient was noted to have mild elevation in his amylase and lipase. Upper endoscopy with endoscopic ultrasound demonstrated a 2.3 cm subepithelial lesion along the lesser curve with surrounding gastritis. Biopsy demonstrated chronic gastritis and benign pancreatic tissue. The patient was placed on proton pump inhibitors and carafate with improvement in his symptoms. He was referred for surgical evaluation but declined intervention as his symptoms had improved with medical therapy.

Results of literature analysis

A systematic review of the literature was conducted to identify published cases of HP lesions. The initial search resulted in 1220 unique publications. Of these, 232 publications described cases of symptomatic lesions and met the inclusion criteria. The majority of these publications (n = 182) were single patient case studies. There were only 20 studies identified which contained more than 10 symptomatic patients in their series. The majority of the larger studies contained cases of both symptomatic and asymptomatic disease with few publications dedicated to describing symptomatic cases. In total, these studies included 1762 patients of which 934 patients were reported to have symptomatic lesions in the stomach or duodenum and comprised the total cohort of this evaluation. Demographic information was provided for 864 patients (94%) of which the mean age at symptom presentation was 43 years old and 564 were male (61%). In total, there were 556 patients with gastric lesions (59%) and 378 with duodenal lesions (41%).

A summary of the clinical cases of HP was performed by individual publication and were tabulated for review in Table 2. The majority of patients presented with epigastric abdominal pain (n = 620, 67%). In addition to pain, the remaining symptoms were noted to fall within one of 4 predominant diagnostic categories: (1) Dyspepsia, which included symptoms of epigastric pain, eructation, nausea or bloating with meals (n = 445, 48%); (2) Pancreatitis within the ectopic lesion (n = 260, 28%); (3) Gastrointestinal bleeding (n = 80, 9%); and (4) Gastric outlet obstruction (n = 80, 9%). There were also 37 patients who presented with jaundice and biliary obstruction due to periampullary lesions (4%), and 3 patients with perforated ulcers. The remaining patients presented with various symptoms which included fever, diarrhea, abscess, carcinoid syndrome and dysphagia but were in single cases or small numbers. The majority of patients who presented with pancreatitis were found to have radiographic evidence of pseudocyst formation or cystic degeneration of an ectopic lesion (n = 251, 97%). These lesions were commonly located within the wall of the stomach or duodenum or were characterized as "groove pancreatitis" with ectopic pancreatic tissue located between the medial duodenal wall and the head of the pancreas. Of the patients with clinical and radiographic evidence of pancreatitis, only 50 cases (19%) reported elevations in biochemical markers such as serum amylase or lipase to correlate with the clinical or radiographic evidence of pancreatitis. One hundred forty-five cases (56%) were reported to be associated with alcohol consumption as the inciting etiology.

The majority of cases (n = 832, 90%) underwent surgical or endoscopic management of their heterotopic pancreatic lesion, while 92 were observed or managed medically. Surgical therapy ranged from radical gastrectomy or pancreaticoduodenectomy to local excision or endoscopic resection. Available data on the therapies provided are listed in Table 3. Data on the effect of treatment on symptom control was available for 645 patients. Of the cases which reported post-intervention follow

Table 2 Summary of systematic literature review listed by publication, number of symptomatic cases, demographics and clinical symptoms

Ref.	Year	Total patients	Symptomatic patients	Mean age	Gastric lesions	Duodenal lesions	Location other than foregut	Abdominal Pain	Dyspepsia	Gastrointestinal Bleeding	Pancreatitis	Gastric outlet obstruction	Perforation	Nausea	Jaundice
Noh *et al*[5]	2019	5	5	32	5			5			5				
Zhou *et al*[6]	2019	93	78	39.75	78			38	41					4	
Betzler *et al*[7]	2017	67	11	53		11		8			7				
Jun *et al*[8]	2017	165	79	45.2	46	18	15	64	64					64	
Zhang *et al*[9]	2016	184	26	49	25	1		26	26						1
Liao *et al*[10]	2014	2	2	36	2				2						
Chou *et al*[11]	2014	13	13	40.8	13			6	7						
Endo *et al*[12]	2014	2	2	74	1	1		1		1					
Liu *et al*[13]	2013	9	9	48.3	9			6	9			1			
Zhong *et al*[14]	2013	60	30	39	30			16	24	6					
Shah *et al*[15]	2011	30	4	53	1	1	2	1			1	1			
Park *et al*[16]	2011	26	9	34	9			4	9						
Wei *et al*[17]	2011	11	11	52	3	8		6	8	3				2	
Ryu *et al*[18]	2010	8	4	36	4				4						
Yuan *et al*[19]	2009	2	2	61		1	1	1	1						
Khashab *et al*[20]	2009	2	2	32	2				1	1					
Casetti *et al*[21]	2009	58	58	44.7		58		46	1		58				
Jovanovic *et al*[22]	2008	13	13	41.5		13		10			6	1		7	4
Chen, H *et al*[23]	2008	39	15	46	12	1	2	9	9	5					
Chen, S *et al*[24]	2008	20	13	39	12	1		9	4						
Rebours *et al*[25]	2007	105	105	46		105		91		5	105			30	13
Tison *et al*[26]	2007	9	9	49.6		9		8			9	9			1

Ormarsson *et al* [4]	2006	30	11	49.2	11			5	5					1	
Pessaux *et al* [27]	2006	12	12	42.4		12		11		1	12			8	
Ayantunde *et al*[28]	2006	3	3	55	3			1	1			2		2	
Chatelain *et al* [29]	2005	2	2	40.5		2		2			2	2			
Zinkiewicz *et al* [30]	2003	12	7	34	6	1		7	7						
Shi *et al*[31]	2002	7	7	41	1	4	2		2	1		1			3
Huang *et al*[32]	2002	2	2	46	2							2			
Otani *et al*[33]	2000	34	2	43	2			1	1						
Hsia *et al*[34]	1999	17	10	47.5	8	2			8	2					
Fekete *et al*[35]	1996	6	6	40	1	5		6		1	6				
Flejou *et al*[36]	1993	10	10	41	1	9		7		1	10				2
Claudon *et al* [37]	1988	2	2	41	2			2	2						
Pang *et al*[38]	1988	32	14	44	3	4	7	6	5	2				1	1
Lai *et al*[39]	1986	37	9	50	6	3		7	7	1		1			
Mollitt *et al*[40]	1984	9	6	6.8	6			3	3			3			
Armstrong *et al* [41]	1981	34	13	51	5	5	3		11	2					
Thoeni *et al*[42]	1980	9	6	43	6			1	1	1	1	1			
Yamagiwa *et al* [43]	1977	64	26	45.2	23	3		17	15	4				8	
Dolan *et al*[44]	1974	212	73	60	43	25	5	52	46	7				1	
Eklof *et al*[45]	1973	4	4	7	3	1		3	2					1	
Nebel *et al*[46]	1973	3	3	28	1	2		3	3						
McGarity *et al* [47]	1971	2	2	33	2				2			1		2	
Abrahams *et al* [48]	1966	9	9	49.8	5	4			9	6					

Tonkin *et al*[49]	1962	4	4	44	2	2		3	4			1			
Dirks *et al*[50]	1961	3	3	44.6	0	3		3	2	1					
Martinez *et al*[51]	1958	51	28	43.1	21		7	16	19	2		3			
Waugh *et al*[52]	1946	5	5	34.8	5			3	3	2					
De Castro Barboso *et al*[1]	1947	41	17	43	10	7		10	11	4					
Single case studies (n = 182)[3,53-233]		182	182	39.1	126	56		96	66	21	38	51	3	57	12
Total		1761	978	43	556	378	44	620	445	80	260	80	3	188	37

up, five hundred forty-nine patients demonstrated symptom resolution with treatment (85%), 63 were reported to have symptom improvement (9.7%) and 33 reported no improvement (5.1%). Based on the level of detail reported in the literature, there was no delineation of whether patients who did not improve were treated medically or *via* surgical or endoscopic intervention. Likewise, there was no study providing a direct comparison of treatment outcomes between medical versus surgical therapies nor was there a study comparing outcomes between different interventional therapies.

DISCUSSION

Heterotopic pancreas is a congenital anomaly, often identified incidentally in asymptomatic patients, yet it has the ability to illicit significant clinical symptoms in some cases. These lesions can arise in tissues throughout the body including stomach, small bowel, gallbladder, esophagus and mediastinum, leading to a wide range of manifestations[234]. Characterization of symptomatic lesions is difficult due to the relative infrequency of this diagnosis and the variability in presentation. This often leads to misidentification and suboptimal management in many cases. Large volume studies characterizing the common presentation of HP are lacking and the aim of this study was to provide a conglomerate population to classify the typical clinical presentation within the foregut and aid in classification. With improved understanding and recognition of this disease process, clinicians can render more appropriate treatment decisions that may save patients morbidity from radical resection.

The clinical manifestations of HP are dictated in large part by two factors, the location and the functionality of the lesion. The anatomic location is often predetermined early in life by embryologic development. The exact mechanism by which this abnormality forms is not well understood however, it is believed to be related to a disruption in the normal embryologic migration of the pancreas. Formation of dorsal and ventral pancreatic buds results from endodermal outpouchings of the duodenum[235]. Aberrant separation of these tissues from the visceral endoderm may be responsible for retained pancreatic tissue within the developing gastrointestinal tract and subsequent migration as the embryo develops[235]. However, this theory does not fully account for the development of HP in all cases as this

Table 3 Treatment of heterotopic pancreas; listing of surgical or endoscopic procedures performed for patients with symptomatic heterotopic pancreas by procedure

Procedure	*n*	Percent (%)
Distal gastrectomy	84	10.50
Billroth I reconstruction	14	1.70
Billroth II reconstruction	64	8.00
Roux-en-Y reconstruction	6	0.75
Gastric wedge resection	81	10.00
Subtotal gastrectomy	18	2.25
Total gastrectomy	5	0.63
Gastrotomy and local excision	42	5.30
Partial gastrectomy NOS	215	27.00
Pancreaticoduodenectomy	168	21.00
Trans-duodenal excision	19	2.40
Partial duodenectomy	7	0.90
Ampullectomy	2	0.25
Endoscopic submucosal excision	158	19.77
Total procedures	799	100.00

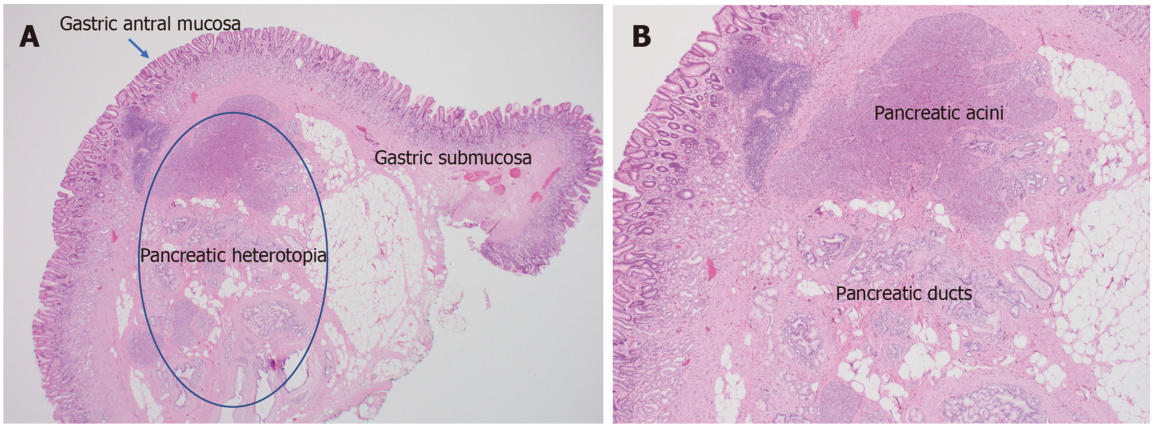
anomaly has also been reported to be found in tissues outside the gastrointestinal tract including the gallbladder, fallopian tube, umbilicus, mediastinum, omentum, mesentery and spleen[9,39]. An alternative explanation in these cases is the "metaplasia" theory in which totipotent endodermal tissues develop into pancreatic tissue during embryologic development and can present in various tissues throughout the body[235].

However, the majority of these lesions are located within the upper gastrointestinal tract with surgical and autopsy data reporting a frequency within the stomach of 25-52%, 27-36% in the duodenum and 15%-17% in the jejunum[9,44,235]. HP symptoms often mimic common upper gastrointestinal pathology and are difficult to identify. The location within the foregut often dictates symptom manifestation with peripyloric lesions more likely to lead to gastric outlet obstruction while periampullary lesions may cause biliary obstruction[17,80,164,236,237].

In addition to the anatomic location, the histologic location within the layers of the visceral wall may also have an effect on symptom presentation. HP occurs most frequently between the submucosa and lamina propria but can be found within all layers of the visceral wall[238]. Submucosal lesions may be more likely to cause ulceration with local gastritis or duodenitis while transmural lesions which involve all layers of the bowel wall can lead to chronic inflammation, and ultimately stricture or perforation[77].

While location may often explain symptom presentation it does not fully account for why some lesions are symptomatic while others found in similar locations are not. The remaining factor can be potentially explained by the functionality of the lesion, and the ability to perform the normal exocrine and endocrine functions of the pancreas. Histopathologic evaluation by Heinrich in 1909 demonstrated differences in the composition of these lesions which were later revised by Fuentes in 1973[239,240]. These classification systems categorized HP lesions based on the presence of all the cellular components of functional pancreas tissue (Type I in both categories) versus presence of ducts, acinar tissue or islet cells alone (Fuentes classification types II, III and IV respectively)[240] (Figure 2). The presence or absence of histologic elements may affect the functionality of the lesion and contribute to its ability (or inability) to produce symptoms. It has been proposed that lesions with functional exocrine potential can produce local chemical irritation of surrounding tissues while those without appropriate ductal drainage may lead to pancreatitis and pseudocyst formation within the lesion[4,60].

The clinical nature of HP may therefore have variable patterns of presentation which are highly dependent on both the location and the functional ability of the lesion. Since the majority of identified cases are located within the upper gastrointestinal tract, the scope of this study was restricted to identifying and characterizing symptoms within these locations. The combined experience from our center and the analysis of the published literature demonstrated that the majority of clinically significant cases can be categorized within 4 main groups. These categories are significant as they are common presentations of other pathology within the upper GI tract and can lead to difficulty in diagnosing and treating this pathology.



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Figure 2 Histopathologic images. A: Histologic appearance of heterotopic pancreas in the stomach; B: High power view of heterotopic pancreas demonstrating pancreatic acinar and ductal architecture.

Dyspepsia

The majority of cases identified in this series reported symptoms consistent with epigastric abdominal pain, bloating, belching and nausea which are typically associated with eating. This category consisted predominantly of patients with gastric lesions. The manifestation of symptoms has been thought to be related to local chemical irritation of the gastric mucosa by products of the exocrine pancreas[87]. This acts as an inciting factor that then may lead to inflammatory mucositis and subsequent gastritis or duodenitis, with or without ulceration. As many of these patients present with identical symptoms to those with peptic ulcer disease (PUD) (and indeed, are often misdiagnosed as such), many authors argue that ulceration is the inciting factor and etiology of the majority of symptoms associated with HP [2,44]. In the series of 34 patients reported by Armstrong *et al*[41] the extent of mucosal involvement on pathology was correlated with symptoms suggesting a causal relationship. In the series published by Lai *et al*[39] comparing the histology of symptomatic and asymptomatic patients, 49% of symptomatic patients had mucosal ulcers while asymptomatic patients in their series lacked objective evidence of ulceration or gastritis.

Mucosal ulceration in some cases may progress to visceral perforation. This is thought to be the result of normal evolution of inflammation seen in peptic ulcer disease, with ulceration of the protective mucosal barrier, caused by heterotopic pancreas, acting as the initial catalyst. Perforation may also be related the degree of penetration within the gastric wall of the ectopic lesion. Extension of the HP lesions through the serosa can be seen in up to 4% of histologically examined specimens[84]. Lesions which penetrate deeper through the layers of the gastric wall can lead to transmural inflammation, necrosis and perforation of the stomach wall. This theory is supported by the histologic findings reported by Martinez *et al*[51] demonstrating inflammation and fibrosis extending through multiple layers of the gastric wall on tissue histology depending on the histologic depth of the lesion. Overall, this is a rare complication of HP, with only three identified publications describing visceral perforation related to HP[77,121,241]. We report the fourth known case above in Case 3 where the transmural gastric ulceration was clearly associated with HP. It is possible that additional cases exist but remain undocumented as they may have been treated with omental patching without resection or biopsy. Still, this remains an uncommon symptomatic presentation of HP compared to local mucosal ulceration.

While there are clear associations for mucosal ulceration with the symptomatic presentations of HP it does not account for all cases. Multiple series including that of Dolan *et al*[44] report on patients with symptoms of dyspepsia who did not exhibit evidence of ulceration or gastritis. This is also supported by reports such as that by Ormarsson *et al*[4] and Barbosa *et al*[242] which demonstrate resolution of symptoms in patients undergoing resection of HP with and without the presence of ulceration. In these patients, the etiology of abdominal pain and/or dyspepsia is not fully clear. It has been proposed that exocrine secretions into the stomach may affect the pH composition of the gastric lumen leading to alterations in the digestive process, gas formation and bloating, although confirmation of this *via* testing of gastric pH in the setting of HP has not been conducted to our knowledge[47]. In addition, it is hard to conceive that these lesions, unless the reach a large size, are able to secrete enough volume to significantly alter the pH composition of gastric juices.

The majority of patients with HP and dyspeptic symptoms are initially misdiagnosed and managed with medical therapy designed for reduction of acid secretion. It is unclear whether traditional treatments for gastritis and PUD is beneficial in these patients. If the inciting physiology is chemical irritation from pancreatic secretions, then traditionally prescribed therapies to reduce acid secretion would be unlikely to help alleviate symptoms. In patients who develop mucosal ulceration there may be some plausible benefit that acid reduction may prevent ulcer progression once breakdown of the

mucosal barrier has already occurred. However, it is not clear whether this can promote adequate ulcer resolution in the setting of HP or prevent future recurrent ulceration since acid secretion is not believed to be the causative factor for inflammation or ulceration in this setting[41]. There were few symptomatic patients in the reviewed studies who were managed with observation or medical therapy and long term follow up was lacking. The single patient treated with medical therapy at our institution (Case 6), demonstrated symptomatic improvement and to date, has not returned with recurrent symptoms over six years of observation. However, an adequate evaluation of medical management compared to surgical or endoscopic resection is lacking and further study is needed to evaluate the optimal treatment modality.

Heterotopic pancreatitis

Manifestations of acute and chronic pancreatitis in HP tissues were the second most common presentation in our review. Clinical symptoms may include abdominal pain, nausea, vomiting, anorexia and weight loss[85,163]. Similar to orthotopic pancreatitis, the most common causes can be related to alcohol, medications, smoking, autoimmune disease or inadequate ductal drainage[243]. According to the histologic classification system proposed by Heinrich and Fuentes, heterotopic pancreas types I-III contain functional pancreatic acini and ductal components. Impaired drainage and outflow obstruction of these ducts may lead to pancreatitis and pseudocyst formation[241]. Cystic formation within the visceral wall of the stomach or duodenum was demonstrated radiographically in the majority of patients with pancreatitis in our series. This was often associated with radiographic signs of stranding, edema and inflammation. This pathophysiology is commonly seen in "groove pancreatitis" where ectopic pancreatic tissue between the duodenal wall and pancreas lack appropriate enteric drainage leading to inflamation and cystic degeneration[244,245].

Diagnosis of heterotopic pancreatitis is made through a combination of clinical presentation, radiographic imaging and biochemical evaluation[243,245]. Serum labs may yield mild elevations of amylase and lipase however, these levels are usually only modestly elevated, even in the setting of severe inflammation due to the relatively small volume of pancreatic tissue within the heterotopic lesion [235]. Leakage of enzyme-rich pancreatic fluid can cause pancreatic pseudocyst formation within the gastric wall producing symptoms of early satiety and gastric outlet obstruction[60]. This finding results in the appearance of a cystic structure within the wall of the stomach or bowel and is often mistaken for a duplication cyst or mucinous neoplasm[208].

Conservative treatment may be successful in mild cases of this disease process. Investigation of the inciting etiology should be performed with cessation of alcohol or smoking when applicable. However, in patients with repeated episodes of abdominal pain, oral intolerance and vomiting (as in Case 1 above), surgical intervention may be required. When accurately diagnosed, drainage of the pseudocyst and local excision is typically adequate. However, in patients with recurrent groove pancreatitis or cystic degeneration of the duodenal wall, more radical surgical intervention is often required. Groove pancreatitis may require pancreaticoduodenectomy in order to resolve recurrent flairs of pancreatitis and avoid complications such as duodenal stricturing and necrosis of the pancreatic head. The largest series of patients with groove pancreatitis and cystic degeneration of the duodenal wall was published by Rebours *et al*[25] and included 105 patients. In this series, 18% resolved with observation, 43% resolved with medical therapy, of which half of these required nutritional support and 39% required surgical intervention. The majority of patients underwent a pancreaticoduodenectomy (N=`17) with the remainder undergoing endoscopic cyst fenestration, biliary bypass or gastric bypass for symptom management[25].

Gastrointestinal bleeding

Gastrointestinal bleeding is a rare but potentially serious presentation of HP. Bleeding may manifest as chronic melena as in the two patients in our series (Case 2 and 5). However, there are also reports of massive gastrointestinal hemorrhage associated with large volume transfusion and hemodynamic instability[86,191]. Martinez *et al*[51] reported a rate of bleeding of 7% in gastric lesions which was supported by the experience of Pang *et al*[38] who also reported a bleeding rate of 7% in symptomatic patients. The largest series of HP published by Dolan *et al*[44] from the Mayo Clinic evaluated 212 cases of HP of which 40 were discovered due to clinical symptoms. Of these, 7 patients (17.5%), presented with anemia and or evidence of gastrointestinal bleeding. Overall, the rate of patients presenting with gastrointestinal bleeding in pooled analysis of the literature was 9%.

The etiology of bleeding in patients with heterotopic pancreas is believed to be related to ulceration of the gastric mucosa overlying the aberrant lesion. As described previously, chemical irritation and inflammation can lead to disruption of the mucosal barrier while pancreatic enzymes, such as elastase, may cause thinning and erosion into the walls of underlying vasculature. Ulceration as the source of gastrointestinal bleeding is supported by the series put forth by Pang and colleagues which reported ulcerated mucosa in pathologic specimens from patients with evidence of bleeding[38,86]. However, this does not account for all reported cases of bleeding related to HP as there are several cases of documented bleeding and anemia in which the mucosa overlying the resected lesion was found to be intact[246]. One proposed explanation for this is through the concept of hemorrhagic "suffusion". This idea, originally proposed by Madinaveita and Loma, and later described by Hudock and colleagues

speculates that chronic inflammation from heterotopic lesions leads to edema and congestion in the gastric submucosa[191,247]. Congestion of the fragile vasculature in the submucosa may lead to bleeding and diapedesis into the gastric lumen.

Definitive management of major bleeding caused by heterotopic pancreatic lesions is primarily through resection. There is no clear role for medical management in gastric bleeding related to HP. Even among those patients with bleeding related to gastric ulceration, traditional medical management for gastric ulceration is of questionable utility as the presumed etiology of mucosal ulceration is not related to acid secretion. However, there may be an argument that medical therapy may reduce the potential of gastric secretions to propagate ulceration in the setting of an already disrupted mucosal barrier from HP. In the setting of acute gastrointestinal bleeding, endoscopic therapies can be utilized to control hemorrhage but ultimately, resection of the offending lesion should be considered. There have been reports of endoscopic submucosal dissection and resection of HP in patients with melena and chronic anemia[14,18]. However, surgical resection remains the preferred management of a bleeding mass in most cases. This may be achieved with localized partial gastrectomy or duodenectomy in the setting of small lesions and more extensive resection and reconstructions can often be avoided.

Gastric outlet obstruction

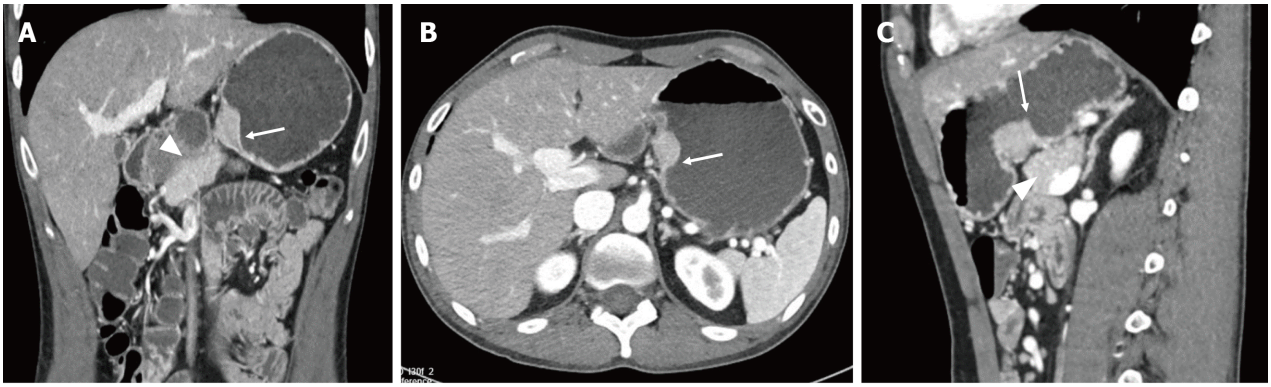
Gastric outlet obstruction is the source of symptomatic presentation in 9% of patients based on our review. The most common location for lesions arising in the stomach is the distal antrum, accounting for 85-96% of cases[94]. Given the proximity to the pylorus, growth of these lesions may lead to obstruction of the pyloric channel. While complete gastric outlet obstruction is possible, the more common scenario is partial or intermittent obstruction from a ball-valve occlusion or protrusion of the mass into the pylorus[51]. Symptoms associated with this process include abdominal pain, nausea, intermittent vomiting, bloating and anorexia[81,162]. Gastric outlet obstruction is a common presentation in infants and pediatric patients where smaller lesions can easily occlude the pyloric channel[32,247]. Nevertheless, obstruction is also seen in adults where ectopic lesions grow to a size that can lead to mechanical obstruction as seen in Case 4[41]. Lesions that are small and otherwise asymptomatic for decades, may also become acutely symptomatic in setting of inflammation and edema[60,92]. Likewise, the development of cysts and fluid collections within the wall of the stomach may grow to a size that can impede gastric emptying[163]. Some have also proposed that HP lesions may cause functional obstruction due to pyloric spasm[28].

Definitive management involves gastric decompression with a nasogastric tube and surgical correction of the obstruction. Surgical resection in these scenarios will typically require a more extensive resection than local excision or wedge resection as lesions causing obstruction are typically large (> 2 cm) and located at or near the pylorus or duodenum. Distal gastrectomy with a Billroth I, Billroth II or Roux-en-Y reconstruction are the most common operations performed for gastric lesions causing obstruction while partial duodenectomy or pancreaticoduodenectomy may be required in the obstructing lesion in the duodenum. If the lesion is small with a large cystic component causing obstruction and the pathology of the lesion is definitively known prior to surgery, a less extensive resection can be accomplished with preservation of the pylorus[95]. In the case series published by Ayantunde *et al*[28], two out of three cases of gastric outlet obstruction underwent distal gastrectomy while one underwent an anterior gastrotomy and local resection of the submucosal lesion. The majority of studies reviewed in the literature however, reported more extensive surgical resection and reconstruction procedures[14,58,64,244].

Evaluation and treatment

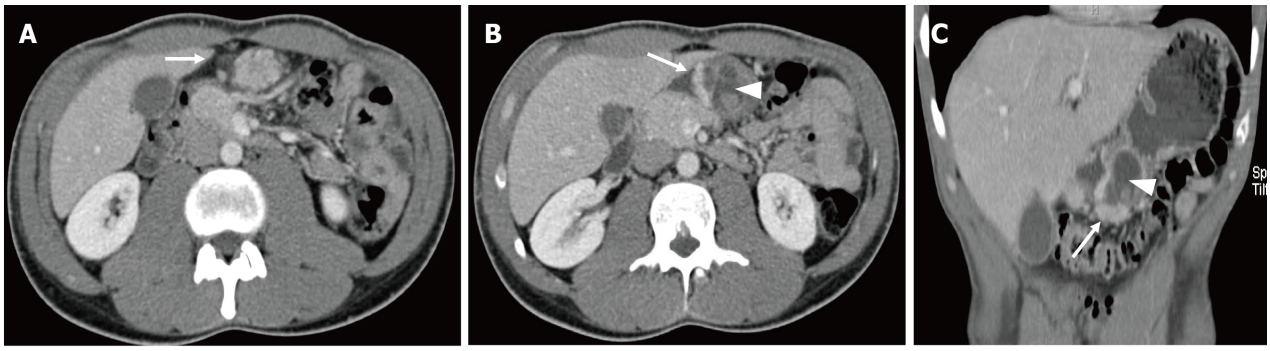
Regardless of the presenting symptoms, accurate diagnosis of HP can alter management and affect surgical decision making. Appropriate imaging and diagnostic studies must be ordered and evaluated carefully if suspicion for HP is high. Lesions of sufficient size can be identified with computed tomography, MRI, and fluoroscopy. When found within the stomach, HP tissue is typically seen along the greater curvature within 6 cm of the pylorus[238]. On CT, uncomplicated HP tissue will present as a soft tissue mass within the wall of the stomach, exhibiting similar enhancement and attenuation characteristics to that of normal pancreatic tissue[17] (Figure 3). However, the attenuation can be variable depending upon the dominant cellular type in the HP tissue, as there are pathologically different structures of heterotopic tissue described and characterized Heinrich's classification system[95,248]. Due to the variable enhancement of the HP tissue, it can sometimes be difficult to differentiate it from a gastrointestinal stromal tumor (GIST) or other common submucosal lesions[163]. In the setting of elevated pancreatic enzymes, sequelae of acute inflammation can be identified surrounding the ectopic tissue (Figure 4). On MRI the imaging of HP tissue will follow the signal intensity and enhancement characteristics of the native pancreas on all sequences. The HP tissue will demonstrate high signal intensity similar to that of the native pancreas on non-contrast T1 weighted imaging and demonstrate avid enhancement on post contrast images[234] (Figure 5). Barium fluoroscopy may also demonstrate a characteristic rounded filling defect with central indentation[241] (Figure 6).

Further diagnostic workup may require biochemical evaluation of serum amylase, lipase or, in some cases, tumor markers and/or endoscopic investigation[249]. EGD will typically demonstrate the presence of a submucosal mass which may be associated with a central umbilication and or ulceration.



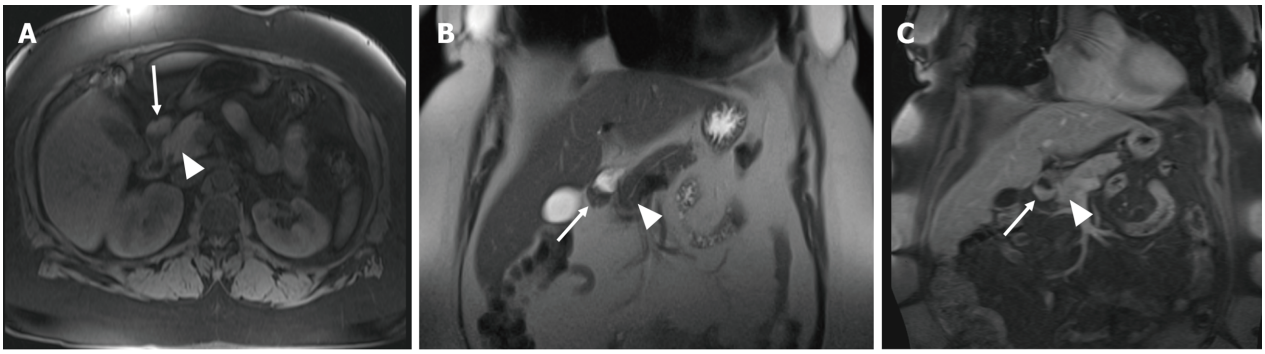
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Figure 3 Coronal (A), axial (B), and (C) sagittal images of the abdomen and pelvis following the administration of IV contrast demonstrates enhancing heterotopic pancreatic tissue within the wall of the stomach along the lesser curvature (white arrows). The heterotopic pancreatic tissue is intramural in location and demonstrates similar attenuation characteristics of the adjacent pancreatic tissue seen on the coronal and sagittal images (white arrowhead).



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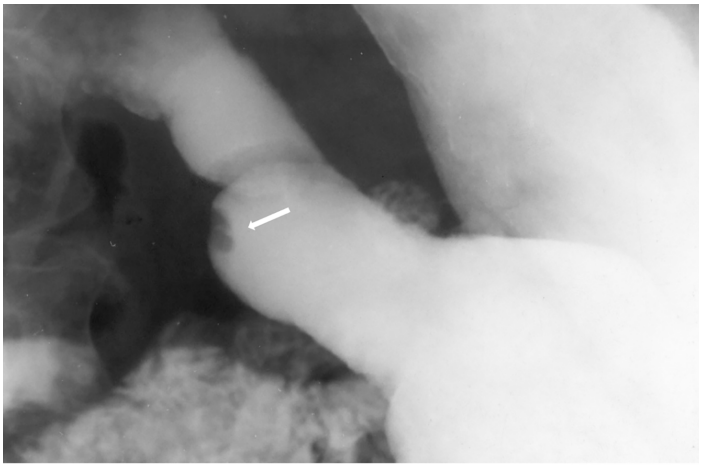
Figure 4 Computed tomography images. A: Axial image demonstrating heterotopic pancreatic tissue within the stomach (white arrow). B: Axial image demonstrating heterotopic pancreas (white arrow) with associated psudocyst (white arrowhead). C: Coronal reformatted image demonstrating heterotopic pancreas tissue (white arrow) with associated pseudocyst (arrowhead) causing gastric outlet obstruction.



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Figure 5 Non-contrast axial T1 weighted (A), coronal T2 weighted (B), and coronal postcontrast T1 weighted (C) images show a lesion within the first portion of the duodenum (white arrow) demonstrating T1 pre-contrast hyperintense signal similar to that of the adjacent pancreas (white arrowhead). This tissue shows similar imaging characteristics of the adjacent pancreatic tissue on the T2 coronal and T1 coronal post contrast images.

At centers with expertise in endoscopic ultrasound, this modality may be helpful in determining the size, location and depth of penetration within the visceral wall. Final determination is achieved by tissue biopsy. This can often be difficult in the setting of ulceration, cystic degeneration and in lesions that are located within the outer wall of the viscera and EUS will may help facilitate accurate targeting and biopsy.



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Figure 6 Single spot image of the stomach on a barium fluoroscopic study demonstrates an intraluminal filling defect within the stomach with a central indentation (arrow) consistent with pancreatic heterotopia.

Accurate identification of HP in the diagnostic workup is an important factor in determining appropriate management. Unfortunately, many patients are misdiagnosed or assumed to have alternative pathology at the time of surgery. The range of clinical and radiographic findings makes proper pre-operative identification difficult, especially in the context of a relatively rare anatomic anomaly. Most lesions are mistakenly identified as malignant or premalignant pathology. This often affects the surgical decision making and may lead to a more extensive resection than would otherwise be required. This is evident in studies such as the one performed by Zhang *et al*[9] which reported that over 54% of patients with HP were misdiagnosed preoperatively. Many of the lesions were presumed to represent malignancy and underwent extensive resections. In our series, 18 patients underwent subtotal gastrectomy, 78 underwent distal gastrectomy and reconstruction and 168 underwent a pancreaticoduodenectomy. As the majority of HP lesions are benign and do not involve the entire visceral wall, many are amenable to local or endoscopic resection. Overall, we identified 158 cases of local endoscopic resection in our series. The largest series was published by Zhou *et al*[6] who reported endoscopic submucosal dissection in 78 symptomatic patients with good results. In this series, the majority of patients had lesions located in the submucosa or lamina propria and were less than 1cm in size. Likewise, the series published by Zhong *et al*[14] included 30 patients of which 90% were under 2cm in size. Thus, an endoscopic approach is a reasonable intervention in pathologically confirmed HP, < 2 cm in size in the submucosa.

Limitations

There are several limitations to this study. First, the relatively rare presentation of the disease process makes it difficult to identify a sufficiently large patient population to analyze and draw conclusions from. For this reason, we combined our single institution experience with that of a cohort derived from a literature review in order to evaluate a meaningful number of symptomatic cases. This type of analysis can be subject to inconsistency due to the differences in reporting, and classification of symptomatic cases in the literature. Likewise, a cohort derived from retrospective review of the literature may lead to selection bias as only those cases which are clearly symptomatic or associated with a higher level of severity are reported, potentially neglecting less symptomatic or milder cases. However, given the paucity of data and characterization of this abnormality, the reporting of the known literature and experience of HP in a conglomerate population may be important in the understanding and management of patients and to help guide future analysis and treatment decisions.

CONCLUSION

Heterotopic pancreatic tissue is a relatively rare anatomic anomaly that is found within the stomach and gastrointestinal tract. While numerous case reports of this pathology exist within the literature, there are few large volume studies that describe and characterize the clinical significance of this disease process. Review of a single institutional series and the available literature identified four categories of symptomatic features that occur within the upper gastrointestinal system. Many of these lesions are misdiagnosed and undergo invasive surgical resection. Better understanding of the clinical presentation and diagnosis of these lesions may lead to improved treatment and reduction of unnecessary surgical resection.

ARTICLE HIGHLIGHTS

Research background

Heterotopic pancreas (HP) is a rare embryologic anomaly that occurs when aberrant pancreatic tissue develops independently from the main body of the main body of the pancreas. This can occur throughout the body and develop in various tissues however, this most commonly occurs in the stomach and duodenum. While the majority of these lesions are asymptomatic, many have the potential to cause harmful clinical manifestations.

Research motivation

Symptomatic heterotopic pancreas is difficult to identify and often mimics common upper gastrointestinal pathology making diagnosis and treatment challenging. Symptomatic cases of HP often require surgical or endoscopic management however, the optimal treatment strategy is poorly understood at this time and patients often undergo radical resection.

Research objectives

The purpose of this study was to review the cases of symptomatic HP at a single, high volume academic institution and compare these with a cohort of patients derived from a systematic review of symptomatic cases in the English literature. This combined cohort was used to classify the common presentation and clinical symptoms for HP and identify the various treatment options.

Research methods

A retrospective review was conducted of pathologic specimens containing heterotopic pancreas at a single, high volume, academic medical center. Symptomatic cases were identified and analyzed for details related to presentation, symptoms and treatment. A systematic review was then conducted utilizing Embase and PubMed databases for publications in English which pertained to heterotopic pancreas. Publications which included symptomatic cases of HP were included for final literature analysis and results were tabulated into a final cohort for review.

Research results

Institutional review: Twenty-nine cases of HP were identified at a single institution, of which, six presented with symptoms (20%). Two patients (7%) presented with elevated lipase and evidence of pancreatitis within the heterotopic lesion, two patients presented with gastrointestinal bleeding or anemia, one patient (3.4%) presented with perforated gastric ulceration related to a heterotopic lesion, and one patient presented with gastric outlet obstruction from a HP lesion located at the pylorus.

Systematic review: Literature review resulted in identification of 1220 unique publications related to HP of which 232 publications met inclusion criteria and were reviewed. The final cohort of patients was 934 patients. Analysis of symptomatic cases revealed that the majority of cases could be classified into the following: (1) Dyspepsia, which included symptoms of epigastric pain, eructation, nausea or bloating with meals (n = 445, 48%); (2) Pancreatitis within the ectopic lesion (n = 260, 28%); (3) Gastrointestinal bleeding (n = 80, 9%); and (4) Gastric outlet obstruction (n = 80, 9%). 37 patients presented with jaundice and biliary obstruction due to periampullary lesions (4%), and 3 patients presented with perforated ulcers. The majority of patients underwent surgical or endoscopic resection (n = 832, 90%) and 85% of reported cases indicated symptom resolution. However, most patients underwent some form of formal anatomic resection (n = 490, 61%) most commonly related to improper diagnosis preoperatively and few benefited from local surgical or endoscopic resection.

Research conclusions

Heterotopic pancreas is a rare condition but has the ability to manifest severe clinical symptoms. These most commonly arise in the stomach and duodenum and can be classified within for main categories. Improved recognition and diagnosis of this disease process may help guide treatment recommendations and improve outcomes for patients.

Research perspectives

Prospective evaluation of medical and interventional therapies is needed to determine the most appropriate treatment for the different manifestations for heterotopic pancreas.

FOOTNOTES

Author contributions: LeCompte M designed the study and performed the research, reviewed the manuscripts and wrote the paper; Mason B and Yano M reviewed the radiographic images and contributed content to the manuscript regarding radiographic identification; Chatterjee D collected and reviewed pathologic specimens; Robbins K assisted

in reviewing medical records and collecting data; Hawkins G, Strasberg S, and Fields R participated in designing the study and contributed to the analysis and editing of the manuscript.

Institutional review board statement: This study was reviewed and approved by the institutional review board of Washington University of St. Louis. IRB number [#210811062].

Informed consent statement: Based on the study protocol and design, informed consent was determined to not be required and was therefore not obtained for this study. Patient information was collected and reviewed based on the parameters laid out by the IRB at our institution and was deidentified for analysis and reporting. The remainder of the study population was constructed based on a cohort of patients obtained through systematic review of the literature and was therefore based on patient information which had already been published and presented in the literature. For further questions or concerns please feel free to contact the corresponding author.

Conflict-of-interest statement: The authors of this study have no related conflicts of interest to disclose.

Data sharing statement: Technical data and information were provided by the corresponding author, Michael LeCompte who can be reached at michael_lecompte@med.unc.edu. Data from research subjects was anonymized and excluded any patient identifiers. Given the retrospective nature of this study, informed consent was deemed not necessary by the IRB. Additional data was collected and reviewed from datasets collected and published in the literature.

STROBE statement: The authors have read and revised the manuscript based on the STROBE guidelines.

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Country/Territory of origin: United States

ORCID number: Michael T LeCompte 0000-0001-8853-2957; Brandon Mason 0000-0003-2102-1233; Keenan J Robbins 0000-0001-6950-175X; Motoyo Yano 0000-002-4837-1906; Deyali Chatterjee 0000-002-3322-1163; Ryan C Fields 0000-0001-6176-8943; Steven M Strasberg 0000-0003-0777-2698; William G Hawkins 0000-0001-7087-3585.

Corresponding Author's Membership in Professional Societies: American Hepatopancreatobiliary Association; Society of Ameican Gastrointestinal and Endoscopic Surgeons; and American College of Surgeons.

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ORIGINAL ARTICLE

Retrospective Study

Radiomics signature: A potential biomarker for β-arrestin1 phosphorylation prediction in hepatocellular carcinoma

Feng Che, Qing Xu, Qian Li, Zi-Xing Huang, Cai-Wei Yang, Li Ye Wang, Yi Wei, Yu-Jun Shi, Bin Song

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Feng Che, Qian Li, Zi-Xing Huang, Cai-Wei Yang, Yi Wei, Bin Song, Department of Radiology, West China Hospital, Sichuan University, Chengdu 610041, Sichuan Province, China

Qing Xu, Yu-Jun Shi, Institute of Clinical Pathology, West China Hospital, Sichuan University, Chengdu 610041, Sichuan Province, China

Li Ye Wang, Department of Research and Development, Shanghai United Imaging Intelligence Co., Ltd, Shanghai 200232, China

Corresponding author: Bin Song, MD, Chief Doctor, Doctor, Professor, Department of Radiology, West China Hospital, Sichuan University, No 37, Guoxue Alley, Wuhou District, Chengdu 610041, Sichuan Province, China. songlab_radiology@163.com

Abstract

BACKGROUND

The phosphorylation status of β-arrestin1 influences its function as a signal strongly related to sorafenib resistance. This retrospective study aimed to develop and validate radiomics-based models for predicting β-arrestin1 phosphorylation in hepatocellular carcinoma (HCC) using whole-lesion radiomics and visual imaging features on preoperative contrast-enhanced computed tomography (CT) images.

AIM

To develop and validate radiomics-based models for predicting β-arrestin1 phosphorylation in HCC using radiomics with contrast-enhanced CT.

METHODS

Ninety-nine HCC patients (training cohort: n = 69; validation cohort: n = 30) receiving systemic sorafenib treatment after surgery were enrolled in this retrospective study. Three-dimensional whole-lesion regions of interest were manually delineated along the tumor margins on portal venous CT images. Radiomics features were generated and selected to build a radiomics score using logistic regression analysis. Imaging features were evaluated by two radiologists independently. All these features were combined to establish clinico-radiological (CR) and clinico-radiological-radiomics (CRR) models by using multivariable logistic regression analysis. The diagnostic performance and clinical usefulness of the models were measured by receiver operating characteristic and decision curves, and the area under the curve (AUC) was determined. Their association

with prognosis was evaluated using the Kaplan-Meier method.

RESULTS

Four radiomics features were selected to construct the radiomics score. In the multivariate analysis, alanine aminotransferase level, tumor size and tumor margin on portal venous phase images were found to be significant independent factors for predicting β-arrestin1 phosphorylation-positive HCC and were included in the CR model. The CRR model integrating the radiomics score with clinico-radiological risk factors showed better discriminative performance (AUC = 0.898, 95%CI, 0.820 to 0.977) than the CR model (AUC = 0.794, 95%CI, 0.686 to 0.901; P = 0.011), with increased clinical usefulness confirmed in both the training and validation cohorts using decision curve analysis. The risk of β-arrestin1 phosphorylation predicted by the CRR model was significantly associated with overall survival in the training and validation cohorts (log-rank test, $P < 0.05$).

CONCLUSION

The radiomics signature is a reliable tool for evaluating β-arrestin1 phosphorylation which has prognostic significance for HCC patients, providing the potential to better identify patients who would benefit from sorafenib treatment.

Key Words: Hepatocellular carcinoma; Sorafenib resistance; β-Arrestin1 phosphorylation; Radiomics; Computed tomography; Overall survival

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Core Tip: The aim of this study was to develop and validate radiomics-based models for predicting β-arrestin1 phosphorylation in hepatocellular carcinoma (HCC). A total of 99 HCC patients (training cohort: n = 69; validation cohort: n = 30) were included, and the final clinico-radiological-radiomics model integrating the radiomics scores and clinico-radiological risk factors showed satisfactory discriminative performance (AUC = 0.898, 95%CI, 0.820 to 0.977). The preoperative prediction model can be used as a noninvasive and effective tool to help predict the outcome of HCC patients treated with sorafenib and identify patients who would benefit most from sorafenib treatment.

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INTRODUCTION

Hepatocellular carcinoma (HCC) was the sixth most common cancer and the third leading cause of cancer-related death worldwide in 2020[1]. Liver resection and transplantation are considered potentially curative methods for early-stage patients with well-preserved liver function. For advanced-stage HCC, systemic therapies such as multikinase inhibitors and immune checkpoint inhibitors, represented by sorafenib, have shown the potential to confer a survival advantage of 2-3 mo[2,3]. However, patients undergoing sorafenib treatment have a high resistance rate, which is still the greatest challenge and leads to a discouraging prognosis[3]. Hence, identifying patients who are more likely to benefit from sorafenib treatment and discovering related biomarkers associated with sorafenib treatment response are urgently needed.

β-Arrestins, including β-arrestin1 and β-arrestin2, are important regulators of seven-transmembrane domain G-protein-coupled receptors. They can block subsequent G protein activation and result in receptor desensitization by phosphorylation/dephosphorylation of β-arrestin1 at a carboxyl-terminal serine, Ser-412[4,5]. The development of sorafenib resistance includes primary and secondary resistance, and the phosphorylation of ERK has been widely accepted to play an important role in both[6-8]. Activation of AKT and epithelial-mesenchymal transition (EMT) also participates in acquired resistance and the signaling pathways mentioned above have strong relationships with β-arrestin1[9-11]. Wu *et al* revealed that β-arrestin1 enhances hepatocellular carcinogenesis by inflammation-mediated Akt signaling[12] and promotes HCC invasion and metastasis through p-ERK1/2 to mediate EMT[13]. The phosphorylation status of β-arrestin1 influences its function in activating downstream receptors such as

ERK1/2, forming a negative feedback loop[14]. All these signals are highly related to sorafenib resistance[9,15], which indicates that the expression of phosphorylated β-arrestin1 (p-β-arrestin1) may correlate with sorafenib resistance in HCC patients. Thus, the preoperative prediction of β-arrestin1 phosphorylation may help identify patients who could benefit from sorafenib treatment.

Radiomics is a newly emerging computational medical imaging method that allows for the quantitative analysis and translation of medical images[16,17]. Additionally, radiomics studies can provide insights into the depth and comprehensive characterization of tumor heterogeneity, with the underlying hypothesis that radiomics can better characterize tumor heterogeneity[18-20]. Preliminary studies have suggested that radiomics features can be useful for tumor lesion detection[18,21] and are potentially predictive of the microenvironment and molecular status of tumors[16,22,23]. Xu *et al*[24] extracted radiomics signatures from contrast-enhanced computed tomography (CECT) images to build a risk model that showed good performance in microvascular invasion stratification and could well predict the clinical outcomes of HCC patients. To the best of our knowledge, the value of radiomics based on CECT images in predicting β-arrestin1 phosphorylation in HCC has not yet been reported.

The purpose of this study was therefore to develop and validate a radiomics-based model combining visual imaging and clinical features for the preoperative noninvasive prediction of β-arrestin1 phosphorylation and to further investigate its association with prognostic outcomes in HCC patients.

MATERIALS AND METHODS

Patients

This retrospective study was approved by the Institutional Review Board of West China Hospital and the requirement for informed consent was waived. Patients who had histologically proven HCC and received systemic treatment with sorafenib after surgery between January 2013 and April 2017 were retrospectively reviewed and consecutively recorded. The inclusion criteria were as follows: (1) Age ≥18 years; (2) Pathologically confirmed HCC; (3) Interval between CECT imaging and surgery less than four weeks; (4) Treatment naive [*i.e.*, no hepatectomy, transcatheter arterial chemoembolization (TACE) or radiofrequency ablation (RFA) before CECT]; and (5) Administration of 400 mg sorafenib twice a day after surgery with up to two dose reductions allowed (from 400 mg once daily to 400 mg every 2 d) for drug-related adverse events. The exclusion criteria were as follows: (1) Incomplete or poor-quality CT images; (2) Interrupted sorafenib treatment for longer than 48 h between the initiation of sorafenib and the first follow-up time point; and (3) Death or loss to follow-up. Among the 146 eligible patients, 47 patients were excluded because CECT imaging was performed more than 4 wk before surgery (n = 13), the CT images were incomplete or of poor quality (n = 11), sorafenib treatment was interrupted for longer than 48 h between the initiation of sorafenib and the first follow-up time point (n = 8), or the patient was lost to follow-up (n = 15). Therefore, 99 patients were ultimately enrolled in this study. In addition, the investigated laboratory data within 7 days of the CT examination and clinical conditions were recorded, as shown in Figure 1.

In this study, consecutive patients who underwent surgery between January 2013 and March 2016 comprised the training cohort and were used to construct the nomograms, and patients who underwent surgery from April 2016 to April 2017 comprised the validation cohort.

Imaging techniques

CT imaging was performed by using multidetector CT scanners (Revolution, GE Healthcare, Milwaukee, United States; SOMATOM definition, Siemens Healthcare, Erlangen, Germany). Precontrast images were first obtained before contrast agent (iodine concentration, 300-370 mg/mL; volume, 1.5-2.0 mL/kg of body weight; contrast type, iopromide injection, Bayer Pharma AG) injection. Then, the arterial phase and portal venous phase were obtained with the following parameters: tube voltage, 100-120 kVp; tube current, 450 mA; slice thickness, 0.625 mm; pitch, 0.992:1; rotation speed: 0.5 s/rot; and ASIR-V: 30%. The arterial phase and portal venous phase were obtained at 25 s and 60 s after contrast injection.

Imaging evaluation

Three abdominal radiologists who were blinded to the histopathological results, clinical data, and survival outcomes reviewed all the CT images. The following imaging features were assessed by these readers: (1) Tumor margin, defined as a non-smooth margin with budding portion protruding into the liver parenchyma or infiltrative appearance at the tumor periphery, otherwise as smooth margin; (2) Tumor size, defined as the maximum diameter, measured on arterial phase transverse images or portal venous phase images; (3) Pseudocapsule, defined as a complete capsule with a uniform border around most or all of the tumor, unequivocally thicker or more conspicuous than the fibrotic tissue around background nodules, otherwise as incomplete integrity or not applicable; (4) Multifocality; (5) Arterial phase hyperenhancement; (6) Portal venous/delay phase hypoenhancement; (7) Radiologic evidence of necrosis; (8) Radiologic evidence of cirrhosis; and (9) Portal vein tumor thrombosis invasion. All examinations were performed using a workstation and recorded on a picture archiving and

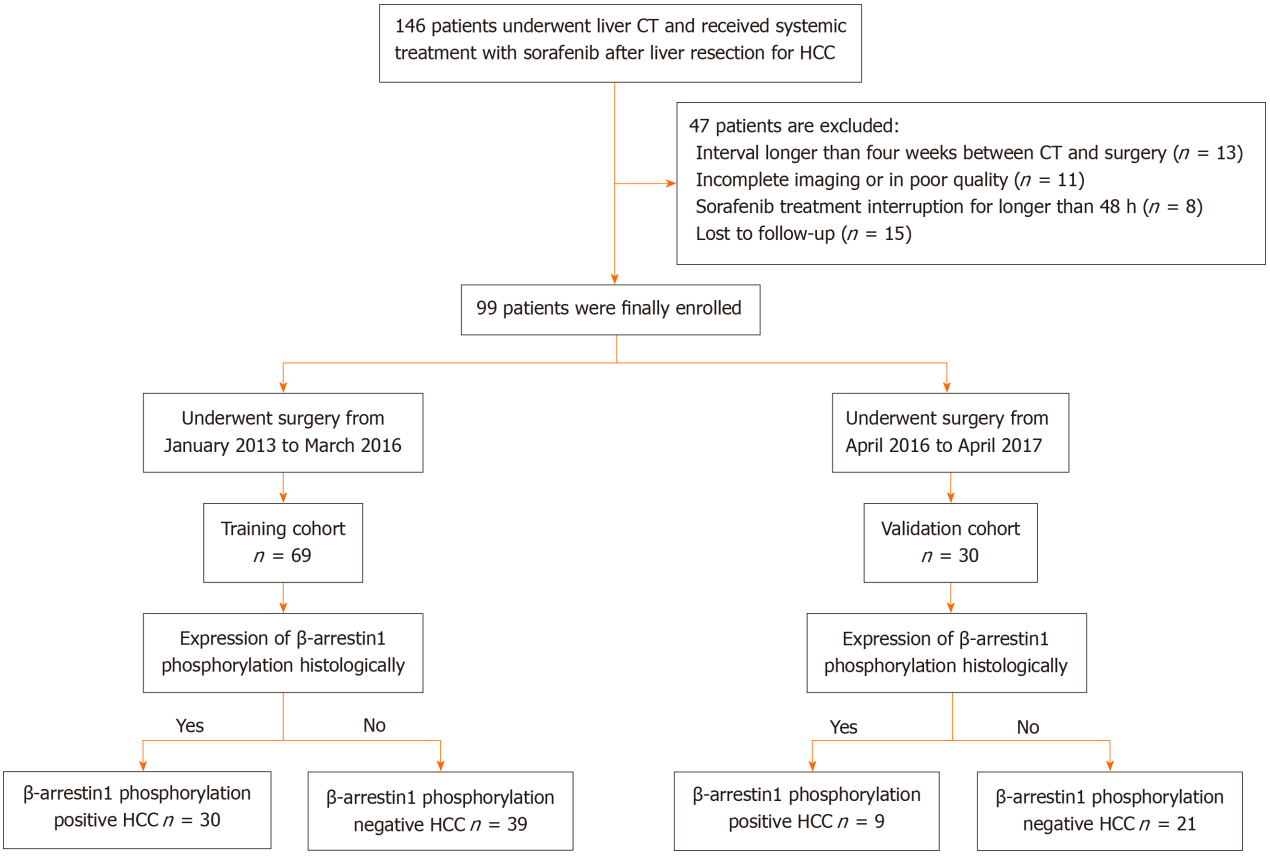


Figure 1 Patient recruitment process.

communication system.

Immunohistochemistry

Surgically resected specimens embedded in paraffin were cut into 4 μm-thick sections dewaxed, hydrated, and subjected to antigen retrieval. Subsequently, the tissue slides were incubated with primary antibodies using rabbit anti-human p-β-arrestin1 polyclonal antibody (Abcam Biotechnology, ab247229; diluted, 1:200) at 4 °C overnight, followed by incubation with secondary antibodies (cat # K5007; Dako). Staining was performed with 3,3'-diaminobenzidine (DAB) and counterstained with hematoxylin. Two senior pathologists who were blinded to all radiological and clinical results independently selected five nonoverlapping and discontinuous regions to calculate the mean for statistical analysis. Variations in the results within a range of 5% were reassessed, and a consensus decision was made. With the threshold value of 5% (p-β-arrestin1 tumor cells/total tumor cells), cases with expression higher than 5% were considered p-β-arrestin1 positive.

Follow-up surveillance after surgical resection

The patients were consistently followed-up after liver resection at intervals of 3 to 6 mo based on α-fetoprotein and imaging examinations, including ultrasound, CT or magnetic resonance imaging (MRI), and the time of disease-specific progression (local recurrence or distant organ metastasis) and time of death were recorded. These survival data were collected by one radiologist using electronic medical records and follow-up imaging studies until June 30, 2020. Overall survival (OS) was measured as the interval from the date of surgery to the date of death from a disease-related cause or the latest follow-up. For patients who were alive at the latest follow-up, the data were censored.

Radiomics workflow

Regions of interest (ROIs) were manually delineated around the outline of the tumor slice by slice using ITK-SNAP software (version 3.6.0) and excluded necrosis and calcification in the tumors. Radiomics features were generated from the images using in-house scientific research 3D analysis software (Analysis Kit, version V3.0.0. R, GE healthcare). Two classes of feature extraction methods were extracted as follows: the original feature class and 14 filter classes (boxmean, additiveGaussiannoise, binomialblurimage, curvatureflow, boxsigmaimage, log, wavelet, normalize, laplaciansharpening, discreteGaussian, mean, specklenoise, recursiveGaussian and shotnoise). A total of 2600 features were extracted from the tumors. Two radiologists (readers 1 and 2) performed ROI segmentation in a blinded

manner to assess interobserver reliability. Reader 1 repeated the feature extraction twice during a 1-wk period to evaluate intraobserver reliability. The interobserver reliability and intraobserver reliability were assessed by obtaining the intraclass correlation coefficient (ICC). Features with ICC values > 0.75 were selected for subsequent investigation. The feature selection process comprised the following three steps in the training group: variance analysis, Spearman correlation, and Lasso regression analysis. The radiomics score of each patient was calculated using this determined multivariable logistic regression model.

Prediction models of β-arrestin1 phosphorylation

For CT radiographic and clinical factors, predictors with $P < 0.05$ in the univariate logistic analysis ($P < 0.05$) were included. Multivariate logistic analysis, which was used to identify significant predictors based on a backward stepwise selection process with the Akaike information criterion, was employed to develop a clinical-radiological (CR) model. In addition, a clinical-radiological-radiomics (CRR) model was constructed by multivariate logistic regression analysis, tests of the association with radiomics scores, clinical factor evaluations and CT imaging findings based on a backward stepwise selection process with the Akaike information criterion.

Statistical analysis

Categorical variables are summarized as frequencies and proportions, while continuous variables are expressed as the means and standard deviations or medians and interquartile ranges (IQRs). The differences in characteristics between groups were evaluated using Student's *t* test (normal distribution) and the Mann-Whitney *U* test (skewed distribution) for continuous variables and the chi-squared test or Fisher's exact test for categorical variables. OS curves were drawn by using the Kaplan-Meier method, and the difference in OS between groups was compared using the log-rank test. Inter-observer agreement was applied to assess the reliability of imaging analysis using the Kappa test; 0-0.2 represents slight, 0.21-0.40: fair, 0.41-0.60: moderate, 0.61-0.80: substantial, 0.81-1: excellent.

The discriminative performance of the prediction models was quantified by the area under the curve (AUC) of receiver operator characteristic (ROC) curves. Differences in the ROC curves were compared by using the DeLong test. Calibration curves were generated to assess the calibration of the prediction model with the Hosmer-Lemeshow test. The probabilities of net benefits were quantified by decision curve analysis to evaluate the clinical application value of the prediction models.

The statistical analyses were implemented using *R* statistical software (version 3.4.2, http://www.R-project.org) and SPSS software (version 22.0, IBM), and two-sided *P* values < 0.05 were considered significant.

RESULTS

Patient characteristics

Of the 99 patients [male/female: 88/11; mean age, 51.53 ± 12.62 years, range 21 to 78 years) included in the study (training ($n = 69$) and validation ($n = 30$)], p-β-arrestin1 was identified in 39 (39.4%) patients. The 3-year survival rates of p-β-arrestin1-positive and p-β-arrestin1-negative HCC patients were 38.5% and 31.7%, respectively. The Kaplan-Meier method showed that p-β-arrestin1-positive patients lived longer than p-β-arrestin1-negative patients ($P < 0.05$ with the log-rank test). The clinical, pathological, and imaging characteristics of patients in the training and validation cohorts are summarized in Table 1.

Development of the radiomics score

Variance analysis using *t* test identified 15 radiomics features, assessed by Spearman rank correlation, and 4 features (boxsigmaimage_glrlm_RunLengthNonUniformity, wavelet_firstorder_wavelet-HLL-Skewness, wavelet_glcm_wavelet-HLH-Correlation, and wavelet_ngtdm_wavelet-LHL-Busyness) were chosen for logistic regression analysis ($P > 0.05$). Variables with $P < 0.1$ in the univariable logistic regression analysis were included in the multivariable regression model with backward stepwise selection using the Akaike information criterion. The radiomics score was calculated with the following formula: radiomics score = -0.3527 + 0.4748 × boxsigmaimage_glrlm_RunLengthNonUniformity + 0.7046 × wavelet_firstorder_wavelet-HLL-Skewness-0.5697 × wavelet_glcm_wavelet-HLH-Correlation + 0.6471 × wavelet_ngtdm_wavelet-LHL-Busyness.

Development of the predictive models

In total, 2 clinical characteristics [alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels], 2 imaging features (tumor size and tumor margin on portal venous phase images) and the radiomics score were identified by univariate analysis (all $P < 0.1$). In the multivariable logistic regression analysis, radiomics score [odds ratio (OR), 3.412; 95%CI: 1.562-7.453, $P = 0.002$], ALT level (OR, 0.159; 95%CI: 0.038-0.673, $P < 0.012$), tumor size (OR, 0.243; 95%CI: 0.059-1.003, $P = 0.05$) and tumor margin (OR, 0.170; 95%CI: 0.044-0.664, $P = 0.011$) significantly predicted β-arrestin1 phosphorylation

Table 1 Baseline characteristics of the patients in the training and validation cohorts

Variables	Training cohort (*n* = 69)	Validation cohort (*n* = 30)	*P* value
Age, mean ± SD, yr	51.00 ± 13.019	52.73 ± 11.776	0.269
Gender			0.751
Male	61 (88.4)	26 (86.7)	
ALT (IU/L)			0.375
< 40	44 (63.8)	16 (53.5)	
AST (IU/L)			0.829
< 35	32 (46.4)	13 (43.4)	
ALB (g/L)			0.612
< 40	15 (21.7)	8 (26.7)	
GGT (μ/L)			0.817
< 45	24 (34.8)	9 (30.0)	
TBIL (μmol/L)			0.828
< 17.1	39 (56.5)	16 (53.3)	
PLT (× 10^9/L)			0.791
< 100	14 (20.3)	7 (23.3)	
PT(s)			0.596
< 9.6 or > 12.8	16 (23.2)	5 (16.7)	
AFP (ng/mL)			0.279
< 400	34 (49.3)	11 (36.7)	
CEA (ng/mL)			0.798
< 3.4	53 (76.8)	24 (80.0)	
HBsAg			0.270
Positive	61 (88.4)	29 (96.7)	
MVI			0.827
Absent	36 (52.2)	17 (56.7)	
Present	33 (47.8)	13 (43.3)	
Differentiation			0.661
Highly	37 (53.6)	18 (60.0)	
Middle-Low	32 (46.4)	12 (40.0)	
BCLC			0.254
0-A	9 (13.0)	8 (26.7)	
B	28 (40.6)	10 (33.3)	
C	32 (46.4)	12 (40.0)	
Child-Pugh Score			0.770
< 3	57 (82.6)	26 (86.7)	
Morphologic CT features			
Tumour size			0.499
< 5 cm	24 (34.8)	13 (43.3)	
Multifocality			0.351
1	49 (71.0)	18 (60.0)	
≥ 2	20 (29.0)	12 (40.0)	

Tumour margin			0.661
Smooth	32 (46.4)	12 (40.0)	
Non-smooth	37 (53.6)	18 (60.0)	
Pseudo–capsule			0.824
Well-defined	27 (39.1)	13 (43.3)	
Ill-defined	42 (60.9)	17 (56.7)	
AP hyperenhancement			0.448
No	5 (7.2)	4 (13.3)	
Yes	64 (92.8)	26 (86.7)	
PVP hypoenhancement			0.430
No	4 (5.8)	3 (10.0)	
Yes	65 (94.2)	27 (90.0)	
Radiologic evidence of necrosis			0.822
Absent	25 (36.2)	12 (40.0)	
Present	44 (63.8)	18 (60.0)	
Radiologic evidence of cirrhosis			0.654
Absent	45 (65.2)	18 (60.0)	
Present	24 (34.8)	12 (40.0)	
Portal vein tumor thrombosis invasion			0.186
Absent	43 (62.3)	14 (46.7)	
Present	26 (37.7)	16 (53.3)	

Note: Unless otherwise indicated, data are the number of patients, and data in parentheses are percentages. AFP: Alpha-fetoprotein; CEA: Carcinoembryonic antigen; HBsAg: Hepatitis B surface antigen; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; TBIL: Total bilirubin; ALB: Albumin; PT: Prothrombin time; PLT: Platelet count; GGT: γ-glutamyl transpeptidase; MVI: Microvascular invasion; BCLC: Barcelona Clinic Liver Cancer; SD: Standard deviation; AP: Arterial phase; PVP: Portal venous phase.

(Table 2). Thus, the CR and CRR models were constructed by using the above aggressive features and the nomograms of the above multiparametric models are shown in Figure 2A and B. Excellent interobserver agreement was observed for the imaging feature evaluation, with Kappa values of 0.890 for tumor size and 0.789 for smooth tumor margin (Figure 3).

Predictive performance of the models

In the training cohort, the AUCs of the radiomics score, CR model and CRR model were 0.754 (95%CI: 0.640-0.868), 0.794 (95%CI: 0.686-0.901) and 0.898 (95%CI: 0.820-0.977), respectively. The CRR model had a significantly higher AUC than the radiomics score (P = 0.007) and the CR model (P = 0.011). In the validation cohort, the AUCs of the radiomics score, CR model and CRR model were 0.704 (95%CI: 0.454-0.953), 0.646 (95%CI: 0.411-0.880) and 0.735 (95%CI: 0.505-0.966), respectively. The diagnostic performance of the radiomics score and two models is shown in Table 3 and Figure 2C and D. The calibration curve of all the models showed excellent agreement between the predictions and observations in both the training and validation cohorts (all P > 0.05) (Figure 2E and F). The decision curve showed that the CRR model had the largest overall net benefit compared with the treat-all-patients as p-β-arrestin1 positive and treat-none patients as p-β-arrestin1 negative across the full range of reasonable threshold probabilities (Figure 4).

Risk stratification with p-β-arrestin1 predicted by the CRR model

According to the risk of β-arrestin1 phosphorylation predicted by the CRR model, patients with p-β-arrestin1 positivity lived longer than those with p-β-arrestin1 negativity using the log-rank test (P < 0.05) in both the training and validation cohorts (Figure 5).

Table 2 Univariate and multivariate regression analyses of the p-β-arrestin1-positive and p-β-arrestin1-negative groups in the training cohort

Variables	Univariable analysis		Multivariable analysis	
	OR (95%CI)	*P* value	OR (95%CI)	*P* value
ALT	0.237 (0.043-1.292)	0.096[a]	0.159 (0.038-0.673)	0.012[a]
AST	0.497 (0.100-2.471)	0.393	-	-
Tumor size	0.245 (0.059-1.019)	0.053[a]	0.243 (0.059-1.003)	0.050[a]
Tumor margin	0.180 (0.046-0.706)	0.014[a]	0.170 (0.044-0.664)	0.011[a]
Radiomics	3.473 (1.574-7.663)	0.002[a]	3.412 (1.562-7.453)	0.002[a]

[a]*P* value < 0.1.

ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; CI: Confidence interval; OR: Odds ratio.

Table 3 Diagnostic performance of the three models for predicting β-arrestin1 phosphorylation-positive hepatocellular carcinoma

	Training group				Validation cohort			
	AUC (95%CI)	SPE	SEN	*P* value	AUC (95%CI)	SPE	SEN	*P* value
RS	0.754 (0.640-0.868)	53.8	86.7	0.007[1]	0.704 (0.454-0.953)	47.6	77.8	0.791[1]
CR	0.794 (0.686-0.901)	87.2	66.7	0.631[2]	0.646 (0.411-0.880)	81.0	33.3	0.713[2]
CRR	0.898 (0.820-0.977)	87.2	86.7	0.011[3]	0.735 (0.505-0.966)	71.4	66.7	0.147[3]

[1]AUCs of the radiomics score and clinico-radiological-radiomics model were compared.

[2]AUCs of the radiomics score and clinico-radiological model were compared.

[3]AUCs of the clinico-radiological model and clinico-radiological-radiomics model were compared.

RS: Radiomics score; CR: Clinico-radiological; CRR: Clinico-radiological-radiomics; SEN: Sensitivity; SPE: Specificity; AUC: Area under the curve; CI: Confidence interval.

DISCUSSION

In this retrospective study, a CT image-based model incorporating qualitative imaging features, clinical characteristics and quantitative radiomics features for predicting β-arrestin1 phosphorylation in HCC was generated. In addition, in patients treated with sorafenib, we found that p-β-arrestin1-positive HCC patients predicted by the CRR model were associated with better prognosis. The CRR model may serve as a noninvasive and effective tool to predict HCC patients β-arrestin1 phosphorylation status and help select patients who are suitable for sorafenib treatment.

For predicting β-arrestin1 phosphorylation in HCC patients, radiomics features provided increased power (AUC = 0.754) and were indicated to be independent predictors for p-β-arrestin1 in the final CRR model (P = 0.005). Utilizing the radiomics method, the proposed CRR model yielded an improved diagnostic performance in the training cohort (AUC from 0.794 to 0.898) and validation cohort (AUC from 0.646 to 0.735), indicating that the combined radiomics approach may have greater value in preoperative β-arrestin1 phosphorylation prediction than clinico-radiological features. The reason why the CRR model achieved the best predictive performance can be explained by the fact that the final model includes both qualitative and quantitative imaging features to provide a comprehensive overview of the correlations of radiomics features with HCC pathological status and genomics characteristics[25]. The radiomics signature includes shape, intensity, and texture information, which can reflect the complexity of the properties of the target tissue. Previous studies have shown that imaging features, including texture features, are informative of the gene expression profiles of HCC lesions, which parallels the diversity of molecular activities[26]. Hectors *et al* found that MRI radiomics features are highly associated with HCC immuno-oncological characteristics and can serve as noninvasive predictors of its status[27]. A predictive nomogram incorporating a radiomics signature and other clinico-radiological factors showed a significantly improved diagnostic performance in cytokeratin19 stratification of HCC[28]. However, to our knowledge, no other studies have investigated the possible value of quantitative analysis integrating clinical factors in predicting β-arrestin1 phosphorylation in HCC. Developed in the training cohort and applied to the validation cohort, the radiomics score based on CECT images combined with clinico-radiological factors could correctly identify the β-arrestin1 phosphorylation status of more than 86.7% of the patients in the training cohort and was well validated

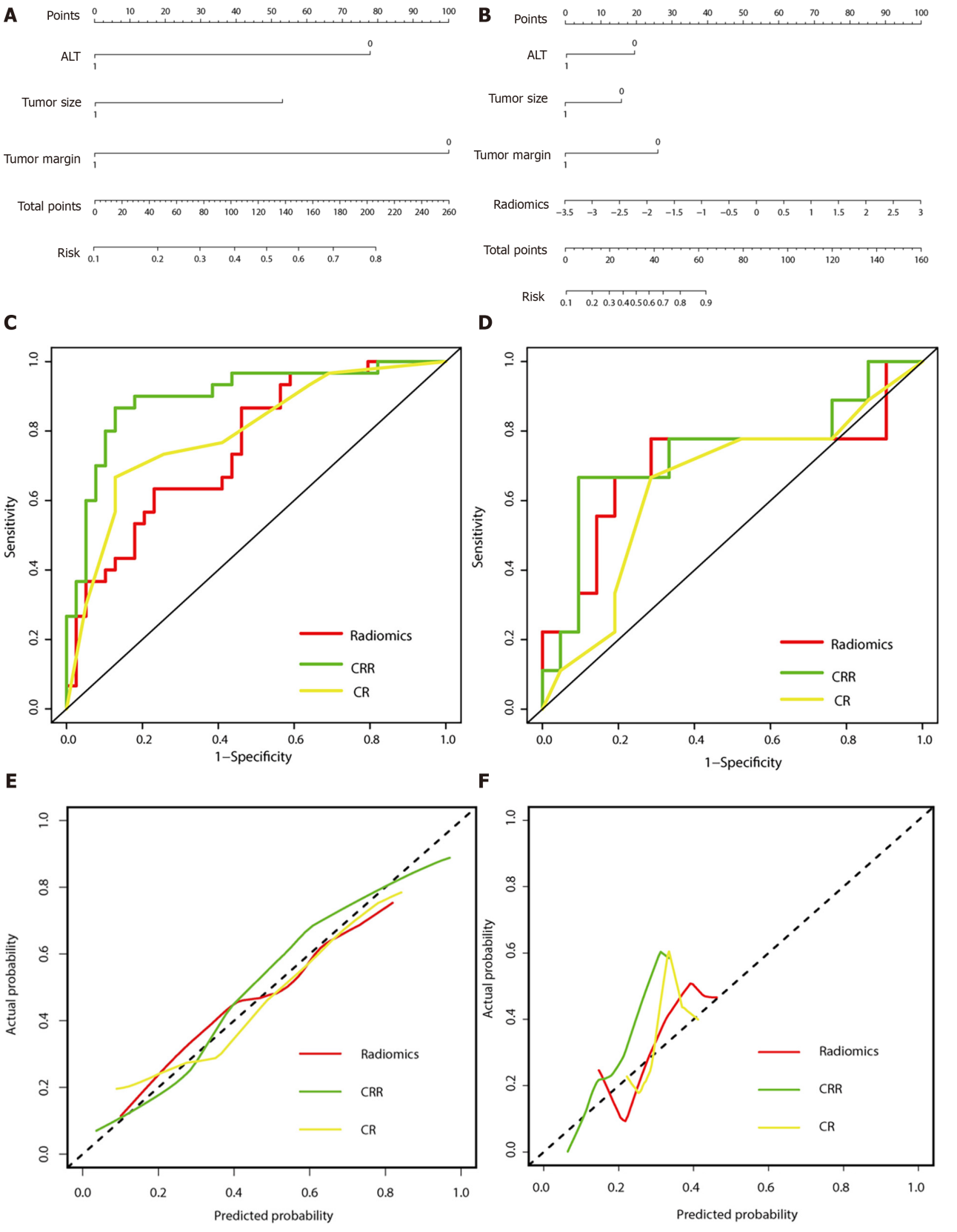


Figure 2 Performance of the three models. A: The developed clinico-radiological (CR) nomogram; B: The developed clinico-radiological-radiomics (CRR) nomogram. Predictor points are found on the uppermost point scale that corresponds to each variable. On the bottom scale, the points for all variables are added and translated into a β-arrestin1 phosphorylation positivity probability. C: Comparison of receiver operating characteristic (ROC) curves of the radiomics model, CR model and CRR model in the training cohort; D: Comparison of receiver operating characteristic (ROC) curves of the radiomics model, CR model and CRR model in the validation cohort. E: Calibration curves of the three models in the training cohort; F: Calibration curves of the three models in the validation cohort. The actual high expression of p-β-arrestin1 is represented on the y-axis, and the predicted probability is represented on the x-axis. The closer fit of the solid line to the ideal black dotted line indicates a better calibration.

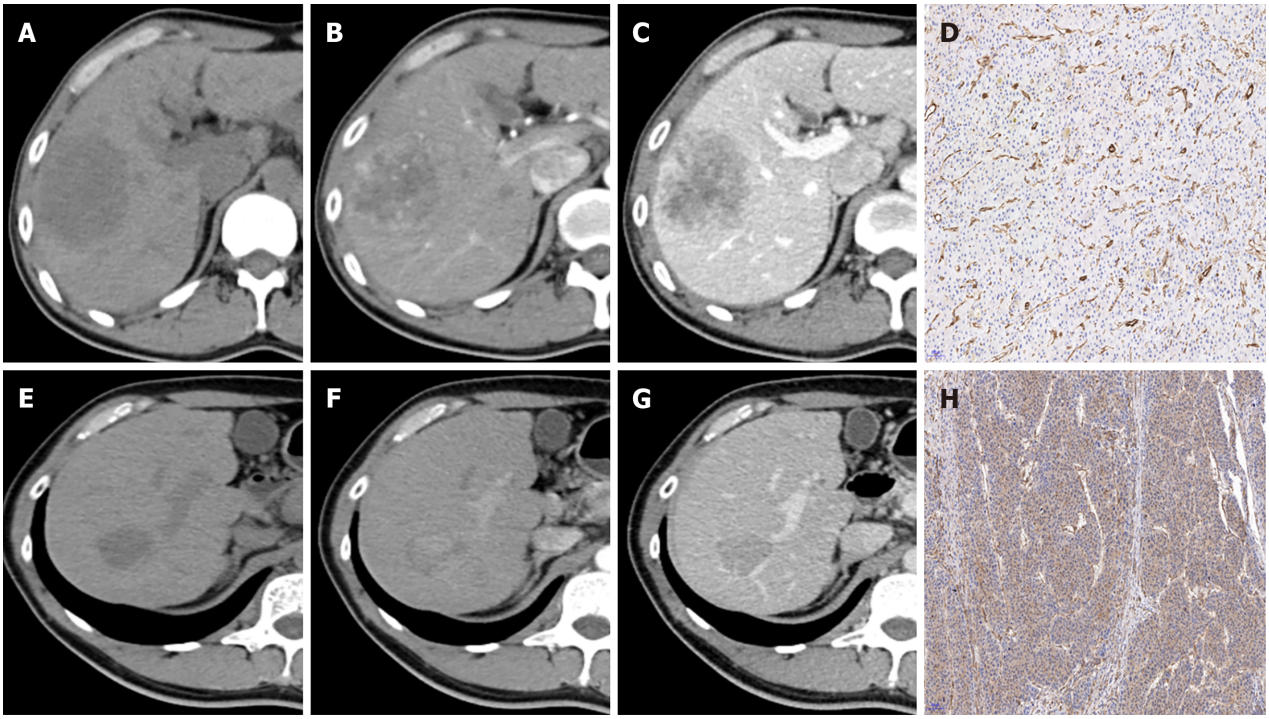


Figure 3 Representative images of contrast-enhanced computed tomography and β-Arrestin1 phosphorylation (magnification, × 100). A: CT images of a 45-year-old man with a 6.3-cm hepatocellular carcinoma (HCC) in the right liver lobe in the plain phase; B: The tumor shows heterogeneous hyperenhancement in the arterial phase; C: The tumor shows washout at the portal venous phase with intratumor necrosis, an ill-defined capsule and a non-smooth tumor margin. D: Immunohistochemical staining shows a β-arrestin1 phosphorylation-negative status at 100× magnification.

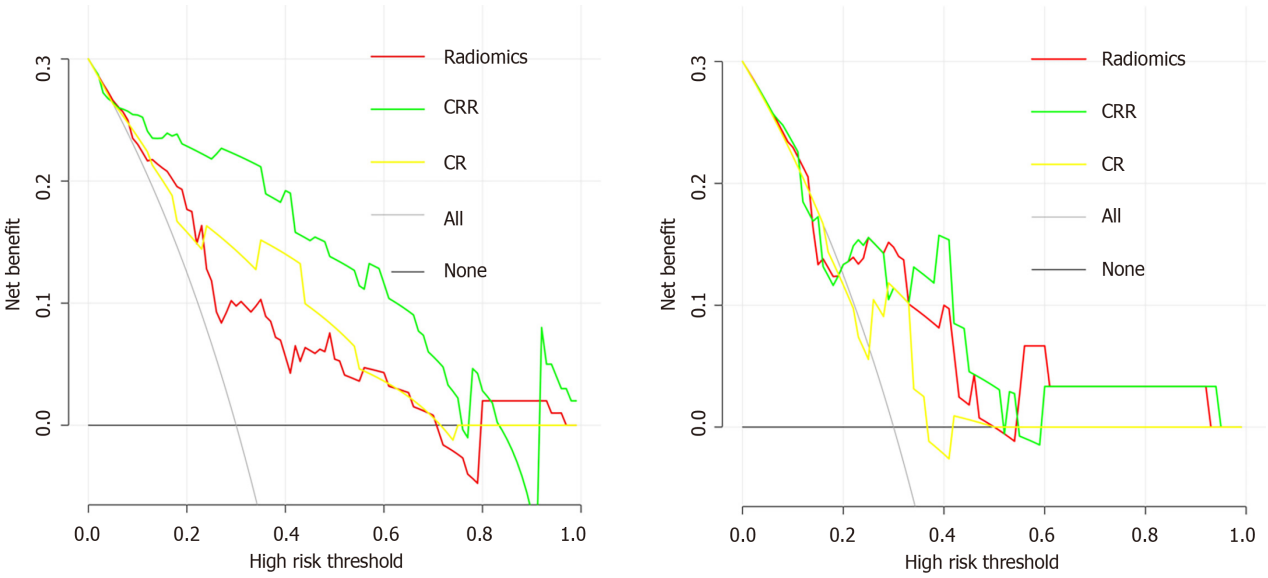


Figure 4 Decision curve analysis for each model. A: Decision curve analysis in the training cohort; B: Decision curve analysis in the validation cohort. The y-axis measures the net benefit, and the x-axis is the threshold probability. The gray line represents the hypothesis that all patients are β-arrestin1 phosphorylation-positive. The black line represents the hypothesis that all patients are β-arrestin1 phosphorylation-negative. Among the three models, the clinico-radiological-radiomics (CRR) model provided the highest net benefit compared with the radiomics and clinico-radiological (CR) models.

to serve as a quantitative multiple-feature parameter for the β-arrestin1 phosphorylation-based risk stratification of HCC patients. Our study investigates the predictive aspects of computational-assisted models for the preoperative prediction of β-arrestin1 phosphorylation status, which currently can now only be attained by invasive biopsy or surgery. This computational method can guide clinical management by identifying patients for targeted therapy, as most patients recommended for systematic treatment according to the Barcelona Clinic Liver Cancer algorithm are not candidates for surgery due to their poor condition[29].

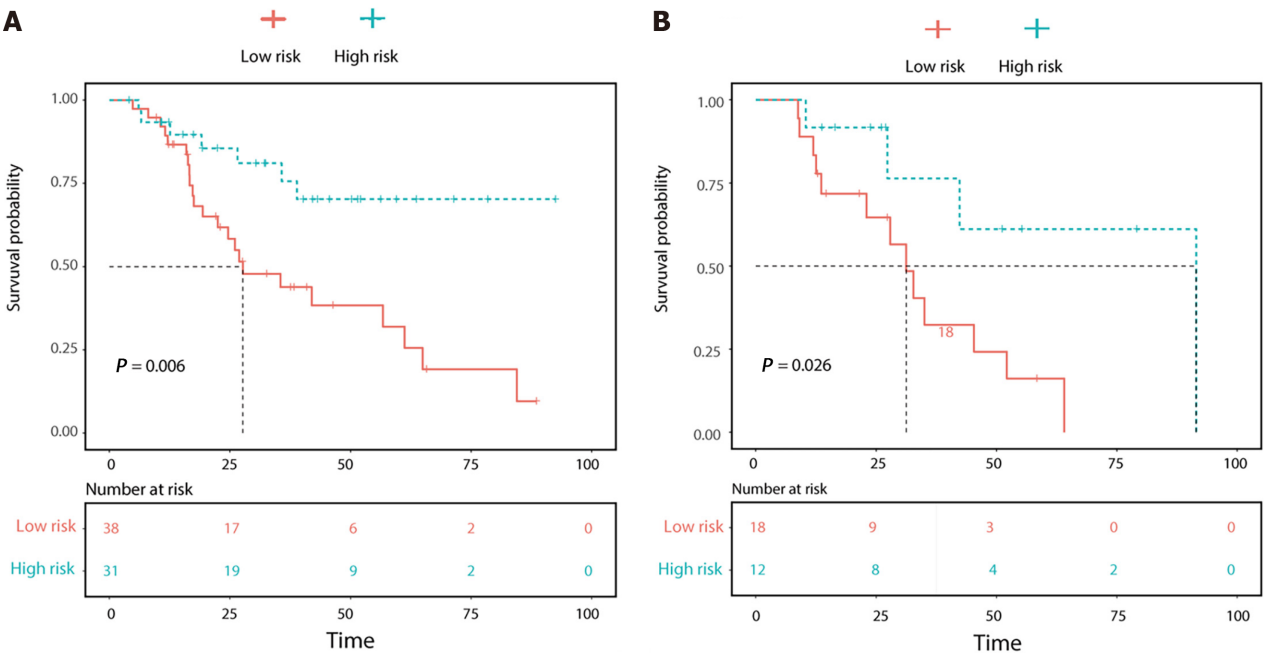


Figure 5 Overall survival (OS) curve analysis. A: The OS curve estimates by clinic-radiological-radiomics model in patients with β-Arrestin1 phosphorylation positive and β-Arrestin1 phosphorylation negative in the training cohort; B: The OS curve estimates by clinic-radiological-radiomics model in patients with β-Arrestin1 phosphorylation positive and β-Arrestin1 phosphorylation negative in the validation cohort.

The clinicopathologic features of preoperative serum ALT levels were significantly different in the p-β-arrestin1-positive and p-β-arrestin1-negative groups in this study. In line with the findings of previous studies, serum ALT is an important hepatic inflammation marker that is correlated with liver function. Hepatitis infection can simultaneously induce serum ALT increases and β-arrestin1 upregulation, and higher serum ALT levels are a feature commonly associated with this subtype of HCC[12]. In clinical practice, serum ALT levels can be easily obtained and incorporated into a radiomics model for individualized risk estimation. A larger tumor size and nonsmooth tumor margins were also shown to be associated with p-β-arrestin1 expression. This finding is in accordance with previous studies showing that β-arrestin1 can promote hepatocellular proliferation *via* the Akt pathway, and HCCs with higher p-β-arrestin1 levels are more likely to have an infiltrative growth pattern[13]. Serum AST levels were associated with p-β-arrestin1 in the univariate analysis but not the multivariate models, probably because of a lack of statistical power due to the insufficient number of patients.

We also found that patients who were p-β-arrestin1-positive lived longer than those who were p-β-arrestin1-negative. Previous studies have revealed that high expression of β-arrestin1 contributes to tumor survival, proliferation, angiogenesis, invasion and metastasis and is associated with the prognosis of epithelial ovarian cancer, prostate cancer and lung cancer[30-34]. Although the correlation of β-arrestin1 with HCC prognosis has not been investigated, β-arrestin1 has been shown to be positively related to HCC carcinogenesis and metastasis[12,13]. Evidence has shown that the sorafenib response is impaired in HCC with dysregulated phosphorylated ERK (p-ERK) and AKT (p-AKT) activation and that suppression of ERK1/2 increases sorafenib sensitivity in several HCC cell lines[35-37], while β-arrestin1 can activate PI3K/Akt signaling by Akt phosphorylation and trigger ERK1/2 phosphorylation-mediated EMT in HCC. Moreover, hyperactive PI3K/AKT signaling has been reported to be one of the primary causes of EMT in HCC resistance to sorafenib[35,36,38]. These studies indicate that PI3K/AKT signaling and p-ERK1/2-mediated EMT signal hyperactivity may function in β-arrestin1-induced HCC resistance to sorafenib and further influence the prognosis of HCC patients treated with sorafenib, which was consistent with a series of studies recently showing that β-arrestin1 expression had some correlation with resistance to therapy in several types of cancers, such as breast[39] ovarian[40,41] and non-small-cell lung cancer[42]. Increased phosphorylation of β-arrestin1 Leads to decreased levels of dephosphorylated β-arrestin1, which influences its function in the activation of downstream factors, such as p-ERK and p-AKT. Therefore, the phosphorylation status of β-arrestin1 has a critical role in HCC sorafenib resistance. Predicting p-β-arrestin1 can help to identify patients who are sensitive to this treatment and prevent unnecessary side effects.

There were some limitations in our study. First, this was a retrospective longitudinal cohort study and selection bias may exist due to the strict inclusion criteria. Although we performed internal validation, additional external validation is needed to facilitate the wider use of this predictive model. Second, our study was performed at a single institution, and the CT scanner in this study was not fixed in their protocol. However, this could be a strength in terms of the generalizability of the findings by reflecting actual clinical practice. Third, p-β-arrestin1 positivity was defined as a cutoff of 5% for tumor

cells to avoid false-positive results. The association between our predictive model and the graded degree of p-β-arrestin1 immunopositivity should be further assessed.

CONCLUSION

In conclusion, CECT-based radiomics combining clinico-radiological factors achieved desirable results in the prediction of β-arrestin1 phosphorylation in HCC which showed prognostic value in patients treated with sorafenib. This finding suggests that CT radiomics may provide promising and noninvasive biomarkers for the evaluation of p-β-arrestin1 expression and may help identify the subset of HCC patients who are more sensitive to sorafenib treatment, thus potentially guiding personalized treatment strategies.

ARTICLE HIGHLIGHTS

Research background

Sorafenib is regarded as a first-line systematic treatment option for patients with advanced hepatocellular carcinoma (HCC), but its efficacy is largely influenced by raising resistance. The phosphorylation status of β-arrestin1 influences its function as a signal strongly related to sorafenib resistance.

Research motivation

Identifying patients who are more likely to benefit from sorafenib treatment and discovering related biomarkers associated with sorafenib treatment response can guide personal management.

Research objectives

The purpose of this study was to develop and validate radiomics-based models for predicting β-arrestin1 phosphorylation in HCC with contrast-enhanced computed tomography (CT).

Research methods

We included ninety-nine HCC patients (training cohort: n = 69; validation cohort: n = 30) who received systemic sorafenib treatment after surgery. Radiomics features were generated and selected to build a radiomics score and then combined with clinical and imaging features to establish clinico-radiological (CR) and clinico-radiological-radiomics (CRR) models. The performance and clinical usefulness of the models were measured by receiver operating characteristic and decision curves. Their association with prognosis was also evaluated using the Kaplan-Meier method.

Research results

Our study found that the ALT level, tumor size and tumor margin were significant independent factors for predicting β-arrestin1 phosphorylation. The CRR model showed better discriminative performance than the radiomic score or the CR model. The β-arrestin1 phosphorylation status predicted by the CRR model was shown to be significantly associated with overall survival.

Research conclusions

The radiomics signature is a reliable tool for evaluating β-arrestin1 phosphorylation, and may help to better identify patients who would benefit from sorafenib treatment.

Research perspectives

The results of this study suggests that CT-based radiomics may provide promising and noninvasive biomarkers for the evaluation of β-arrestin1 phosphorylation and may help to identify the subset of HCC patients who are more sensitive to sorafenib treatment, thus potentially guiding personalized treatment strategies.

FOOTNOTES

Author contributions: Che F, Xu Q, Shi YJ and Song B designed the research; Che F, Li Q and Xu Q conducted literature search and analysis; Yang CW, Huang ZX, Wang LY and Wei Y provided material support; Song B provided funding for the article; Che F and Xu Q wrote the paper; Che F and Xu Q contributed equally to this work.

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

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

Conflict-of-interest statement: We have no financial relationships to disclose.

Data sharing statement: No additional data are available.

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Country/Territory of origin: China

ORCID number: Feng Che 0000-0002-8008-2696; Qing Xu 0000-0001-9438-5535; Qian Li 0000-0001-8330-7654; Zi-Xing Huang 0000-0001-7967-5948; Cai-Wei Yang 0000-0003-3335-3948; Li-Ye Wang 0000-0002-5535-4514; Yi Wei 0000-0003-3993-9747; Yu-Jun Shi 0000-0003-0494-6023; Bin Song 0000-0002-7269-2101.

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LETTER TO THE EDITOR

Comment on review article: Chronic hepatitis C virus infection cascade of care in pediatric patients

Nouhoum Bouare, Mamadou Keita, Jean Delwaide

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Nouhoum Bouare, Biomedical Sciences, National Institute of Public Health, Bamako 1771, Mali

Mamadou Keita, Department of Prevention, Medical and Psychosocial Management, CSLS-HIV-TB-H, Bamako E 595, Mali

Jean Delwaide, Department of Gastroenterology and Hepatology, CHULiege, Liege 4000, Belgium

Corresponding author: Nouhoum Bouare, DSc, PhD, Research Scientist, Biomedical Sciences, National Institute of Public Health, Hippodrome 235, Bamako 1771, Mali.
nouhoumsamakoro@yahoo.fr

Abstract

An enhanced cascade of care should include a younger population, helping to achieve the goal of the World Health Organization with a focus on elimination in the pediatric population. Furthermore, enhanced screening and awareness efforts and continued education of health care providers will improve the outcomes of chronic hepatitis C virus (HCV) infection in the pediatric population. The present work discusses and comments on the topic "cascade of care in HCV chronic pediatric patients".

Key Words: Cascade of care; Hepatitis C virus; Chronic patients; Pediatric population; Disease management; Commentary

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Core Tip: Worldwide disparities exist regarding the chronic hepatitis C virus (HCV) infection cascade of care, and it is most evident between high-income countries and areas with scarce resources. An integrative strategy encompassing efficient pediatric HCV diagnosis and treatment as well as prevention is needed. Addressing health care disparities by insightfully applying successful outcomes from high-income countries in certain disadvantaged regions with poor cascade of care may help to achieve the elimination goal of HCV set by the World Health Organization.

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TO THE EDITOR

To eliminate viral hepatitis worldwide by 2030, the World Health Organization (WHO) focused on strategic objectives to reach a decrease of the disease incidence and mortality by 90% and 65%, respectively[1,2]. The cascade of care (CoC) originates from HIV management aimed in some an extent to achieve sustained virological response[2]. It addresses the diagnosis, treatment and possible cure of hepatitis C virus (HCV)[1,2]. HCV chronic infection is reported as affecting 71 million persons globally, and many of them are unaware of their infectious status[3]. The global HCV and hepatitis B virus populations remain largely underdiagnosed and undertreated[4]. For worldwide heterogeneity, reliable HCV prevalence estimates (adjusted to age and geographic area) are needed. These country-specific estimates can highly improve the intervention by accelerating HCV elimination[1].

Rogers and Balistreri[1] reported a number of field epidemiological and/or clinical factors impacting disease evolution. The perinatal route is mainly responsible for the HCV infection propagation in the pediatric population. This route is reported as favorable to genotype 1a that is even associated with decreased clearance, persistent viremia and the risk of liver damages such as end-stage liver disease in treatment-naïve children. High grade fibrosis or cirrhosis patients are still at high risk to develop hepatocellular carcinoma even if achieving sustained virological response. Histological studies are still needed to support direct acting antiviral (DAA) treatment efficacy. The authors noticed the need of fibrosis control in children regarding the constant risk of portal hypertension and hepatocellular carcinoma occurrence. Moreover, poor social act and health-related quality of life were described in adolescent HCV patients[1]. Delaying treatment until 18 years of age favors lifetime risk of avoidable late liver complications. Hence early treatment is economic and lifesaving[1].

However, HCV CoC reports, similar to that of Rogers and Balistreri[1], mostly emanate from high-income countries[2]. Extending an HCV CoC strategy to low- and middle-income countries (LMIC) must initially and insightfully focus on disease awareness and education to disseminate the desired global health goal. Moreover, addressing the differences between a CoC applicable in high-income countries and another adjusted for low-income areas is central to achieving the ultimate HCV elimination goal.

Regarding HCV therapy, DAAs highly impact CoC. However, in 2016 the treatment was less accessible for the patients. As for CoC, updated estimates are needed to assess the impact of newly implemented approaches aimed to promote hepatitis C elimination[5]. Furthermore, improving the linkage to care is lifesaving given the precipitous dropouts occurring in HCV CoC[6]. Rogers and Balistreri[1] reported innovative intervention approaches to promote HCV management through an academic mentorship program. This program intended to build a model that could improve the health of underserved communities in the Appalachian region. It has even reinforced the competences of primary health care providers to treat patients on site, thus reducing the need for travel to seek a DAA therapy specialist. The advent of telemedicine has also had a positive impact on HCV treatment. Extension for community health care outcomes through videoconferencing technology may be useful for HCV treatment in underserved communities[1].

This educative, extensive and informative model, combined with efficient and reliable HCV diagnostic and treatment efforts among pediatric patients along with preventive tools, should thoroughly address the hepatitis C elimination goal worldwide. The simultaneous screening of three viral infections (HIV, HCV and hepatitis B virus) using a multiplex immunochromatographic rapid diagnosis test [HCV/hepatitis B surface antigen/HIV Combo RDT Cassette (ITHD-C43), Biotest Biotech Inc, Hangzhou, China] in childbearing aged women may reinforce the prevention against these diseases [7]. This multiplex test may be useful in sub-Saharan Africa because it improves the "cascade of screening" and linkage to care with reduced cost[7].

The electronic medical record-based screening programs are also recommended for low-income areas since it was determined to be an effective method to manage patients for HIV and HCV in the Appalachian region[8]. Among the primary preventive means, we can enumerate campaigns for behavioral changes in high-risk groups, such as persons who inject drugs, and surveillance of pregnant women and diabetic and non-hepatic cancer patients.

However, high treatment costs and small numbers of trained providers are the primary reasons for slow HCV comprehensive elimination in low-income areas[9]. Successful efforts toward HCV elimination among persons who inject drugs contrast with the relative lack of attention to scarce resource settings where the hopes and potential for elimination are less clear, such as in many LMICs [10]. In addition, pretherapeutic tests and treatment access are still challenging in developing countries [11]. CoC is a major challenge in regard to the prison setting. Post release interventions that integrate

HCV care are highly effective. The treatment observance can be highly improved by a close collaboration between community and prison health care programs. Investigations are still needed to determine predictors of linkage to HCV care after release[12].

To overcome these barriers mentioned above, an efficient model for HCV CoC should be simple, targeted, pluralistic, scalable, integrated, patient-centered and affordable[13]. Efforts should focus on linkage to care to capitalize on DAA treatment advances and increase patient access to Medicaid assurance[14]. Regular follow-up of patients greatly increases HCV therapy effectiveness. Modelling studies show that a perfect follow-up would be cost-effective. Multilevel interventions along the cascade should be prioritized rather than single level[15]. Interventions that promote chronic hepatitis B/C elimination can be summarized as follow: Prioritize management of patients at multiple levels of the health care setting; define the required supports enabling to notice unassessed patients; expand disease management skill in a variety of settings; and promote harm reduction services[16].

Moreover, if major price reduction achieved by the HIV/AIDS community can be applied to HCV, a similar success story could emerge[17]. Domestic program and funding are continually implemented in many middle-income areas to support large scale DAA treatment, intended to achieve the WHO HCV elimination objective. However, investments required to achieve the WHO related elimination targets are insufficient given the feeble contribution from domestic financing sources and weakness of international funding for HCV programs. In fact, Unitaid partnership is starting with some countries to simplify and decentralize HCV CoC for a large scale feasibility. This donor is even encouraging coordination among other partners working in the field to support the Global Hepatitis Program for better response[18]. Models of service delivery are being piloted and implemented throughout the Western Pacific Region, which will support "learning by doing," in the delivery of hepatitis testing, care and treatment tailored to countries' unique health contexts[19].

Malaysia launched universal coverage of free testing and treatment for HCV in March 2018, with rapid expansion of services to decentralize testing and treatment in primary care clinics in 2019. In Vietnam, pilot programs are in progress to assess the feasibility of decentralizing HCV testing using point-of-care diagnostics to screen in different settings. In China, the entry of DAAs into the market changed conversations on the possibility of eliminating HCV and improving large-scale access to treatment. Many high-income countries in the Asian region universally covered hepatitis testing, care and treatment through government financing and health insurance.

However, challenges remain regarding vulnerable populations, including persons who inject drugs, incarcerated populations, migrants and indigenous people. These challenges involve increasing testing access and connection to care after diagnosis, improving public awareness of hepatitis B and C, training health care providers, particularly at the primary care level and addressing stigma and discrimination.

Medicines (DAAs) have revolutionized treatment for HCV, and they are becoming available in many LMICs, even if the cost can barely be borne by patients in a large majority of these countries. The market for generics has developed quickly but is volatile due to fluctuations in demand combined with inconsistent and insufficient financing. In the Asian region, affordability has improved as generic medicines have become more widely available. In countries where medicine prices remain high, access is limited by rationing and cost containment. On the other hand, where cheaper generics are available, financing and affordability by those most affected by HCV can still be a major barrier to access.

Out-of-pocket expenses vary widely across the Asian region and are still a significant barrier to access. There are significant differences in how countries control the prices of medicines, ranging from free pricing to single-payer, controlled pricing arrangements. Countries with single-payer systems achieved significant price controls for the new DAAs by using their monopoly purchasing power. For example, the Australian Department of Health negotiated a 5-year volume-based price deal based on treating 62000 people for AUS$ 1 billion. Over 38000 people were treated in the 1st year. This innovative approach removed price as a barrier to treatment. In New Zealand, the advantage of the Pharmaceutical Management Agency helps to secure favorable prices for medicines in negotiations with suppliers. Similar purchasing strategies could be used in other countries where medicines are financed by the government. Greater sharing of information on prices and negotiation strategies across the region is important to improve access to expensive medicines.

Another option for accessing affordable hepatitis medicine has been to take advantage of a commercial agreement related to intellectual property rights *a fortiori* in LMICs[19]. As far as HCV CoC is concerned, interesting and successful decentralized public health programs and government support have been tested in Africa (Rwanda, for instance) and Asia[20]. Moreover, Rwanda and Egypt have incredibly developed their national HCV programs in line with the WHO elimination targets, as reported by Shah *et al*[21]. However, to reach the above targets, the sub-Saharan African region must still overcome several barriers. Important investments and political engagement are needed to overcome these challenges[21]. From the period of 2014-2020, Egypt undertook a vast campaign of HCV screening and treatment in line with the disease elimination purpose. In fact, more than 50 million residents were screened for chronic HCV (4 million were treated). The key successful elements were as follow: Reliable epidemiological surveillance; robust public health system; inclusive care open to all social sectors; increased health care spending; and innovative research and use of new technologies of communication. Egypt is suggested to be the first country to eliminate HCV out of its territory. This expertise can be useful for other LMICs with high HCV burdens[22].

As far as the WHO HCV elimination goal is concerned, Mali has included viral hepatitis in the category of priority diseases through the promulgation of Law No. 2019-021 of July 3, 2019, which creates the sectorial unit to fight HIV/AIDS, tuberculosis and viral hepatitis and takes these diseases into account in the 10-year social health development plan 2014-2023. In this country, despite the political will that has been shown, there are still difficulties such as low level of knowledge of health workers on viral hepatitis, insufficient human resources, infrastructure and reagents for the management of viral hepatitis and financial and geographic (for the patients who reside outside Bamako) inaccessibility to antivirals[23]. The care centers for HCV patients, mainly made up of hepato-gastroenterology, infectious diseases and internal medicine departments, are concentrated in Bamako (the capital of Mali). DAA medicines such as sofosbuvir in combination with ledipasvir or velpatasvir, which are not provided in management centers, are sold at a high price in private pharmacies. These drugs are not covered by governmental compulsory health insurance. Other factors limiting access to treatment are the high costs of pretreatment investigations (170000 XOF by patient for HCV)[23].

The next key steps to strengthen interventions include: Sustaining efforts to reach major cost reduction of HCV management (for instance promotion of both generic DAAs and diagnosis tests) in all countries at a level comparable to HIV pricing; recording potential generic drug manufacturers at the country level; improving domestic and donor funding; reinforcing of monitoring and evaluation system by using digital tools to tract patients and manage stock; integrating programs such as HIV and sexually transmitted diseases, harm reducing service, primary health care and reproductive health; decentralizing and task delegation service; and further community engagement. Furthermore, early management of disease is important because it is cheaper to treat HCV early than treat complications *a fortiori* in LMICs where resources are scarce[20].

Pediatric patients with liver fibrosis evidence must be regularly monitored given the constant risk of portal hypertension and hepatocellular carcinoma occurrence. Early treatment can save money and lives. Furthermore, the actions taken to improve access to health care in certain disadvantaged regions of high-income countries, as seen in the Appalachian region, can be usefully employed in low-income countries.

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FOOTNOTES

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Country/Territory of origin: Mali

ORCID number: Nouhoum Bouare 0000-0002-8362-6740; Mamadou Keita 0000-0001-5572-8996; Jean Delwaide 0000-0001-7894-8123.

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LETTER TO THE EDITOR

Comments on "Effect of type 2 diabetes mellitus in the prognosis of acute-on-chronic liver failure patients in China"

Wei Wang, Chen-Chen Pan, Wen-Ying Zhao, Jin-Yu Sheng, Qi-Qi Wu, Si-Si Chen

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Wei Wang, Department of Interventional Oncology, Taizhou Municipal Hospital, Taizhou 318000, Zhejiang Province, China

Chen-Chen Pan, Wen-Ying Zhao, Jin-Yu Sheng, Qi-Qi Wu, School of Medicine Taizhou University, Taizhou 318000, Zhejiang Province, China

Si-Si Chen, Department of Obstetrics and Gynecology, Taizhou Women and Children's Hospital of Wenzhou Medical University, Taizhou 318000, Zhejiang Province, China

Corresponding author: Si-Si Chen, MD, Occupational Physician, Department of Obstetrics and Gynecology, Taizhou Women and Children's Hospital of Wenzhou Medical University, No. 188 Qianjin Village, Jiajia Street, Jiaojiang District, Taizhou 318000, Zhejiang Province, China. chensisiobstetrics@126.com

Abstract

A study addressing the influence of type 2 diabetes on the prognosis of acute-on-chronic liver failure patients was reviewed. Some statistical deficiencies were found in the reviewed article, and the sample size was too small to support the study. In addition, age should have been considered as one of the prognostic factors.

Key Words: Type 2 diabetes mellitus; Liver failure; Complication; Prognosis; Age

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Core Tip: This is a comment on a study of the influence of type 2 diabetes on the prognosis of patients with acute-on-chronic liver failure. We believe that the conclusion of this study can provide more significant data.

Citation: Wang W, Pan CC, Zhao WY, Sheng JY, Wu QQ, Chen SS. Comments on "Effect of type 2 diabetes mellitus in the prognosis of acute-on-chronic liver failure patients in China". *World J Gastroenterol* 2022; 28(14): 1499-1502
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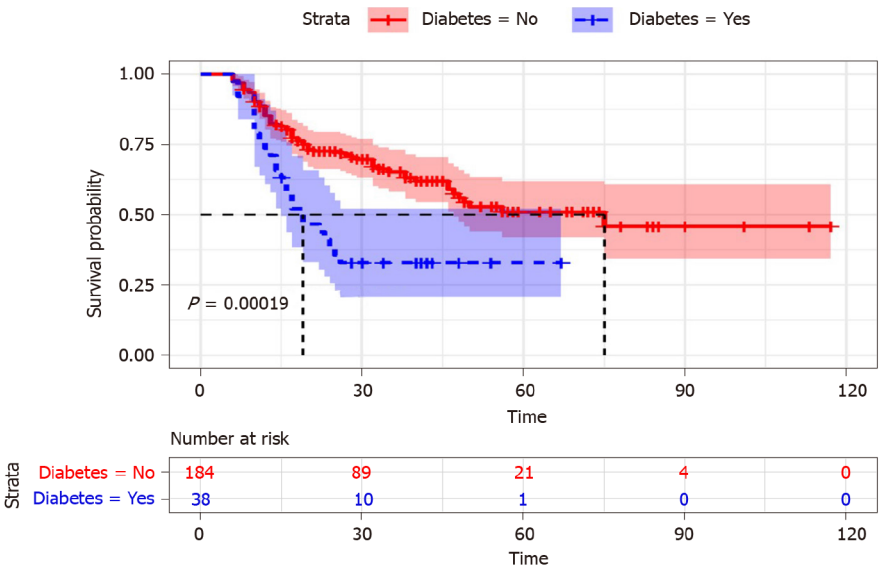


Figure 1 Cumulative survival time in acute-on-chronic liver failure patients with diabetic mellitus and non-diabetic mellitus[1]. Citation: Lai RM, Chen TB, Hu YH, Wu G, Zheng Q. Effect of type 2 diabetic mellitus in the prognosis of acute-on-chronic liver failure patients in China. *World J Gastroenterol* 2021; 27: 3372-3385. Copyright© The Authors 2020. Published by Baishideng Publishing Group Inc.

TO THE EDITOR

We were pleased to read the high-level article published by Lai *et al*[1]. The results of their study showed a significant relationship between the prognosis of diabetes mellitus (DM) and acute-on-chronic liver failure (ACLF) patients. ACLF patients with DM have higher in-hospital mortality and infection rates than patients without DM. This is an important study that has made a significant contribution to the study of prognostic indicators for ACLF patients. However, there are still questionable issues in this study that we would like to discuss with the authors.

First, the Materials and Methods section needs to be more detailed and refined. The author should list the number of diabetic patients included and the number of people after grouping. The author should state the time for follow-up of the patients and redefine the concept of follow-up. If the author regards the discharge time as the end of the follow-up event, it may cause data loss. Therefore, another reference could be added for loss to follow-up. We notice that they performed multivariate analysis on parameters with $P < 0.1$ in univariate analysis, which is not convincing. A P value less than 0.05 would make the results more convincing. In addition, a sample of 200 for a study period of 7 years is small; the author should calculate the population size and analyze the sample size.

There are problems in the statistical analyses. Statistical analyses should be described in detail. In the table in the Results section, the author should provide more accurate statistical values, such as Student's t-values or χ^2 values. Table 1 shows that there are statistical differences in age between the DM group and non-DM group. Therefore, the author should describe the frequency and distribution of age in more detail and discuss the possible impact of age as a potential risk factor on the disease. For example, the author could divide the patients into different levels in the DM and non-DM groups according to age and statistically analyze the impact of different ages in each level on the mortality and infection rate of related diseases. According to the criteria of the World Health Organization in 2012[1,2], a younger age was defined as less than 45 years, and an older age was defined as greater or equal to 45 years. Therefore, the author could divide the original two groups into four groups. In Figure 1 (Figure in the manuscript "Effect of type 2 diabetes mellitus in the prognosis of acute-on-chronic liver failure patients in China"[1]), the author should indicate the time unit. We do not know how long the patients lived. In addition, we also noticed that the author did not indicate the corresponding P value when stating some conclusions.

In the paper, the author repeatedly proposed that DM could predict ACLF. This is a confusing statement. The study only proves that DM has a certain influence on ACLF. To further predict ACLF through DM, a survival model would need to be established. The author should provide more detailed data, such as median follow-up and survival times, to build up the survival model.

In the Discussion section, the author explained that albumin (ALB) has nothing to do with the prognosis of liver failure in this study and believed that exogenous injection of ALB interfered with the experimental results. Inferring from this, we believe that the author neglected interference caused by the patients' medications on the experimental results during treatment. ALB constitutes about half of serum proteins. ALB is involved in scavenging free radicals, maintaining colloidal osmotic pressure and protecting neuronal cells and is closely related to nutritional level[3] and systemic inflammatory

Table 1 Demographic characteristics and clinical features of the patients between diabetic mellitus and non-diabetic mellitus[1]

	DM (yes) (*n* = 38)	DM (no) (*n* = 184)	*P* value
Age (yr)	56.32 ± 14.23	49.16 ± 12.84	0.002
Gender, *n* (%)			0.309
Male	28 (73.68)	149 (80.98)	
Female	10 (26.32)	35 (19.02)	
Cause of disease, *n* (%)			0.201
Hepatitis B virus	26 (68.42)	139 (75.54)	
Hepatitis B virus + other	5 (13.16)	20 (10.87)	
Alcohol	2 (5.26)	15 (8.15)	
Others	5 (13.16)	10 (5.44)	
WBC (10^9/L)	6.17 ± 4.03	7.35 ± 3.58	0.07
RBC (10^{12}/L)	3.68 ± 0.87	3.94 ± 0.84	0.084
Hb (g/L)	117.21 ± 24.71	121.95 ± 23.13	0.257
PLT (10^9/L)	100.34 ± 42.20	118.79 ± 59.09	0.069
PT (s)	23.01 ± 5.38	24.45 ± 6.95	0.229
INR	1.97 ± 0.45	2.10 ± 0.59	0.229
ALT (U/L)	396.08 ± 448.56	560.36 ± 693.06	0.163
AST (U/L)	365.95 ± 391.18	419.99 ± 513.42	0.541
γ-GGT (U/L)	174.16 ± 305.61	137.57 ± 127.33	0.231
TBIL (μmol/L)	320.71 ± 141.31	309.56 ± 134.00	0.664
ALB (g/L)	29.25 ± 4.51	30.73 ± 4.03	0.045
Scr (μmol/L)	56.37 ± 22.00	63.45 ± 27.28	0.134
BUN (mmol/L)	3.94 ± 2.65	4.25 ± 2.98	0.56
TCHO (mmol/L)	2.67 ± 0.81	2.65 ± 1.05	0.919
TG (mmol/L)	1.45 ± 0.67	1.26 ± 0.70	0.124
Na^+ (mmol/L)	136.98 ± 3.97	136.86 ± 4.43	0.878
K^+ (mmol/L)	3.90 ± 0.46	4.08 ± 0.58	0.072
AMON (μmol/L)	3.90 ± 0.46	4.08 ± 0.58	0.332
AFP (ng/mL)	64.40 ± 40.39	128.19 ± 192.02	0.784
BMI (kg/m^2)	24.99 ± 3.32	22.78 ± 3.03	< 0.001
FBG (mmol/L)	5.34 ± 1.87	3.83 ± 1.07	< 0.001
Scoring systems			
CTP	10.79 ± 1.49	10.40 ± 1.35	0.115
MELD	19.38 ± 4.52	20.74 ± 5.06	0.128
MELD-Na	20.89 ± 5.00	22.27 ± 6.84	0.239
Death, *n* (%)	25 (65.79)	69 (37.5)	0.001

γ-GGT: Gamma-glutamyl transpeptidase; AFP: Alpha fetal protein; ALB: Albumin; ALT: Alanine aminotransferase; AMON: Ammonia; AST: Aspartate aminotransferase; BMI: Body mass index; BUN: Blood urea nitrogen; CTP: Child-Turcotte-Pugh; DM: Diabetic mellitus; FBG: Fasting blood-glucose; Hb: Hemoglobin; INR: International normalized ratio; K+: Kalium; MELD: Model for End-Stage Liver Disease; MELD-Na: Model for End-Stage Liver Disease with serum sodium; Na+: Natrium ion; PLT: Platelet count; PT: Prothrombin time; Scr: Serum creatinine; TBIL: Total bilirubin; TCHO: Total cholesterol; TG: Triglyceride; RBC: Red blood cell; WBC: White blood cell. Citation: **Lai RM**, Chen TB, Hu YH, Wu G, Zheng Q. Effect of type 2 diabetic mellitus in the prognosis of acute-on-chronic liver failure patients in China. *World J Gastroenterol* 2021; **27**: 3372-3385. Copyright© The Authors 2020. Published by Baishideng Publishing Group Inc.

response[4]. The author should describe in detail in the Methods section the type, dosage and method of drug injection during the treatment process. Actually, well-established indications for the use of human ALB in patients with cirrhosis pertain to conditions that are characterized by an acute deterioration of effective hypovolemia[5], such as renal dysfunction secondary to spontaneous bacterial peritonitis and hepatorenal syndrome, as shown by the international guidelines on the management of decompensated cirrhosis[6]. The current established indications for ALB use in patients with cirrhosis, such as preventing renal dysfunction induced by systolic blood pressure, suggest that patients should receive 1.5 g/kg body weight at diagnosis +1 g/kg body weight at day 3[7].

All in all, this is a high-level article in the field of diabetes and liver cirrhosis. Although there are some statistical deficiencies in the article, the author may be able to expand the sample and establish a multicenter prospective cohort study. For example, age factors could be considered for related diseases to evaluate the impact of DM on the prognosis in ACLF patients.

FOOTNOTES

Author contributions: Wang W and Chen SS contributed equally to this work; Wang W, Chen SS, Pan CC, Zhao WY, Sheng JY and Wu QQ designed the research study; Wang W, Chen SS, Pan CC and Zhao WY performed the research; Wang W and Chen SS contributed to new reagents and analytic tools; Wang W, Pan CC and Zhao WY analyzed the data and wrote the manuscript; All authors have read and approve the final manuscript.

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Country/Territory of origin: China

ORCID number: Wei Wang 0000-0001-5630-3287; Chen-Chen Pan 0000-0001-8234-6870; Wen-Ying Zhao 0000-0002-8190-2561; Jin-Yu Sheng 0000-0002-6132-4637; Qi-Qi Wu 0000-0002-4997-1022; Si-Si Chen 0000-0003-0125-8022.

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Editorial Board Member of *World Journal of Gastroenterology*, Masahito Nakano, MD, PhD, Assistant Professor, Division of Gastroenterology, Department of Medicine, Kurume University School of Medicine, 67 Asahi-machi, Kurume, Fukuoka 830-0011, Japan. nakano_masahito@kurume-u.ac.jp

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EDITORIAL

Liquid biopsy in colorectal cancer: No longer young, but not yet old

Giandomenico Roviello, Daniele Lavacchi, Lorenzo Antonuzzo, Martina Catalano, Enrico Mini

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Giandomenico Roviello, Enrico Mini, Department of Health Sciences, Section of Clinical Pharmacology and Oncology, University of Florence, Firenze 50139, Italy

Daniele Lavacchi, Clinical Oncology Unit, AOU Careggi, Firenze 50134, Italy

Lorenzo Antonuzzo, Department of Experimental and Clinical Medicine, University of Firenze, Firenze 50134, Italy

Martina Catalano, School of Human Health Sciences, University of Florence, Florence 50134, Italy

Corresponding author: Giandomenico Roviello, MD, PhD, Assistant Professor, Department of Health Sciences, Section of Clinical Pharmacology and Oncology, University of Florence, Viale Pieraccini, 6, Firenze 50139, Italy. giandomenico.roviello@unifi.it

Abstract

Colorectal cancer (CRC) is one of the most prevalent cancers and the second leading cause of cancer-related deaths worldwide. The treatment strategy employed in CRC patients is becoming highly dependent on molecular characteristics present at diagnosis and during treatment. Liquid biopsy is an emerging field in the management of this cancer, and its relevance as a potential diagnostic, prognostic, monitoring, and therapeutic tool makes it a viable strategy in the clinical management of CRC patients. Liquid biopsy also has certain limitations, but these limitations seem to be at the reach of near-future technological development. In this letter, we focus on the clinical perspectives of liquid biopsy in CRC with particular regard to the various biomarkers recently identified that have been shown to be potentially useful in multiple aspects of early stage or metastatic CRC.

Key Words: Colorectal cancer; Liquid biopsy; Circulating tumor DNA; Diagnosis; Prognosis; Targeted therapy

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Core Tip: Liquid biopsy through analysis of biological components, such as circulating nuclear acids, circulating tumor cells, and more recently exosomes in body fluids, has shown good capacity to overcome several limitations faced by conventional tissue biopsies, in particular invasiveness and unrepeatability. Liquid biopsy has shown significant results in clinical applications in different types of cancer, especially colorectal cancer (CRC). Indeed, liquid biopsy can be used to detect CRC at an early stage, make treatment decisions, monitor response to treatment, predict relapses and metastases, reveal tumor heterogeneity, and detect minimal residual disease.

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INTRODUCTION

The strategy employed for the treatment of colorectal cancer (CRC) patients is becoming highly dependent on molecular characteristics at diagnosis and during the course of treatment. New therapeutic options have been shown to be effective for metastatic disease, including immune checkpoint inhibitors (ICIs), HER2-directed antibody-drug conjugates, chemotherapy-free regimens for BRAFV600-mutated tumors, and new agents targeting the RAS signaling pathway[1,2].

In recent years, peripheral blood has been extensively studied as a new source of information and alternative to tumor biopsy samples, but its potential has not yet been elucidated. Among the most studied components, circulating tumor cells (CTCs), circulating tumor DNA (ctDNA), and, more recently, exosomes have been considered promising biomarkers for monitoring treatment response, longitudinal molecular profiling, prognostication, and detection of minimal residual disease[3].

These advances coincided with the progressive increase in the accessibility and cost-effectiveness of next-generation sequencing (NGS) and with the development of new therapeutic approaches, including tumor-agnostic treatments[4].

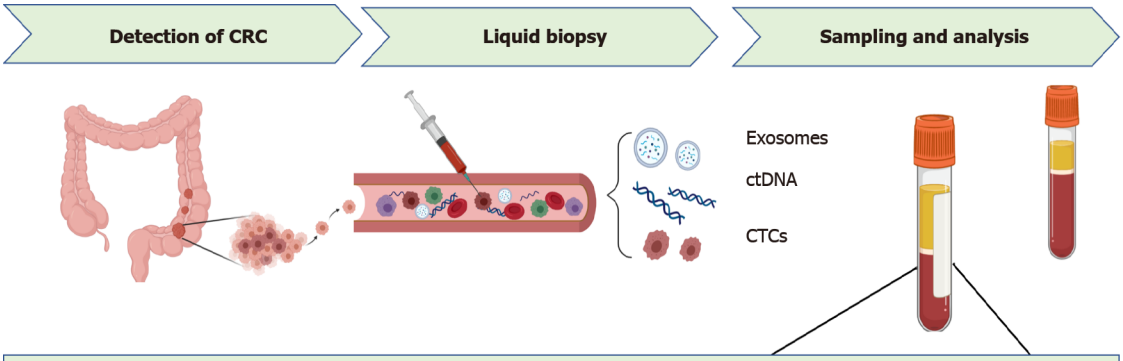
LIQUID BIOPSY APPLICATION

CTC detection and enumeration have been studied as prognostic markers in several cancers. Sastre *et al*[5] evaluated the prognostic effect of the presence of CTCs detected using the CellSearch System in CRC patients (n = 97). The authors showed an association between CTC detection and stage. With a mean number of 3.4/7.5 mL CTCs, a higher rate of CTC positivity was observed in patients with stage IV disease (60.7%) than in those with stage II-III disease (20.7%-24.1%). Interestingly, no CTCs were detected in the healthy population[5]. Although CTC determination could represent a reliable surrogate for tumor burden, the possibility of longitudinal profiling of the disease offers far greater advantages for clinical practice than mere enumeration.

One of the most promising applications of liquid biopsy is the detection of early-stage CRC. For this purpose, CTC detection and quantification were studied with conflicting results regarding the discrimination of cancerous, precancerous, and other benign lesions[6]. Then, epigenetic changes were investigated to achieve a greater specificity. Methylated SEPT9 promoter DNA has been shown to be a potential biomarker with some limitations[7]. The interpretation of the results was, indeed, highly dependent on the choice of a favorable balance between sensitivity and specificity[8]. The detection of methylated SEPT9 promoter DNA through a real-time polymerase chain reaction assay was validated in a prospective study and recently received FDA approval for CRC screening[9]. However, several limitations deserve to be considered, including the rates of false-positive and negative results, reproducibility, need for confirmatory tests, and schedule of tests over time.

In the last few years, ctDNA has been extensively studied to identify minimal residual disease. A prospective multicenter study conducted by Henriksen *et al*[10] evaluated the preoperative and postoperative ctDNA status in stage I-III CRC. Overall, postoperative ctDNA was associated with relapse-free survival (P < 0.001). In addition, the detection of ctDNA made it possible to anticipate radiological relapse by approximately eight months[10]. Consistent with this finding, Kasi *et al*[11] evaluated the ctDNA status in a cohort of 250 patients using Signatera liquid biopsy. The ctDNA detection rate was significantly associated with stage and response to treatment[11].

Another application of liquid biopsy for the treatment of metastatic CRC involves the monitoring of RAS mutational status to select patients for anti-EGFR rechallenge. In the CRICKET trial, no responses were observed with cetuximab-based chemotherapy in RAS and RAF wild-type mCRC patients who had RAS-mutated ctDNA at the time of progression. Given that RAS mutations in ctDNA could be



Diagnosis

Gene methylation status on ctDNA (*e.g.* methylated SEPT9 promoter DNA)

Prognosis

Isolation and enumaration of CTCs

Quantification of exosomal miRNAs (*e.g.* exosome miR-17-92°)

Detection of microsatellite instability status and tumor mutationl burden

Treatment decision

Molecular assessment of ctDNA, CTCs or exosomes to predict primary resistance to EGFR blockade

Identification of patients amenable to rechallenge with anti-EGFR

Monitoring response

Real time identification of tumor cell sub-clones conferring acquired resistance to anti-EGFR agents

Residual disease

ctDNA detection after surgery I-III stage CRC

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Figure 1 Potential clinical uses of liquid biopsy in colorectal cancer. ctDNA: Circulating tumor DNA; CTC: Circulating tumor cell; CRC: Colorectal cancer.

detected in approximately half of the patients at the beginning of third-line treatment[12], longitudinal monitoring of the disease is essential to offer potentially effective treatments to patients with few residual therapeutic opportunities. In addition, molecular selection in chemorefractory patients allows us to avoid unnecessary side effects for ineffective treatments. However, at the time of diagnosis, the molecular alterations in tissue biopsy cannot be replaced with those in liquid biopsy until the key issue of the concordance of RAS mutational status between plasma and tumor tissue is completely resolved. The results may differ depending on the methods used. To date, several prospective trials have demonstrated a high concordance between the two techniques that is approximately equal to 85%-95% [13,14].

Exosome quantification was initially studied as a prognostic factor associated with clinical and pathological parameters and, consequently, survival outcomes[15]. Then, several studies analyzed specific exosomal miRNAs. Among these, Matsumura *et al*[16] showed that CRC patients had higher levels of exosomal miR-17-92a than the control group. In addition, the authors identified the expression of exosomal miR-17-92a in peripheral blood as a prognostic factor for CRC patients[16]. Additionally, exosomal RNAs are used in the early diagnosis of cancers. For example, a panel consisting of two mRNAs (KRTAP5-4 and MAGEA3) and one lncRNA (BCAR4) serves as a promising candidate for CRC diagnosis[17-19].

Other studies are exploring the possibility of detecting other predictors of response, including microsatellite instability status and tumor mutational burden. The noninvasive detection of biomarkers predictive of response to ICIs is eagerly awaited to personalize treatments. However, their identification is profoundly limited by the amount of ctDNA and validation of specific assays. To date, there are no data to support their routine use[20,21].

CONCLUSION

In conclusion, liquid biopsy is no longer considered a mere surrogate of tumor biopsy with minimal invasiveness (Figure 1). New perspectives could radically change clinical practice[22]. Further advances will include refining serum sequencing techniques to gain a deeper understanding of tumor temporal heterogeneity and promote accessibility to tumor-agnostic treatments. In addition, the real-time monitoring of drug resistance beyond RAS may offer the opportunity to guide and monitor new therapies, such as anti-RAS agents, BRAFV600-directed or HER2-directed treatments, and ICIs. In addition, monitoring plasma with amplicon-based NGS in CRC patients may offer high sensitivity in detecting low-frequency mutations and promote the identification of clones with potentially targetable alterations. All these aspects will be crucial to ensure the paradigm of a continuum of care for metastatic CRC patients.

FOOTNOTES

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Country/Territory of origin: Italy

ORCID number: Giandomenico Roviello 0000-0001-5504-8237; Daniele Lavacchi 0000-0001-9749-9511; Lorenzo Antonuzzo 0000-0003-3349-5604; Martina Catalano 0000-0003-1856-2848; Enrico Mini 0000-0003-1853-8557.

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REVIEW

Novel approaches in search for biomarkers of cholangiocarcinoma

Lavinia-Patricia Mocan, Maria Ilieș, Carmen Stanca Melincovici, Mihaela Spârchez, Rareș Crăciun, Iuliana Nenu, Adelina Horhat, Cristian Tefas, Zeno Spârchez, Cristina Adela Iuga, Tudor Mocan, Carmen Mihaela Mihu

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Lavinia-Patricia Mocan, Carmen Stanca Melincovici, Carmen Mihaela Mihu, Department of Histology, Faculty of Medicine, "Iuliu Haţieganu" University of Medicine and Pharmacy, Cluj-Napoca 400012, Romania

Maria Ilieș, Cristina Adela Iuga, Department of Proteomics and Metabolomics, MedFUTURE Research Center for Advanced Medicine, "Iuliu Haţieganu" University of Medicine and Pharmacy, Cluj-Napoca 400349, Romania

Mihaela Spârchez, 2nd Pediatrics Department, Faculty of Medicine, "Iuliu Haţieganu" University of Medicine and Pharmacy, Cluj-Napoca 400012, Romania

Rareș Crăciun, Iuliana Nenu, Adelina Horhat, Cristian Tefas, Zeno Spârchez, Tudor Mocan, 3rd Medical Department, Faculty of Medicine, "Iuliu Haţieganu" University of Medicine and Pharmacy, Cluj-Napoca 400012, Romania

Rareș Crăciun, Iuliana Nenu, Adelina Horhat, Cristian Tefas, Zeno Spârchez, Tudor Mocan, Department of Gastroenterology, "Prof. dr. Octavian Fodor" Institute for Gastroenterology and Hepatology, Cluj-Napoca 400162, Romania

Cristina Adela Iuga, Department of Pharmaceutical Analysis, Faculty of Pharmacy, "Iuliu Haţieganu" University of Medicine and Pharmacy, Cluj-Napoca 400012, Romania

Corresponding author: Maria Ilieș, PhD, Academic Research, Department of Proteomics and Metabolomics, MedFUTURE Research Center for Advanced Medicine, "Iuliu Haţieganu" University of Medicine and Pharmacy, Marinescu 23, Cluj-Napoca 400349, Romania. ilies.maria@umfcluj.ro

Abstract

Cholangiocarcinoma (CCA) arises from the ductular epithelium of the biliary tree, either within the liver (intrahepatic CCA) or more commonly from the extrahepatic bile ducts (extrahepatic CCA). This disease has a poor prognosis and a growing worldwide prevalence. The poor outcomes of CCA are partially explained by the fact that a final diagnosis is challenging, especially the differential diagnosis between hepatocellular carcinoma and intrahepatic CCA, or distal CCA and pancreatic head adenocarcinoma. Most patients present with an advanced disease, unresectable disease, and there is a lack in non-surgical therapeutic modalities. Not least, there is an acute lack of prognostic biomarkers which further complicates disease management. Therefore, there is a dire need to find alternative diagnostic and follow-up pathways that can lead to an accurate

result, either singlehandedly or combined with other methods. In the "-omics" era, this goal can be attained by various means, as it has been successfully demonstrated in other primary tumors. Numerous variants can reach a biomarker status ranging from circulating nucleic acids to proteins, metabolites, extracellular vesicles, and ultimately circulating tumor cells. However, given the relatively heterogeneous data, extracting clinical meaning from the inconsequential noise might become a tall task. The current review aims to navigate the nascent waters of the non-invasive approach to CCA and provide an evidence-based input to aid clinical decisions and provide grounds for future research.

Key Words: Cholangiocarcinoma; Biomarker; Proteomics; Metabolomics; Extracellular vesicles; Circulating nucleic acids

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Core Tip: The current review paper aims to critically analyze the most recent developments in non-invasive cholangiocarcinoma diagnosis and prognosis. The article takes an in-depth look at the fields of circulating nucleic acids, proteomic and metabolomic-derived biomarkers, extracellular vesicles, and circulating tumor cells in an attempt to outline promising results for future research and clinical use.

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INTRODUCTION

Cholangiocarcinoma (CCA) is a malignant tumor arising from the biliary epithelial cells. The latest World Health Organization Classification of Tumors-Digestive System Tumors acknowledges the heterogeneous nature of CCA, emphasizing the importance of tumor localization. In this matter, there are two main types of CCAs: Intrahepatic CCA (iCCA) and extrahepatic CCA (eCCA) [including both: Perihilar CCA (pCCA) and distal (dCCA)], featuring different aspects in etiology, molecular alterations, pathogenesis, behavior, potential diagnostic or prognostic biomarkers and hence a different clinical management[1].

iCCAs represent approximately 10%-15% of liver tumors and the second primary liver malignancy, after hepatocellular carcinoma (HCC)[2], while eCCAs account for 0.5-2 cases/10.000 person-years[3]. Although considered a relatively rare type of cancer, the incidence of CCA is rising in most geographic areas[4]. Both HCC and iCCA, although they are considered different diseases, do share some common risk factors including hepatitis B or C, non-biliary hepatic cirrhosis, alcoholic and non-alcoholic steato-hepatitis, or metabolic syndrome. On the other hand, eCCA typically occurs in conditions associated with chronic biliary inflammation, such as primary sclerosing cholangitis, lithiasis, cysts, or liver fluke infections. In most cases, the exact etiology remains difficult to pinpoint[5].

To this point, CCA is notoriously difficult to diagnose. Diagnosing these tumors requires the correlation of clinical, imaging, and, when available, histopathologic data. In terms of treatment, surgical resection with curative intent remains the best option. However, most patients with CCA (approximately 70%) are diagnosed at late stages due to lack of specific symptoms[6]. Mortality rates are high, and thus the prognosis is poor[7], especially in the case of large tumors, satellite nodules, vascular or lymphatic invasion, positive resection margins, or advanced pathological tumor-node-metastasis stages (TNM)[8,9]. For surgically resectable tumors, the 5-year survival rate reaches 20%-30%, but the percentage drops to a bitter 0% for the rest of the cases[10]. After surgery, the recurrence rate is relatively high, reaching from 49% to 70%[11] and relapse occurs early, typically within 2 or 3 years after surgery[8].

These circumstances emphasize the necessity of novel, clinical-suited tools that would serve for early diagnosis, as prognostic indicators or in treatment guidance, such as biomarkers.

Biomarkers were defined by the Food and Drug Administration-National Institute of Health Biomarker Working Group back in 2016 as “a defined characteristic that is measured as an indicator of normal biological processes, pathogenic processes, or responses to an exposure or intervention, including therapeutic interventions. Molecular, histologic, radiographic, or physiologic characteristics are types of biomarkers.”. While α-fetoprotein (AFP) is the most convenient and non-invasive serum

biomarker for detecting HCC, elevated AFP was observed only in approximately 20% of a subgroup of CCA, namely iCCA patients[12].

The charbohydrate antigen 19-9 (CA19-9) is currently used worldwide in clinical practice as a non-specific serum marker for orientation in diagnosing CCA, but it bears a low sensitivity in European patients[13]. In terms of prognosis there are some validated tools that are useful in the clinical practice. These markers are not specific for CCA but rather apply to all human malignancies. Tumor size and differentiation, vascular involvement, lymph node status, margin status and presence of occult metastasis were all shown to be good predictors for overall survival (OS) for both iCCA and eCCA[14].

One option could be the study of tumor tissue in search of novel biomarkers. This strategy appeared to be fruitful, as several tumor tissue-based biomarkers were already identified. Mutations in TP53 and KRAS proto-oncogene are associated with an impaired outcome-lower OS and higher tumor recurrence than other mutations in resected CCA while several other genetic signatures with prognostic potential include epidermal growth factor receptor (EGFR), mucin 1 (MUC1), MUC4, and fascin (FSCN) expression[15]. Moreover, alteration in targetable pathways [*e.g.*, fibroblast growth factor receptor 2 gene (FGFR2) involved in MAP kinase signaling, isocitrate dehydrogenase 1 and 2 (IDH1, IDH2)] were also depicted in CCA patients[16] and currently, several clinical trials are actively recruiting patients. Nevertheless, several microRNAs (miRs) expressions in tissue or deregulated immune responses [expression levels of cytotoxic T-lymphocyte antigen 4 (CTLA-4), forkhead box P3 (FOXP3), and programmed death-ligand 1 (PD-L1)] might have predictive capabilities in CCA patients[17,18]. Many other diagnostic and prognostic tissue-derived biomarkers have already been previously described[19].

Unfortunately, biopsy collection for tissue analysis is not an ideal biospecimen for biomarker assessment and translation to clinical practice. Although it offers absolute insights into tumor biology, the collection procedure presents several caveats and poses the risk of serious clinical complications [20]. As an alternative to tissue biopsy, a much more reliable biospecimen, already implemented in the clinical practice with several advantages over tissue, is the liquid biopsy (blood). Serum, plasma, or urine, collected non-invasively using well-established low-cost techniques are considered "ideal fluids" in biomarker research. Moreover, liquid biopsy encloses molecules from the whole body, and a single sample can offer a wide range of information and is enough for multiple measurements.

The current review aims to explore the nascent waters of the non-invasive biomarkers reported for CCA by taking an in-depth look at the fields of circulating nucleic acids, proteomic and metabolomic-derived biomarkers, extracellular vesicles, and circulating tumor cells (Figure 1) and provide an evidence-based input which could provide grounds for future research to pave the way for prospective validation and translation into the clinical practice of novel biomarkers. In this review the term CCA will make reference to all types of CCA, while the terms iCCA, hCCA, pCCA, and dCCA will stand for intrahepatic, hilar, perihilar and distal CCA, respectively.

CIRCULATING NUCLEIC ACIDS

Circulating nucleic acids represent snippets of genetic material, either DNA (cell-free DNA–cfDNA) or RNA (usually miR), reaching various fluid compartments (serum, urine, bile) through active cellular export or following cell death. The road from bench to bedside for circulating nucleic acids has taken a relatively long time and has not quite reached the point of clinical applicability in cancer diagnosis. However, more than four decades have passed between the initial proof-of-concept[21] and present-day genome-wide cfDNA mutational integration[22]. The world of cfDNA and miRs seems to be emerging more promising than ever, as the highly effervescent field has started to deliver on the early expectations. Moreover, CCA might provide a unique setting for the method to flourish: A conventional diagnostic challenge, sometimes a hard to biopsy tumor, all while having a relatively underdeveloped therapeutic arsenal.

CfDNA–the mutational fingerprint

The analysis of circulating cfDNA can provide a quick, complete, and non-invasive mutational profile of any tumor, by amplifying each mutation encountered throughout the tumor burden. The method reflects the entirety of mutations, thus not being the subject of selection bias in the case of heterogeneous cancers. More specifically, tissue samples can provide the mutational palette only for the available specimen. Therefore, the genetic fingerprint of a tumor might be incomplete, as metastases or distant regions of a tumor might have additional alterations. Consequently, cfDNA provides (at least in principle) a better understanding of the disease, with a substantial impact in disease management, from diagnostics to guide therapeutic choices.

This concept has been recently validated for CCA, using plasma samples of patients with fully characterized mutation status[23]. According to the study design, 31 mutations in the *KRAS*, *NRAS*, *BRAF*, and *PIK3CA* genes were screened using multiplex polymerase chain reaction (PCR) and further quantified. The results were then compared with the mutational profile of the primary tumor, resulting in a perfect match. These results were partially reinforced by the work of a German team that performed the deep sequencing of 15 genes involved in CCA (n = 32), revealing a 74% overall blood-tissue sample

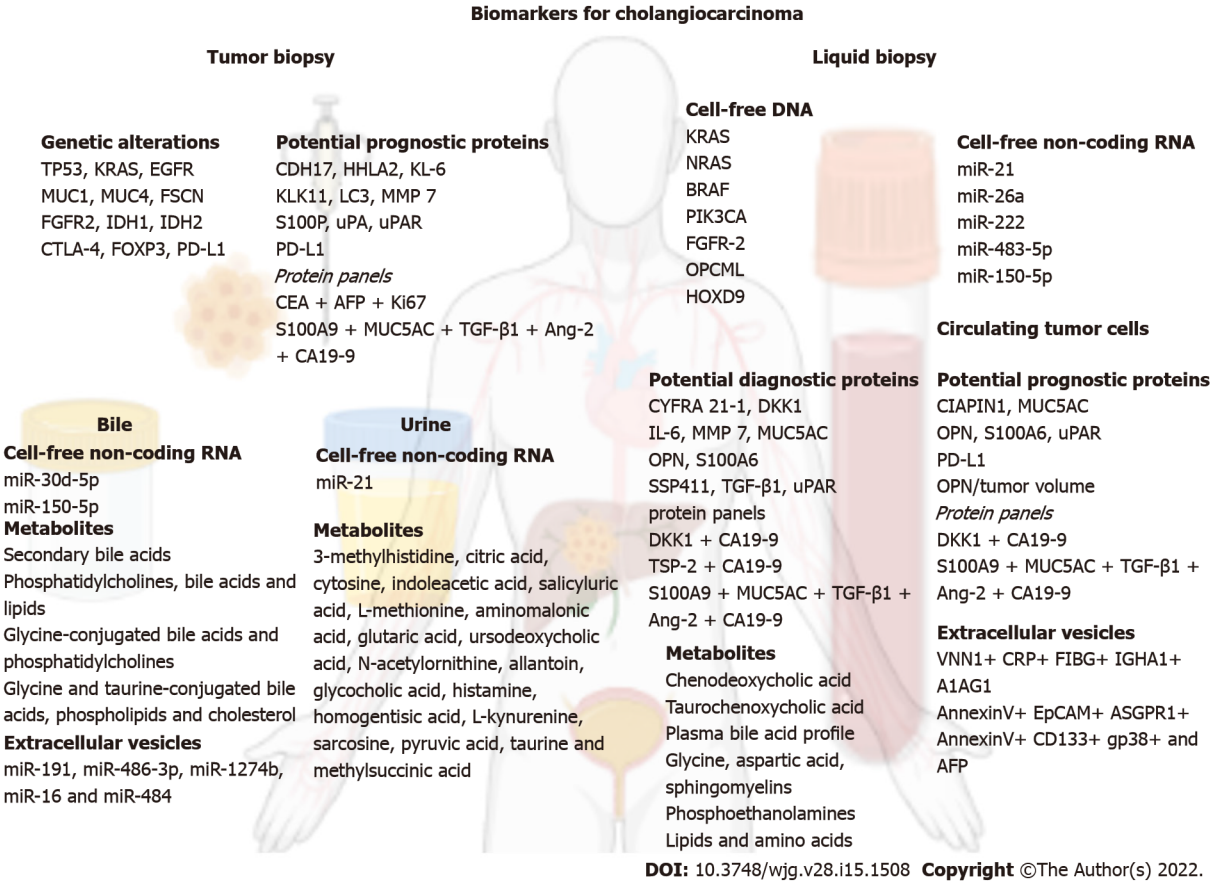


Figure 1 An overview on the biomarkers for cholangiocarcinoma. Created with biorender.com. A1AG1: Alpha-1 acid glycoprotein; AFP: Alpha fetoprotein; Ang-2: Angiopoietin-2; ASGPR1: Asialoglycoprotein receptor 1; CA19-9: Charbohydrate antigen 19-9; CDH17: Cadherin-17; CEA: Carcinoembryonic antigen; CIAPIN1: Cytokine-induced apoptosis inhibitor 1; CRP: C-reactive protein; CTLA-4: Cytotoxic T-lymphocyte antigen 4; CYFRA 21-1: Cytokeratin 19 fragment; DKK1: Dickkopf-1; EGFR: Epidermal growth factor receptor; EpCAM: Epithelial cell adhesion molecule; FGFR2: Fibroblast growth factor receptor 2; FIBG: Fibrinogen gamma chain; FOXP3: Forkhead box P3; FSCN: Fascin; HHLA2: Human endogenous retrovirus-H long terminal repeat-associating protein 2; IDH1: Isocitrate dehydrogenase 1; IDH2: Isocitrate dehydrogenase 2; IGHA1: Immunoglobulin heavy constant alpha 1; IL-6: Interleukin 6; Ki67: Proliferation marker protein Ki67; KL-6: Krebs von den Lungen 6; KLK11: Kallikrein related peptidase 11; LC3: Microtubule-associated protein 1A/1B-light chain 3; MMP-7: Metalloproteinase 7; MUC1: Mucin 1; MUC4: Mucin 4; MUC5AC: Mucin 5AC; OPN: Osteopontin; PD-L1: Programmed death-ligand 1; S100A6: S100 calcium-binding protein A6; S100A9: S100 calcium-binding protein A9; S100P: Tissue protein S100P; SSP411: Spermatogenesis-associated protein 20; TGF-β1: Transforming growth factor-β1; TSP-2: Thrombospondin-2; uPA: Urokinase-type plasminogen activator; uPAR: Urokinase-type plasminogen activator receptor; VNN1: Pantetheinase.

concordance and 92% for intrahepatic tumors. Moreover, the patients were followed throughout chemotherapy, during which 63% of the patients had their mutational fingerprint altered[24]. This finding might have particular implications regarding treatment selection, especially in the case of loss of response. There is evidence of resistance to BGJ398, a pan-FGFR inhibitor, due to *de novo* point mutations in the FGFR-2 kinase domain, revealed by cfDNA analysis[25].

Another promising subfield of cfDNA in CCA is the study of cell-free epigenetics. A recently published report, which analyzed 40 samples of each patient group, hints towards distinct methylation profiles between benign biliary tract disease (BTD) and CCA. The methylation pattern of opioid-binding protein/cell adhesion molecule (OPCML) and homeobox D9 (HOXD9) had a promising discriminative potential, with an area under the receiver operating characteristic (AUROC) of 0.85 for diagnosing CCA [26].

However, we believe that, to this point, there is a dire need for more data to support these initial findings. Barriers regarding study design: Method synchronization, number of patients included, data heterogeneity, cost, and lack of validation prevent their use in clinical settings, while also preventing the funding of large-scale translational endeavors.

Cell-free non-coding RNA

Research in the past decade has revealed an increasing role of miRs as cancer biomarkers for multiple primary tumors, including CCA[27-31]. There are several qualities that, at least in theory, favor miRs as useful biomarkers: Relative specificity, long-term stability, presence in multiple fluids, as well as relative ease of detection and amplification through ever more accessible PCR techniques[19]. To this point, numerous studies[32-36] have investigated the role of miRs in CCA, some showing substantial promise [37-41]. These studies are briefly analyzed in Table 1. The viability of miRs as biomarkers in CCA was

Table 1 The role of microRNAs as diagnostic and prognostic biomarkers in cholangiocarcinoma

Circulating microRNA	Biofluid	Comparison	Statistics	Discriminant specificity	Valuable considerations	Subjective rating
miR-21	Increased in serum[27,28]; plasma[34]; urine[35]	iCC (n = 74), HC (n = 74)[27]; CCA (n = 11), HC (n = 9)[28]. iCC (n = 25), HC (n = 7); CCA (n = 22), HC (n = 21)	AUROC *vs* HC: Serum: 0.91[27]; serum: 0.80[28]; plasma 0.94[34]. Combined miR-21 + miR 192. Urine: 0.85 [35]	LOW. Also increased in HCC[36] and other malignancies[37,38]	Corelates well with tumor stage and survival[39]. Most data support	Useful
miR-150-5p	Decreased in serum and bile[40]	CCA (n = 28), PSC (n = 30), HC (n = 50)	Significantly decreased *vs* HC and PSC[40] association with	LOW. Upregulation suppresses tumor progression in colorectal cancer[41]	Appears to correlate with tumor staging. Added value of the CA19-9 combination. Contradictory findings: Report of being upregulated in iCCA (AUROC: 0.76)[27]	Debatable
miR-26a	Increased in serum[29]	CCA (n = 66), HC (n = 66)	AUROC *vs* HC: 0.90 [29]	Moderate involved in HCC[30]	Correlates well with tumor stage, metastases, differentiation, and survival. Reliable decrease following curative surgery[29]	Promising
miR-30d-5p	Increased in bile[31]	CCA (n = 48), benign BTD (n = 58)	AUROC *vs* benign biliary obstruction 0.730[31]	Moderate downregulated in gastric cancer[32]	Increased sensitivity and specificity compared to CA19-9	Debatable
miR-222; miR-483-5p	Increased in serum[33]	CCA (n = 70), PSC (n = 70), HC (n = 70)	AUROC *vs* PSC; miR-222: 0.71; miR-483-5p: 0.70 combined miR-222 and 483-5p: 0.74 [33]	No evidence of overlap with other cancers	Might be useful for monitoring patients with PSC	Promising

AUROC: Area under a receiver operating characteristic; CA19-9: Carcinoembrionic antigen 19-9; CCA: Cholangiocarcinoma; HC: Healthy controls; HCC: Hepatocellular carcinoma; iCCA: Intrahepatic cholangiocarcinoma; miRs: Micro RNAs; PSC: Primary sclerosing cholangitis.

tested in two meta-analyses, each including approximately 500 patients and testing the diagnostic capabilities of the cell-free non-coding RNA method, without focusing on specific miRs. Overall, the results were promising, with an AUROC ranging between 0.88 and 0.90 for CCA detection[42,43].

However, there are some nuances in the study of miRs, which are worth addressing since the clinical future of the method might be at stake. Of critical relevance is the problem of specificity. Most biomarkers fare relatively well against healthy controls (HC), which is helpful for initial validation, yet far from desirable in a clinical scenario where the setting is less straightforward. This issue has been partially addressed in some study designs by comparisons with other benign BTD conditions, probably the most relevant being primary sclerosing cholangitis (PSC), which shares a common natural history pathway with CCA. However, in such conditions, the specificity and AUROCs tend to drop at least by 0.10-0.20 (as shown in Table 1). Consequently, their diagnostic biomarker value remains only slightly above the threshold for utility in the clinical scenario where the discriminative capabilities were most sought after. Moreover, there is the issue of overlapping with other cancers, which might further complicate the matter. In response, some designs have tried to implement a panel of up to eight miRs to generate distinct profiles depending on CCA subtypes (n = 14) and tumor progression[44].

The use of circulating nucleic acids in CCA diagnosis and prognosis is undoubtedly promising. Nevertheless, the field is still nascent, and most of the data come from studies with heterogeneous designs, most of which are proof-of-concept. Therefore, a potential research direction might be to stimulate reproducibility instead of novelty to provide the grounds for a quicker clinical application.

PROTEINS

Protein-based biomarkers in the clinical practice

Proteomics is a rapidly growing field of biomedical research in the postgenomic era, given the ever-expanding role of personalized medicine. Proteome-based biomarker studies target proteins that could serve as agents to fit a patient's molecular profile in the clinical practice for diagnostic, prognostic, and predictive molecules, their levels being measured from serum samples usually by ELISA.

There are three protein-based biomarkers currently used in the clinical practice towards assisting CCA diagnosis and prognosis: CA19-9 and CA125, and carcinoembryonic antigen (CEA)[45,46].

CA19-9 is a circulating high molecular weight glycoprotein produced by the biliary duct and pancreatic cells and secreted by the gastric and colonic epithelia. Up to 7% of the general population is not producing CA19-9 because of blood cell Lewis antigen deficiency. For CCA, CA19-9 is by far the most frequently used biomarker. Concerning CCA diagnosis, CA19-9 showed a somewhat limited diagnostic accuracy, with following performances: Sensitivity: 72% and specificity: 84%[13]. Hence its promise resides in assessing CCA prognosis. As recently reviewed by Lang *et al*[45] CA19-9 appears to be an independent prognostic biomarker associated with treatment outcome, as elevated CA19-9 serum levels pre- and postoperatively after systemic therapy show impaired OS. Nevertheless, several factors hamper CA19-9 use as a unique CCA prognostic biomarker[47], thus making its clinical use tumor-associated rather than tumor-specific.

Also known as MUC16, CA125 is the largest membrane-associated mucin, which is also, a glycoprotein. Being a well-known biomarker, CA125 is primary used for the ovarian cancer clinical management[48]. CA125 showed incipient potential diagnostic and prognostic value towards clinical management of CCA[45]. However, CA125 proves its predictive power only in combination with other biomarkers, such as CA19-9, CAE and AFP.

Being produced by the gastrointestinal tissue during fetal development, CEA is a cell surface glycoprotein and functions as an intracellular adhesion molecule. In clinical practice, CEA is extensively used in colorectal cancer monitoring[49]. CEA proved its potential value as a diagnostic biomarker, with rages of sensitivity reported between 40% and 79%, and specificity between 48% and 90%. CEA was also reported as a prognostic indicator for CCA, with expanded predictive capabilities in several biomarker combinations, such as with CA19-9[45].

The three glycoproteins are the most used biomarkers in the clinical management of CCA, and their role is to assist rather than provide a definite diagnostic or prognostic statement. Various other protein-based biomarker candidates reported as single molecules, combined with CA19-9 or as biomarker panels, have been spotlighted in several CCA studies. Towards identifying the potential biomarkers, several approaches have been used. With respect to the study design, CCA patient samples have been compared to (1) Only HC; (2) Only to benign BTD; (3) To benign BTD and HC; and (4) To other disease related conditions and HC. Other studies were interested only in searching biomarkers for iCCA and only one approach was headed towards subtypes of CCA, such as perihilar iCCA, hCCA, and eCCA. While the biospecimens are limited to serum and tissue, and the methods to ELISA or immunohistochemistry, the number of samples included appears to be very heterogenous, ranging from around 20 to up to over 200. The proteins associated with favorable diagnostic and with poor prognostic, potential protein-based biomarkers, are desciphered in Tables 2 and 3.

Proteins associated with favorable diagnostic potential in CCA patients

Multiple proteins appeared to have a role in CCA diagnosis, typically showing increased serum levels. These findings were reported in studies using serum as biospecimen and ELISA assays for their absolute quantification (Table 2). Such examples are osteopontin (OPN)[50] and S100 calcium-binding protein A6 (S100A6)[51] which efficiently discriminated between CCA and HC, and dickkopf-1 (DKK1) between iCCA and HC[52]. Studies reporting serum cytokine interleukin 6 (IL-6)[53], spermatogenesis-associated protein 20 (SSP411)[54] and MUC5AC[55] also included groups of metastatic liver cancer, HCC, and benign BTD disease. Metalloproteinase 7 (MMP-7) was assessed only in groups of CCA *vs* benign BTD[56,57].

After several reports, cytokeratin 19 fragment (CYFRA 21-1) was included in a comprehensive meta-analysis[58] and the pooled diagnostic indices showed a sensitivity of 81% and a specificity of 86% for iCCA diagnosis. More recently, the urokinase-type plasminogen activator receptor (uPAR)[59], reported as a single protein-based biomarker, proved to be a reliable tool for differentiating CCA from HC with sensitivity of 95% and specificity close to 90%, while transforming growth factor-β1 (TGF-β1) appears to help distinguishing CCA from other pro-inflammatory conditions and HC.

Moreover, the combination of MMP-7[57], DKK1[60], thrombospondin-2 (TSP-2)[61] and uPAR[59] assessed together with CA19-9 showed higher values of sensitivity and specificity than the markers measured individually to diagnose CCA patients. Not least, a biomarker panel consisting of five proteins investigated in a decision tree algorithms based study, namely S100A9, MUC5AC, TGF-β1, angiopoietin-2 (Ang-2), and CA19-9, showed to have the greatest diagnostic potential among all mentioned proteins towards CCA *vs* HC (sensitivity: 95%, specificity: 90%) and towards CCA *vs* non-CCA (sensitivity: 70%, specificity: 83%) differentiation[62].

Proteins associated with poor outcome in CCA patients

Concerning prognosis, several protein-based potential biomarkers have shown increased levels in CCA, most frequently by employing immunohistochemistry in tissue samples. The serum has also emerged as a biospecimen towards prognostic biomarkers exploration (Table 3). As such, high levels of tissue Krebs von den Lungen 6 (KL-6 mucin)[63], cadherin-17 (CDH17)[64], kallikrein-11[65], uPAR[59] and high levels of serum cytokine-induced apoptosis inhibitor 1 (CIAPIN1)[66], MUC5AC[55], OPN[50], S100A6 [67] and uPAR[59,68] were found adverse prognostic factors for CCA patient's survival. Subjected to meta-analysis, high levels of tissue PD-L1[17] and tissue protein S100P[69] were also proposed as potential prognostic markers of CCA. Out of a protein multimarker panel consisting of serum S100A9,

Table 2 Proteins associated with favorable cholangiocarcinoma diagnostic potential

Protein	Comparison	SEN (%)	SPE (%)	AUC	Ref.
	Tissue				
CYFRA 21-1	iCCA (n = 217) *vs* HC (n = 514) meta-analysis	81.0	86.0	0.904	[58]
DKK1	iCCA (n = 37) *vs* HC (n = 50)	75.7	100.0	0.872	[52]
DKK1 + CA19-9	iCCA(n = 79) *vs* HC (n = 160)	74.7	56.3	0.793	[60]
IL-6	CCA (n = 26), HCC (n = 26) and HC (n = 23)	73.0	92.0	0.875	[53]
MMP-7	CCA (n = 44) *vs* benign BTD (n = 36)	76.3	46.8	0.730	[56]
	CCA (n = 59) *vs* benign BTD (n = 128)	75.0	78.0	0.840	[57]
MUC5AC	CCA (n = 49), benign BTD (n = 23), HC (n = 16)	71.0	94.7	0.909	[55]
OPN	CCA (n = 107) *vs* HC (n = 55)	87.5	100.0	0.964	[50]
S100A6	CCA (n = 112) *vs* HC (n = 42)	86.2	90.9	0.909	[51]
SSP411	CCA (n = 30), benign BTD (n = 13) and HC (n = 23)	90.0	83.3	0.913	[54]
TGF-β1	CCA (n = 45), other disease conditions related inflammation (n = 25) and HC (n = 45)	71.1	68.9	0.668	[78]
TSP-2 + CA19-9	dCCA (n = 51), pancreatic ductal adenocarcinoma (n = 52), benign pancreatic diseases (n = 27) and HC (n = 52)	79.0	96.0	0.920	[61]
uPAR	CCA (n = 118), and HC (n = 76)	95.3	89.7	0.969	[59]
Biomarker panel: S100A9, MUC5AC, TGF-β1, Ang-2, and CA19-9	CCA (n = 40), non-CCA (n = 40) and HC (n = 40)	95.0	90.0	0.975	[62]

AUC: Area under the curve; BTD: Biliary tract disease; CCA: Cholangiocarcinoma; dCCA: Distal CCA; HC: Healthy controls; HCC: Hepatocellular carcinoma; iCCA: Intrahepatic cholangiocarcinoma; SEN: Sensitivity; SPE: Specificity; CYFRA 21-1: Cytokeratin 19 fragment; DKK1: Dickkopf 1; IL-6: Interleukin 6; MMP-7: Metalloproteinase 7; MUC5AC: Mucin 5AC; OPN: Osteopontin; S100A6: S100 calcium binding protein A6; SSP411: Spermatogenesis-associated protein 20; TGF-β1: Transforming growth factor-β1; TSP-2: Thrombospondin-2; uPAR: Urokinase-type plasminogen activator receptor; S100A9: S100 calcium binding protein A9; Ang-2: Angiopoietin-2.

MUC5AC, TGF-β1, Ang-2, and CA19-9, serum levels of TGF-β1 and Ang-2 provided predictive potential for both metastasis and TNM stage prognosis in CCA patients[62].

For patients with tumors of combined HCC and CCA (cHCC-CC), microtubule-associated protein 1A/1B-light chain 3 (LC3) increased tissue expression was found to predict postresection OS (5-year OS, 61.2%) and disease-free survival (74.6%)[70].

iCCA prognostic biomarkers were also of particular interest in some studies. High tissue levels of urokinase-type plasminogen activator (uPa)[71], MMP-7[72,73] and human endogenous retrovirus-H long terminal repeat-associating protein 2 (HHLA2), reported from a recent meta-analysis[74], were associated with adverse outcomes in iCCA patients. High serum DKK1 in combination with CA19-9 was independently associated with shorter survival[60]. Recently, Qiang *et al*[75] found that the biomarker panel consisting of CEA, AFP, and proliferation marker protein Ki67 are significant prognostic indicators in iCC patients.

Proteins that showed decreased levels in association with CCA were PD-L1 and OPN. The lack of serum PD-L1 level normalization after surgery seems to identify patients at high risk for recurrence and adverse outcomes[76]. By applying an innovative approach, decreased serum OPN *per* tumor volume was associated with invasive behavior and early recurrence of iCCA[77].

There is vast evidence of protein-based biomarkers reported in CCA diagnosis and prognosis, but only CA19-9 and CEA are currently employed in routine clinical practice. The data above reveals exciting results for new potential protein-based biomarkers used as single molecules (*e.g.*, uPAR) or biomarker panels (*e.g.*, S100A9, MUC5AC, TGF-β1, Ang-2, and CA19-9) for CCA.

Extrapolating from proteomic-derived biomarker studies in other diseases, it appears that using multiple-molecule panels instead of individual proteins provides better predictive results and shows more promise for a translation to clinical practice. However, future validation studies on large patient cohorts are needed towards establishing the real applicability and the subsequent translation of these

Table 3 Proteins associated with poor outcome in cholangiocarcinoma patients

Protein	Comparison	Outcome	Ref.
Tissue			
CDH17	CCA (n = 180)	High CDH17 was associated with a worse OS and recurrence-free survival	[64]
HHLA2	iCCA (n = 218) meta-analysis	High HHLA2 expression was significantly associated with shorter OS	[74]
KL-6	CCA (n = 21), cHCC-CCA (n = 12), HCC (n = 78)	A key molecule for tumor cell adhesion and invasion	[63]
KLK11	CCA and adjacent normal tissues (n = 18)	OS of CCA patients with a high expression of KLK11 was significantly shorter than those with a low expression of KLK11 (414 d *vs* 809 d, respectively; P = 0.048)	[65]
LC3	cHCC-CC (n = 40)	The 5-yr OS and disease-free survival rates were 61.2% and 74.6% in high LC3 expression patients and 0% and 0% in those with low LC3 expression	[70]
MMP-7	Perihilar iCCA, hCCA, and eCCA (n = 66)	Patients with moderate to marked expression of MMP-7 had a significantly poorer prognosis, as compared to those with negative to focal expression	[72]
	iCCA (n = 35)	The 5-yr survival rates of MMP-7(+) and MMP-7(−) patients were 72.7% and 18.3%, respectively	[73]
PD-L1	CCA (n = 2012) meta-analysis	Overexpression of PD-L1 was significantly associated with worse OS	[17]
S100P	CCA (n = 1925) meta-analysis	S100 calcium binding protein P overexpression was associated with poor OS	[69]
uPa	iCCA (n = 174)	High uPa expression was correlated with lymphatic invasion and metastasis of CCA patients	[71]
uPAR	CCA (n = 108) *vs* normal tissue (n = 108)	The median OS was 890 d for patients with uPAR positive vs 1.321 d for patients with uPAR negative	[59]
Biomarker panel: CEA, AFP, and Ki67	iCCA (n = 92)	higher AFP, CEA, and Ki67, as well as more advanced TNM staging were associated with worse OS	[75]
Serum			
CIAPIN1	CCA (n = 159) *vs* HC (n = 93)	Higher CIAPIN1 level was significantly associated with shorter OS time	[66]
DKK1 + CA19-9	iCCA (n = 79) *vs* HC (n = 160)	DKK-1 in combination with CA19-9 showed a better diagnostic performance than CA19-9 alone; low DKK-1 and CA19-9 were associated with longer OS	[60]
MUC5AC	CCA (n = 49), benign BTD (n = 23), HC (n = 16)	High MUC5AC level was related to a worse prognosis compared with patients with lower levels, with 3-yr survival rates of 21.5% and 59.3%, respectively	[55]
OPN	CCA (n = 107) *vs* HC (n = 55)	Poor postoperative survival	[50]
OPN/tumor volume	iCCA (n = 124)	Low circulating OPN *per* tumor volume was associated with shorter OS and disease-free survival	[77]
PD-L1	CCA (n = 73) *vs* HC (n = 42)	Low PD-L1 levels displayed a strong trend towards an impaired prognosis	[76]
S100A6	CCA (n = 112) *vs* HC (n = 42)	S100A6 potential was like those of the clinically established biomarkers CEA and CA19-9	[67]
uPAR	CCA (n = 168)	Baseline level of uPAR was an independent predictor of survival; a high level of uPAR after 2 cycles of chemotherapy was associated with poor survival	[68]
	CCA (n = 117), HC (n = 76)	Multivariate Cox-regression analysis revealed	[59]

		circulating uPAR levels as an independent prognostic marker following biliary tract cancer resection	
Biomarker panel: S100A9, MUC5AC, TGF-β1, Ang-2, and CA19-9	CCA (n = 40), and non-CCA patients (n = 40) and HC (n = 40)	TGF-β1 and Ang-2 are predictors of higher TNM stages	[62]

BTD: Biliary tract disease; CCA: Cholangiocarcinoma; cHCC-CC: Combined hepatocellular carcinoma and CCA; HC: Healthy controls; HCC: Hepatocellular carcinoma; iCCA: Intrahepatic cholangiocarcinoma; OS: Overall survival; CDH17: Cadherin-17; CYFRA 21-1: Cytokeratin 19 fragment; DKK1: Dickkopf 1; IL-6: Interleukin 6; MMP-7: Metalloproteinase 7; MUC5AC: Mucin 5AC; OPN: Osteopontin; S100A6: S100 calcium binding protein A6; SSP411: Spermatogenesis-associated protein 20; TGF-β1: Transforming growth factor-β1; TSP-2: Thrombospondin-2; uPAR: Urokinase-type plasminogen activator receptor; S100A9: S100 calcium binding protein A9; Ang-2: Angiopoietin-2; PD-L1: Programmed death-ligand 1; CIAPIN1: Serum cytokine-induced apoptosis inhibitor 1; AFP: Alpha-feto protein; LC3: Microtubule-associated protein 1A/1B-light chain 3; KLK11: Kallikrein related peptidase 11; KL-6: Mucin KL-6; HHLA2: Human endogenous retrovirus-H long terminal repeat-associating protein 2.

biomarkers into the clinical practice.

METABOLITES

Metabolomics, another branch of omics-derived technologies, analyzes low molecular weight metabolites (< 1500 Da) in various biological fluids. One of the hallmarks of cancer is energy metabolism reprogramming. In order to promote cancer survival and subsequently cancer growth, there are several shifts in normal metabolic pathways (*e.g.*, a higher glucose uptake rate and an increase in lactate production)[78,79]. A different or "wiser" use of metabolic pathways in cancer cells leads to the release of several metabolites in various body fluids, providing an opportunity for diagnosis and monitoring. Metabolic profiling is, therefore, a promising approach for the identification of potential biomarkers in several cancers, including CCA[80]. To date, several studies have investigated the potential of metabolomics in CCA diagnosis or prognosis in various body fluids.

We believe that investigating the molecular composition of the bile could provide more crucial information than other fluids due to at least two reasons. Firstly, it could unravel mechanistic information regarding the pathological alteration of the biliary epithelium. Secondly, it could identify biomarkers from nearby tumor cells, markers that might or might not be present in other body fluids. Several metabolite profiling studies of human bile have been performed over the past few years. One such study reported a reduction in the proportion of secondary bile acids in patients with CCA compared to those with biliary tract stones and healthy individuals[81]. Another study showed that changes in phosphatidylcholines, bile acids, and lipids could discriminate CCA from PSC and benign BTD (sensitivity: 88.9%; specificity: 78.1%)[82]. When comparing inoperable eCCA to non-malignant, non-cholestatic biliary diseases (including PSC), CCA was associated with increased levels of glycine-conjugated bile acids and phosphatidylcholines. Moreover, constructed models could discriminate CCA patients from those with non-malignant biliary diseases with an 80% sensitivity and 95% specificity: 95%[83].

Unfortunately, the impact of cholestasis on the metabolic profile was not investigated, and it is difficult to reach a solid conclusion. In contrast, the analysis of metabolites in patients with CCA, HCC, and non-malignant liver diseases showed a decrease in glycine and taurine-conjugated bile acids, phospholipids, and cholesterol in patients with CCA compared to control groups but only to a certain extent when compared to HCC[84].

In theory, if one biomarker is detected and validated in bile, it might provide sufficient grounds further to test it in more accessible and less invasive fluids (*e.g.*, serum, urine, plasma). Nevertheless, metabolomics studies can also be performed directly on serum, plasma, or urine. Using serum, one study from the United Kingdom failed to show any differences between profiles from patients with benign biliary strictures and CCA[85]. In contrast, one study from China showed that two bile acids, chenodeoxycholic acid (CDCA) and taurochenoxycholic acid (TCDCA) (from plasma), had higher sensitivity and specificity than CA19-9 for CCA *vs* benign bile duct disease and CCA *vs* HC[86]. Furthermore, a study from Europe (Italy), using an artificial intelligence approach, found a plasma bile acid profile that could discriminate between CCA and benign BTD with an accuracy of 86.4%[87]. However, all the beforementioned studies could not offer more answers to some of the most critical clinical dilemmas when caring for patients with liver cancer.

In the liver cancer community, there are at least two primary clinical necessities. The first clinical dilemma is probably the most common scenario: One patient with advanced liver disease and focal liver lesions: Is it cancer? If the answer is yes, is it HCC or iCCA? In this setting, one study (on serum) has shown that the development of an algorithm combining glycine, aspartic acid, sphingomyelin (SM) (42:3), and SM (43:2) permitted accurate discrimination between HCC and iCCA with a sensitivity of 75% and specificity of 90%. In the same study, another algorithm discriminated PSC from iCCA with a sensitivity of 100% and specificity of 70%. Of note, these results were further validated in an

independent cohort[88]. A similar finding was also reported in one study from China. A panel of four metabolites {PE (19:0/0:0), PE [18:2 (9Z, 12Z)/0:0], PC (14:0/0:0) and PC (18:0/0:0)} attained a diagnostic accuracy (HCC *vs* iCCA) of 99.7%[89].

The second clinical dilemma is: One patient with distal bile duct obstruction: Is it cancer? dCCA or pancreatic ductal adenocarcinoma (PDAC)? A combination of serum levels of nine metabolites [acylcarnitine AC (16:0), ceramide Cer (d18:1/24:0), phosphatidylcholines PC (20:0/0:0) and PC (O-16:0/20:3), lysophosphatidylcholines PC (20:0/0:0) and PC (0:0/20:0), lysophosphatidylethanolamine PE (P-18:2/0:0), and sphingomyelins SM (d18:2/22:0) and SM (d18:2/23:0) and CA 19-9] could discriminate between dCCA and PDAC with a sensitivity of 55.9% and specificity of 89.5%[90]. Metabolic profiling of urine in patients with CCA was also applied, showing some metabolic differences in the urine of CCA compared to controls. As such, a urine metabolomic panel consisting of 3-methylhistidine, citric acid, cytosine, indoleacetic acid, salicyluric acid, L-methionine, aminomalonic acid, glutaric acid, ursodeoxycholic acid, N-acetylornithine, allantoin, glycocholic acid, histamine, homogentisic acid, L-kynurenine, sarcosine, pyruvic acid, taurine and methylsuccinic acid were identified as potential biomarkers for primary extrahepatic CCA[91]. Nevertheless, in terms of prognosis, only a few studies have shown the potential of metabolites to predict recurrence or OS[91,92].

The road ahead is still long for metabolomics in CCA. Metabolome studies in CCA have just begun, and some promising metabolites have already been identified. However, a shift from bench to bedside is not expected to appear in the next few years. First, identifying a specific metabolite with diagnostic or prognostic properties is a challenging goal due to the presence of many confounding factors (*e.g.*, age, gender, diet, underlying liver disease, concomitant disease, drugs, and others). Secondly, the results from untargeted metabolomics might be different from targeted metabolomics, according to at least one recent metabolomics study, investigating plasma fetal bile acids towards assessing liver cirrhosis severity[93]. Not least, the reproducibility of many of these studies is a genuine concern (due to multiple analytical platforms, different sample preparation protocols), and standardized procedures are urgently needed.

EXTRACELLULAR VESICLES

In terms of minimally invasive biomarkers, EVs are the "new kids on the market". They hold great promise in the diagnosis and prognosis of cancer. EVs are encountered in all body fluids including blood[94], urine[95] and bile[96]. According to their size and biogenesis there are two classes: (1) Large EVs [also called microvesicles (MVs)] roughly ranging from 100 to 1000 nm in size, which directly bud from the plasma membrane of their parental cell; and (2) Small EVs (also called exosomes) are considerably smaller (below 100 nm) and originate from accumulated intraluminal vesicles within the endomembranous system, forming so-called multivesicular bodies[97].

The function of EVs depends on the type and content (*e.g.*, lipids, proteins, nucleic acids) of their parent cells. They orchestrate many of the processes described by Hanahan *et al*[98] as "Hallmarks of Cancer"[98] either *via* paracrine signaling or horizontal transfer of bioactive agents[99]. In the initial steps of cancer genesis, EVs (released by cancer cells) appear to be responsible for the differentiation of mesenchymal stem cells into fibroblasts, contributing to stroma generation ant thus preparing their tumor niche[100]. Furthermore, EVs could transport miR species from human CCA cells to cancer-associated fibroblasts, a communication between cancer cells and the cancer microenvironment responsible for tumor growth and, later on, CCA cells-derived EVs can transfer oncogenes to normal cholangiocytes, increasing their migration and invasive potential [*via* increasing the expression of beta-catenin (CTNNB1) and decreasing the expression of E-cadherin (CDH1)], hence preparing the final processes of carcinogenesis: Tumor invasion and metastasis[101].

Some studies have already revealed the great potential of EVs content or surface markers in terms of diagnosis. Proteomic profiling of serum EVs has identified a panel of five proteins that could assist CCA diagnosis. The study design included CCA (n = 43), PSC (n = 30), HCC (n = 29) patients and HC (n = 32). As such, pantetheinase (VNN1), C-reactive protein, fibrinogen gamma chain (FIBG), immunoglobulin heavy constant alpha 1 (IGHA1) and alpha-1 acid glycoprotein (A1AG1) showed to have an increased concentration in serum EVs of CCA compared to all PSC, HCC and HC. Moreover, a panel of three EVs proteins, namely ficolin-2 (FCN2), inter-alpha-trypsin inhibitor heavy chain H4 (ITIH4), and FIBG showed to be able to discriminate between early-stage CCA and PSC patients with an AUC > 0.88[102].

A major challenge nowadays is the differential diagnosis between HCC and iCCA or between dCCA and PDAC. More often, the final diagnosis (HCC *vs* cCCA) in clinical practice is based on liver biopsy. In terms of EVs surface antigens, one study enrolling 172 patients with liver cancer (HCC or CCA), 54 with cirrhosis and no liver neoplasia, and 202 control subjects, found a combination of tumor-associated microparticles (AnnexinV+ epithelial cell adhesion molecule (EpCAM+) and asialoglycoprotein receptor 1 (ASGPR1+)) could diagnose CCA from healthy individuals and other cancer entities with up to 90% sensitivity. However, it was unable to differentiate between CCA and HCC[103]. Interestingly, later on, the same group in another study including a large set of patients, including 77 CCA, 67 HCC, identified a combination between EVs surface antigens (AnnexinV+ CD44v6, cut-off = 34 number *per* 10^3

AnnexinV+ EVs) together with AFP (cut-off = 30 ng/mL) that could discriminate between HCC and CCA (iCCA and eCCA) with both sensitivity and specificity of 100%[104]. Indeed, it is a novel potential diagnostic biomarker that could help clinicians diagnose CCA non-invasively and accurately. Further large multicenter studies are urgently necessary. In search of novel biomarkers for the differential diagnoses between dCCA and PDAC, one study including 50 patients (n = 20 pancreatic cancer, n = dCCA, n = 15 chronic pancreatitis, n = 10 common bile duct obstruction due to biliary stones patients) reported that the concentration of EVs *per se* in bile and serum could discriminate malignant from non-malignant pancreaticobiliary diseases with 100% sensitivity in bile and 47% in serum[105]. EVs cargo profile could also have diagnosis potential. In particular, a panel of 5 miRs (miR-191, miR-486-3p, miR-1274b, miR-16 and miR-484) isolated from bile EVs showed good diagnostic values for CCA diagnosis compared to non□malignant biliary diseases (sensitivity: 67%; specificity: 96%)[106].

CIRCULATING TUMOR CELLS

Circulating tumor cells (CTCs) have been evaluated as a diagnostic marker in pancreatic, colorectal, breast or prostate cancer, and are associated with poor survival rates. However, only a handful of studies have assessed their potential in CCAs. CTCs are cancer-derived cells released from a primary solid tumor or local lymphoid reservoirs into the bloodstream, harboring tumor-initiation properties, and possibly enabling distant metastasis. Even after primary tumor resection, the permanence of viable CTCs in the portal venous blood seems to be a consequence of T-cell suppression by myeloid-derived suppressor cells and CTC-induced apoptosis[107]. Subsequently, CTCs proliferate and cluster, possibly under the influence of cell adhesion molecules such as plakoglobin, leading to tumor growth and immune resistance[107,108].

Identification of CTCs in peripheral blood relies on their overexpression of EpCAM. It has been performed using immunocytochemistry, reverse transcriptase-PCR, flow cytometry, or an enzyme-linked immunosorbent spot assay. The most used assay is CellSearch™, which uses ferrofluid nanoparticles with antibodies that target EpCAM, which is expressed in various of human epithelial tissues, carcinomas, and stem cells, and is involved in cell signaling, migration, proliferation, and differentiation[109-111]. In a study of 26 CCA patients, targeting CTCs using antibodies against EpCAM, DAPI, cytokeratin 8, 18, and/or 19, Al Ustwani *et al*[112] showed that 25% of patients with CCA had a significant amount of CTCs (≥ 2/7.5 mL of blood). Similar results were reported in another study, where out of 95 CCA patients, 24% had a count of two cells or higher *per* 7.5 mL blood, while 22% had a count of one cell, and the remainder of 54% no detectable cells[113]. Since CTCs seem to be relatively rare in peripheral blood, their potential as a diagnostic marker might be more evident in patients with metastatic disease and less in early tumors.

CTCs are also seemingly associated with more aggressive tumors, as patients with no CTCs in their blood sample had the best survival rate. In contrast, the presence of two or more CTCs was strongly associated with worse OS (median 18.1 mo *vs* 8.7 mo)[113]. However, their presence does not seem to predict treatment outcome, as evidenced in the ABC-03 trial[113,114].

The high degree of variability in detection rates might be explained by suboptimal EpCAM levels for detection, loss of epithelial surface antigens, or epithelial-mesenchymal transition[112,115,116]. To overcome these shortcomings, a novel glycosaminoglycan-SCH45-probe on a microfluidic platform has been employed to isolate CCA CTCs by combining multiple-capture approaches in a shorter period and using lower blood volumes compared to the traditional method. Using EpCAM as a conventional protein biomarker, the authors showed, by analyzing peripheral blood of 65 metastatic CCA patients, that CTCs could be detected in all advanced or metastatic CCA, suggesting that CTCs may maximize the predictive performance of liquid biopsies if the proper diagnostic tool is used[117]. Reduzzi *et al*[118] assessed an alternative to improving detection rates in a prospective study of 21 patients with advanced-stage biliary tract cancer. Using non-conventional CTCs lacking epithelial and leukocyte markers, but presenting aberrant genomes, the detection rate increased from 19% to 83%.

CONCLUSION

A non-invasive approach towards diagnosis and prognosis is the path forward in CCA, a type of cancer that sometimes appears to be hiding in plain sight. The previously discussed methods aim to provide the necessary leap forward towards a personalized approach and might allow for a refined characterization of the disease. However, most available reports are deeply heterogeneous, study protocols are not harmonized, and the number of included patients is inconsistent. These caveats appear to be the primary reasons for the gap between the wide range of cancer biomarkers that appear to be effective in individual studies and the relatively low number of biomarkers ready to be translated into the clinic. Consequently, the most challenging task in the short term might be not to find new molecules and pathways but rather to validate or infirm the role of current methods to shorten the bench to bedside gap.

FOOTNOTES

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Country/Territory of origin: Romania

ORCID number: Lavinia-Patricia Mocan 0000-0003-4957-4390; Maria Ilieş 0000-0002-0659-8926; Carmen Stanca Melincovici 0000-0002-2757-2565; Mihaela Spârchez 0000-0001-8620-9160; Rareş Crăciun 0000-0002-5872-8630; Iuliana Nenu 0000-0002-1690-6689; Adelina Horhat 0000-0002-8701-8750; Cristian Tefas 0000-0002-8263-7923; Zeno Spârchez 0000-0002-3813-1677; Cristina Adela Iuga 0000-0002-0345-0993; Tudor Mocan 0000-0001-7785-6403; Carmen Mihaela Mihu 0000-0002-9419-4824.

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MINIREVIEWS

COVID-19 and liver dysfunction: What nutritionists need to know

Ming-Ke Wang, Xue-Lu Yu, Li-Yun Zhou, Hong-Mei Si, Ju-Fen Hui, Deng-Yong Hou, Wei-Peng Li, Ji-Shun Yang

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Ming-Ke Wang, Xue-Lu Yu, Li-Yun Zhou, Hong-Mei Si, Ju-Fen Hui, Deng-Yong Hou, Wei-Peng Li, Department of Disease Control and Prevention, Naval Medical Center of PLA, Naval Medical University, Shanghai 200052, China

Ji-Shun Yang, Medical Care Center, Naval Medical Center of PLA, Naval Medical University, Shanghai 200052, China

Corresponding author: Ji-Shun Yang, MD, PhD, Director, Medical Care Center, Naval Medical Center of PLA, Naval Medical University, No. 338 Huaihai West Road, Shanghai 200052, China. jasunyang@foxmail.com

Abstract

The coronavirus disease 2019 (COVID-19) pandemic caused by severe acute respiratory syndrome coronavirus-2 has brought serious challenges for the medical field. Patients with COVID-19 usually have respiratory symptoms. However, liver dysfunction is not an uncommon presentation. Additionally, the degree of liver dysfunction is associated with the severity and prognosis of COVID-19. Prevention, diagnosis, and treatment of malnutrition should be routinely recommended in the management of patients with COVID-19, especially in those with liver dysfunction. Recently, a large number of studies have reported that nutrition therapy measures, including natural dietary supplements, vitamins, minerals and trace elements, and probiotics, might have potential hepatoprotective effects against COVID-19-related liver dysfunction *via* their antioxidant, antiviral, anti-inflammatory, and positive immunomodulatory effects. This review mainly focuses on the possible relationship between COVID-19 and liver dysfunction, nutritional and metabolic characteristics, nutritional status assessment, and nutrition therapy to provide a reference for the nutritionists while making evidence-based nutritional decisions during the COVID-19 pandemic.

Key Words: COVID-19; SARS-CoV-2; Liver dysfunction; Nutritional status assessment; Nutrition therapy

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Core Tip: Patients with coronavirus disease 2019 (COVID-19) usually have respiratory symptoms, but liver dysfunction is not an uncommon presentation. The degree of liver dysfunction is associated with COVID-19 severity and prognosis. Nutrition has played a critical therapeutic and prognostic role in the management of patients with COVID-19-related liver dysfunction. This review mainly focuses on the possible relationship between COVID-19 and liver dysfunction, nutritional and metabolic characteristics, nutritional status assessment, and nutrition therapy in patients with COVID-19 to provide a reference for the nutritionists while making evidence-based nutritional decisions in the era of COVID-19.

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INTRODUCTION

Since December 2019, novel coronavirus disease 2019 (COVID-19) caused by severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) has been a major global health-related issue and has caused an unprecedented pandemic[1,2]. As of January 2, 2022, a total of 288867634 confirmed cases of COVID-19 and 5437636 deaths have been reported worldwide[3]. The liver is one of the main organs involved in nutrient metabolism, including protein synthesis, glycogen storage, and detoxification[4]. Studies have shown that angiotensin-converting enzyme 2 (ACE2), a functional receptor that allows the entry of SARS-CoV-2 into host cells, is expressed in cholangiocytes (59.7% of cells) and hepatocytes (2.6% of cells), indicating that COVID-19-related liver dysfunction may occur through direct cytotoxicity due to continuous viral replication within liver cells[5-8]. Additionally, COVID-19-related liver dysfunction was observed in approximately 20%-30% of the infected patients, especially in critically ill patients, and it was associated with poor outcomes[9-12]. Therefore, COVID-19-related liver dysfunction should not be ignored.

Meanwhile, almost all patients with liver disease, especially those at an advanced stage, have signs of malnutrition, including mineral and vitamin deficiency[13]. Micronutrient deficiencies may lead to impaired immune responses, including improper cytokine secretion, secretory antibody alterations, and antibody affinity, which increase the susceptibility to SARS-CoV-2 infection[14]. The malnutritional status of the host can also be a virulence factor for SARS-CoV-2 infection[15]. In addition, the nutritional status of COVID-19 patients with liver dysfunction is significantly related to the disease severity. An evaluation of the patient's nutritional status should not be ignored owing to the implications of nutritional status on the susceptibility, course, severity, and responsiveness to therapies[16,17]. Tailored nutritional therapy prescribed after evaluating the nutritional status has also been an integral part of the comprehensive treatment for patients with COVID-19. This paper mainly focuses on a possible relationship between COVID-19 and liver dysfunction, nutritional and metabolic characteristics, nutritional status assessment, and nutrition therapy in patients with COVID-19.

COVID-19 AND LIVER DYSFUNCTION

Liver dysfunction has been reported in a significant proportion of COVID-19 patients, especially in those with a severe illness[5]. Additionally, 2%-11% of patients with COVID-19 suffer from chronic liver disease. The prevalence of liver dysfunction in COVID-19 ranged from 3.75% to 59.04%; most studies reported a prevalence of 20%-30%[9-12]. A cross-sectional study reported that the prevalence of liver dysfunction in patients with COVID-19 was 59.04%; of the 62 patients, 44 (70.9%) were male and 18 (29.03%) were female. The average hospital stay of patients with liver dysfunction was 15 d (range, 10-16 d) compared with 10 d (range, 7-11 d) for patients with normal liver function[12]. In general, patients with COVID-19 who develop liver dysfunction are mostly male, elderly, and obese[5]. Another retrospective cohort study reported that of the 2273 COVID-19 patients at three hospitals in the NewYork-Presbyterian network, 45% suffered from mild liver injury, 21% from moderate liver injury, and 6.4% from severe liver injury. Patients with severe liver dysfunction had elevated levels of inflammatory markers, including ferritin and interleukin-6. They also suffered a worse clinical course, including higher rates of intensive care unit (ICU) admission (69%), intubation (65%), renal replacement therapy (33%), and mortality (42%)[10]. Several studies reported that the presence of liver dysfunction was closely related to higher admission, as well as higher ICU admission and/or death. The measurement of liver biochemical indexes might help the clinicians to evaluate the severity and prognosis of patients with COVID-19[18-23]. The mechanisms of COVID-19-related liver dysfunction may include direct viral cytopathic impairment, secondary liver injury resulting from a systemic inflam-

matory response or hypoxia-reperfusion, stress-induced liver injury, drug-induced liver damage, and, finally, exacerbation of the pre-existing liver diseases (Figure 1)[5,24-27]. Although COVID-19-related liver injuries are often transient and reversible, physicians, dietitians, and nutritionists need to take notice of the pre-existing liver damage, monitor liver function, improve supportive treatment, and prevent the occurrence of drug-induced liver injury[28].

NUTRITIONAL AND METABOLIC CHARACTERISTICS OF PATIENTS WITH COVID-19

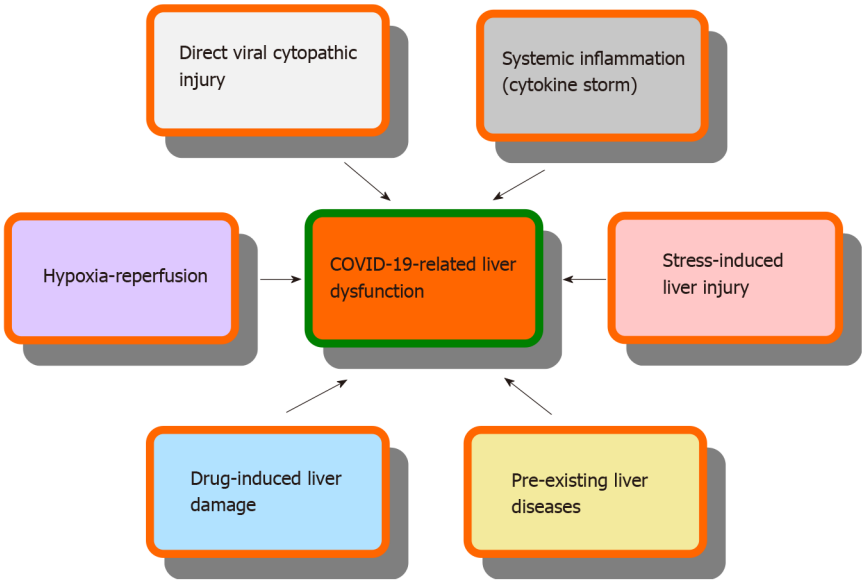
The main manifestation of patients with COVID-19 is high fever (range, 37.5-39.0 °C), which induces a catabolic state, including impaired glucose utilization, and increased energy utilization and protein breakdown. The metabolic effect of the temperature increase is said to be 10%-13% for every 1 °C increase, which should be considered in the nutritional recommendations[29]. Additionally, great influence on appetite and consciousness, and direct gastrointestinal damage may lead to nausea, vomiting, diarrhea, and feeding intolerance, which may adversely affect the nutrient intake and nutritional status[29]. Rouget *et al*[30] reported a high prevalence of malnutrition (37.5%) with 26% severe malnutrition according to the Global Leadership Initiative on Malnutrition (GLIM) criteria in a general cohort of patients with COVID-19. Bedock *et al*[31] reported that the overall incidence of malnutrition in COVID-19 patients was 42.1% (moderate: 23.7%; severe: 18.4%), while the incidence of malnutrition in patients admitted to the ICU reached 66.7% using the GLIM criteria. They found that lower albumin levels were related to a higher risk of admission to the ICU, and this association was independent of age and C-reactive protein levels. Li *et al*[32] found a high incidence (52.7%) of malnutrition according to the Mini Nutritional Assessment (MNA) in 182 elderly patients with COVID-19. Additionally, further regression analysis indicated that diabetes, low calf circumference, and low albumin level were independent risk factors for malnutrition. Malnutrition can impair the hepatic metabolic functions, and malnutrition alone can result in severe fatty liver[33].

NUTRITIONAL STATUS ASSESSMENT

According to the expert statements and practical guidance of the European Society for Clinical Nutrition and Metabolism for the nutritional management of individuals with SARS-CoV-2 infection, COVID-19 patients at risk of poor outcomes and high mortality, namely, the elderly and individuals with multiple comorbidities, should be assessed for malnutrition using the Malnutrition Universal Screening Tool (MUST) criteria; for hospitalized patients, the Nutrition Risk Screening 2002 (NRS-2002) criteria should be used[34]. Identification of the risk and presence of malnutrition should be conducted early in the overall assessment of all patients with COVID-19 using criteria such as MUST or NRS-2002. Ganatra *et al*[35] investigated and analyzed the nutritional risk and dietary intake of patients with COVID-19 and provided data supporting nutritional intervention using the NRS-2002 criteria. The Subjective Global Assessment criteria, the MNA criteria for geriatric patients, and the Nutrition Risk in Critically ill (NUTRIC) criteria for ICU patients have been used to further assess patients with COVID-19 and are accepted in clinical practice[36,37]. Zhang *et al*[38] reported that the modified NUTRIC score could be applied to nutritional risk evaluation and prognosis indication in critically ill patients with COVID-19 [39]. Recently, the GLIM criteria for malnutrition diagnosis endorsed by clinical nutrition societies worldwide have been used to assess the nutritional status of patients with COVID-19[30,31].

NUTRITION THERAPY FOR COVID-19-RELATED LIVER DYSFUNCTION

Currently, the fight against the COVID-19 epidemic is entering a decisive stage[38]. Evidence-based and logical nutrition interventions can effectively improve the nutritional status and enhance the immunity, and they are essential for preventing and managing viral infections[40]. Patients with mild clinical manifestations or recovered patients who have returned home should rest in bed, carefully choose foods and recipes, maintain an adequate supply of energy and nutrients (including drinking water), improve their immune status, and speed up their recovery process. Severe, critically ill patients with COVID-19 often have loss of appetite and insufficient diet, which worsens their already weak immune system. For these patients, subsequent nutritional support should be adopted, and specific nutritional treatment plans should be formulated according to the general condition of the patient's body, fluid intake, liver and kidney functions, and glucose and lipid metabolism[41]. Ten expert recommendations for medical nutritional therapy for patients with COVID-19 have been proposed by the Chinese Society for Parenteral and Enteral Nutrition to further promote patient recovery, improve their treatment effects, and reduce the mortality rate[42]. The main nutritional therapy recommendation is a five-step method, including diet and nutrition education, oral nutritional supplements, tube feeding, supplemental parenteral nutrition, and total parenteral nutrition[42].



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Figure 1 Mechanisms of coronavirus disease 2019-related liver dysfunction. COVID-19: Coronavirus disease 2019.

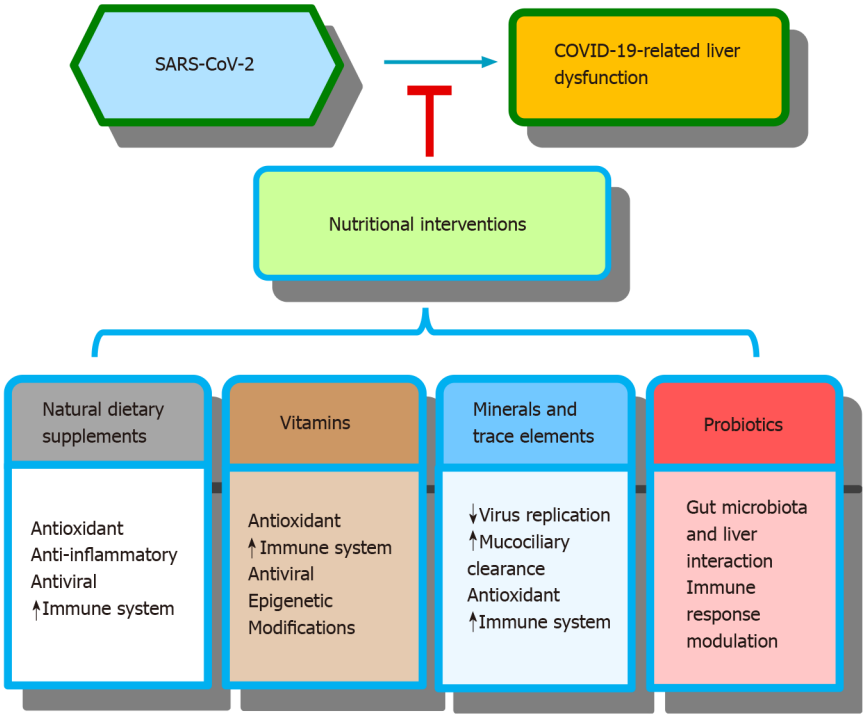
Numerous studies have found that nutrition therapy measures for patients with COVID-19 mainly include natural dietary supplements, vitamins, minerals, trace elements, and probiotics. A schematic summarizing the nutritional interventions for COVID-19-related liver dysfunction and their possible mechanisms is shown in Figure 2.

Natural dietary supplements

Rizzo *et al*[43] reviewed the properties of some vegetal products and their derivatives, including Lupin, salvia, garlic, and extra-virgin olive oil (EVOO), and they found that intake of these products or their extracts might prevent SARS-CoV-2 infection or improve the patients' nutritional status. Lupin, salvia, garlic, and EVOO have anti-oxidant, anti-inflammatory, or antiviral properties and can recover the expression of ACE2 on the cell membrane, otherwise suppressed by SARS-CoV-2 binding and entry into the cytoplasm. Sikander *et al*[6] provided information and summarized the effects of natural bioactive antiviral, immunomodulatory, and hepatoprotective nutraceuticals (*Silybum marianum*, *Solanum nigrum*, *Cichorium intybus*, *Allium sativum*, *Glycyrrhiza glabra*, *Phyllanthus amarus*, *Withania somnifera*, *Curcuma longa*, and other hepatoprotective agents) that might be explored in managing COVID-19-induced liver dysfunction. Additionally, omega-3 long-chain polyunsaturated fatty acids (omega-3 LC-PUFAs) might also have effects on different stages of viral infection, including virus entry and replication, and help improve the inflammatory balance. An optimized omega-3 PUFA status, considering both the omega-3 precursor alpha-linolenic acid and long-chain derivatives, such as eicosapentaenoic acid and docosahexaenoic acid, might be helpful in preventing infectious diseases, including COVID-19[44].

Vitamins

Accumulating data have demonstrated that vitamin deficiency could be a risk factor for SARS-CoV-2 infection and it affects the COVID-19 susceptibility and prognosis[45]. For instance, vitamin A deficiency increases the severity of the disease, and appropriately timed intake during recovery reduces the death risk and speeds up the recovery. Studying interactions of vitamin A metabolism with SARS-CoV-2 infection may thus provide improved COVID-19 treatment[46]. Vitamin D deficiency may decrease the ability of the immune system to defend against COVID-19 and cause progression to severe disease[47]. Vitamin K deficiency may be a potentially modifiable risk factor for severe COVID-19; the mechanism is pneumonia-induced extrahepatic vitamin K consumption, resulting in accelerated elastic fiber damage and thrombosis[48]. Vitamins A, B, C, D, and E have been shown to be potentially beneficial in fighting against COVID-19 by exerting antioxidant and immunomodulatory effects, increasing natural barriers, and causing local paracrine signaling[49]. Additionally, vitamins can serve as epigenetic modifiers to enhance the immunity and reduce the inflammatory response in patients with COVID-19 and noncommunicable diseases. Combined vitamin therapy can improve the health in a more personalized manner or help in the prevention of infectious diseases in patients at risk for COVID-19[50]. Molecular simulations also suggest that vitamins, steroids, and retinoids may serve as ligands in the free fatty acid pocket of the SARS-CoV-2 spike protein and may thus provide a promising strategy for prophylaxis or therapeutics[51]. Supplements with vitamins A, B, C, D, E, and K may represent a cheap and safe approach and can be used as adjuvant therapy together with antiviral medicines in managing COVID-19[45,52]. However, caution must be exercised when recommending vitamin supple-



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Figure 2 Scheme showing the effects of nutritional interventions against coronavirus disease 2019-related liver dysfunction and their possible mechanisms. SARS-CoV-2: Severe acute respiratory syndrome coronavirus-2; COVID-19: Coronavirus disease 2019.

mentation during the COVID-19 pandemic because the effects of hypervitaminosis can be serious, especially of fat-soluble vitamins A, D, and E[49]. More robust data from randomized controlled trials are needed in the near future.

Minerals and trace elements

Zinc (Zn) supplementation may inhibit SARS-CoV-2 virus replication, increase mucociliary clearance, and stimulate and activate the natural antiviral response of the immune system in patients with COVID-19[52]. Derwand *et al*[53] have also reported that the application of the combination therapy of Zn, low-dose hydroxychloroquine, and azithromycin to COVID-19 outpatients as early as possible after symptom onset resulted in significantly fewer hospitalizations and mortality rates.

Selenium (Se), one of the essential trace elements in the human body, has antioxidant and immunity-boosting effects that may induce a strong antiviral effect[54]. Recently, it was reported that the Se status was positively correlated with the survival rate of COVID-19 patients according to an exploratory study on the Se status in healthy individuals and patients with COVID-19 in the southern part of India[55]. Se has long been considered to help fight different viruses, such as herpes simplex virus type 1, influenza virus, Coxsackie virus, hepatitis C virus, and human immunodeficiency virus; the potential application of nano-Se may play an important role in combating COVID-19 in the future[54]. Nutrition interventions that ensure an adequate status of Zn, Se, and vitamin D could protect against infection with the novel coronavirus SARS-CoV-2 and retard the course of COVID-19. Meanwhile, the initiation of adequate supplementation of Se in high-risk population immediately after the time of suspected SARS-CoV-2 infection is recommended[56].

Magnesium (Mg) is important for the activation of vitamin D and plays a protective role against oxidative stress. Mg deficiency increases the endothelial cell susceptibility to oxidative stress, induces endothelial dysfunction, decreases fibrinolysis, and increases coagulation. Mg-deficiency in animals and humans may lead to suppressed immune responses. However, upon supplementation with Mg, a partial or nearly full reversal of immunodeficiency occurs. Since Mg and vitamin D are important for immune function and cellular resilience, deficiency of either of the two micronutrients may contribute to cytokine storms in COVID-19 infection[57]. Furthermore, a low Mg status may induce the transition from mild to critical clinical manifestations of COVID-19[58]. Additionally, a recent review summarized the effect of Mg supplementation on various types of disorders and diseases, providing a reference supporting the possibility of Mg supplementation for supportive therapy of COVID-19 patients[59]. Additional epidemiological, basic, and clinical research on the potential role of Mg deficiency in COVID-19 is needed.

Copper (Cu) is an important micronutrient for both pathogens and hosts during viral infection. It has the capability of contact killing of several viruses, including SARS-CoV-2[60]. Enrichment of plasma Cu

levels was hypothesized to boost both innate and adaptive immunity; Cu may have preventive and therapeutic effects against COVID-19[60]. A better understanding of Cu signaling, safety, assessment and interpretation methods, administration route, and dosage could open up new perspectives regarding the administration of therapeutic Cu to critically ill patients with COVID-19. Andreou *et al*[61] found that the combined use of Cu, colchicine, N-acetylcysteine, and nitric oxide (NO) with candidate antiviral agents, such as remdesivir or EIDD-2801, might be a potential treatment scheme for COVID-19. Physicians should consider Cu insufficiency in critically ill patients with COVID-19 and pay attention to Cu toxicity and estimate the adverse responses according to the Cu dose, and severity of Cu limitation, as well as the duration of Cu imbalance[62].

Probiotics

Beneficial live microbes in humans and animals are known as probiotics, and the chemical compounds that increase the probiotic growth rate are termed prebiotics[63]. SARS-CoV-2 infection is closely related to immune dysfunction and gut microbiota alterations. Delineating the mechanisms of probiotics, prebiotics, and a diet that promotes immunity and protects against SARS-CoV-2 presents possibilities of identifying microbial therapies to prevent and treat COVID-19[64]. Probiotics can exert beneficial effects by manipulating the gut microbiome, suppressing the gut opportunistic pathogens, decreasing the translocation of opportunistic organisms in the gut, activating the mucosal immunity, and modulating the innate and adaptive immune responses. Probiotics may be used as potential candidates to treat moderate and severe COVID-19 patients due to their benefits, including safety, ease of administration, high availability, and cost-effectiveness[65]. Emerging evidence has shown the role of gut microbiota in liver diseases through immune system cross-talk[66]. There is a lack of evidence that probiotics can directly inhibit SARS-CoV-2 infection, and probiotic therapy in COVID-19-related liver dysfunction is also not very effective. However, probiotics may be potentially helpful in the treatment of patients with severe COVID-19 and liver dysfunction[67].

CONCLUSION

Patients with COVID-19 usually have respiratory symptoms, but liver dysfunction is not an uncommon presentation and can lead to a delay in diagnosis and management[68]. Nutrition and immune statuses are two critical aspects of the successful fight against COVID-19[55]. Prevention, diagnosis, and treatment of malnutrition should be routinely recommended in the management of patients with COVID-19, especially in those with liver dysfunction[34,69]. Nutritional therapy is a basic treatment and one of the core contents of comprehensive treatment measures for patients with COVID-19. Evidence-based effective nutritional therapy should be based on reasonable and indexed nutritional evaluation [70].

Studies have shown that nutrition therapy measures, including natural dietary supplements, vitamins, minerals, trace elements, and probiotics, might have potential hepatoprotective effects against COVID-19-related liver dysfunction *via* their antioxidant, antiviral, anti-inflammatory, and positive immunomodulatory effects. Combination therapy strategies and personalized nutritional and behavioral approaches can be developed in the COVID-19 era[52,71]. Additionally, the risk of excessive intake of some nutrients due to the popularity of dietary supplements exists, and dietitians' use of foods with protective effects against diseases has increased during the pandemic. Hence, consumers, patients, and nutritionists should be educated on the rational use of dietary supplements and health-protecting behaviors that can protect against COVID-19 for acute treatment, recovery, and prevention of chronic condition[72,73]. Moreover, additional tools and training are needed to optimize remote nutritional consultations, except for telemedicine, which have good prospects for dietary consultation[74]. Currently, many patients with COVID-19 have liver dysfunction, but nutritional studies related to this topic are not adequate. As nutritionists, it is our responsibility and obligation to facilitate further research in this area.

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Country/Territory of origin: China

ORCID number: Ming-Ke Wang 0000-0001-9918-0491; Xue-Lu Yu 0000-0002-8527-2093; Li-Yun Zhou 0000-0003-1413-8679; Hong-Mei Si 0000-0003-1175-6594; Ju-Fen Hui 0000-0001-7816-0635; Deng-Yong Hou 0000-0002-1683-6502; Wei-Peng Li 0000-0002-8993-5536; Ji-Shun Yang 0000-0001-7160-706X.

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ORIGINAL ARTICLE

Basic Study

Establishing a rabbit model of perianal fistulizing Crohn's disease

Shuang-Shuang Lu, Wen-Jia Liu, Qiu-Ya Niu, Chun-Yan Huo, Yu-Qing Cheng, En-Jing Wang, Rong-Nan Li, Fang-Fang Feng, Yi-Ming Cheng, Rong Liu, Jin Huang

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Shuang-Shuang Lu, Wen-Jia Liu, Qiu-Ya Niu, Chun-Yan Huo, Yu-Qing Cheng, En-Jing Wang, Rong-Nan Li, Fang-Fang Feng, Yi-Ming Cheng, Jin Huang, Gastroenterology Center, The Affiliated Changzhou Second People's Hospital of Nanjing Medical University, Changzhou 213000, Jiangsu Province, China

Shuang-Shuang Lu, Wen-Jia Liu, Yu-Qing Cheng, Rong-Nan Li, Fang-Fang Feng, Yi-Ming Cheng, Jin Huang, Graduate School, Dalian Medical University, Dalian 116044, Liaoning Province, China

En-Jing Wang, Graduate School, Nanjing Medical University, Nanjing 210000, Jiangsu Province, China

Rong Liu, Jin Huang, Medical Statistics Center, Changzhou University, Changzhou 213000, Jiangsu Province, China

Corresponding author: Jin Huang, PhD, Professor, The Affiliated Changzhou Second People's Hospital of Nanjing Medical University, No. 68 Gehu Road, Wujin District, Changzhou 213000, Jiangsu Province, China. hj042153@hotmail.com

Abstract

BACKGROUND

Crohn's disease (CD) is a chronic nonspecific intestinal inflammatory disease. The aetiology and pathogenesis of CD are still unclear. Anal fistula is the main complication of CD and is a difficult problem to solve at present. The main limitation of developing new therapies is bound up with the short of preclinical security and effectiveness data. Therefore, an ideal animal model is needed to establish persistent anal fistula and an inflamed rectal mucosa.

AIM

To improve the induction method of colitis and establish a reliable and reproducible perianal fistulizing Crohn's disease animal model to evaluate new treatment strategies.

METHODS

Twenty male New Zealand rabbits underwent rectal enema with different doses of 2,4,6-trinitrobenzene sulfonic acid to induce proctitis. Group A was treated with an improved equal interval small dose increasing method. The dosage of group B was constant. Seven days later, the rabbits underwent surgical creation of a transsphincteric fistula. Then, three rabbits were randomly selected from each

group every 7 d to remove the seton from the fistula. The rabbits were examined by endoscopy every 7 days, and biopsy forceps were used to obtain tissue samples from the obvious colon lesions for histological analysis. The disease activity index (DAI), colonoscopy and histological scores were recorded. Perianal endoscopic ultrasonography (EUS) was used to evaluate the healing of fistulas.

RESULTS

Except for the DAI score, the colonoscopy and histological scores in group A were significantly higher than those in group B ($P < 0.05$). In the ideal model rabbit group, on the 7th day after the removal of the seton, all animals had persistent lumens on EUS imaging, showing continuous full-thickness high signals. Histological inspection of the fistula showed acute and chronic inflammation, fibrosis, epithelialization and peripheral proctitis of the adjoining rectum.

CONCLUSION

The improved method of CD colitis induction successfully established a rabbit perianal fistula CD preclinical model, which was confirmed by endoscopy and pathology.

Key Words: Crohn's disease; Perianal fistula; Model; Endoscopy; Histology

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Core Tip: In this work, we improved the method of Crohn's disease (CD) colitis induction and successfully established a rabbit perianal fistula CD preclinical model, which was confirmed by endoscopy and pathology. The anatomy of this mid- to large-sized animal can simulate the human intestinal environment and tolerate examination and operation. This model may be used to assess perianal fistulizing CD treatments and their effectiveness.

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INTRODUCTION

Crohn's disease (CD) is a chronic, nonspecific intestinal inflammatory disease. The aetiology and pathogenesis of CD are still unclear[1]. Since the 1950s, the incidence rate of CD has been steady-state growth of industrialization nations. CD is a common digestive disease with an incidence of 12.7/100000 residents very year in Europe[2]. In the course of CD, there are different types of perianal diseases, including fistula, abscess, anal fissure, stricture and dermatophyte. These lesions may appear prior to or accompanied by CD intestinal symptoms and are factors affecting the prognosis of CD[3]. Anal fistula is the main complication of CD. Studies have shown that 15%-45% of CD patients have perianal lesions such as anal fistula[1].

Fistulizing anoperineal lesions represent a complex disease phenotype for which the treatment requires a multidisciplinary approach[4]. Modern medical concepts describe that patients with CD anal fistula should be treated with drugs first, and surgical treatment should be considered when necessary to control intestinal inflammation[5]. The main therapeutic drugs used are antibiotics, immunosuppressants, biological agents, *etc*[6-9]. In recent years, many studies have shown that the use of mesenchymal stem cells can be a new treatment for specific cases of complex fistulas[10,11]. In addition, some scholars have suggested other new treatments, such as hyperbaric oxygen therapy, as a potential adjuvant treatment for patients with inflammatory bowel diseases (IBDs)[12,13]. However, these new treatments have not been fully developed into routine and safe technical procedures. Major constraints on the development of update therapeutic schedules is obviously correlated with the short of preclinical security and effectiveness data. Up to now, an ideal animal model that can reproduce sustaining anal fistula and an inflamed rectal mucosa is needed.

The main purpose of this research is to improve the colitis induction method and develop a simple, reliable and reproducible fistula animal model to assess new treatment strategies.

MATERIALS AND METHODS

Animals and groups

Twenty male New Zealand rabbits, weighing about 2.0 kg, were chosen and numbered after weighing (the grouping methods are listed in Table 1). They were raised and placed under the condition of no special pathogen. The laboratory was clean, with good light and ventilation. The indoor temperature was controlled between 24 and 28 ℃, and the relative humidity was maintained between 50% and 70%, with 10-15 air changes per hour and 12 h of light each day. On the day before and the day of the operation, the rabbits were fed formula and drank freely. Cages of rabbits were disinfected and kept separate. Sufficient water and food were given. The rabbits were kept in cages for 7 d to adapt to the environment. The rabbits were weighed on the day of operation and then every 7 d. This study was approved by the ethics committee of Changzhou University.

For each procedure (enema, surgery or endoscopy), 1.5% pentobarbital sodium (3.5 mL/kg) was used for ear vein anaesthesia, and dyclonine hydrochloride mucilage was locally applied around the anus to reduce the pain associated with surgery.

Model induction process

Proctitis: A total of 100 mg/kg 2,4,6-trinitrobenzenesulfonic acid solution (TNBS) was dissolved in 50% ethanol (the total volume of solution is shown in Table 1) and was used for the induction of CD[14,15]. After 7 d of adaptive feeding, the experimental rabbits were fasted for 48 h and injected with 1.5% pentobarbital sodium through the ear vein. After anaesthesia, the rabbits were administered enemas with a TNBS + ethanol mixture by a 5 mL syringe through a central venous catheter every week according to the dose in Table 1 and then injected with air in a section of approximately 0.5 mm in length to remove the drug adhering to the syringe and enema tube wall as much as possible. Then, the rabbits were assigned to intervention groups A, B or C, where group C was used as a control.

Perianal fistula: On the 7th day after enema with TNBS, an anal fistula was caused by a minor operation. in the state of anaesthesia, the rabbits was fixed supine. Their perianal hair was shaved, and the area was disinfected with iodophor solution and then smeared with dyclonine hydrochloride mucilage. The elastic surgical seton (rubber band, diameter = 1.2 mm), soaked with TNBS solution in advance, was inserted into the needle core. For the experimental group, the seton was placed 1 cm from the anal margin at the same site, and a straight needle with a rubber band was used to puncture the rectum and then remove the punctured tissue from the body. A needle holder was used to clip the rubber band from the outside of the anus through the whole tunnel to make the rubber band pass through the perianal puncture opening, and a thin thread was used to fix the rubber band to prevent slippage and anal congestion. The external orifice is approximately 1 cm from the anus, as shown in Figure 1A. The surgical loop must be released without any tension. Finally, after the operation, the rabbits were returned to the feeding room, where they were observed and their vital signs were monitored until they woke up.

After the operation, a 1-mL syringe was used to inject TNBS mixed solution (diluted with 5% TNBS and absolute ethanol 1:1, total volume of 200 μL) into the fistula. Different doses of TNBS mixed solution (Table 1) were infused into the intestine once a week for three weeks, three times in total. To determine the best surgical procedure and reproducibility, 3 rabbits were randomly selected from each of groups A and B every 7 d, and the fistula setons were removed for endoscopic ultrasonography (EUS) assessment to evaluate the lumen. By the 28th day, the setons of all rabbits were removed. The characteristics of the two intervention groups and the different stages of the study are summarized in Figure 1B.

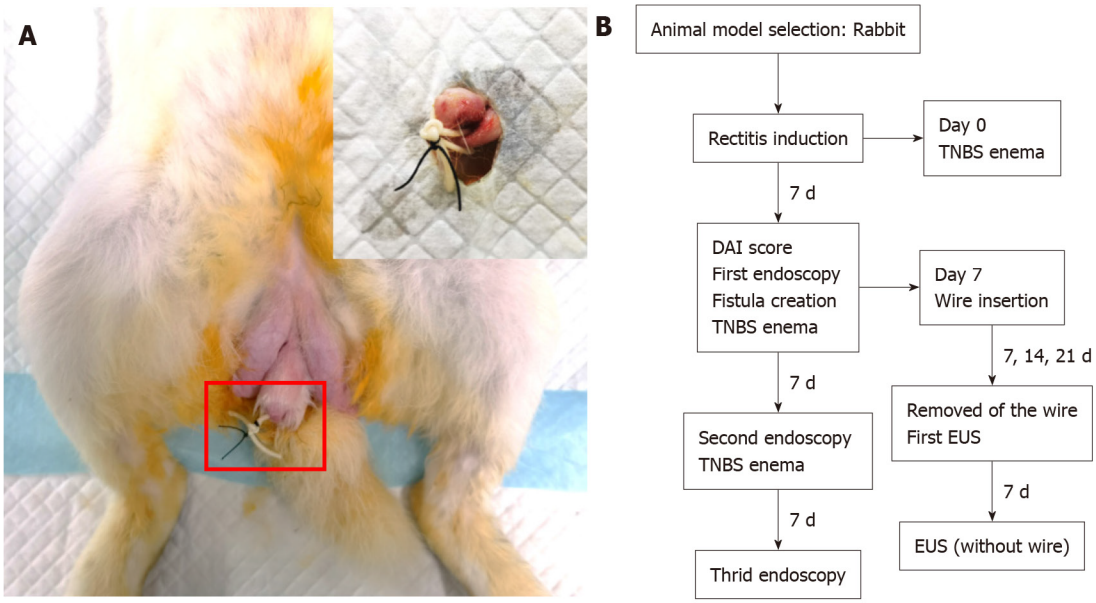
Model assessment

Clinical assessment: Clinical observation included: (1) recording the changes in daily activity, food intake, stool characteristics and body weight of the experimental animals and determining the disease activity index (DAI) score (Supplementary Table 1)[16]; (2) recording the number of deaths of the experimental animals in each group every day; and (3) checking whether the operation seton existed every day. In autonomous shedding, the new seton was inserted into the primary lumen again.

Endoscopic assessment: The colon macroscopic damage index (CMDI) was used for endoscopic assessment[17]. The CMDI was assessed according to the criteria described in Supplemental Table 2. Before the start of the study (TNBS enema administration), we performed an endoscopic examination of the rabbits to determine that the colon before treatment was normal, and these results were not included in the final statistics. After the study, the first intestinal endoscopy was performed on the 7th day (the day of surgical seton insertion). Morphological damage to the intestinal wall after the first intestinal administration was observed and scored. Then, endoscopy was performed every 7 d, and intestinal injury was observed and recorded. The last endoscopy was performed 21 d after the first enema. Endoscopy and scoring were performed by two experienced gastroenterologists (19 and 22 years of experience in the diagnosis and treatment of IBD, respectively).

Table 1 The volume of 2,4,6-trinitrobenzene sulfonic acid mixture administered by enema in each group

	Day 1	Day 7	Day 14
Group A (n = 9)	4.0 mL	5.0 mL	6.0 mL
Group B (n = 9)	5.0 mL	5.0 mL	5.0 mL
Group C (n = 2)	-	-	-



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Figure 1 Photograph of the external opening of the anal fistula, which was approximately 1 cm away from the anus, and a flowchart of the study protocol. A: The rubber band was used to hang the seton, and the leather band was fixed with a No. 0 operation seton to prevent slippage; B: One rabbit died one week after surgery. The remaining 17 rabbits were marked according to the length of insertion time. Three rabbits each were randomly selected from groups A and B. TNBS: 2,4,6-Trinitrobenzene sulfonic acid; DAI: Disease activity index; EUS: Endoscopic ultrasonography.

Histological examination: The tissue damage index (TDI) was used for the histological examination. The TDI was assessed using a modified version of the histological grading system described by MacPherson *et al*[18], as shown in Supplementary Table 3. At the same time as the endoscopic examination, 2-4 pieces of tissue with obvious inflammation and/or ulcers were clipped with biopsy forceps, fixed with neutral formaldehyde solution for 24 h, and stored at -4 ℃. Then, the specimens were embedded in paraffin, sliced continuously with a slicer, stained with haematoxylin-eosin, and finally scored histologically. Two experienced gastrointestinal pathologists performed blinded histological analyses.

The histological diagnosis of fistulas was ground on the following criteria: the internal orifice of the lumen is located on the rectal mucosa, and the external orifice of the lumen is located on the perineal skin. At the same time, it has the histological characteristics of proctitis (neutrophils, B and T lymphocytes, macrophages) were present. The feature of fistulas was decided by the presence or absence of epithelialization, fibration, and inflammation[19].

EUS assessment: The time of the insertion of the anal fistula operation thread was different in each group. After anal fistula formation, on the day of the removal of the thread inserted into the fistula, the perianal fistula of experimental rabbits in each group was examined by EUS for the first time, including mainly the observation of the fistula inner mouth, outer mouth, and course and the inflammation of the surrounding mucosa. The second EUS was performed on the 7th day after the removal of the thread. Spontaneous healing of the fistula was observed and recorded. Image recording and parameter interpretation were accomplished by a gastroenterologist (12 years of experience in diagnosis and treatment in IBD) and a ultrasound engineer (14 years of experience in interpreting ultrasound imaging). While they were blinded to groups of animals and histological results.

Statistical analysis

All the data were handled and analyzed by statistical software (SPSS 19.0), and the results are rendered as the mean ± SD. $P < 0.05$ was have been viewed as statistically critical.

RESULTS

Colitis model assessment

Clinical examination: In groups A and B, there were different degrees of loose stool and bloody stool visible to the naked eye. Rabbits ate less and were slow, low spirited, and occasionally irritable. Their weight gradually decreased with time. In group C, the body weight increased significantly with time, the activity was normal, and although there was occasional diarrhoea, there was no bloody stool. This condition was followed by the expected gradual weight recovery phase after the discontinuation of TNBS, which confirmed the healing of the colon injury. No rectal prolapse was observed. One week after the operation, the seton was removed from the perianal area of the experimental rabbits, and all the experimental rabbits showed two visible healing holes, which demonstrated the existence of the inner and outer holes. The rate of spontaneous seton shedding was approximately 17.6% (3/17) in each group. Also new setons were inserted again in the primary lumen of each animal. A total of 1 experimental rabbit died one week after surgery (group B) throughout the duration of experiments.

Endoscopy and pathology: Endoscopy was used to assess the modelling results. The process of colitis induction was smooth, and all rabbits underwent anal fistula surgery. One rabbit died one week after surgery. The rabbit was excluded from the results analysis. The remaining 17 rabbits were marked according to the length of insertion time.

After colitis was induced in the intervention group, the scores were determined, and the results are shown in Table 2. According to the statistical analysis (Table 2), except for the DAI score, the scores in group A were significantly higher than the scores in group B ($P < 0.05$).

In addition, we performed endoscopy in the process of colitis induction in rabbits of groups A and B and used biopsy forceps to obtain intestinal specimens for histological analysis. We found that although the rabbits in group B had obvious intestinal inflammation on the 7th day after the first TNBS enema, the intestinal inflammation at the last endoscopic examination was weaker than the intestinal inflammation in group A (Figures 2 and 3), showing that the inflammation of group A was higher than the inflammation of the other groups, and the modelling method of a TNBS dose increase in group A was better than the modelling method of other groups.

Model assessment of perianal fistulizing CD

EUS: All rabbits in groups A and B underwent two EUS scans of perianal fistulas. At the first EUS scan, all 17 rabbits (100%) had visible fistulas, also the external and internal orifice were noticeable in just about the greater part rabbits. At the second perianal EUS, that is, 7 d after the surgical seton was removed from the rabbits, the healing of the fistula in each group was different, as shown in Figure 4. Fistula was observed in 100% (6/6) of 6 rabbits with a surgical seton insertion time of 21 d. A scar was seen at the outer mouth of the fistulas, and granulation tissue hyperplasia was seen at the inner mouth. Other rabbits showed spontaneous healing of the fistula lumen and the disappearance of the fistula inner and outer orifices (Figure 4).

Pathology: Fibrosis has been distinguished in the connective tissue contiguous of the fistula 7 d after the insertion of the surgical seton. In addition, there were signs of acute (neutrophil infiltration and abscess formation) and chronic inflammation (lymphoplasmacyte infiltration, granuloma). On the 21st day after the insertion of the suture, granulation tissue was identified on the perianal orifice of the fistula. The pathologist positioned the rectal mucosa and thread through the anal sphincter as the fistula,which shown in Figure 5.

Immunohistochemistry confirmed that group A was acute inflammation. Neutrophils and other inflammatory cells infiltrated in the fistula (Figure 6).

DISCUSSION

One of the most challenging phenotypes of CD is perianal fistula. The combination of perianal disease and CD predicts a significantly worse course[20]. The pathogenesis of CD and its complications are unclear. At present, there is no ideal curative treatment[21]. It is difficult to treat perianal fistulizing CD, which usually requires more active medical and surgical intervention.

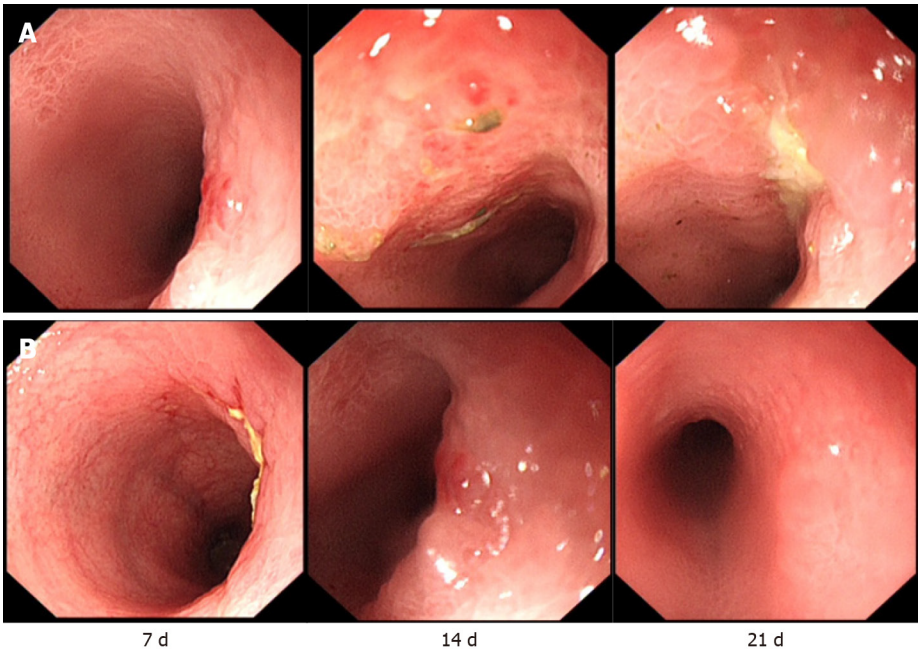
Compared with rats, rabbits are mid- to large-sized animals. The rabbit anal and rectal anatomy is similar to that of humans and is of appropriate size[22,23]. The rabbit anatomy can maximize and tolerate the simulation of human perianal fistulizing CD-related auxiliary examinations, such as endoscopy, EUS, computed tomography, and magnetic resonance imaging (MRI). A preclinical model of rectal histological inflammation with perianal sphincteric fistula was established and observed continuously under endoscopy and confirmed by EUS. The diagnosis of fistula depends on EUS and histology.

As to the improvements in CD animal modelling through the use of this method involving TNBS[24], the method started with the administration of a small dose of TNBS, and then an increasing dose was

Table 2 Identification of the rabbit model of Crohn's disease induced by 2,4,6-trinitrobenzene sulfonic acid

	Group	Day 7	*P* value	Day 14	*P* value	Day 21	*P* value	Day 28	*P* value
DAI	A	4.22 ± 2.33	0.026	6.88 ± 0.93	0.020	8.00 ± 2.00	0.810	7.78 ± 1.56	0.021
	B	7.13 ± 2.53		8.75 ± 1.91		8.25 ± 2.25		5.75 ± 1.67	
Endoscopy scores	A	1.11 ± 0.78	0.002	2.44 ± 0.88	0.319	3.89 ± 0.93	< 0.001		
	B	2.88 ± 1.13		2.88 ± 0.83		1.63 ± 0.92			
Histology scores	A	1.44 ± 0.88	< 0.001	3.11 ± 0.93	0.017	4.11 ± 0.78	< 0.001		
	B	3.50 ± 0.93		2.00 ± 0.76		1.88 ± 0.99			

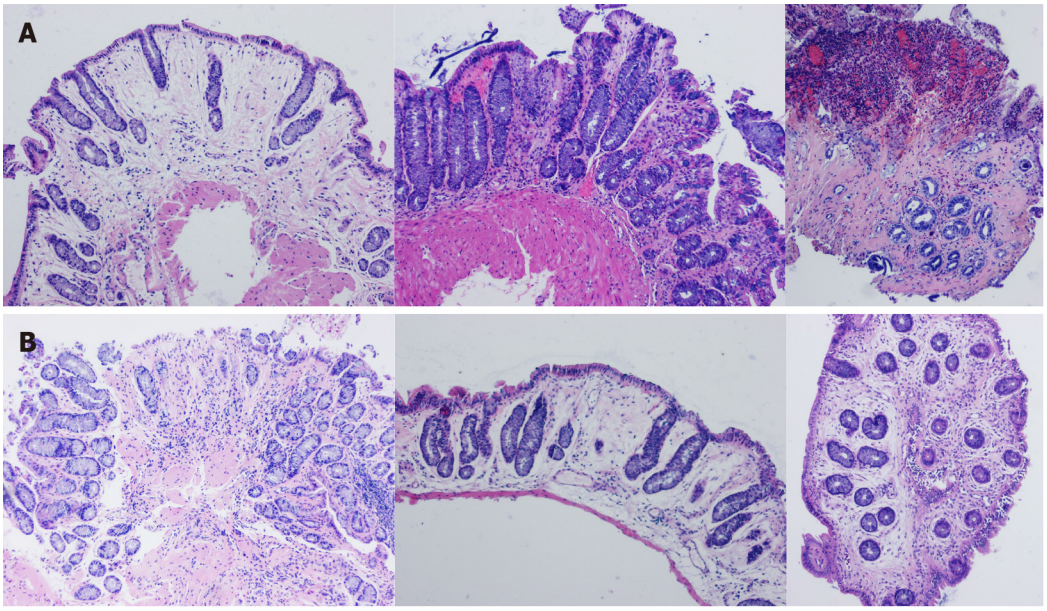
DAI: Disease activity index.



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Figure 2 The rabbits in groups A and B underwent endoscopy 3 times. A: In group A, mild mucosal erosion was observed on the 7th day. On the 14th day, a large area of mucosal oedema and erosion appeared. On the 21st day, the surrounding mucosa was swollen, and a central ulcer had formed; B: In group B, ulceration occurred on the 7th day. On the 14th day, the mucosa was swollen, and the surface was hyperaemic and eroded. On the 21st day, the main manifestation was local congestion.

administered at the same intervals. This procedure not only simulated the characteristics human CD recurrence through repeated stimulation of the intestinal mucosa but also prolonged the duration of inflammation, showing that TNBS could induce a stronger inflammatory response by being administered at an increasing dose in a step-by-step manner. Group B showed improvements in bowel inflammation, possibly due to the initially established tolerable doses, but further investigation is needed. In our model, persistent inflammation is induced, and slight changes may occur with longer healing time, with histological changes replicating the unpredictability of CD. In the process of making an anal fistula, we used an elastic rubber band, which ensured the tension-free state of the fistula. At the end of the study, all rabbit models still had visible fistulas 7 d after the removal of the seton, and EUS showed continuous full-thickness high signal. However, the rabbits in which setons were inserted by 7 and 14 d had fistula healing, and 50% and 67% of the rabbits had fistulas visible on EUS after setons were removed 7 d later. Pathology revealed the same results for the longest seton insertion. The best option to obtain a preclinical model of perianal fistula consisted of low-dose incremental administration of TNBS and 21-d seton insertion. The induced fistula might has been described by mucosal ulcers extending to the perianal dermis. Pathologic examination showed augmented chronic submucosal infiltration of inflammatory cells, granuloma formation, and neutrophilic aggregation of colonic mucosa. At the same time, ultrasound endoscopy revealed continuous high signals in the skin around the anus, indicating the presence of an inflammatory response. These features are similar to those found in human perianal fistula CD and there is active inflammation in the fistula[19].



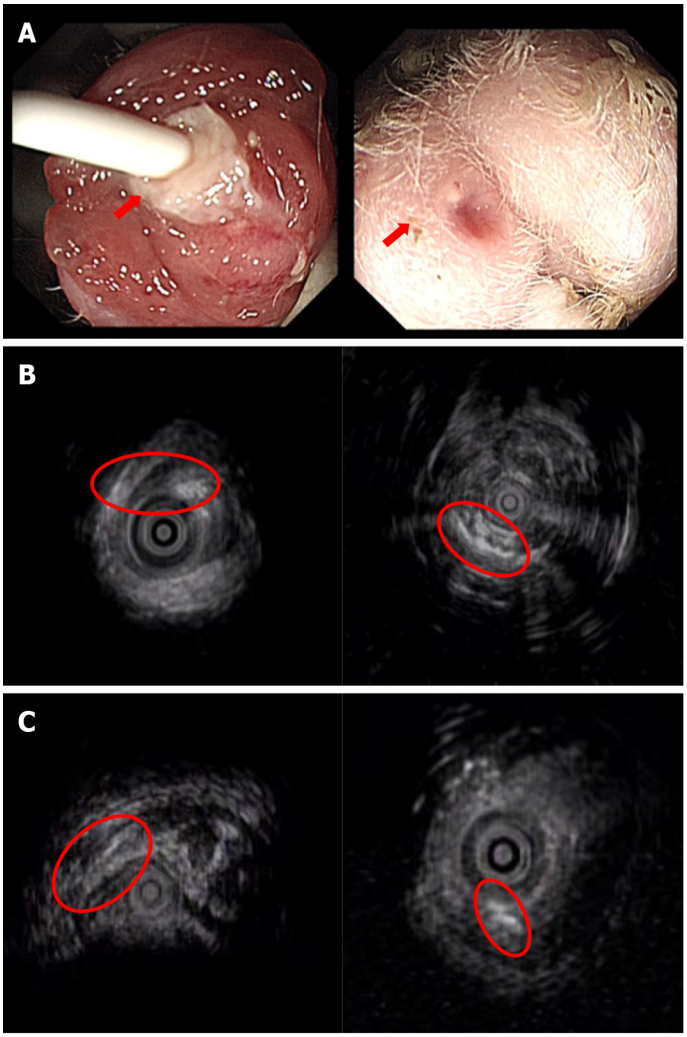
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Figure 3 The rabbits in groups A and B underwent three consecutive endoscopic biopsies, and samples were stained with haematoxylin-eosin. A: Group A exhibited mild to moderate inflammation on day 7, manifested by fewer crypts, marked mucosal oedema, and minimal inflammatory cell infiltration; Group A exhibited fibrosis, hyperaemia, and moderate inflammatory cell infiltration on day 14, and severe inflammatory mucosal ulceration on day 21; B: On the 7th day, there was a higher number of shallow crypts, less oedema and less inflammatory cell infiltration in group B. On the 14th day, there was severe oedema, decreased crypts and less inflammatory cell infiltration in the mucosa, and on the 21st day, there was moderate fibrosis and oedema, mild inflammatory cell infiltration and a complete crypt structure (magnification ×100).

Perianal fistulizing CD models have been described differently by several authors. For example, the SAMP1/YitFc mouse model[25] or anal furunculosis model in canines[26] generate fistulas at random. A unprompted model similar to CD terminal ileitis was found in SAMP1/YitFc mice[25]. In this study, 5% of mice were found to form anal fistula naturally, which may be partially similar to human CD anal fistula. Whereas, only a small proportion of mice form fistula, which restricts application of this model in the study of CD. Size considerations make the murine model[27] more suitable for testing medical therapies than surgical interventions. For the canine model, considering the unpredictable onset time and the difficulty of inducing fistula, the applicability of the model is limited. A previous study developed another experimental model in 16 rabbits[28] by the surgical creation of a high transsphincteric fistula. Another study developed a pig anal fistula model[29] (6 pigs) to test a new biological plug, which was confirmed by histology. A recent article in 2019[19] described induced proctitis in rats by rectal enema using TNBS. Seven days later, the sphincter fistula was established using a surgical seton. In all these cited studies, unlike our research, previously published models either did not have proctitis or fistula inflammation, or a single high-dose enema caused high mortality. In addition, it is to some extent unethical that tissues can be obtained from these animals only after they are killed.

According to the current literature, we first applied endoscopy and EUS to the study of animal models of anal fistula in CD. Previous reports have used endoscopy to study oesophageal diseases in rabbits[30]. We performed colonoscopy with a small-diameter endoscope on rabbits, which can clearly show intestinal mucosa lesions. In addition, biopsy was performed in this mid- to large-sized animal for pathology assessment. In recent years, less invasive imaging modalities, such as pelvic MRI and EUS, have been used for fistula evaluation[31]. According to the latest available guidelines, contrast-enhanced pelvic MRI is generally considered the initial procedure for assessing perianal fistulizing CD[32]. However, EUS is considered a good alternative to MRI and has good accuracy when anal stenosis is excluded[32]. Although this method cannot obtain complete fistula tissue for histological analysis, we believe that, to a certain extent, this method is more in line with ethical requirements. Moreover, this simple, reproducible animal model can be used to evaluate the new treatment and efficacy of CD anal fistula.

With that in mind, we chose a small dose of TNBS as the initial stimulus and then repeated administration at the same intervals with dose increases. This procedure mimics to some extent the characteristics of human CD recurrence with repeated stimulation of the intestinal mucosa, prolongs the duration of inflammation, and imitates the process of CD recurrence. Second, the longest period of seton insertion was 21 d, an ideal result. Although in other studies, fistula-related thread insertion times were longer than ours[28],the prolongation of the seton insertion time may lead to longer fistula removal.



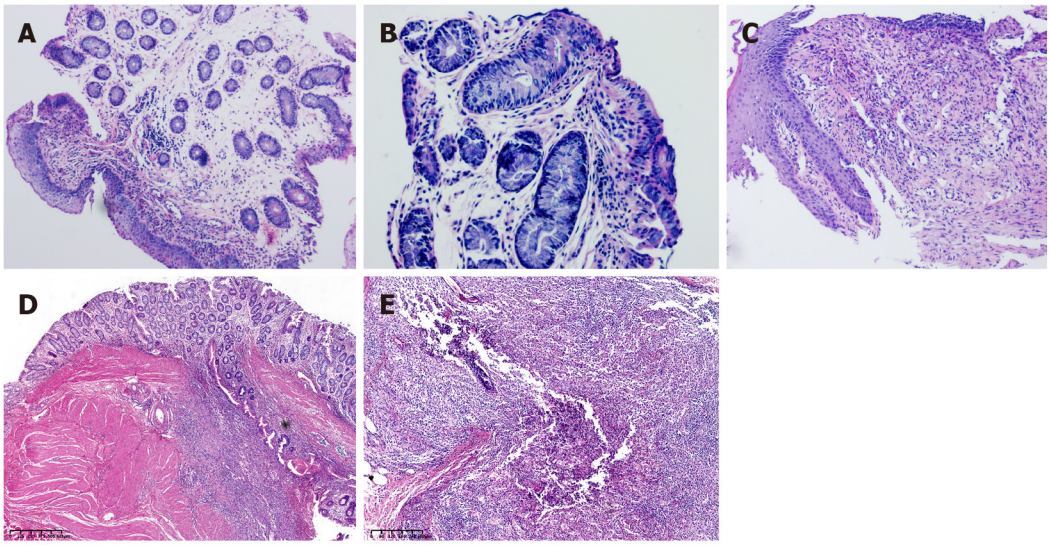
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Figure 4 Visualization of a transsphincteric anal fistula *via* endoscopic ultrasonography. A: The internal and external openings of the experimental fistula can be directly observed; B: All rabbits with a 21-d insertion time of the surgical thread showed a complete fistula; C: The rabbits with short thread insertion times had different degrees of fistula healing or the disappearance of internal and external fistulas. D0: Day 0; D7: Day 7.

However, given that our study required continuous weekly bowel administration, extending the duration of the study increased the damage to the animals. Moreover, the criteria for the difference between the two experimental conditions (constant-dose or increased-dose TNBS) introduced in the study still need to be verified by larger animal experiments, and the molecular mechanism involved should be investigated. Thus, there are some limitations to this study.

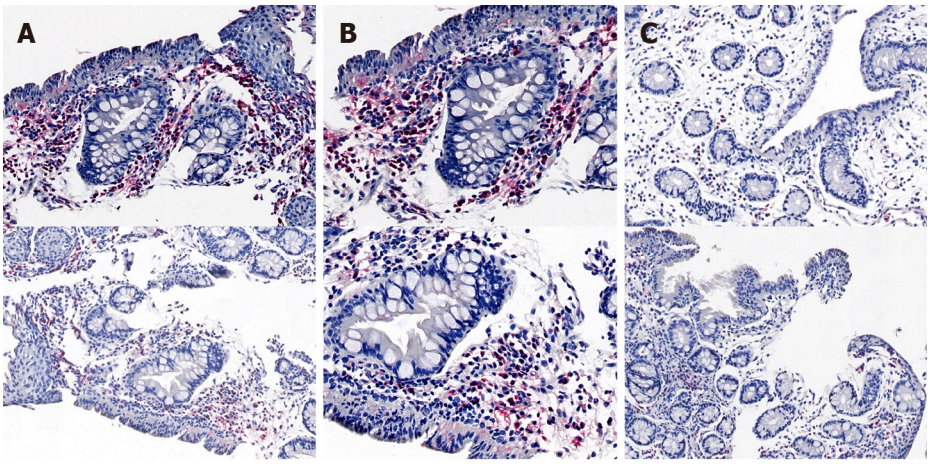
CONCLUSION

In this study, a simple preclinical animal model of perianal fistulizing CD in rabbits was established by using an improved method of CD colitis induction. The model can simulate the human condition, and the intestinal and fistula lesions induced can be evaluated by EUS, endoscopic and histological examinations to assess new therapeutic strategies.



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Figure 5 The histological characteristics of a fistula tract. A: Magnification ×100; B: Magnification ×200; C: Magnification ×100. The early histological changes of fistula are shown; D and E: Longitudinal sections; histological results (rabbits with setons inserted 21 d) showing the inflamed fistula tract. The fistula lumen is visible with internal (digestive side) and external (perineal skin with adipocytes) orifices. There were local inflammatory signs of suppurative inflammation with abscess formation around the fistula.



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Figure 6 Immunohistochemistry of a fistula tract. A: Magnification ×10; B: Magnification ×20; C: Control. Immunohistochemistry of the fistula tract using anti-CD68 antibodies (upper panel) or anti-MPO antibodies (lower panel).

ARTICLE HIGHLIGHTS

Research background

Crohn's disease (CD) is a chronic nonspecific intestinal inflammatory disease. The aetiology and pathogenesis of CD are still unclear. Anal fistula is the main complication of CD and is a difficult problem to solve at present. In recent years, there has been an increasing number of potential treatments for patients with inflammatory bowel diseases. However, these new treatments have not been fully developed into routine and safe technical procedures.

Research motivation

The main limitation in developing new therapies for CD with anal fistula is connected with the deficiency of preclinical safety and credible experimental data records. Therefore, an ideal animal model is needed to establish models of persistent anal fistula and an inflamed rectal mucosa.

Research objectives

The aim of this study was to improve the induction method of colitis and establish a reliable and reproducible perianal fistulizing CD animal model to evaluate new treatment strategies.

Research methods

Twenty male New Zealand rabbits underwent rectal enema with different doses of 2,4,6-trinitrobenzene sulfonic acid (TNBS) to induce proctitis. Group A was treated with an improved equal interval small dose increasing method. The dosage of group B was constant. Seven days later, the rabbits underwent surgical creation of a transsphincteric fistula. Then, three rabbits were randomly selected each group every 7 d to remove the seton from the fistula. The rabbits were examined by endoscopy every 7 d, and biopsy forceps were used to obtain tissue samples from the obvious colon lesions for histological analysis. The disease activity index (DAI), colonoscopy and histological scores were recorded. Perianal endoscopic ultrasonography (EUS) was used to evaluate the healing of fistulas.

Research results

Except for the DAI score, the colonoscopy and histological scores in group A were significantly higher than those in the other groups ($P < 0.05$). In the ideal model rabbit group, on the 7th day after the removal of the seton, all animals had persistent lumens on EUS imaging, showing continuous full-thickness high signals. Acute and chronic inflammation, epithelialization, fibrosis, and peripheral proctitis of consecutive rectum are the histological features of fistula.

Research conclusions

A preclinical model of perianal fistulizing CD in rabbits was established by using an improved method of CD colitis induction. The model can simulate the human environment, and intestinal and fistula lesions can be evaluated by EUS, endoscopic and histological examinations to assess new therapeutic strategies.

Research perspectives

The establishment of a model of fistula associated with colitis allows the evaluation of different therapeutic approaches. However, fistula formation in animal models does not fully reflect the condition in humans. We hope that the simple, reliable and repeatable fistula animal model established by this improved colitis induction method can be used to evaluate new treatment strategies. The criteria for the difference between the two experimental conditions (constant-dose or increased-dose TNBS) introduced in the study still need to be verified by larger animal experiments, and the molecular mechanism involved should be investigated. The optimal animal model should include genetically mediated development of CD with anal fistula. However, an ideal model for preclinical research is difficult to establish due to the long experimental period required.

FOOTNOTES

Author contributions: These authors contributed to this work. Huang J and Niu QY conceived the concept; Lu SS designed the method; Lu SS completed the entire study with the help of Liu WJ, Huo CY, Li RN, Wang EJ, Feng FF, Liu R and Cheng YM; the histological sections were completed with the help of Cheng YQ and Huo CY; endoscopy was performed by Huang J and Niu QY; Lu SS, Huang J and Liu WJ discussed the results and revised the manuscript.

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Institutional animal care and use committee statement: All animal experiments conformed to the internationally accepted principles for the care and use of laboratory animals.

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

Data sharing statement: No additional data are available.

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

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Country/Territory of origin: China

ORCID number: Shuang-Shuang Lu 0000-0002-3791-0361; Wen-Jia Liu 0000-0002-5502-0709; Qiu-Ya Niu 0000-0001-5689-9615; Chun-Yan Huo 0000-0002-8082-8953; Yu-Qing Cheng 0000-0003-1806-9893; En-Jing Wang 0000-0002-6569-4627;

Rong-Nan Li 0000-0002-6271-0468; Fang-Fang Feng 0000-0002-1730-0523; Yi-Ming Cheng 0000-0001-8415-1030; Rong Liu 0000-0001-7574-0685; Jin Huang 0000-0001-7235-7381.

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ORIGINAL ARTICLE

Case Control Study

Reevaluation of the expanded indications in undifferentiated early gastric cancer for endoscopic submucosal dissection

Jiyoung Yoon, Seung-Yeon Yoo, Young Soo Park, Kee Don Choi, Beom Su Kim, Moon-Won Yoo, In Seob Lee, Jeong Hwan Yook, Ga Hee Kim, Hee Kyong Na, Ji Yong Ahn, Jeong Hoon Lee, Kee Wook Jung, Do Hoon Kim, Ho June Song, Gin Hyug Lee, Hwoon-Yong Jung

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Jiyoung Yoon, Kee Don Choi, Ga Hee Kim, Hee Kyong Na, Ji Yong Ahn, Jeong Hoon Lee, Kee Wook Jung, Do Hoon Kim, Ho June Song, Gin Hyug Lee, Hwoon-Yong Jung, -Department of Gastroenterology, Asan Medical Center, University of Ulsan College of Medicine, Seoul 05505, South Korea

Seung-Yeon Yoo, Young Soo Park, Department of Pathology, Asan Medical Center, University of Ulsan College of Medicine, Seoul 05505, South Korea

Beom Su Kim, Moon-Won Yoo, In Seob Lee, Jeong Hwan Yook, Department of Surgery, Asan Medical Center, University of Ulsan College of Medicine, Seoul 05505, South Korea

Corresponding author: Kee Don Choi, MD, PhD, Doctor, Department of Gastroenterology, Asan Medical Center, University of Ulsan College of Medicine, 88 Olympic-ro 43-gil, Songpa-gu, Seoul 05505, South Korea. keedon@amc.seoul.kr

Abstract

BACKGROUND

Although the criteria for the indication of endoscopic submucosal dissection (ESD) for undifferentiated early gastric cancer (UD-EGC) have been recently proposed, accumulating reports on the non-negligible rate of lymph node metastasis (LNM) after indicated ESD raise questions on the reliability of the current criteria.

AIM

To investigate the prevalence and risk factors of LNM in UD-EGC cases meeting the expanded indication for ESD.

METHODS

We retrospectively reviewed 4780 UD-EGC cases that underwent surgical resection between January 2008 and February 2019 at Asan Medical Center, a tertiary university hospital in Korea. To identify the risk factors of LNM of UD-EGC meeting the expanded criteria for ESD, we performed a case-control study by matching the cases with LNM to those without at a ratio of 1:4. We reviewed the clinical, endoscopic, and histologic features of the cases to identify features with a significant difference according to the presence of LNM. Univariate and

multivariate logistic regression analyses were performed to estimate the odds ratios (ORs).

RESULTS

Of the 4780 UD-EGC cases, 1240 (25.9%) were identified to meet the expanded indication for ESD. Of the 1240 cases, 14 (1.1%) cases had LNM. Among the various clinical, endoscopic, and histopathological features that were evaluated, mixed histology (tumors consisting of 10%-90% of signet ring cells) had a marginally significant association (P = 0.059) with the risk of LNM. Moreover, diffuse blurring of the muscularis mucosae (MM) underneath the tumorous epithelium, a previously unrecognized histologic feature, had a significant association with the absence of LNM (P = 0.028). Multivariate logistic regression analysis showed that the blurring of MM was the only explanatory variable significantly associated with a reduced risk of LNM (OR: 0.12, 95%CI: 0.02-0.95; P = 0.045).

CONCLUSION

The risk of LNM is higher than expected when using the current expanded indication for UD-EGC. Histological evaluation could provide useful clues for reducing the risk of LNM.

Key Words: Gastric cancer; Undifferentiated carcinoma; Endoscopic submucosal dissection; Lymph node metastasis

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Core Tip: This was a retrospective study investigating the prevalence and risk factors of lymph node metastasis (LNM) in cases with undifferentiated early gastric cancer meeting the expanded indication for endoscopic submucosal dissection (ESD). We found that the incidence rate of LNM was 1.1% (14/1240), which was higher than expected for indicated ESD. A subsequent case-control study revealed that two histological features-histologic purity of tumors and blurring of the muscularis mucosae underneath the tumorous epithelium-are promising factors for predicting the risk of LNM. Combining these histologic features could improve the current expanded indication criteria for ESD.

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INTRODUCTION

Endoscopic submucosal dissection (ESD) has gained popularity in the treatment of early gastric cancer (EGC) due to the benefits of organ preservation and maintenance of the quality of life. However, ESD cannot dissect lymph nodes around the stomach and diagnostic modalities such as endoscopic ultrasonography, computed tomography, and positron emission tomography cannot provide adequate data for detecting lymph node metastasis (LNM); as such, the indication for ESD for EGCs has been suggested based on the analysis of the risk of LNM in a large number of surgically resected specimens [1], and ESD is usually considered for tumors with a very low risk of LNM.

Undifferentiated EGC (UD-EGC) accounts for 40%-50% of EGCs, and has been reported to show a higher incidence of LNM than EGCs with differentiated histology[2,3]. Thus, gastrectomy with lymphadenectomy has been used as the standard treatment for UD-EGCs, and ESD for UD-EGC remains an investigational treatment[3,4]. In an attempt to expand the indication of ESD in UD-EGC, some researchers have reported that a select group of UD-EGCs had a very low possibility of LNM[1]. Accordingly, it has been suggested that ESD could be considered for UD-EGC cases when the tumor is an intramucosal lesion with a size of less than 2 cm and no sign of ulcer, and no further surgery is indicated when pathologic evaluation of the ESD specimen does not reveal lymphovascular invasion (LVI) or positive vertical and horizontal margin[1,4]. However, there have been several reports of lymph node or distant metastases arising after curative ESD in UD-EGC cases meeting the expanded criteria[5, 6]. These conflicting data raise a question on the reliability of the current expanded criteria for UD-EGC, and necessitates further efforts to identify more clinicopathologic features associated with the risk of LNM in UD-EGC.

Because LNM-negative patients can be curatively treated with minimally invasive ESD, evaluating the risk factor of LNM is crucial for determining the appropriate treatment. Although many studies have been performed to identify the clinicopathological factors associated with LNM in UD-EGC[7,8], the only risk factors that were identified include tumor size, depth of invasion, presence of LVI, and ulcer. Therefore, to obtain clarity regarding the treatment of UD-EGC, we investigated the risk of LNM of UD-EGCs meeting the criteria for the expanded indication for ESD, and performed a case-control study to identify the clinical, endoscopic, and histopathologic features related to the risk of LNM.

MATERIALS AND METHODS

Patients

We retrospectively reviewed the medical records of all patients who underwent curative gastrectomy with extended lymphadenectomy for UD-EGC at Asan Medical Center between January 2008 and February 2019. To focus on the histologic types that are most frequently encountered in clinical practice, we only included tumors diagnosed as "adenocarcinoma, poorly differentiated (with or without signet ring cell component)," "poorly cohesive carcinoma," and "signet ring cell carcinoma (SRCC)," and excluded rare variants such as mucinous adenocarcinoma and gastric carcinoma with lymphoid stroma. We also excluded patients with multiple tumors, tumors in the remnant stomach, any synchronous malignancy in other organs, a history of preoperative treatment such as ESD, and those who received neoadjuvant chemotherapy. Cases with less than 15 lymph nodes harvested were also excluded. Based on the original pathology reports of the remaining cases, we included those meeting all of the following criteria for the expanded indication of ESD: (1) confinement to the mucosal layer (pT1a); (2) size ≤ 2 cm; (3) absence of ulcer; and (4) absence of LVI[3,4].

To identify the clinical, endoscopic, and pathologic findings associated with the risk of LNM, we performed a case-control study by matching the patients with LNM to those without at a ratio of 1:4 in terms of sex, age at gastrectomy (± 2 years), and tumor size. Histologic review was conducted to confirm whether the cases indeed satisfy the expanded criteria. The selection process for our study population is shown as a flowchart (Figure 1).

Data collection

Clinical data, endoscopic features, and pathological characteristics of the study patients were evaluated. Endoscopic characteristics (*e.g.*, tumor location, macroscopic type of the lesion, endoscopic presence of ulcer, converging folds, exudates, and tumor island) were evaluated by two endoscopists (JY and KDC); the endoscopists independently reviewed the initial endoscopic images obtained before biopsy, and discussed with each other until a consensus was reached. The tumor locations were specified both longitudinally (upper *vs* middle *vs* lower third) and cross-sectionally (lesser curvature *vs* posterior wall *vs* greater curvature *vs* anterior wall). By referring to the classification system of the Japanese Research Society for Gastric Cancer[3], the macroscopic type of the lesion was evaluated based on the predominant type into three categories as follows: elevated type (including the protruded type and superficial elevated type), flat type (the superficial flat type), and depressed type (the superficial depressed type and excavated type)[3]. Endoscopic ulcer was defined as the presence of a mucosal defect of ≥ 3 mm. Converging folds were indicated by the presence of any centripetal folds in the EGC lesions. The representative endoscopic appearance is depicted in Supplementary Figure 1.

Histologic evaluation of the tumor and background stomach

Hematoxylin and eosin (HE)-stained glass slides produced at the time of initial diagnosis were evaluated to double-check the size of tumors in the greatest dimension, depth of invasion, and the status of lymph node metastasis. Entire tumors and adjacent non-tumor areas were serially sectioned at 3-to-4-millimeter intervals and made into paraffin blocks, which were then used to generate slides for histologic evaluation. Histologic mapping was performed to explicitly measure the size of a tumor, and the entirely embedded tumor and adjacent normal area were examined for the percentage of signet ring cells (SRCs), and status of background gastric mucosa. The percentage of SRCs was evaluated by examining the entire tumor sections. Definition of SRCs was established by the agreement between two pathologists (SYY and YSP) based on a recent consensus guideline on poorly cohesive gastric carcinoma [9]; according to the guideline, tumors almost exclusively (≥ 90%) consisting of SRCs were designated as SRCC, and those with < 10% of SRC components as poorly differentiated carcinomas (PDs), which encompass both poorly differentiated adenocarcinomas and non-SRC type of poorly cohesive carcinomas. The remaining tumors in which SRCs comprise 10%-90% of the components were designated as mixed tumors (*e.g.*, adenocarcinoma, poorly differentiated with SRC component and adenocarcinoma, moderately differentiated with SRCC). The status of background gastric mucosa was evaluated based on the likelihood of *Helicobacter pylori* (*H. pylori*) colonization as follows: less likely [minimal to mild chronic gastritis (CG) with no intestinal metaplasia (IM)], indeterminate (moderate CG or presence of IM), possible [chronic active gastritis (CAG)], and definite (CAG with clearly visible *H. pylori*). For each case, the section that seemed most likely to harbor *H. pylori* was selected for immuno-

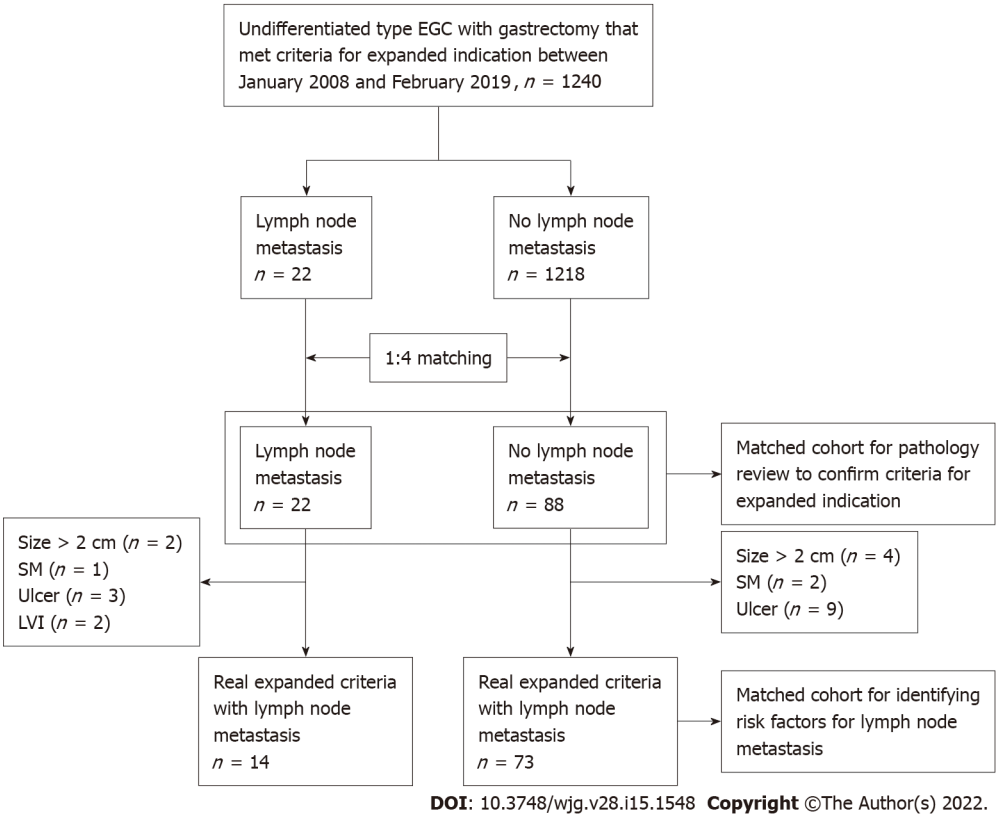


Figure 1 Flow chart of study patients. EGC: Early gastric cancer; SM: Submucosa; LVI: Lymphovascular invasion.

histochemical studies.

Immunohistochemistry

Immunohistochemical staining was performed on 4 μm-thick serial tissue sections of formalin-fixed paraffin-embedded (FFPE) blocks. For *H. pylori* evaluation, tissue sections were stained using the antibody against *H. pylori* (1:500, rabbit polyclonal, catalog No. 215A-76, Cell Marque, Rocklin, CA) using the OptiView DAB IHC Detection Kit on the BenchMark XT automatic immunostaining device (Ventana Medical Systems, Tucson, AZ, United States) according to the manufacturer's instructions. The abundance of immunohistochemically highlighted *H. pylori* was evaluated based on the Sydney system [10] as follows: 0 (absent), 1+ (*H. pylori* occupies < 1/3 of mucosa), 2+ (*H. pylori* occupies 1/3–2/3 of mucosa) and 3+ (*H. pylori* occupies > 2/3 of mucosa). *H. pylori* stain could not be performed in two cases due to the unavailability of FFPE blocks. TP53 staining was performed on representative sections at the time of initial diagnosis following the same protocol described above (1:1000, mouse monoclonal, clone DO-7, catalog No. M7001, Dako, Glostrup, Denmark). The degree of TP53 nuclear immunoreactivity was graded as 0 (no positive cells), 1+ (focal, faint positivity), 2+ (focal, moderate positivity), and 3+ (unanimously strong positivity); 0 was interpreted as the loss of expression, 1+ and 2+ as wildtype pattern, and 3+ as overexpression. TP53 status could not be evaluated in four cases due to the loss of stained slides.

Histologic evaluation of the tumor microenvironment

The abundance of tumor-infiltrating lymphocytes (TILs) was evaluated in HE slides in a semi-quantitative manner according to the proposed standardized methodology described in a recent consensus guideline[11].

The degree of peritumoral fibrosis was evaluated by Masson's trichrome (MT) staining. For 85 cases with accessible FFPE blocks, two sections per case-the deepest section and the edge of the tumor-were selected, and MT stain was performed on 4 μm-thick serial tissue sections of FFPE blocks using Trichome III Blue Staining Kit (Ventana Medical Systems) at BenchMark Special Stains platform (Ventana Medical Systems). The degree of fibrosis was visually graded as mild, moderate, or marked (Supplementary Figure 2C). For computer-aided image analysis, slides were scanned by the Pannoramic 250 Flash slide scanner (3D HISTECH, Budapest, Hungary) at 20× magnification with a resolution of 0.22 μm per pixel.

The degree of MT staining was quantified by pixel classification functionality of QuPath, an open-source software for analyzing digital pathology images (Supplementary Figure 2D)[12]. Briefly, the interface between MM and submucosa underneath the tumorous epithelium was manually annotated as

the region of interest (ROI). Two different types of pixel classifiers were trained and sequentially applied. The first classifier was trained to classify the ROI into empty (empty space and fat vacuoles) and non-empty areas. Non-empty areas were placed into the second classifier that graded the intensity of MT as 0 (vessel and MM), 1+ (mild), 2+ (moderate), or 3+ (marked). To express the intensity and extent of MT staining, a metric named "fibrosis score" was defined as follows:

$$\text{Fibrosis score} = \frac{3 \times \text{Area}_{3+} + 2 \times \text{Area}_{2+} + 1 \times \text{Area}_{1+}}{\text{Area of total non - empty area}}$$

Evaluation of blurring of muscularis mucosa

Blurring of MM underneath tumorous epithelium was primarily evaluated at scanning magnification. A case was judged to have blurred MM when any of the two MT-stained slides showed a focus of blurring or disruption of MM by fibrosis that caused loss of integrity relative to adjacent MM underneath the non-tumorous epithelium. If a case had more than one of such foci or the disruption was prominent enough to localize the tumor at scanning magnification, the case was judged to show diffuse blurring of MM (Figure 2A and B). At the foci of MM blurring, the distance from the invasive front to MM was measured. The value of 0 was assigned for tumors touching or invading into the MM. The interobserver reproducibility of MM blurring (non-diffuse or diffuse) was assessed by independent assessment of two pathologists (SYY and YSP). MM blurring assessed by SYY was used for subsequent statistical analysis.

Statistical analysis

Categorical variables are expressed as numbers with percentages and continuous variables are expressed as medians with interquartile ranges (IQRs). The Chi-squared test or Fisher's exact test was used to compare categorical variables as appropriate, and the *t*-test or Wilcoxon rank-sum test was used to compare continuous variables depending on the result of the Shapiro–Wilk normality test. Univariable and multivariable logistic regression analyses were performed to identify the risk factors by estimating the ORs and 95%CIs. Cohen's kappa was computed as a metric of interobserver reproducibility of MM blurring. *P* values < 0.05 were considered statistically significant. Statistical evaluations were performed using SAS 9.4 (SAS Institute, Inc., Cary, NC, United States) and R version 3.6.2 for Windows (R Foundation for Statistical Computing, Vienna, Austria). The statistical methods of this study were reviewed by Kim HJ from the Department of Clinical Epidemiology and Biostatistics at Asan Medical Center, University of Ulsan College of Medicine.

RESULTS

Risk of LNM in UD-EGCs meeting the criteria for expanded indication for ESD

During the study period, 4780 patients underwent curative gastrectomy with extended lymphadenectomy for EGCs whose histology showed SRCs, PD, or a mixed type of both tumors. Of the 4780 patients, 1240 satisfied the criteria for the expanded indication for ESD. Among them, 22 patients had LNM and the remaining 1218 patients did not.

To identify the risk factors of LNM in patients with UD-EGC satisfying the expanded indication, a 1:4 case-control study was designed. According to the matching conditions, 22 patients with LNM were matched to 88 patients without. Subsequent pathology review was conducted for case and control patients to ensure that they satisfied the criteria for the expanded indication; as a result, 8 cases in the case group revealed histologic features inconsistent with the original pathology report and did not meet the criteria for the expanded indication as follows: size > 2 cm (n = 2), presence of submucosal invasion (n = 1), ulcer (n = 3), and LVI (n = 2). Similarly, 15 cases in the control group were excluded from the study owing to the following discrepancies from the original pathology report: size > 2 cm (n = 4), presence of submucosal invasion (n = 2), and ulcer (n = 9). Consequently, 14 (1.1%) patients among 1240 patients with UD-EGC were designated to have LNM and were included in the case group (LNM+), and 73 patients without LNM were included in the control group (LNM-).

Clinical and endoscopic features of the study patients

Clinical and endoscopic features of the 87 UD-EGC cases are summarized in Table 1. The median tumor size of the LNM+ group was 1.5 cm (IQR 1.3-1.8 cm), and the size of 11 (78.6%) LNM+ lesions exceeded 1 cm. Ten (71.4%) patients showed macroscopically depressed morphology, and the median number of harvested lymph nodes in the LNM+ group was 30.5. There were no significant differences between the LNM- and LNM+ groups in terms of the tumor location, gross type, and the number of retrieved LNs. Also, no significant differences were noted between the two groups in the preoperative endoscopic findings such as exudate, endoscopic ulcer, converging fold, and tumor island.

Detailed information on the 14 LNM+ cases is presented in Table 2; of those, cases 1, 3, 5, and 9 did not show endoscopic findings such as exudate, mucosal break, converging fold, and tumor island (Figure 3). Case 5 had six lymph node metastasis, but the endoscopic findings showed only a flat lesion with hyperemic mucosa (Figure 3C).

Table 1 Clinical and endoscopic features of the patients according to the presence of lymph node metastasis

Variables	LNM- (*n* = 73)	LNM+ (n = 14)	*P* value
Age at diagnosis, yr (median, IQR)	47.0 (41.0-52.0)	43.5 (37.0-51.0)	0.276
Lesion size, cm (median, IQR)	1.5 (1.2-1.7)	1.5 (1.3-1.8)	0.485
Male, *n* (%)	25 (34.2)	4 (28.6)	0.918
Longitudinal location, *n* (%)			0.269
Upper	4 (5.5)	0 (0.0)	
Middle	22 (30.1)	2 (14.3)	
Lower	47 (64.4)	12 (85.7)	
Cross-sectional circumference, *n* (%)			0.421
Anterior wall	16 (21.9)	6 (42.9)	
Great curvature	16 (21.9)	2 (14.3)	
Posterior wall	23 (31.5)	3 (21.4)	
Lesser curvature	18 (24.7)	3 (21.4)	
Gross type, *n* (%)			0.440
Depressed	42 (57.5)	10 (71.4)	
Flat	25 (34.2)	4 (28.6)	
Elevated	6 (8.2)	0 (0.0)	
Number of retrieved LNs (median, IQR)	30.0 (25.0-37.0)	30.5 (25.5-37.0)	0.862
Endoscopic appearances, *n* (%)			
Exudate	6 (8.2)	1 (7.1)	> 0.999
Endoscopic ulcer	30 (41.1)	7 (50.0)	0.747
Converging fold	11 (15.1)	1 (7.1)	0.715
Tumor island	14 (19.2)	3 (21.4)	> 0.999

LNM: Lymph node metastasis; IQR: Interquartile range.

Histologic features of tumor and background stomach

Histopathologic features of the study patients and their background gastric mucosa were evaluated from HE stains and immunohistochemical stains for *H. pylori* and TP53 (Table 3). Except for the diagnostic category according to the proportion of SRCs, none of the histologic features showed significant differences between the LNM- and LNM+ groups. Although no significant difference was noted in the average percentage of SRCs, the LNM+ group tended to have more patients with mixed histology (consisting of 10–90% of SRCs) than pure SRCC or PD carcinoma ($P = 0.059$).

Histologic features of the tumor microenvironment

We further evaluated histologic features of the tumor microenvironment, abundance of TILs, and degree of peritumoral fibrosis (Table 4). We specifically focused on fibrosis because while evaluating histologic features of tumors and the background stomach, we observed that some tumors showed marked peritumoral fibrosis (Supplementary Figure 2A and B). We performed MT staining and analyzed the slides visually and computationally to investigate the degree, extent, and pattern of peritumoral fibrosis (Supplementary Figure 2C-E). However, neither TIL abundance nor the pattern and degree of peritumoral fibrosis showed a statistically significant association with the risk of LNM.

Blurring of MM as an independent risk factor for LNM

During the evaluation of MT stain, we noticed a peculiar pattern of fibrosis disrupting the MM, which could be categorized into diffuse and non-diffuse (focal plus no disruption) types (Figure 2). Importantly, the diffuse blurring of MM (Figure 2A and B) was significantly associated with the invasion of MM, shorter distance between the invasive front and MM, and higher fibrosis score (all $P < 0.001$, Supplementary Table 1). Some cases that lacked diffuse MM blurring had substantial peritumoral fibrosis (Supplementary Figure 3A) or invading MM (Supplementary Figure 3B-C), and other cases showed diffuse blurring of MM while being confined to the lamina propria or devoid of peritumoral

Table 2 Detailed clinical information of the 14 patients with lymph node metastasis

Case No.	Age (yr)	Sex	Type	Size (cm)	Location	Histology	Depth of invasion	Total number of dissected LNs	Number of metastatic LNs
1	59	Female	IIb	1.5	Middle	PD with SRC	LP	31	3
2	41	Female	IIc	1.3	Lower	PD with SRC	LP	18	3
3	57	Male	IIc	1.3	Lower	SRC	LP	21	1
4	47	Female	IIc	1.8	Middle	PD with SRC	LP	21	3
5	46	Female	IIb	2.0	Lower	PD with SRC	LP	25	6
6	37	Female	IIc	1.5	Lower	PD with SRC	MM	31	3
7	48	Female	IIc	1.5	Lower	PD with SRC	LP	37	1
8	35	Male	IIc	0.9	Lower	PD with SRC	LP	38	1
9	35	Female	IIc	1.5	Lower	PD with SRC	LP	27	1
10	52	Female	IIb	0.7	Lower	PD with SRC	LP	29	1
11	60	Male	III	0.6	Lower	PD with SRC	MM	30	1
12	39	Female	IIc	2.0	Lower	PD with SRC	LP	37	1
13	37	Female	IIb	1.8	Lower	PD with SRC	LP	42	1
14	33	Male	IIc	2.0	Lower	PD with SRC	LP	48	3

LNM: Lymph node metastasis; PD: Poorly differentiated carcinoma; SRC: Signet ring cell; SRCC: Signet ring cell carcinoma; LP: Lamina propria; MM: Muscularis mucosa.

fibrosis (Supplementary Figures 3D-E). Most importantly, we found a significant association between diffuse MM blurring and the absence of regional LNM (P = 0.028, Table 4). Multivariate logistic regression analysis with backward variable selection revealed that of the multiple clinical and histological variables, diffuse blurring of MM was the only statistically significant explanatory variable associated with the risk of LNM (OR: 0.12, 95%CI: 0.02–0.95; P = 0.045, Table 5).

Potential clinical utility of MM blurring

Independent assessment of MM blurring by a second pathologist revealed strong interobserver reproducibility with a Cohen's Kappa coefficient of 0.90 (95%CI: 0.80–0.96, Supplementary Table 2). Furthermore, MT staining on the ESD specimens of UD-EGC cases demonstrated that the presence of MM blurring could be readily evaluated in ESD specimens (Supplementary Figure 4). Collectively, these results suggest that MM blurring could serve as a feasible histologic marker that aids the decision on the follow-up strategy after ESD for UD-EGC cases meeting the expanded indication criteria.

DISCUSSION

In this study, we investigated the risk factors for LNM in patients with UD-EGC meeting the criteria for the expanded indication for ESD by using surgically resected cases. Our results demonstrated that the incidence rate of LNM in cases of UD-EGC meeting the criteria for the expanded indication for ESD was 1.1% (14/1240). By reviewing the clinical, endoscopic features, and pathologic results, we found that the LNM- and LNM+ groups did not show significant differences in terms of preoperative clinical and endoscopic features. On the other hand, histologic features such as mixed histology (P = 0.059) and blurring of MM (P = 0.028) showed a notable difference according to the presence of LNM, suggesting

Table 3 Histologic features of the tumors and background stomach according to the presence of lymph node metastasis

Variables	LNM- (*n* = 73)	LNM+ (*n* = 14)	*P* value
Depth of invasion			0.503
LP	53 (72.6)	12 (85.7)	
MM	20 (27.4)	2 (14.3)	
Size, cm	1.5 (1.2-1.7)	1.5 (1.3-1.8)	0.642
% of SRCs			0.157
< 10%	17 (23.3)	0 (0)	
≥ 10% and < 50%	35 (47.9)	10 (71.4)	
≥ 50% and < 90%	14 (19.2)	3 (21.4)	
≥ 90%	7 (9.6)	1 (7.1)	
Diagnostic category according to the proportion of SRCs			0.059
Non-mixed (SRCC and PD)	24 (32.9)	1 (7.1)	
Mixed (PD with SRC component)	49 (67.1)	13 (92.9)	
Background stomach			0.278
Mild CG	8 (11.0)	0 (0)	
Moderate CG or IM	24 (32.9)	3 (21.4)	
CAG	22 (30.1)	4 (28.6)	
CAG with visible *H. pylori*	19 (26.0)	7 (50.0)	
H. pylori abundance, *n*/total *n*			0.263
0	23/71 (32.4)	2/14 (14.3)	
1+	14/71 (19.7)	3/14 (21.4)	
2+	14/71 (19.7)	6/14 (42.9)	
3+	20/71 (28.2)	3/14 (21.4)	
TP53 expression, *n*/total			> 0.999
Loss (0)	3/70 (4.3)	0/14 (0)	
Wildtype pattern (1+/2+)	63/70 (90.0)	13/14 (92.9)	
Overexpression (3+)	4/70 (5.7)	1/14 (7.1)	

LNM: Lymph node metastasis; IQR: Interquartile range; LP: Lamina propria; MM: Muscularis mucosa; SRC: Signet ring cell; SRCC: Signet ring cell carcinoma; PD: Poorly differentiated carcinoma encompassing adenocarcinoma and non-signet ring cell type of poorly cohesive carcinoma; CG: Chronic gastritis; IM: Intestinal metaplasia; CAG: Chronic active gastritis; *H. pylori*: *Helicobacter pylori*.

that histologic evaluation could be useful for improving patient stratification.

To date, ESD for UD-EGC has required an expanded indication, and surgery has been the standard treatment because the risk of LNM has been shown to be relatively higher in UD-EGCs than in differentiated EGCs, thus raising concern about the long-term outcomes. However, in the recent guidelines in Japan, UD-EGC lesions have been integrated into the absolute indication for ESD[13]. Li *et al*[14] reported that there were no cases of LNM in patients with UD-EGC meeting the expanded indications of ESD. Another study revealed that LNM was not found in intramucosal cancer when the lesion was 20 mm or less without LVI and ulcerative findings[1]. However, these studies have the limitations of small sample sizes and retrospective study design. In a recent multicenter clinical trial, Takizawa *et al*[15]. reported that patients who were followed after undergoing curative ESD for UD-EGC showed neither local/distant recurrence nor deaths due to gastric cancer, thereby suggesting favorable clinical outcomes of ESD for UD-EGC; however, this study had a single-arm design and the outcomes after ESD were not compared with those after surgery. In addition, other studies reported contrasting results in that up to 2.3% of patients with UD-EGC meeting the criteria of expanded indications (intramucosal cancer, size of ≤ 20 mm without LVI and ulcerative findings) were found to have LNM[16]. Our study also showed that 1.1% of 1240 patients meeting the criteria for the expanded indication for ESD showed LNM. Considering these conflicting results, further targeted investigations are required regarding the risk of

Table 4 Histologic features of the tumor microenvironment according to the presence of lymph node metastasis

Variables	LNM- (*n* = 73)	LNM+ (*n* = 14)	*P* value
TIL abundance			0.438
< 10%	25 (34.2)	7 (50.0)	
10%-20%	33 (45.2)	6 (42.9)	
≥ 20%	15 (20.5)	1 (7.1)	
Degree of central fibrosis			0.522
Mild	6 (8.5)	2 (14.3)	
Moderate	35 (49.3)	8 (57.1)	
Marked	30 (42.3)	4 (28.6)	
Degree of peripheral fibrosis			
Mild	11 (15.5)	2 (14.3)	0.495
Moderate	53 (74.6)	9 (64.3)	
Marked	7 (9.9)	3 (21.4)	
Distribution of fibrosis			
Central = peripheral	32 (45.1)	7 (50.0)	0.204
Central > peripheral	33 (46.5)	4 (28.6)	
Central < peripheral	6 (8.5)	3 (21.4)	
Fibrosis score	0.55 (0.38-0.83)	0.53 (0.31-0.77)	0.273
Blurring of MM, *n*/total			0.028
Non-diffuse (absent/focal)	43/71 (60.6)	13/14 (92.9)	
Diffuse	28/71 (39.4)	1/14 (7.1)	

LNM: Lymph node metastasis; TIL: Tumor-infiltrating lymphocytes; IQR: Interquartile range; MM: Muscularis mucosa.

LNM in UD-EGC cases meeting the expanded criteria.

LNM is the most important factor for deciding the treatment strategy in cases of UD-EGC. For the appropriate usage of ESD in UD-EGC, the characteristics of UD-EGCs with LNM should be clarified so that such cases should be excluded from consideration for ESD. Hence, we reviewed the original pathologic reports of 4781 surgically resected UD-EGC and found that 1240 cases met the criteria for the expanded indication for ESD, 22 of whom exhibited regional LNM. However, a subsequent histologic review revealed that 8 of the 22 cases with LNM and 15 out of the matched 88 control cases did not satisfy the criteria for expanded indications due to deviation in size from the original pathology reports (n = 2 in the case group, n = 4 in the control group) and the presence of ulcer (n = 3 in the case group, n = 9 in the control group). The discrepancy likely occurred because unlike mucosectomy specimens, gastrectomy specimens do not mandatorily undergo systematic evaluation for the tumor size and the presence of an ulcer[3,17]. In our study, three cases with submucosal invasion that had been misdiagnosed as mucosal cancer showed deceptive histologic features that called for careful examination. In one case, it seemed that the site of submucosal invasion had been missed because the tumor was located in an extensively thick and undulating mucosa. The tumor in another case was accompanied by a massively lymphoid stroma so that the tumor cells in the submucosa were barely visible. Tumor cells of the remaining case were almost indistinguishable from macrophages. TP53 immunostaining, which is routinely performed for all stomach cancer cases at our institution, was useful in highlighting the tumor cells in the submucosa in the latter two cases. Careful attention is needed to diagnose ESD cases showing features of discrepant cases.

This discrepancy after the second histologic review implies a more serious message. Considering that the number of patients in the LNM+ group decreased from 22 to 14 after the second histologic review, the total number of UD-EGC patients satisfying the expanded criteria could decrease from 1240 if the entire case cohort was reviewed. As a consequence, the actual incidence rate can be actually higher than 1.1%. Therefore, our results suggest that UD-EGC cases meeting the criteria for the expanded indication does have a risk of LNM, which may be too higher to consider endoscopic resection. Therefore, further research is needed to discover clinical, endoscopic, and histologic features of UD-EGC that can aptly supplement the current expanded criteria.

Table 5 Logistic regression analysis for the risk of Lymph node metastasis

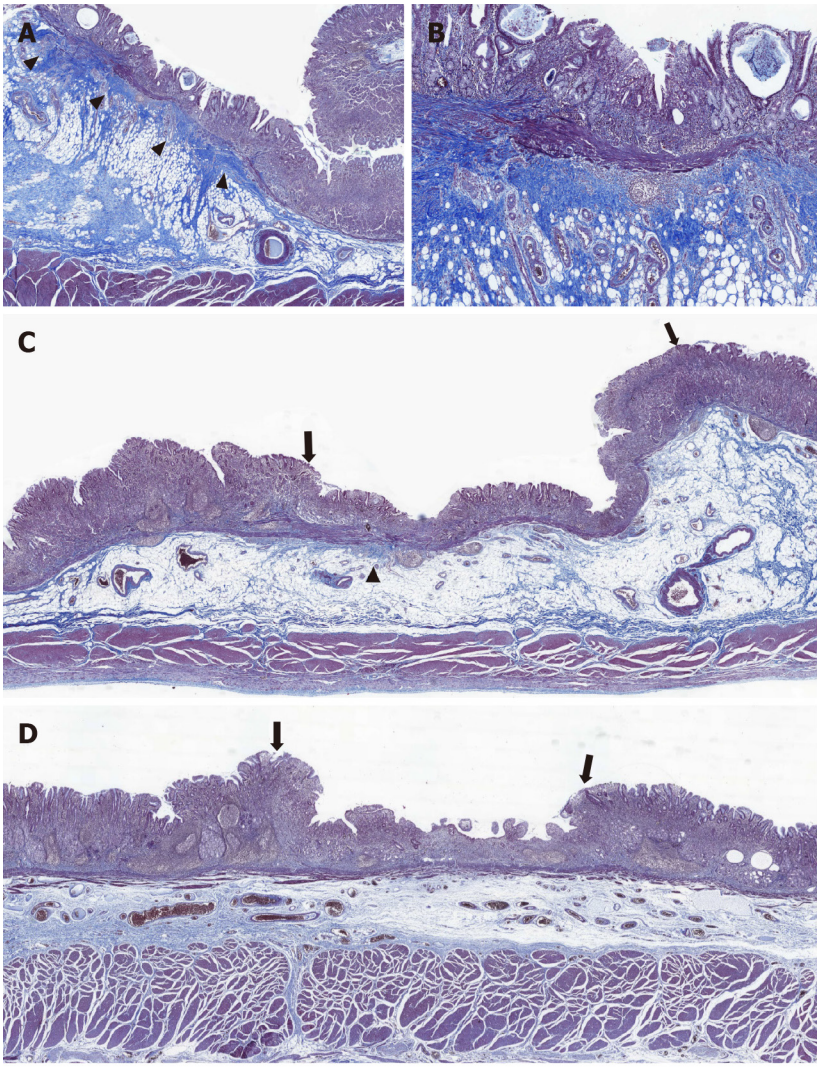
Variable	OR	95%CI	*P* value
Depth of invasion			
LP	1		
MM	0.44	0.09-2.15	0.312
Diagnostic category according to % of SRCs			
Non-mixed (SRC and PD)	1		
Mixed (PD with SRC component)	6.37	0.79-51.6	0.083
Background stomach			
CG	1		
CAG	2.86	0.74-11.1	0.129
Presence of *H. pylori*			
Absent (0)	1		
Present (≥ 1+)	2.88	0.59–13.9	0.190
TIL abundance			0.409
< 10%	1		
10%-20%	0.65	0.19-2.17	0.484
≥ 20%	0.24	0.03-2.13	0.199
Fibrosis score	0.34	0.05-2.49	0.285
Blurring of MM			
Non-diffuse (absent/focal)	1		
Diffuse	0.12	0.02-0.95	0.045

LNM: Lymph node metastasis; OR: Odds ratio; LP: Lamina propria; MM: Muscularis mucosa; SRC: Signet ring cell; SRCC: Signet ring cell carcinoma; PD: Poorly differentiated carcinoma encompassing adenocarcinoma and non-signet ring cell type of poorly cohesive carcinoma; CG: Chronic gastritis; CAG: Chronic active gastritis; TIL: Tumor-infiltrating lymphocytes; *H. pylori*: *Helicobacter pylori*.

For this purpose, the tumor characteristics of 14 patients with LNM were evaluated. The tumor characteristics including tumor location, gross type, the number of retrieved LNs, and endoscopic appearances did not have significant associations with LNM. According to histologic analysis, among various histologic features, mixed histology (consisting of 10%-90% SRCs) in comparison with non-mixed histology (*i.e.*, pure SRCC, poorly cohesive carcinoma, or poorly differentiated adenocarcinoma) was the only variable with a marginal statistical significance ($P = 0.059$). This is consistent with previous studies suggesting more aggressive biology of EGC by using mixed histology rather than pure adenocarcinoma and SRCC[18,19]. Therefore, it is likely that we would have reached statistical significance if a larger number of cases were analyzed.

Inspired by the recent interest in the role of the tumor microenvironment on metastasis[20], we further investigated the histologic features of the tumor microenvironment, especially the pattern and degree of peritumoral fibrosis. Previous studies on submucosal fibrosis of EGCs have mostly focused on its negative effect on successful ESD[21-25]. Conversely, we focused on whether the extent or pattern of submucosal fibrosis had an impact on LNM. While the degree of submucosal fibrosis did not show a significant association with LNM, we unexpectedly discovered a significant association between MM blurring and LNM. A structural study on the distribution of lymphatic and blood capillaries of human gastric mucosa showed that lymphatic capillaries were present in the deep lamina propria adjacent to and within the MM[26]. As such, we hypothesize that the blurring of MM is a consequence of an exaggerated anti-tumoral reaction against tumor cells trying to invade the lymphatics within the MM. In contrast with the traditional concept of the pro-tumorigenic role of fibrosis, recent studies have suggested that tumor-related fibrosis can also restrain cancer initiation, proliferation, and metastasis [27]. Therefore, it is possible that tumors that managed to elicit anti-tumoral fibrosis against the tumor cells' attempt to invade lymphatics are seen as having blurred MM, and are hence less likely to metastasize into the regional lymph nodes.

Considering the intimate relationship between lymphatics and the MM, it can be assumed that the tumor's proximity to the MM would be significantly associated with the risk of LNM. Indeed, it has

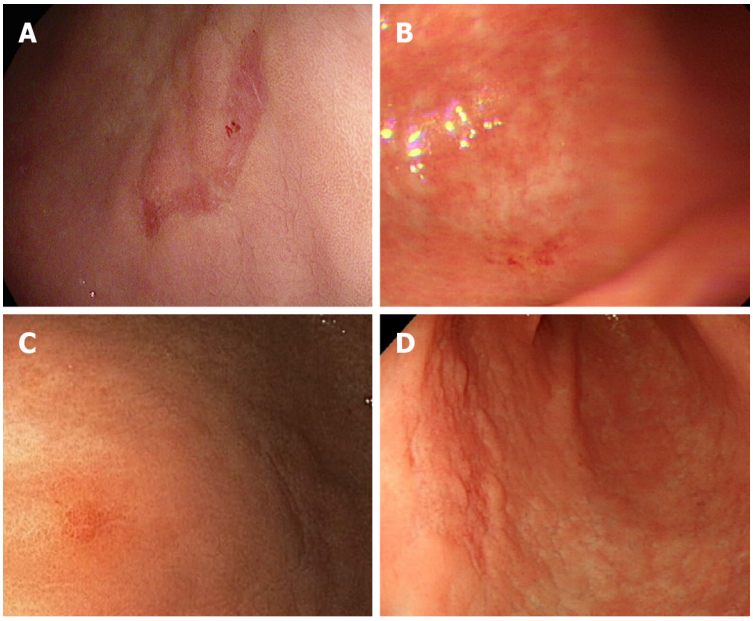


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Figure 2 Blurring of muscularis mucosa underneath the tumorous epithelium. Representative images of tumors with diffuse, focal, and no blurring of muscularis mucosa. A: Diffuse blurring of muscularis mucosa (MM) was prominent enough to localize the tumor at scanning magnification (arrowhead); B: At higher magnification (40´), the thickness of MM appeared irregular due to collagen fibers disrupting the muscle fibers of MM; C: The majority of MM underneath the tumorous epithelium (both ends are marked by arrows) was undisrupted compared with adjacent MM underneath the non-tumorous epithelium, making the foci of MM blurring focal (arrowhead); D: With no blurring of MM, it was difficult to localize the tumor (both ends are marked by arrows) at scanning magnification based on the status of MM.

been reported that tumors invading the MM are more likely to metastasize into regional lymph nodes than those limited to the lamina propria[28]. However, we failed to reach the same conclusion in our current study, and our study might suggest the opposite conclusion considering the significant association between MM invasion and MM blurring. This may be due to the fact that our control group is biased toward tumors invading the MM; however, unlike the two previous studies, we only focused on UD-EGC cases meeting the expanded criteria for ESD.

We hypothesize that the seemingly counterintuitive result of our study might be explained by the differences in the mode of invasion between differentiated and undifferentiated GCs, considering the results of recent studies that elucidated the various modes of cancer cell invasion, ranging from single-cell migration to collective invasion[29]. Because poor differentiation often involves the loss of cellular adhesion molecules[30], undifferentiated GCs might preferentially invade as single cells. For this reason, the main body of poorly differentiated tumors does not necessarily need to be in close proximity to lymphatics to invade them, and desmoplasia is more likely attributable to anti-tumoral microenvironmental responses rather than invading tumor cells. On the other hand, differentiated GCs would invade the adjacent normal structures by forming glands. Glandular structures are likely more destructive than scattered cells, and massive violation of the normal structure itself is sufficient enough to elicit fibrosis; for this reason, desmoplasia in differentiated GCs would more reflect the invasiveness of the tumors than anti-tumoral responses. Further studies in independent cohorts are needed to validate the hypothesis on the differential significance of MM blurring in differentiated and undifferentiated GCs.



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Figure 3 Endoscopic images of the cases with lymph node metastasis without exudate, mucosal break, converging fold, and tumor island. A: Case 1, B: Case 3, C: Case 5, D: Case 9.

There are several limitations to our study. First, because we excluded cases that showed deviation from the original pathology reports (*e.g.*, larger tumor size, ulcer, LVI, submucosal invasion), the study population was reduced and the case-control study could not be performed as originally intended. Second, the validity of MM blurring may benefit from further scrutiny; aside from the four tumors that were small enough to be embedded in single blocks, we examined two representative sections per each case and judged a case as having blurred MM when such foci were identified in any of the two sections. As such, it is possible that the cases classified as clear MM might have disrupted MM in unexamined sections. Finally, this study had limitations inherent to the nature of a retrospective, single-center study. Although the number of patients with LNM was small, this is because the incidence of LNM in patients with UD-EGC meeting the expanded indications of ESD is low. Considering the low incidence, a large-scale, prospective, multicenter study is needed to confirm our findings. Despite these limitations, we believe that our study appropriately highlights the fact that diffuse blurring of MM may be a potential predictive factor for the risk of LNM in cases of UD-EGC meeting the expanded criteria for ESD. Further research is needed to validate our results and to elucidate its mechanistic basis.

CONCLUSION

In conclusion, our results show that cases of UD-EGC meeting the criteria for the expanded indication have the risk for LNM, albeit low (1.1%), and that routine histological examination has practical limitations for identifying such cases. When ESD is planned for a case of UD-EGC, obtaining detailed informed consent after the disclosure of the risk of LNM is necessary, and careful observation is essential. A model for patient stratification based on histologic evaluation of the proportion of SRCs and MM blurring in ESD specimens could be useful for identifying the patients with a higher risk of LNM.

ARTICLE HIGHLIGHTS

Research background

There have been several reports of lymph node or distant metastases arising after curative endoscopic submucosal dissection (ESD) in undifferentiated early gastric cancer (UD-EGC) cases meeting the expanded criteria.

Research motivation

The clinicopathologic features associated with the risk of lymph node metastasis (LNM) in UD-EGC have not been well-studied.

Research objectives

To investigate the prevalence and risk factors of LNM in UD-EGC cases meeting the criteria for the expanded indication for ESD.

Research methods

In this retrospective study, we investigated the risk of LNM of UD-EGC meeting the criteria for the expanded indication for ESD, and performed a matched case-control study to identify the clinical, endoscopic, and histopathological features associated with the risk of LNM. Univariate and multivariate logistic regression analyses were performed to identify the risk factors by estimating the odds ratios.

Research results

The incidence rate of LNM in UD-EGC cases meeting the criteria for the expanded indication for ESD was 1.1% (14/1240). No significant differences existed between the LNM group and the matched non-LNM group in terms of preoperative clinical endoscopic features and conventional histologic features. In the tumor microenvironment, blurring of muscularis mucosa (MM) underneath the tumorous epithelium was associated with the risk of LNM.

Research conclusions

The risk of LNM was higher than expected when using the current expanded indication for UD-EGC. Evaluation of blurring of MM could provide useful clues for reducing the risk of LNM.

Research perspectives

Further studies are needed to validate the significance of MM blurring and elucidate its mechanistic basis. Eventually, an improved model for patient stratification based on detailed histologic evaluation of ESD specimens should be established to identify patients with a high risk of LNM.

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FOOTNOTES

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Country/Territory of origin: South Korea

ORCID number: Jiyoung Yoon 0000-0001-7448-4296; Seung-Yeon Yoo 0000-0002-7881-9038; Young Soo Park 0000-0001-

5389-4245; Kee Don Choi 0000-0002-2517-4109; Beom Su Kim 0000-0002-3656-2086; Moon-Won Yoo 0000-0003-0346-9042; In Seob Lee 0000-0003-3099-0140; Jeong Hwan Yook 0000-0002-7987-5808; Ga Hee Kim 0000-0002-7652-2580; Hee Kyong Na 0000-0001-6764-9099; Ji Yong Ahn 0000-0002-0030-3744; Jeong Hoon Lee 0000-0002-0778-7585; Kee Wook Jung 0000-0002-3771-3691; Do Hoon Kim 0000-0002-4250-4683; Ho June Song 0000-0001-9255-1464; Gin Hyug Lee 0000-0003-3776-3928; Hwoon-Yong Jung 0000-0003-1281-5859.

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ORIGINAL ARTICLE

Retrospective Cohort Study

Validation model of fibrosis-8 index score to predict significant fibrosis among patients with nonalcoholic fatty liver disease

Thaninee Prasoppokakorn, Wah-Kheong Chan, Vincent Wai-Sun Wong, Panyavee Pitisuttithum, Sanjiv Mahadeva, Nik Raihan Nik Mustapha, Grace Lai-Hung Wong, Howard Ho-Wai Leung, Pimsiri Sripongpun, Sombat Treeprasertsuk

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Thaninee Prasoppokakorn, Sombat Treeprasertsuk, Department of Medicine, Division of Gastroenterology, Faculty of Medicine, Chulalongkorn University and King Chulalongkorn Memorial Hospital, Thai Red Cross Society, Bangkok 10330, Thailand

Wah-Kheong Chan, Sanjiv Mahadeva, Department of Medicine, Division of Gastroenterology, Division of Gastroenterology and Hepatology Unit, Faculty of Medicine, University of Malaya, Kuala Lumpur 50603, Malaysia

Vincent Wai-Sun Wong, Grace Lai-Hung Wong, Department of Medicine and Therapeutics, The Chinese University of Hong Kong, Hong Kong, China

Panyavee Pitisuttithum, Department of Medicine, Division of General Internal Medicine, Faculty of Medicine, Chulalongkorn University and King Chulalongkorn Memorial Hospital, Thai Red Cross Society, Bangkok 10330, Thailand

Nik Raihan Nik Mustapha, Department of Pathology, Hospital Sultanah Bahiyah, Alor Setar, Kedah 05460, Malaysia

Howard Ho-Wai Leung, Department of Anatomical and Cellular Pathology, The Chinese University of Hong Kong, Hong Kong, China

Pimsiri Sripongpun, Department of Internal Medicine, Prince of Songkla University, Hat Yai 90110, Thailand

Pimsiri Sripongpun, Department of Medicine, Division of Gastroenterology and Hepatology, Stanford University School of Medicine, Palo Alto, CA 94305, United States

Corresponding author: Sombat Treeprasertsuk, MD, PhD, Instructor, Professor, Department of Medicine, Division of Gastroenterology, Faculty of Medicine, Chulalongkorn University and King Chulalongkorn Memorial Hospital, Thai Red Cross Society, King Chulalongkorn Memorial Hospital 1873 Rama 4 Road, Pathumwan District, Bangkok 10330, Thailand. battan5410@gmail.com

Abstract

BACKGROUND

Identifying hepatic fibrosis is crucial for nonalcoholic fatty liver disease (NAFLD)

management. The fibrosis-8 (FIB-8) score, recently developed by incorporating four additional variables into the fibrosis-4 (FIB-4) score, showed better performance in predicting significant fibrosis in NAFLD.

AIM

To validate the FIB-8 score in a biopsy-proven NAFLD cohort and compare the diagnostic performance of the FIB-8 and FIB-4 scores and NAFLD fibrosis score (NFS) for predicting significant fibrosis.

METHODS

We collected the data of biopsy-proven NAFLD patients from three Asian centers in three countries. All the patients with available variables for the FIB-4 score (age, platelet count, and aspartate and alanine aminotransferase levels) and FIB-8 score (the FIB-4 variables plus 4 additional parameters: The body mass index (BMI), albumin to globulin ratio, gamma-glutamyl transferase level, and presence of diabetes mellitus) were included. The fibrosis stage was scored using nonalcoholic steatohepatitis CRN criteria, and significant fibrosis was defined as at least fibrosis stage 2.

RESULTS

A total of 511 patients with biopsy-proven NAFLD and complete data were included for validation. Of these 511 patients, 271 (53.0%) were female, with a median age of 51 (interquartile range: 41, 58) years. The median BMI was 29 (26.3, 32.6) kg/m^2, and 268 (52.4%) had diabetes. Among the 511 NAFLD patients, 157 (30.7%) had significant fibrosis (≥ F2). The areas under the receiver operating characteristic curves of the FIB-8 and FIB-4 scores and NFS for predicting significant fibrosis were 0.774, 0.743, and 0.680, respectively. The FIB-8 score demonstrated significantly better performance for predicting significant fibrosis than the NFS (P = 0.001) and was also clinically superior to FIB-4, although statistical significance was not reached (P = 0.073). The low cutoff point of the FIB-8 score for predicting significant fibrosis of 0.88 showed 92.36% sensitivity, and the high cutoff point of the FIB-8 score for predicting significant fibrosis of 1.77 showed 67.51% specificity.

CONCLUSION

We demonstrated that the FIB-8 score had significantly better performance for predicting significant fibrosis in NAFLD patients than the NFS, as well as clinically superior performance *vs* the FIB-4 score in an Asian population. A novel simple fibrosis score comprising commonly accessible basic laboratories may be beneficial to use for an initial assessment in primary care units, excluding patients with significant liver fibrosis and aiding in patient selection for further hepatologist referral.

Key Words: Nonalcoholic fatty liver disease; Fibrosis-8 score; Fibrosis-4 score; Nonalcoholic fatty liver disease fibrosis score

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Core Tip: Noninvasive diagnosis of hepatic fibrosis is crucial for nonalcoholic fatty liver disease (NAFLD). The fibrosis-8 (FIB-8) score was recently developed by incorporating four additional variables into the fibrosis-4 (FIB-4) score. The diagnostic performance of the FIB-8 score exhibited higher accuracy in diagnosing significant fibrosis (≥ F2) than the NAFLD fibrosis score but was not superior to the FIB-4 score in our Asian cohort population. We postulated that gamma-glutamyl transferase might be an additional variable that predicts significant fibrosis in NAFLD patients.

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INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) is a global health issue and has become the most common liver disease in Western countries, accounting for an estimated 25% of the adult population[1] and affecting an estimated 25%–30% of the adult population in the Asia Pacific region[2]. A meta-analysis in Asia during 1999 to 2019, described the overall pooled incidence rate was 50.9 *per* 1000 person-years[3]. According to our previous study, the prevalence of significant fibrosis (defined as ≥ F2 fibrosis) is 18.4% in asymptomatic NAFLD patients[4]. Nonalcoholic steatohepatitis (NASH) has emerged as the most common cause of cryptogenic cirrhosis and hepatocellular carcinoma worldwide. The presence of hepatic fibrosis is the major determinant of future risk of mortality and liver-related morbidity[5], and detecting significant fibrosis is crucial for NAFLD because no well-accepted and proven therapy is available for this disease to date[6]. However, patients with F2 or higher are at a higher risk of long-term liver-related death than patients with F0-1. Those with significant fibrosis should be intensively followed up or considered to participate in the therapeutic trial for NAFLD.

Liver biopsy remains the gold standard for evaluating hepatic fibrosis. However, because of several drawbacks, including invasiveness, the risk of bleeding complications, intrinsic sampling and pathologist reader variability[7], and cost, noninvasive tests are more practical. Thus, the 2018 American Association for the Study of Liver Diseases (AASLD) practice guidance recommends the use of the fibrosis-4 (FIB-4) score, the NAFLD fibrosis score (NFS), vibration-controlled transient elastography, and magnetic resonance elastography[8] to identify those at low or high risk for advanced fibrosis [bridging fibrosis (F3) or cirrhosis (F4)]. Noninvasive tests using only clinical and routine laboratory parameters are inexpensive and particularly important in primary care or resource-limited settings where the pretest probability of advanced fibrosis is low because these scores have good negative predictive values (NPVs) to exclude advanced fibrosis[9]. Therefore, using simple fibrosis scores as an initial assessment in primary care is reasonable. The FIB-4 score comprises four parameters, age, aspartate aminotransferase (AST), alanine aminotransferase (ALT), and platelets, while the NFS score comprises six parameters in addition to those comprising the FIB-4 score, such as the body mass index (BMI), presence of diabetes, and serum albumin level[10].

According to Sripongpun *et al*[11], their AASLD 2019 abstract reported a new model for a fibrosis-8 score (FIB-8) score developed by incorporating the following four additional variables: BMI, albumin/globulin (A/G) ratio, gamma-glutamyl transferase (GGT) level, and diabetes. The subjects were enrolled in the PIVENS and FLINT trials, of which 522 participants all had histologically confirmed NASH[12,13]. The optimal low and high cutoffs for the FIB-8 score to exclude and include F ≥ 2 were < 0.88 and ≥ 1.77, respectively, with a sensitivity of 95.3% and a specificity of 79.2%. The areas under the receiver operating characteristic curves (AUROCs) of the FIB-8 score were 0.79 and 0.78 in the training and validation datasets, respectively. The FIB-8 score provided significantly better AUROCs than the FIB-4 score ($P < 0.001$) and NFS ($P = 0.005$) in the validation dataset for predicting significant and advanced fibrosis in NAFLD patients. Following the study, the field test and validation of the FIB-8 score in a real-world cohort of NAFLD patients revealed that the AUROCs of the FIB-8 score were 0.84 with imputed data ($n = 130$) and 0.91 when only patients with complete data without imputation were included ($n = 31$). The FIB-8 score again outperformed the FIB-4 score and NFS, with AUROCs of 0.86 *vs* 0.80 and 0.77, respectively, for diagnosing advanced fibrosis (F3)[14].

To our best knowledge, no validation of the FIB-8 score has been reported in a larger cohort. Therefore, this study was to validate the FIB-8 score in a biopsy-proven NAFLD cohort and compare the diagnostic performance of the FIB-8 and FIB-4 scores and NFS for predicting significant fibrosis.

MATERIALS AND METHODS

Study population and data collection

We collected the data of biopsy-proven NAFLD patients from the following three Asian centers in three countries: (1) Chulalongkorn University, Thailand; (2) The Chinese University of Hong Kong, Hong Kong; and (3) University of Malaya, Malaysia. The data from Thailand were collected from April 2008 to May 2019, those from Hong Kong were collected from July 2006 to November 2017, and those from Malaysia were collected from November 2012 to October 2015.

NAFLD was diagnosed based on ultrasonographic findings of fatty liver as well as transient elastography and the exclusion of viral hepatitis B and C infection, significant alcohol intake, and current usage of medications causing hepatic steatosis. Only patients with biopsy-proven NAFLD were included. Patients with other causes of chronic liver disease, incomplete histological data, and without significant hepatic steatosis were excluded. The laboratory data for the FIB-4 score (age, platelet count, and aspartate and ALT levels), FIB-8 score [the FIB-4 variables plus 4 additional parameters: The BMI, albumin to globulin ratio, gamma-glutamyl transferase level, and presence of diabetes mellitus (DM)], and the NFS were collected. The time interval between the enrolled laboratories and the date of liver biopsy was within 1 year. The fibrosis stage was scored using the NASH Clinical Research Network (CRN) criteria, and significant fibrosis was defined as at least fibrosis stage 2 (F ≥ 2).

Noninvasive methods

We validated the noninvasive methods from the FIB-8 score, FIB-4 score, and NFS and the test variables for predicting significant fibrosis (Table 1)[11,15,16].

Outcomes

We aimed to validate the FIB-8 score in a biopsy-proven NAFLD cohort and compare the diagnostic performance of the FIB-8 score, FIB-4 score, and NFS for predicting significant fibrosis (≥ F2) in an Asian cohort.

Ethical permission

The study was reviewed and approved by the Institutional Review Board, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand (IRB number 238/59). This is a retrospective study, and signed informed consent was waived by the Ethics Committee. The analysis used anonymous clinical data after each patient agreed to treatment by written consent.

Statistical analysis

Categorical and continuous variables were compared between patients with and without significant fibrosis using Chi-squared and Student's t-test or the Wilcoxon rank-sum test (according to the distribution of the data), respectively. Most of the numerical values did not follow a normal distribution and were expressed as medians and interquartile ranges. The diagnostic performance of each scoring system was then evaluated using receiver operating characteristic curves, and comparisons between the correlated AUROCs were performed using DeLong's test[17]. The sensitivities (Sens) and specificities (Spec) of each scoring system were analyzed using the given low and high cutoffs for predicting F2, as reported previously-*i.e.*, 0.88 and 1.77 for the FIB-8 score, 0.81 and 1.81 for the FIB-4 score, and -2.45 and 0.03 for the NFS, respectively[11,18]. All statistical analyses were performed using the SPSS statistical analysis package (version 18.0.0; SPSS Inc., Chicago, Illinois, United States), Stata (version 15; StataCorp), and R program version 4.1.1. A P value < 0.05 was considered statistically significant.

RESULTS

Baseline characteristics

A total of 1013 patients with biopsy-proven NAFLD were included in the database. Of those, 511 patients had complete data on variables, including the NFS and FIB-4 and FIB-8 scores, and were eligible for the current study (Figure 1). Of the 511 patients, 271 (53.0%) were female, with a median age of 51 [interquartile range (IQR): 41, 58] years. The median BMI was 29 (26.3, 32.6) kg/m^2, and 268 (52.4%) had diabetes. Among the 511 NAFLD patients, 157 (30.7%), 88 (17.2%), and 16 (3.1%) patients had significant fibrosis (≥ F2), advanced fibrosis (≥ F3), and cirrhosis (F4), respectively. The baseline characteristics comparing NAFLD F0–1 and significant fibrosis (F ≥ 2) are shown in Table 2. The significant factors associated with significant fibrosis were an older age [55 (48, 61) *vs* 49.5 (39, 57) years; P < 0.001], the presence of diabetes (71.3% *vs* 44.0%; P < 0.001), higher levels of AST [53.5 (36, 75) *vs* 35 (26, 52) U/L; P < 0.001], ALT [75 (50, 111) *vs* 59.5 (40, 98) U/L; P < 0.001] and GGT [81 (48, 151) *vs* 56.5 (35, 92) U/L; P < 0.001], a lower platelet count [230 (189, 277) *vs* 266 (226.8, 302) × 10^9/cu.mm; P < 0.001], lower levels of total cholesterol [182 (159, 209) *vs* 193 (170, 220) mg/dL; P = 0.004] and LDL-cholesterol [107 (85, 132) *vs* 116 (96, 143) mg/dL; P = 0.003], and a higher median Controlled Attenuation Parameter (CAP) [324 (294, 347) *vs* 299 (211, 339) dB/m] (Table 2).

Performance of the FIB-8 score, FIB-4 score, and NFS for predicting significant fibrosis (≥ F2)

The AUROCs of the FIB-8 score, FIB-4 score, and NFS for predicting significant fibrosis were 0.774 (95%CI: 0.729-0.820), 0.743 (95%CI: 0.695-0.791), and 0.680 (95%CI: 0.630-0.730), respectively (Figure 2). The FIB-8 score showed a significantly better performance for predicting significant fibrosis (≥ F2) than the NFS (P = 0.001) and was numerically higher than the FIB-4 score, but the difference was not statistically significant (P = 0.073). The sensitivities and specificities of the cutoffs specified to exclude and include significant fibrosis for each score are reported in Table 3.

Diagnostic accuracy of the FIB-8 score, FIB-4 score, and NFS for predicting significant fibrosis (≥ F2) by age group

The cohort was stratified by age into three groups: Age < 35 (n = 66), 35-65 (n = 412), and > 65 years (n = 33). The AUROCs of the FIB-8 score, FIB-4 score, and NFS in patients aged 35-65 years for predicting significant fibrosis were 0.79, 0.76, and 0.68, respectively. This patient group comprised most of the cohort and had similar diagnostic performance results as the entire cohort. However, the FIB-8 score, FIB-4 score, and NFS were poor in patients aged < 35 years (AUROC: 0.55, 0.59, and 0.70, respectively) and > 65 years (AUROC: 0.66, 0.71, and 0.54, respectively). The number of patients in each age group

Table 1 Details of the three noninvasive methods used in this study

Index	Number of parameters and variables	Formula	Ref.
FIB-8 index	8; age, AST, ALT, platelets, BMI, albumin/globulin, GGT, diabetes	FIB4 + 0.025 × BMI (kg/m^2) - 0.702 × (albumin/globulin ratio) + 0.004 × GGT (U/L) + 0.858 × diabetes (yes = 1, no = 0)[1]	Sripongpun *et al*[11], 2019
FIB-4 index	4; age, AST, ALT, platelets	Age (years) × AST (U/L)/[platelet count (10^9/L) × √ALT (U/L)]	Sterling *et al*[15], 2006
NFS	6; age, BMI, diabetes, AST/ALT, platelets, albumin	-1.675 + 0.037 × age (years) + 0.094 × BMI (kg/m^2) + 1.13 × impaired fasting glucose/diabetes (yes = 1, no = 0) + 0.99 × AST/ALT ratio - 0.013 × platelet count (× 10^9/L) - 0.66 × albumin (g/dL)	Angulo *et al*[16], 2007

[1]The FIB-8 score in the abstract was calculated by 1.3 × FIB-4 + 0.03 × BMI - 0.93 × (albumin/globulin ratio) + 0.005 × GGT (U/L) + 1.1 × diabetes (yes = 1, no = 0), which is slightly different from the actual formula presented at the liver meeting congress (the score in the table). We have contacted the authors of the abstract and were informed that the actual formula to use is the one shown in the table.

NAFLD: Nonalcoholic fatty liver disease; FIB-8: Fibrosis-8 score; FIB-4: Fibrosis-4 score; NFS: NAFLD fibrosis score; AUROC: Areas under the receiver operating characteristic curves; GGT: Gamma-glutamyl transferase; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; BMI: Body mass index.

and center is shown in Supplementary Table 1. A detailed summary of the AUROC, sensitivity, specificity, positive predictive value, and NPV for the FIB-8 score, FIB-4 score, and the NFS is shown in Supplementary Table 2.

DISCUSSION

Based on the results of the present study, we validated the diagnostic performance of the FIB-8 score, FIB-4 score, and NFS score in 511 biopsy-proven NAFLD patients for predicting significant fibrosis. The main issue affecting the diagnostic ability of new methods for detecting liver fibrosis in NAFLD patients is the prevalence of fibrosis among the particular population. Our results demonstrated that the overall prevalence rates of significant fibrosis (≥ F2), advanced fibrosis (≥ F3), and cirrhosis (F4) were 157 (30.7%), 88 (17.2%), and 16 (3.1%), respectively. The mean incidence rates of significant fibrosis from previous publications were 52.5% and 35.4% in the PIVENS plus FLINT trials and a Stanford University trial, respectively[11,14] (Table 4). The remarkable aspects were as follows: (1) Our study had a lower incidence of fibrosis than the first cohort; (2) Among the noninvasive methods, the FIB-8 score and NFS included the BMI in their models, and our cohort had a lower mean BMI than previous reports (30.4 kg/m^2 *vs* 34.0 and 31.5 kg/m^2), which might have resulted in lower percentages of sensitivity and specificity in our cohort than those previously reported; and (3) GGT is a uniquely incorporated variable in the new FIB-8 scoring system. Some reported studies have demonstrated that a higher GGT level is a risk factor for advanced fibrosis in NAFLD[19,20]. Additionally, considering NAFLD patients with type 2 DM, a serum GGT level over 82 U/L was independently associated with advanced fibrosis using noninvasive methods in multivariate analysis (P = 0.004)[21]. In our study, the baseline characteristics correlatively showed that a higher level of median GGT was a significant factor associated with significant fibrosis [81 (IQR: 48, 151) *vs* 56.5 (35, 92); P < 0.001]. We postulated that GGT may be an additional variable predicting significant fibrosis in NAFLD patients. The diagnostic performance of the FIB-8 score exhibited higher accuracy for diagnosing significant fibrosis (≥ F2) than the NFS but was not superior to the FIB-4 score in previous studies or our study; the AUROCs for the FIB-8 score, FIB-4 score, and NFS for predicting significant fibrosis were 0.774, 0.743, and 0.680, respectively (FIB-8 *vs* NFS, P = 0.001; FIB-8 *vs* FIB-4, P = 0.073). The sensitivities of the low cutoff of FIB-8 score to exclude significant fibrosis was 92.36%. Consequently, the high sensitivity and NPV for excluded significant fibrosis may be beneficial in primary care units and to select patients for further hepatologist referral. However, the limited specificity of the high cutoff of FIB-8 score to include significant fibrosis may require further step assessment instance transient elastography.

Furthermore, our results demonstrated that the FIB-4 score offered better diagnostic performance than the NFS score (P < 0.001). According to meta-analysis results from Castera[10], the FIB-4 score and NFS showed the best diagnostic performance for detecting advanced fibrosis compared with other blood-based models. However, this meta-analysis included studies that used different cut-off thresholds. Furthermore, a recent meta-analysis from Castellana *et al*[22] reported a head-to-head comparison of the FIB-4 score and NFS from 18 studies that used consistent cutoffs. The FIB-4 score offered higher performance for including and NFS for excluding advanced fibrosis. However, our studies used different cutoffs and aimed to predict significant fibrosis, not advanced fibrosis. Consequently, our cohort was not suitable to compare the FIB-4 score and NFS.

Table 2 Characteristics of patients with F0-1 fibrosis compared to those with F ≥ 2 fibrosis stage (*n* = 511)

Variables	Total (*n* = 511)	Fibrosis stage F0-1 (*n* = 354)	Fibrosis stage ≥ F2 (*n* = 157)	*P* value
Age (yr), median (IQR)	51 (41, 58)	49.5 (39, 57)	55 (48, 61)	< 0.001
Sex				0.138
Male, *n* (%)	240 (47.0)	174 (49.2)	66 (42.0)	
Female, *n* (%)	271 (53.0)	180 (50.8)	91 (58.0)	
BMI (kg/m^2), median (IQR)	29.0 (26.3, 32.6)	28.8 (26.2, 31.9)	29.5 (26.3, 33.8)	0.099
Diabetes, *n* (%)	268 (52.4)	156 (44.0)	112 (71.3)	< 0.001
Albumin (g/dL), median (IQR)	4.4 (4.1, 4.6)	4.4 (4.2, 4.6)	4.30 (4.0, 4.6)	0.053
Globulin (g/dL), median (IQR)	3.4 (3.0, 3.8)	3.4 (3.0, 3.8)	3.5 (3.1, 3.8)	0.21
AST (U/L), median (IQR)	39 (28, 60)	35 (26, 52)	53.5 (36, 75)	<0.001
ALT (U/L), median (IQR)	65 (42, 101)	59.5 (40, 98)	75 (50, 111)	< 0.001
GGT (U/L), median (IQR)	63 (37, 108)	56.5 (35, 92)	81 (48, 151)	< 0.001
Platelet (× 10^9/μL), median (IQR)	254 (213, 297)	266 (226.8, 302)	230 (189, 277)	< 0.001
Hemoglobin (g/dL), median (IQR)	14.2 (13.3, 15.2)	14.2 (13.4, 15.2)	14.1 (13.3, 15.2)	0.393
White blood cells (cells/μL), median (IQR)	7430 (6060, 8700)	7400 (6100, 8725)	7500 (5950, 8695)	0.768
INR, median (IQR)	1.01 (0.96, 1.06)	1.00 (0.95, 1.07)	1.01 (0.97, 10.6)	0.625
Total cholesterol (mg/dL), median (IQR)	189.5 (166, 217)	193 (170, 220)	182 (159, 209)	0.004
LDL-cholesterol (mg/dL), median (IQR)	115 (92, 139)	116 (96, 143)	107 (85, 132)	0.003
HDL-cholesterol (mg/dL), median (IQR)	46 (39, 52)	46 (39, 53)	44 (38, 50)	0.168
Triglyceride (mg/dL), median (IQR)	120 (77, 157)	120 (77, 155)	119 (80, 159)	0.483
HbA1C (%), median (IQR)	6.1 (5.6, 7.2)	5.9 (5.5, 6.8)	6.8 (5.8, 7.6)	< 0.001
Fibrosis stage, *n* (%)				< 0.001
0	151 (29.5)	151 (42.7)	0 (0)	
1	203 (39.7)	203 (57.3)	0 (0)	
2	69 (13.5)	0 (0)	69 (43.9)	
3	72 (14.1)	0 (0)	72 (45.9)	
4	16 (3.1)	0 (0)	16 (10.2)	
Median CAP (dB/m), median (IQR)	308.5 (230, 342)	299 (211, 339)	324 (294, 347)	< 0.001
Median TE (kPa), median (IQR)	7.6 (5.6, 10.9)	6.6 (5.1, 8.8)	11.1 (8.6, 15.5)	< 0.001
FIB-8, median (IQR)	2.0 (1.2, 2.9)	1.8 (1.1, 2.4)	3.0 (2.2, 4.0)	< 0.001
FIB-4, median (IQR)	1.0 (0.7, 1.5)	0.8 (0.6, 1.2)	1.5 (1.0, 2.1)	< 0.001
NFS, mean ± SD	-1.8 ± 1.5	-2.0 ± 1.4	-1.2 ± 1.3	< 0.001

NAFLD: Nonalcoholic fatty liver disease; FIB-8: Fibrosis-8 score; FIB-4: Fibrosis-4 score; NFS: NAFLD fibrosis score; AUROC: Areas under the receiver operating characteristic curves; GGT: Gamma-glutamyl transferase; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; BMI: Body mass index; CAP: Controlled Attenuation Parameter.

Additionally, our results demonstrated the performance of the FIB-8 score, FIB-4 score, and NFS in patients aged > 65 years (AUROC: 0.66, 0.71, and 0.54, respectively). The performance was poor in patients aged < 35 years (AUROC: 0.55, 0.59, and 0.70, respectively). Thus, these scores have insufficient accuracy for use in NAFLD patients in extreme age groups. Similarly, McPherson *et al*[23] demonstrated age as a confounding factor for the accurate noninvasive scoring system predicting advanced fibrosis [23]. The FIB-8 score has low accuracy for predicting significant fibrosis in NAFLD patients, similar to

Table 3 Performance of fibrosis-8, fibrosis-4, and nonalcoholic fatty liver disease fibrosis score for predicting significant fibrosis (F ≥ 2) in the Asian population (*n* = 511)

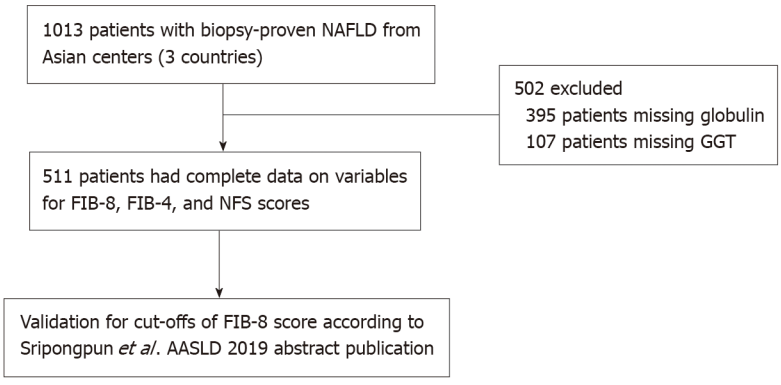
	FIB-8 score	FIB-4 score	NFS
AUC for predicting ≥ F2 fibrosis	0.77[a,b]	0.74	0.68
95% confidence interval	0.73-0.82	0.70-0.79	0.63-0.73
Low and high cutoffs for ≥ F2 fibrosis	0.88 and 1.77	0.81 and 1.81 (17)	-2.45 and 0.03 (17)
Sensitivity (according to the low cutoff)	92.36%	80.25%	80.89%
Specificity (according to the high cutoff)	67.51%	93.50%	93.20%
Proportion of patients in low/indeterminate/high group	18.8/35.4/45.8%	38.2/47.1/14.7%	31.9/58.1/10%

[a]*P* = 0.001 compared with NFS.
[b]*P* = 0.073 compared with FIB-4.
NAFLD: Nonalcoholic fatty liver disease; FIB-8: Fibrosis-8 score; FIB-4: Fibrosis-4 score; NFS: NAFLD fibrosis score; AUROC: Areas under the receiver operating characteristic curves.

Table 4 Comparison of study population using the fibrosis-8 score for predicting significant fibrosis (F ≥ 2)

Variable	Data from AASLD 2019, *n* = 522	FIB-8 score validation (EASL 2020), *n* = 130	FIB-8 score validation (Our cohort), *n* = 511
Population	Mean age: 49 ± 12. Female: 62.5%; BMI: 34 ± 7 kg/m^2; DM: 30%; ≥ F2: 52.5%	Mean age: 52.4; Female: 53.1%; BMI: 31.5 kg/m^2; DM: 34%; ≥ F2: 35.4%	Mean age: 49.3 ± 11.9; Female: 53.0%; BMI: 30.4 ± 7.1 kg/m^2; DM: 52.4%; ≥ F2: 30.7%
Sensitivity and specificity %	86.7% and 82.7%, respectively, Validation set	> 90%, 80.6%	92.3%, 67.5%
AUC for predicting > F2 fibrosis	0.78, Validation set	0.84	0.77
Performance superior to	> FIB-4 score; *P* < 0.001; > NFS; *P* = 0.005	> FIB-4 score (AUC 0.80); > NFS (AUC 0.77)	> FIB-4 score; *P* = 0.073; > NFS; *P* = 0.001

NAFLD: Nonalcoholic fatty liver disease; FIB-8: Fibrosis-8 score; FIB-4: Fibrosis-4 score; NFS: NAFLD fibrosis score; AASLD: American Association for the Study of Liver Diseases; EASL: European Association for the Study of the Liver; AUROC: Areas under the receiver operating characteristic curves; BMI: Body mass index; DM: diabetes mellitus.

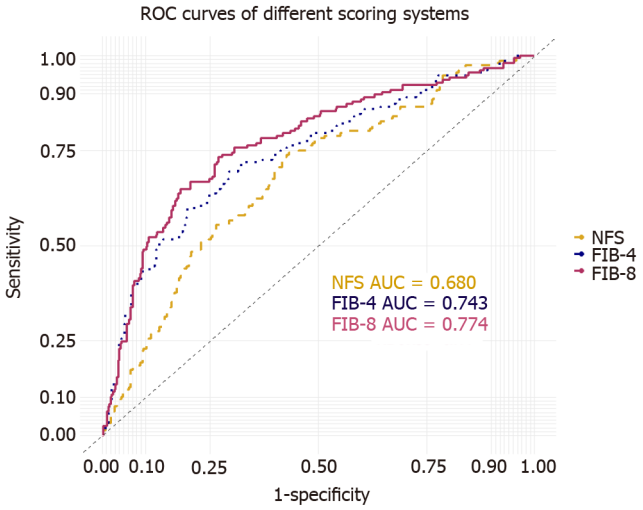


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Figure 1 Flow diagram of the study population. NAFLD: Nonalcoholic fatty liver disease; FIB-8: Fibrosis-8 score; FIB-4: Fibrosis-4 score; NFS: NAFLD fibrosis score; AASLD: American Association for the Study of Liver Diseases; GGT: Gamma-glutamyl transferase.

the FIB-4 score and NFS in patients aged < 35 and > 65 years.

Our study had limitations. First, we had limited complete data for half of our database because of the lack of either globulin or GGT. In usual clinical practice, clinicians do not routinely check both laboratory parameters, and no added value exists for observing or monitoring these values in patients. The second limitation of our study was the lower incidence of fibrosis in our cohort *vs* other cohorts. The differences in fibrosis may have diagnostic value for novel fibrosis scores for validation. Validations



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Figure 2 Receiver operating characteristic curves of the fibrosis-8 score, fibrosis-4 score, and nonalcoholic fatty liver disease fibrosis score for predicting significant fibrosis (F ≥ 2) in the Asian population (*n* = 511). NAFLD: Nonalcoholic fatty liver disease; FIB-8: Fibrosis-8 score; FIB-4: Fibrosis-4 score; NFS: NAFLD fibrosis score; AUROC: Areas under the receiver operating characteristic curves.

in larger cohorts are needed.

To our best knowledge, our study is the first to report a new validation model of the FIB-8 score for predicting significant fibrosis among patients with NAFLD in an Asian population. The FIB-8 score yielded higher accuracy in diagnosing significant fibrosis than the NFS. Additionally, the FIB-8 score was non-inferior but insignificantly superior to the FIB-4 score. A novel simple fibrosis score comprising commonly accessible basic laboratories may be additionally used to add previous fibrosis scores for an initial assessment in primary care units and to select patients for further hepatologist referral.

CONCLUSION

The new, simple fibrosis FIB-8 score had significantly better performance for predicting significant fibrosis in NAFLD patients than the NFS and was non-inferior but insignificantly superior to the FIB-4 score in the Asian population. A simple fibrosis score comprising commonly accessible basic laboratories may be used for an initial assessment in primary care units and to select patients for further hepatologist referral.

ARTICLE HIGHLIGHTS

Research background

In the nonalcoholic fatty liver disease (NAFLD) population, noninvasive fibrosis scores, such as the fibrosis-4 (FIB-4) score and NAFLD fibrosis score (NFS), are generally applied in clinical practice guidelines. The novel fibrosis-8 (FIB-8) score yielded higher accuracy in diagnosing significant fibrosis in a previously reported cohort. A larger cohort may provide more reliability and benefit in clinical practice.

Research motivation

A noninvasive fibrosis score in NAFLD patients using only routine laboratory parameters is particularly important in initial assessment in the primary care unit or resource-limited conditions. We proposed the novel FIB-8 score, which incorporates the additional variables body mass index (BMI), the A/G ratio, gamma-glutamyl transferase (GGT), and diabetes into the FIB-4 score. The additional variables, particularly GGT, may provide better diagnostic accuracy for predicting significant fibrosis in NAFLD patients.

Research objectives

We aimed to validate the FIB-8 score among patients with a biopsy-proven NAFLD cohort and to compare the diagnostic performance of the FIB-8 and FIB-4 scores and NFS for predicting significant fibrosis.

Research methods

This was a retrospective study involving 1013 biopsy-proven NAFLD patients from 3 Asian centers in 3 countries in an Asian population. All the patients with available baseline biochemical tests for the FIB-8 score calculation and all related variables for predicting liver fibrosis were included.

Research results

A total of 1013 patients were included in the final analysis. Of those, 511 patients had complete data on the variables, including the NFS and FIB-4 and FIB-8 scores. One hundred fifty-seven (30.7%) patients had significant fibrosis (≥ F2). The areas under the receiver operating characteristic curves of the FIB-8 and FIB-4 scores and NFS for predicting significant fibrosis were 0.774, 0.743, and 0.680, respectively. The FIB-8 score had significantly better performance for predicting significant fibrosis than the NFS (P = 0.001) but was not superior to the FIB-4 score (P = 0.073). The low cutoff point of the FIB-8 score for predicting significant fibrosis of 0.88 showed 92.36% sensitivity, and the high cutoff point of the FIB-8 score for predicting significant fibrosis of 1.77 had 67.51% specificity.

Research conclusions

The FIB-8 score, which incorporates the additional variables of the BMI, A/G ratio, GGT level, and diabetes into the FIB-4 score, yielded better performance for predicting significant fibrosis in NAFLD patients than the NFS but was not superior to the FIB-4 score in the Asian population. A simple fibrosis score comprising commonly accessible basic laboratories may be used for an initial assessment in primary care units.

Research perspectives

Future prospective studies are needed to compare the diagnostic accuracy of various noninvasive scores for predicting significant fibrosis and staging fibrosis.

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FOOTNOTES

Author contributions: Treeprasertsuk S designed the study; Pitisuttithum P, Chan WK, Wong VWS, and Treeprasertsuk S contributed to data acquisition; Mahadeva S and Wong GLH recruited and managed the patients; Mustapha NRN and Leung HHW performed the histological assessment; Prasoppokakorn T, Pitisuttithum P, Sripongpun P, and Treeprasertsuk S analyzed and interpreted the data; Prasoppokakorn T drafted the manuscript; Chan WK, Sripongpun P, Wong VWS, and Treeprasertsuk S revised the manuscript critically for important intellectual content; all the authors read and approved the final manuscript.

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Country/Territory of origin: Thailand

ORCID number: Thaninee Prasoppokakorn 0000-0002-1012-9874; Wah-Kheong Chan 0000-0002-9105-5837; Vincent Wai-Sun Wong 0000-0003-2215-9410; Panyavee Pitisuttithum 0000-0002-3530-3621; Sanjiv Mahadeva 0000-0003-0021-8596; Nik Raihan Nik Mustapha 0000-0002-4326-882X; Grace Lai-Hung Wong 0000-0002-2863-9389; Howard Ho-Wai Leung 0000-0001-9930-1783; Pimsiri Sripongpun 0000-0003-0007-8214; Sombat Treeprasertsuk 0000-0001-6459-8329.

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ORIGINAL ARTICLE

Retrospective Study

Prognostic factors of recurrent intrahepatic cholangiocarcinoma after hepatectomy: A retrospective study

Zi-Bo Yuan, Hong-Bo Fang, Quan-Kai Feng, Tao Li, Jie Li

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Zi-Bo Yuan, Hong-Bo Fang, Quan-Kai Feng, Tao Li, Jie Li, Department of Hepatobiliary and Pancreatic Surgery, the First Affiliated Hospital of Zhengzhou University, Zhengzhou 450052, Henan Province, China

Zi-Bo Yuan, Hong-Bo Fang, Quan-Kai Feng, Jie Li, Henan Research Centre for Organ Transplantation, the First Affiliated Hospital of Zhengzhou University, Zhengzhou 450052, Henan Province, China

Corresponding author: Jie Li, MD, PhD, Director, Doctor, Professor, Department of Hepatobiliary and Pancreatic Surgery, the First Affiliated Hospital of Zhengzhou University, No. 1 Jianshe Road, Zhengzhou 450052, Henan Province, China. ljljdoctor@126.com

Abstract

BACKGROUND

Intrahepatic cholangiocarcinoma (ICC) is a highly malignant tumour. Hepatectomy is an effective treatment for early ICC, but postoperative recurrence greatly affects patient survival. Studies on recurrent ICC after hepatectomy are lacking.

AIM

To investigate the clinical characteristics of patients with recurrent ICC after hepatectomy, analyse prognostic factors and explore diagnosis and treatment strategies.

METHODS

A retrospective analysis was performed on all ICC patients undergoing hepatectomy from January 2013 to August 2021. Patients with postoperative recurrence were selected according to the inclusion and exclusion criteria. Cumulative overall survival was plotted by the Kaplan-Meier method, and differences were assessed by univariate survival analysis using the log-rank test. Multivariate analysis of cumulative survival was performed using the Cox proportional risk model.

RESULTS

During the 8-year study period, 103 patients underwent ICC-related hepatectomy, and 54 exhibited postoperative recurrence. The median disease-free survival (DFS) was 6 mo, the median overall survival (OS) was 9 mo, and the cumulative OS rates at 1, 2 and 3 years after the operation were 40.7%, 14.8% and 7.4%,

respectively. The median OS after recurrence was 4 mo, and the cumulative OS rates at 1, 2 and 3 years after recurrence were 16.1%, 6.7% and 3.4%, respectively. Multivariate analysis showed that alcohol consumption [hazard ratio (HR) = 4.64, 95% confidence interval (CI): 1.53-14.04, P = 0.007] and DFS < 6 mo (HR = 3.47, 95%CI: 1.59-7.60, P = 0.002) were independent risk factors for the cumulative survival of patients with recurrence, while treatment after recurrence (HR = 0.21, 95%CI: 0.08-0.55, P = 0.001) was an independent protective factor. The median OS time of patients receiving multimodality therapy after recurrence of ICC was 7 mo, which was significantly higher than that of patients receiving only local therapy (3 mo), patients receiving systematic therapy (4 mo) and patients receiving the best supportive therapy (1 mo). Patients with recurrent ICC who received multimodality therapy had a significantly better long-term survival after recurrence than those who did not (P = 0.026).

CONCLUSION

The prognosis of patients with recurrence after ICC-related hepatectomy is poor. Alcohol consumption and DFS < 6 mo are independent risk factors in terms of the cumulative survival of patients with recurrence, while treatment after recurrence is an independent protective factor. Multimodality therapy can effectively improve the prognosis of patients.

Key Words: Intrahepatic cholangiocarcinoma; Hepatectomy; Recurrence; Multimodality therapy; Prognosis

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Core Tip: With this 8-year retrospective study, we aimed to investigate the clinical characteristics, analyse the prognostic factors, and discuss therapeutic strategies for patients with recurrent intrahepatic cholangiocarcinoma (ICC) after hepatectomy. Multivariate analysis showed that alcohol consumption [hazard ratio (HR) = 4.64, 95% confidence interval (CI): 1.53-14.04, P = 0.007] and disease-free survival < 6 mo (HR=3.47, 95%CI: 1.59-7.60, P = 0.002) were independent risk factors for cumulative survival for patients with recurrence, while treatment after recurrence (HR=0.21, 95%CI: 0.08-0.55, P = 0.001) was an independent protective factor. We propose that multimodality therapy should be developed to improve long-term outcomes through the combined approach of local therapy, chemotherapy, targeted therapy, and immunotherapy.

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INTRODUCTION

Intrahepatic cholangiocarcinoma (ICC) is a highly malignant tumour originating from intrahepatic bile duct epithelial cells[1]. Liver cancer ranks sixth in the world in terms of incidence rate and third in terms of mortality rate[2]. ICC accounts for 10% to 15% of primary liver cancers[1]. In the last 30 years, the incidence and mortality rates of ICC have significantly increased worldwide[3]. Hepatectomy is an effective method for the treatment of early ICC[4]. However, ICC has highly malignant biological behaviour, and early recurrence and metastasis are extremely common, so the prognosis is poor[5]. The postoperative 5-year survival rate is only 20%-35%, and the recurrence rate is as high as 50%-70%, and these rates are much worse than those for hepatocellular carcinoma[6,7].

Prevention of ICC recurrence and treatment strategies after recurrence are extremely important to improve the overall survival (OS) time. The early recurrence of ICC is related to the characteristics of the tumour, while late recurrence is related to underlying liver diseases[8]. Studies[9] have shown that the presence of multiple tumours, microvascular invasion, and lymph node metastasis are risk factors for recurrence after hepatectomy. Age, liver disease, lymph node involvement, vascular invasion, multiple tumours, and tumour size are related to prognosis[10]. However, the risk factors affecting the long-term prognosis of patients with recurrent ICC after hepatectomy are not clear. The European Association for Liver Research[11] and the Italian Clinical Practice Guide[12] have pointed out that the treatment strategy for recurrent ICC is based on the clinical characteristics of the site of tumour recurrence. Recently, some studies[6,13] have reported various treatments for different types of recurrence. However, the best treatment strategy for the postoperative recurrence of ICC is still unclear.

In this study, the clinical characteristics and treatment statistics of patients with recurrent ICC after hepatectomy in our hospital were assessed to identify survival-related factors and explore strategies for diagnosis and treatment.

MATERIALS AND METHODS

Patients

The clinical data of 103 ICC patients who underwent hepatectomy in the First Affiliated Hospital of Zhengzhou University were analyzed retrospectively from January 2013 to August 2021. The diagnosis of ICC was based on liver pathological examination, and histological grading was based on the WHO grading system[14]. The tumour stage was determined according to the American Joint Council on Cancer (AJCC) 8th edition tumour-node-metastasis classification system[15]. The inclusion criteria were as follows: (1) Primary intrahepatic cholangiocarcinoma was confirmed by postoperative histopathology; (2) Liver function was considered Child-Pugh grade A or B; (3) Preoperative evaluation indicated that the patient could tolerate surgery without serious heart, lung, brain, and kidney vital organ lesions; and (4) Relapse was observed after hepatectomy. The exclusion criteria were as follows: (1) The patient had a preoperative history of malignant tumour; (2) Postoperative histopathology confirmed hepatocellular carcinoma or mixed liver cancer; or (3) Clinical records and follow-up information were incomplete. Finally, a total of 54 patients with recurrent ICC after hepatectomy were included (Figure 1). The study was approved by the Scientific Research and Clinical Trial Ethics Committee of the First Affiliated Hospital of Zhengzhou University (Ethical number 2021-KY-0464-001).

Treatment strategy for primary ICC-related hepatectomy

The mode of operation was determined according to the location and size of the tumour and the patient's liver function. The scope of resection was classified according to the international consensus standard[16]: Extended hepatectomy was performed in 16 cases (resection of liver tissue more than 3 segments), and local hepatectomy was performed in 38 cases (marginal partial hepatectomy or resection of liver tissue no more than 3 segments). Abnormal enlargement of lymph nodes was found during the operation or imaging examination before the operation, and the hepatic hilum, hepatoduodenal ligament, and posterior pancreatic lymph nodes were dissected. Lymph node dissection was performed in 19 of the 54 patients. According to National Comprehensive Cancer Network (NCCN) practice guidelines[17], 20 ICC patients were treated with adjuvant therapy after hepatectomy.

Follow-up and recurrence

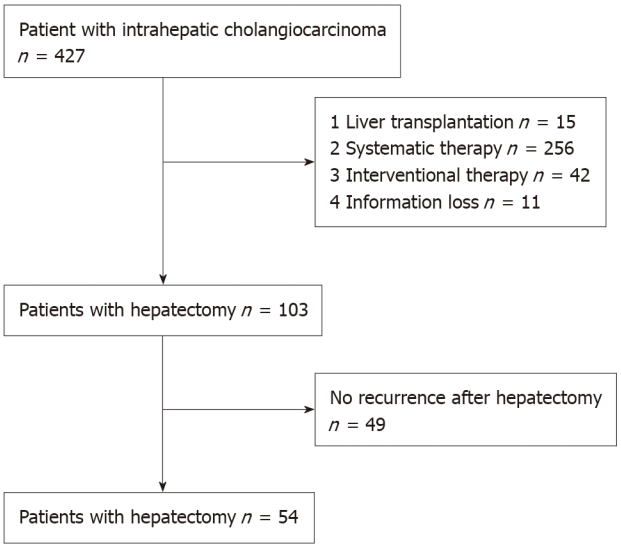
After hepatectomy, all patients were followed up *via* outpatient visits or telephone calls. Follow-up was initiated 1 mo after intrahepatic cholangiocarcinoma resection, followed by follow-up visits every 3 mo for 2 years and every 6 mo after 2 years. Patients with postoperative recurrence of ICC were followed up once a month, and the last follow-up was in August 2021. During the follow-up period, the patient examinations included (1) Haematology examination, including assessment of liver and kidney function, serum tumour markers, and hepatitis viral load; and (2) Imaging examination, including chest plain film or nonenhanced CT, abdominal enhanced CT or MRI. To evaluate the progression of the disease, patients with recurrent ICC were examined by whole-body bone scan or PET-CT. Follow-up began at the time of hepatectomy and ended at the time of death or the last follow-up. Disease-free survival (DFS) was defined as the time from the date of surgery to the first recurrence of ICC. OS was defined as the time from the first recurrence after hepatectomy to death or the last follow-up.

Treatment strategies after relapse

For those who are diagnosed with tumour recurrence or metastasis, the treatment plan is determined according to the evaluation of the reserve function of the liver, the condition of the whole body, and the site of recurrence. The inclusion criteria of secondary hepatectomy were the same as those of primary hepatectomy. Patients with unresectable ICC are treated with local therapy, chemotherapy, targeted therapy, immunotherapy, and multimodality therapy.

Statistical methods

For descriptive statistics, continuous variables are expressed as medians, and categorical variables are expressed as numbers (%). Cumulative survival was plotted by the Kaplan-Meier method. The log-rank test was used to assess differences in the univariate survival analysis. Multivariate analysis of cumulative survival was performed using the Cox proportional risk model. Statistical analysis was performed using SPSS 26.0 software (IBM, Armonk, NY, United States). Differences were considered statistically significant at $P < 0.05$. An online tool (http://www.bioinformatics.com.cn) was applied to draw Venn digrams related to recurrent patterns.



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Figure 1 Patient flowchart.

RESULTS

Follow-up results and clinical characteristics of patients with recurrent ICC

By the end of follow-up, 54 ICC patients (54/103, 52.4%) had recurrence after hepatectomy. Patients were followed up for 2-94 mo, with a median DFS of 6 mo and a median OS of 9 mo. The 1-year, 2-year, and 3-year cumulative OS rates were 40.7%, 14.8%, and 7.4%, respectively (Figure 2). The median OS after recurrence was 4 mo, and the 1-year, 2-year and 3-year cumulative survival rates after recurrence were 16.1%, 6.7%, and 3.4%, respectively (Figure 3). The majority of patients (45/54, 83.3%) relapsed within 1 year, and the recurrence rate was 50% (27/54) within 6 mo after surgery. Venn diagrams showed that intrahepatic lesions (25/54, 46.3%) were the most common recurrence sites, followed by concurrent liver and lymph lesions (13/54, 24.1%) (Figure 4). The clinical and pathological features of patients with recurrence are shown in Table 1. Most of the patients were male (34/54, 63.0%), smokers (34/54, 63.0%), and alcohol consumers (44/54, 81.5%). Sixteen patients had hypertension, and 9 patients had diabetes. Twenty-four patients were associated with hepatitis B virus (HBV), and 9 patients associated with hepatitis C virus (HCV). Sixteen patients were treated with extensive hepatectomy, and lymph node dissection was performed in 19 patients. Postoperative pathological reports showed that 46 patients had single tumours, 22 patients had poorly differentiated tumours, and 12 patients had vascular tumour thrombi.

Prognostic factors in patients with ICC recurrence

According to the univariate analysis of patients with recurrent ICC, nine factors significantly affected the survival of patients (Table 1). Age, alcohol consumption, histological grade, biliary invasion, vascular tumour thrombi, DFS, preoperative and post-recurrence CA19-9 level, and treatment after recurrence were significant favorable prognostic indicators in patients with recurrent ICC. Multivariate Cox regression analysis showed that alcohol consumption, DFS < 6 mo and treatment after recurrence were independent factors affecting cumulative survival in patients with recurrence (Table 2). Early recurrence in ICC patients was associated with biliary invasion, vascular tumour thrombi, and high post-recurrence CA19-9 levels. Multivariate analysis proved that the risk of death from alcohol consumption was 4.64 times that of non-alcohol consumption, and this was independent of other prognostic factors. The mortality risk of patients with DFS < 6 mo was 3.47 times that of patients with DFS > 6 mo. Treatment after recurrence could significantly reduce the mortality risk.

Treatment after recurrence

Treatment patterns for patients with recurrent ICC are shown in Table 3. Fourteen patients received local therapy, 22 patients received systematic therapy, 6 patients received multimodality therapy, and 12 patients received supportive care therapy based on their condition. Figure 5 shows patients with recurrent ICC who received multimodality therapy had a significantly better long-term survival after recurrence than those who did not (P = 0.026, log-rank test). Among the patients who received local treatment, 2 patients had hepatectomy after recurrence; 1 patient had received local liver resection, and hepatectomy was performed again after 7 mo. the patient died due to multiple metastases. Another patient underwent laparoscopic hepatectomy for the primary lesion, but the tumour recurred 13 mo

Table 1 Univariate analysis of prognostic factors after intrahepatic cholangiocarcinoma recurrence following hepatectomy

Factors	Cases, *n* (%)	Median survival after recurrence, mo (95%CI)	*P* value[1]
Sex			0.123
Male	34 (63.0)	4.0 (1.2-6.8)	
Female	20 (37.0)	3.0 (1.1-4.9)	
Age (yr)			0.031
< 65	42 (77.8)	4.0 (2.3-5.8)	
≥ 65	12 (22.2)	3.0 (1.3-5.1)	
Smoking			0.059
No	20 (37.0)	5.0 (2.0-8.0)	
Yes	34 (63.0)	3.0 (1.0-4.1)	
Alcohol consumption			0.004
No	10 (18.5)	10.0 (3.0-17.0)	
Yes	44 (81.5)	3.0 (1.9-4.2)	
Hypertension			0.309
No	38 (70.4)	4.0 (2.4-5.6)	
Yes	16 (29.6)	4.0 (0.0-11.3)	
Diabetes			0.193
No	45 (83.3)	4.0 (2.5-5.5)	
Yes	9 (16.7)	8.0 (1.0-15.0)	
Hepatitis B			0.904
Negative	30 (55.6)	3.0 (1.3-4.7)	
Positive	24 (44.4)	5.0 (2.0-8.0)	
Hepatitis C			0.175
Negative	45 (83.3)	4 .0 (2.2-5.8)	
Positive	9 (16.7)	5.0 (2.1-7.9)	
Anti-hepatitis-virus			0.969
No	29 (53.7)	4.0 (2.4-5.6)	
Yes	25 (46.3)	5.0 (1.9-8.1)	
Cholelithiasis			0.181
No	44 (81.5)	4.0 (2.1-5.9)	
Yes	10 (18.5)	2.0 (0.0-4.8)	
CA19-9 (U/mL) (initial)			0.002
< 200	33 (61.1)	5.0 (1.8-8.2)	
≥ 200	21 (38.9)	2.0 (1.3-2.7)	
CEA (ng/mL) (initial)			0.356
< 5	34 (63.0)	4.0 (2.1-5.9)	
≥ 5	20 (37.0)	3.0 (0.8-5.2)	
Hepatectomy			0.855
Limited	38 (70.4)	4.0 (2.1-5.9)	
Extended	16 (29.6)	3 .0 (0.0-6.9)	
Lymph node dissection			0.909

No	35 (64.8)	4.0 (2.2-5.8)	
Yes	19 (35.2)	5.0 (2.1-7.9)	
Adjuvant treatment			0.619
No	34 (63.0)	3 .0 (0.1-5.9)	
Yes	20 (37.0)	4 .0 (2.7-5.3)	
Tumor size (cm)			0.884
< 5	14 (25.9)	4.0 (0.4-7.6)	
≥ 5	40 (74.1)	4 .0 (2.0-6.0)	
Multiplicity			0.803
Solitary	46 (85.2)	4.0 (1.4-6.6)	
Multiple	8 (14.8)	3.0 (1.3-4.7)	
Satellite nodules			0.953
No	34 (63.0)	4.0 (2.1-5.9)	
Yes	20 (37.0)	4.0 (2.4-5.6)	
Histological grade			0.021
PD	22 (40.7)	2.0 (1.2-2.8)	
WD or MD	32 (59.3)	5.0 (3.5-6.5)	
Vascular invasion			0.705
No	49 (90.7)	4.0 (2.1-5.9)	
Yes	5 (9.3)	5.0 (1.7-8.3)	
Lymph node metastasis			0.531
No	29 (53.7)	3.0 (0.9-5.1)	
Yes	25 (46.3)	5.0 (2.6-7.4)	
Perineural invasion			0.428
No	45 (83.3)	4.0 (2.5-5.5)	
Yes	9 (16.7)	2.0 (1.3-2.7)	
Biliary invasion			0.003
No	35 (64.8)	5.0 (2.6-7.4)	
Yes	19 (35.2)	2.0 (1.2-2.8)	
Vascular tumour thrombi			0.002
No	42 (77.8)	5.0 (3.2-6.8)	
Yes	12 (22.2)	2.0 (1.2-2.6)	
AJCC T category			0.196
T1–2	14 (25.9)	4.0 (0.0-11.3)	
T3–4	40 (74.1)	4.0 (2.1-5.9)	
DFS			0.003
< 6 mo	27 (50.0)	2.0 (0.7-3.3)	
≥ 6 mo	27 (50.0)	6.0 (2.3-9.7)	
CA19-9 (U/mL) (recurrence)			0.002
< 200	35 (64.8)	4.0 (1.3-6.7)	
≥ 200	19 (35.2)	2.0 (1.2-2.8)	
CEA (ng/mL) (recurrence)			0.378
< 5	41 (75.9)	4.0 (2.3-5.7)	

≥ 5	13 (24.1)	7.0 (1.7-12.3)	
NLR (recurrence)			0.804
< 2	24 (44.4)	5.0 (2.8-7.2)	
≥ 2	30 (55.6)	2.0 (0.9-3.1)	
Recurrent site			0.334
Intrahepatic	24 (44.4)	4.0 (2.3-5.7)	
Extrahepatic	6 (11.2)	3.0 (0.0-11.6)	
Intrahepatic + extrahepatic	24 (44.4)	2.0 (0.4-3.6)	
Treatment after recurrence			< 0.001
No	12 (22.2)	2.0 (1.0-2.8)	
Yes	42 (77.8)	5.0 (2.9-7.1)	

[1]Data are based on log-rank test.

N: Number; CI: Confidence interval; CA19-9: Serum carbohydrate antigen 19-9; CEA: Carcinoembryonic antigen; PD: Poor-differentiated; WD: Well-differentiated; MD: Moderate differentiated; NLR: Neutrophil-to-lymphocyte ratio; AJCC: the American Joint Committee on Cancer; DFS: Disease free survival.

Table 2 Multivariate analysis of prognostic factors after intrahepatic cholangiocarcinoma recurrence following hepatectomy

Factors	Multivariate analysis		
	HR	95%CI	*P* value[1]
Age (≥ 65 yr)	2.12	0.88-5.12	0.096
Alcohol consumption (Yes)	4.64	1.53-14.04	0.007
CA19-9 (≥ 200 U/mL) (initial)	2.63	0.94-7.35	0.065
Histological grade (PD)	1.18	0.59-2.35	0.646
Biliary invasion (Yes)	1.04	0.41-2.63	0.940
Vascular tumor thrombus (Yes)	1.80	0.70-4.65	0.222
DFS (< 6 mo)	3.47	1.59-7.60	0.002
CA19-9 (≥ 200 U/mL) (recurrence)	1.13	0.47-2.71	0.785
Treatment after recurrence (Yes)	0.21	0.08-0.55	0.001

[1]Data are based on Cox regression model.

CI: Confidence interval; CA19-9: Serum carbohydrate antigen 19-9; PD: Poor-differentiated; DFS: Disease free survival.

after surgery. Local hepatectomy and lymph node dissection were performed for the recurrent lesion. Table 4 shows the clinicopathological features of the 6 patients who received multimodality therapy for recurrence, of which 2 patients (No. 4, 6) survived, and 4 patients (No. 1, 2, 3, 5) died due to tumour-related complications. Three patients with recurrence (No. 1, 2, 3) received the GEMOX regimen (1 g/m² gemcitabine on d 1 and 8 + 100 mg/m² oxaliplatin on d 1 with 21 d/cycle) after transarterial chemoembolization (TACE). One patient (No. 4) received local therapy after intrahepatic recurrence. Due to extrahepatic metastasis, the patient was switched to the SOX regimen (60 mg/d tegafur on d 1-14 + 130 mg/m² oxaliplatin on d 1 with 21 d/cycle) maintenance therapy. One patient with recurrence (No. 5) received the FOLFOX-4 regimen (400 mg/m² fluorouracil on d 1 and 2 + 200 mg/m² calcium folate on d 1 and 2 + 85 mg/m² oxaliplatin on d 1 with 14 d/cycle) after RFA. One patient (No. 6) received a tyrosine kinase inhibitor regimen + PD-1 inhibitor (250 mg/d apatinib mesylate + 200 mg camrelizumab on d 1 with 21 d/cycle) after RFA, and no disease progression was observed up to the submission date.

DISCUSSION

ICC is a rare invasive biliary tract tumour and primary liver malignancy with an increasing incidence worldwide[3]. Most patients are initially diagnosed with advanced ICC, and only 30% of ICCs can be

Table 3 Treatment of recurrent intrahepatic cholangiocarcinoma

Treatment	*n*	Median DFS, mo (range)	MS after recurrence, mo (range)
Local therapy	14	6 (1-86)	3 (1-36)
Hepatectomy	2	10 (7-13)	25 (13-36)
TACE	8	3 (1-23)	2 (1-7)
RFA	4	7 (2-86)	3 (1-7)
Systemic therapy	22	5 (1-29)	4 (1-38)
Chemotherapy	3	11 (10-29)	4 (1-38)
Targeted therapy	12	3 (1-23)	4 (1-10)
Targeted + immunization therapy	7	4 (1-13)	7 (2-24)
Multimodality therapy	6	4 (2-11)	7 (4-24)
Best supportive care	12	6 (3-20)	1 (1-6)

N: Number; DFS: Disease-free survival; MS: Median survival; TACE: Transarterial chemoembolization; RFA: Radiofrequency ablation.

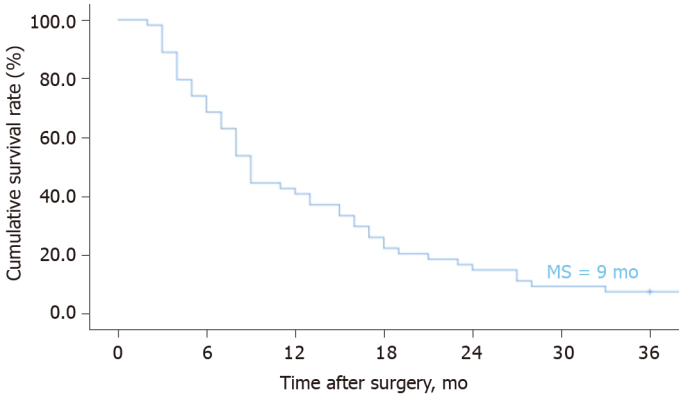
Table 4 Individual characteristics of patients receiving multimodality therapy for recurrent intrahepatic cholangiocarcinoma

Clinical features					Pathological characteristics				Recurrent		Survival		
Case	Age (yr)	Sex	Hepatitis	Surgery	Lymph node metastasis	Tumour size (cm)	Tumour number	Histological grade	Recurrent site	Treatment	DFS (mo)	SAR (mo)	Outcome
1	62	M	HBV	ERH	Yes	12	1	MD	Liver + lymph node	TACE + GEMOX	4	3	Dead
2	48	M	HBV	ERH	None	8	1	MD	Liver	TACE + GEMOX	11	13	Dead
3	40	M	HBV	ERH	None	5	2	MD	Liver + lymph node + bone	TACE + GEMOX	2	5	Dead
4	53	M	None	LH	None	11	1	MD	Liver + lymph node	RFA + TACE + SOX	6	6	Alive
5	44	M	HBV	RH	None	14	1	MD	Liver + bone	RFA + FOLFOX-4	3	2	Dead
6	64	M	None	ERH	Yes	22	1	MD	Liver	RFA + PD-1 + TKI	4	1	Alive

DFS: Disease-free survival; SAR: Survival after recurrence; M: Male; HBV: Hepatitis B virus; ERH: Extended right hepatectomy; LH: Left hepatectomy; RH: Right hepatectomy; MD: Moderate differentiated; TACE: Transarterial chemoembolization; RFA: Radiofrequency ablation; GEMOX: Gemcitabine + oxaliplatin; FOLFOX-4: Fluorouracil + calcium folate + oxaliplatin; SOX: Tegafur + oxaliplatin; PD-1: Programmed death-1 inhibitor; TKI: Tyrosine kinase inhibitor.

surgically resected[18]. Surgical principles include negative margins and tumour-related lymph node resection[19-21]. Due to the biological characteristics of ICC and the vascular system of the liver, local recurrence and lymphatic metastasis are highly likely to occur after surgery. Even after R0 resection, the recurrence rate 5 years after hepatectomy is as high as 70%[6]. At present, the choice of treatment for recurrent ICC remains controversial. Since ICC after recurrence seriously affects the postoperative survival of patients, it is necessary to determine the risk factors for survival after recurrence and explore diagnosis and treatment strategies.

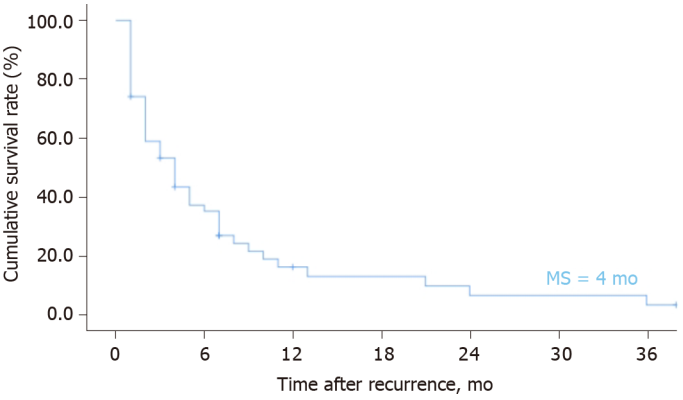
Previous studies[22,23] have explored the clinical characteristics and prognostic factors of the postoperative recurrence of ICC, but few studies have shown the prognosis of ICC patients after recurrence. Chan *et al*[22] reported that tumour diameter > 5 cm, tumour type, lymph node invasion, and vascular invasion are independent risk factors for recurrence in patients after hepatectomy. In other studies, Addeo *et al*[23] found that the risk factors influencing patient recurrence were related to the degree of tumour differentiation and the number of tumours. Regarding the prognosis of patients with recurrent ICC, Ohira *et al*[10] reported that tumour type and nonsurgical treatment were related to a



Number at risk	54	40	22	14	8	5	4
Cumulative survival rate	100.0	74.1	40.7	25.9	14.8	9.3	7.4

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Figure 2 Kaplan-Meier curves of ICC recurrence: Time after surgery. ICC: Intrahepatic cholangiocarcinoma; MS: Median survival.



Number at risk	54	17	6	5	2	1	1
Cumulative survival rate	100.0	35.1	16.1	13.4	6.7	3.4	3.4

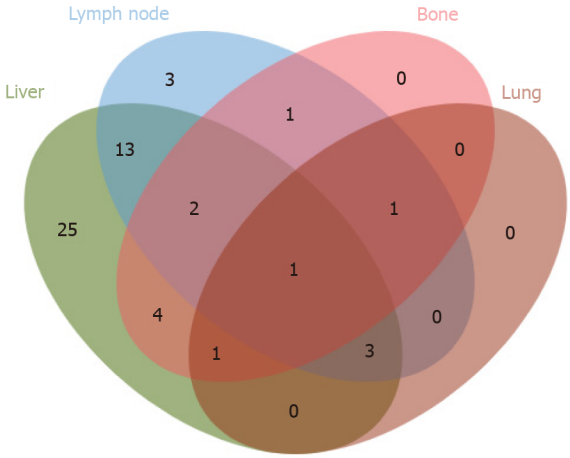
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Figure 3 Kaplan-Meier curves of ICC recurrence: Time after recurrence. ICC: Intrahepatic cholangiocarcinoma; MS: Median survival.

poor prognosis. Our study found that alcohol consumption and DFS < 6 mo were independent risk factors affecting the cumulative survival rate of patients with recurrence, and treatment after recurrence was an independent protective factor.

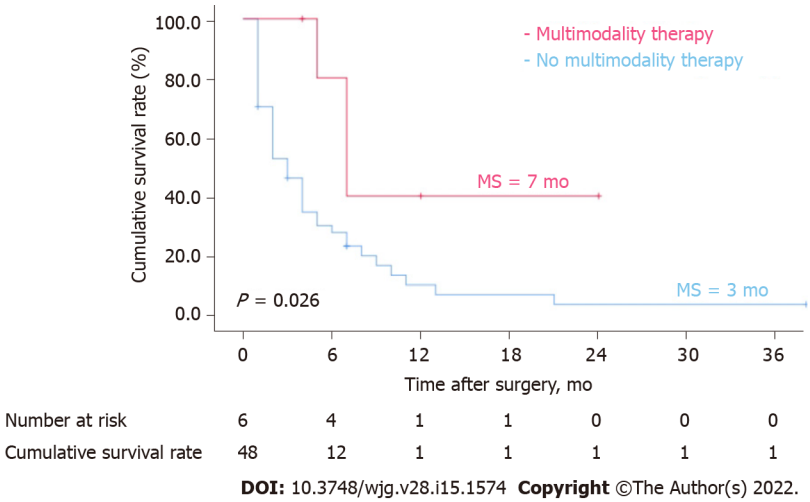
Previous studies[24] have shown that alcohol consumption is a risk factor for ICC. Alcohol may interfere with DNA synthesis and repair through the mechanism of acetaldehyde, a product of ethanol oxidation, to promote the occurrence of liver cancer[25]. Although alcohol drinking is associated with the aetiology of ICC, it is not clear whether alcohol drinking affects the prognosis and survival of patients with recurrent ICC. In this study, multivariate analysis showed that alcohol consumption may be an independent risk factor for recurrent ICC. For patients with recurrent ICC, we recommend reducing alcohol consumption as much as possible to improve the prognosis and survival time of patients.

In this study, the first recurrence of most patients after hepatectomy occurred within 1 year after surgery. Multivariate analysis showed that DFS < 6 was an independent risk factor for survival after ICC recurrence. Park *et al*[26] and Si *et al*[27] also reported that DFS was associated with prognosis. Compared with the clinical characteristics of patients with advanced recurrence, early recurrence is often accompanied by bile duct invasion and lymph node metastasis, and the median survival time after recurrence is 2 mo, which is much lower than the time of late recurrence. Currently, immunohistochemical markers commonly used to predict early recurrence after hepatectomy include B-lymphocyte chemokine 13 (CXCL13)[28], pancreatic secreted trypsin inhibitor (PSTI)[29], and insulin-like growth factor-II mRNA binding protein 3 (IMP3)[30]. Even if the prognosis of patients with early recurrence of ICC after primary hepatectomy is poor, surgical treatment should be considered to improve the prognosis.



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Figure 4 Venn diagram of recurrent location pattern. Intrahepatic lesions (25/54, 46.3%) were the most common recurrence sites, followed by concurrent liver and lymph nodes (13/54, 24.1%).



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Figure 5 Kaplan-Meier curves of ICC recurrence: multimodality therapy versus no multimodality therapy. Patients with recurrent ICC who received multimodality therapy had a significantly better long-term survival after recurrence than those who did not (P = 0.026, log-rank test). ICC: Intrahepatic cholangiocarcinoma; MS: Median survival.

The survival time of patients with recurrent ICC after surgical resection is higher than that of patients without surgical resection. Furthermore, compared with other treatments, secondary hepatectomy significantly improved the OS time of patients with recurrent ICC. Studies[31] have shown that the prognosis of recurrent intrahepatic resection of ICC is comparable to that of primary resection. In a multicentre study of 356 patients with ICC who underwent hepatectomy, approximately 60% exhibited postoperative recurrence, and 37 of them underwent reresection, with a 5-year survival rate of 44%[32]. Recent studies[33] have reported that repeat resection after recurrence significantly prolongs OS compared with palliative treatment. Therefore, we suggest that patients with resectable intrahepatic recurrent ICC can undergo reoperation to improve patient outcomes.

Most recurrent ICCs are highly invasive and have limitations, such as insufficient remaining liver, making patients ineligible for secondary hepatectomy. Multimodality therapies include strategies that combine regional therapy, systemic chemotherapy, targeted therapy, and immunotherapy. In this study, 6 patients with recurrent ICC who were mainly treated with multimodality therapies achieved a higher postoperative median OS (7 mo) than those with local treatment (3 mo), systemic treatment (4 mo), and supportive treatment (1 mo).

Systemic chemotherapy combined with local therapy can significantly improve patient prognosis. Intra-arterial therapies combined with chemotherapy can shrink the lesion to achieve R0 resection[34]. In this study, the median survival of 3 patients with recurrent ICC treated with TACE combined with the GEMOX regimen was 5 mo, which was longer than that of patients with recurrent ICC using chemotherapy alone (median OS, 4 mo). RFA is suitable for local tumours with diameters < 5 cm, and tumour numbers < 3. RFA was superior to systematic chemotherapy in this study[35]. Among the 6

patients with recurrent ICC, 2 patients underwent RFA combined with chemotherapy. One patient had recurrence 3 mo after hepatectomy, and local intrahepatic lesions were treated with RFA combined with the FOLFOX-4 regimen. The other patient relapsed 6 mo after surgery, and 2 mo after RFA, multiple intrahepatic metastases occurred. TACE combined with the SOX regimen was performed again. Currently, the patient is still alive. A new approach of radiotherapy combined with chemotherapy in the open treatment of advanced ICC. Studies[36] have shown that radiotherapy with chemotherapy can not only relieve pain and other complications in patients with advanced ICC but can also improve the disease control rate and patient survival time. Japanese researchers[37] found that 60% of patients with advanced ICC underwent radical hepatocellular carcinoma after radiotherapy combined with systemic chemotherapy, and the 5-year survival rate was 24%. Radiotherapy was not included in our treatment strategy for patients with recurrent ICC. Due to the lack of reliable evidence-based medical data, the NCCN practice guidelines[17] did not recommend radiotherapy as routine treatment for recurrent ICC.

Among the 6 patients with recurrent ICC in our centre, one patient was treated with a tumour immune checkpoint inhibitor combined with targeted therapy after RFA; this patient was still alive without disease progression at the time of submission. PD-L1 expression was found in interstitial cells in 30% of ICC patients[38]. In the tumour microenvironment of connective tissue hyperplasia and immune system deficiency in ICC, the clinical efficacy of a single drug PD-1/PD-L1 inhibitor in tumour suppression is poor[39]. Targeted therapy combined with immunotherapy is being explored[40]. Targeted drugs can induce the death of tumour cells, leading to the release of their own antigens, which are then taken up by antigen-presenting cells to activate specific T cells. However, they also upregulate inhibitory factors such as CTLA-4 and PD-1. Therefore, the combination of PD-1 inhibitors can strengthen the killing effect, reduce the attack of nontumour antigens, and reduce the adverse reactions of immunotherapy[41]. The combination of tumour immune checkpoint inhibitors and targeted therapy is still a hotspot in the field of tumour therapy.

There are several limitations to this study. First, this is a retrospective study, and there may be selection and detection bias in patients with recurrent ICC. Second, ICC is a rare disease. Although the clinical study lasted for 8 years, the number of patients with recurrence is small, and there are not enough randomized controlled trials of recurrent patients. Finally, this is a single-centre study, so multicentre and prospective trials are needed to confirm our results.

CONCLUSION

The prognosis of patients with recurrence after ICC-related hepatectomy is poor. Alcohol consumption and DFS < 6 mo are independent risk factors in terms of the cumulative survival of patients with recurrence, while treatment after recurrence is an independent protective factor. We propose that multimodality therapy should be developed to improve long-term outcomes through the combined approach of local therapy, chemotherapy, targeted therapy, and immunotherapy.

ARTICLE HIGHLIGHTS

Research background

Intrahepatic cholangiocarcinoma (ICC) is a highly malignant tumour originating from intrahepatic bile duct epithelial cells. Recurrence is very common after hepatectomy.

Research motivation

There are few reports on the clinical features and prognostic factors of recurrent ICC, and the treatment strategies for recurrent ICC have not been fully clarified.

Research objectives

The objective of this study was to analyze the prognostic factors of recurrent ICC and to explore treatment strategies.

Research methods

We retrospectively analyzed all ICC patients who underwent hepatectomy at the First Affiliated Hospital of Zhengzhou University between January 2013 and August 2021. We summarized the clinical characteristics of patients with recurrent ICC and assessed prognostic factors by univariate and multivariate analyses.

Research results

Recurrence occurred in 54 of 103 patients with ICC after hepatectomy during the study period. The median OS of patients with recurrent ICC was 4 mo, and the cumulative OS rates at 1, 2, and 3 years

after recurrence were 16.1%, 6.7%, and 3.4%, respectively. Multivariate analysis of cumulative survival by the Cox proportional risk model showed that alcohol consumption [hazard ratio (HR) = 4.64, 95% confidence interval (CI): 1.53-14.04, P = 0.007], DFS < 6 mo (HR = 3.47, 95%CI: 1.59-7.60, P = 0.002) and treatment after recurrence (HR = 0.21, 95%CI: 0.08-0.55, P = 0.001) were independent factors for recurrence. Patients who received multimodality therapy had higher survival rates than those who did not (P = 0.026).

Research conclusions

The prognosis of recurrent patients is related to alcohol consumption, DFS < 6 mo and treatment after recurrence. Active and effective multidisciplinary treatment is beneficial to improve the prognosis of patients.

Research perspectives

Multicentre prospective studies are needed to evaluate the efficacy of multidisciplinary treatment in recurrent ICC.

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FOOTNOTES

Author contributions: Yuan ZB contributed to collect data and draft the manuscript; Fang HB contributed to data analysis; Feng QK and Li T contributed to clinical advice and follow-up survey; Li J contributed to the conception and critically revised the manuscript; all authors read and agreed the final manuscript to be published.

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

Conflict-of-interest statement: We declare that they have no conflicting interests.

Data sharing statement: No additional data are available.

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Country/Territory of origin: China

ORCID number: Zi-Bo Yuan 0000-0002-5563-2924; Hong-Bo Fang 0000-0001-7861-0427; Quan-Kai Feng 0000-0003-3468-0975; Tao Li 0000-0001-6826-7535; Jie Li 0000-0001-9249-886X.

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ORIGINAL ARTICLE

Retrospective Study

Development and validation of a prediction model for moderately severe and severe acute pancreatitis in pregnancy

Du-Jiang Yang, Hui-Min Lu, Yong Liu, Mao Li, Wei-Ming Hu, Zong-Guang Zhou

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Du-Jiang Yang, Yong Liu, Zong-Guang Zhou, Department of Gastroenterological Surgery, West China Hospital, Sichuan University, Chengdu 610041, Sichuan Province, China

Hui-Min Lu, Mao Li, Wei-Ming Hu, Department of Pancreatic Surgery, West China Hospital, Sichuan University, Chengdu 610041, Sichuan Province, China

Corresponding author: Zong-Guang Zhou, FACS, PhD, Chief Doctor, Department of Gastroenterological Surgery, West China Hospital, Sichuan University, No. 37 Guoxue Alley, Chengdu 610041, Sichuan Province, China. zhou767@163.com

Abstract

BACKGROUND

The severity of acute pancreatitis in pregnancy (APIP) is correlated with higher risks of maternal and fetal death.

AIM

To develop a nomogram that could predict moderately severe and severe acute pancreatitis in pregnancy (MSIP).

METHODS

Patients with APIP admitted to West China Hospital between January 2012 and December 2018 were included in this study. They were divided into mild acute pancreatitis in pregnancy (MAIP) and MSIP. Characteristic parameters and laboratory results were collected. The training set and test set were randomly divided at a ratio of 7:3. Least absolute shrinkage and selection operator regression was used to select potential prognostic factors. A nomogram was developed by logistic regression. A random forest model was used to validate the stability of the prediction factors. Receiver operating characteristic curves and calibration curves were used to evaluate the model's predictive performance.

RESULTS

A total of 190 patients were included in this study. A total of 134 patients (70.5%) and 56 patients (29.5%) were classified as having MAIP and MSIP, respectively. Four independent predictors (lactate dehydrogenase, triglyceride, cholesterol, and albumin levels) were identified for MSIP. A nomogram prediction model based on these factors was established. The model had areas under the curve of 0.865 and 0.853 in the training and validation sets, respectively. The calibration curves showed that the nomogram has a good consistency.

CONCLUSION
A nomogram including lactate dehydrogenase, triglyceride, cholesterol, and albumin levels as independent predictors was built with good performance for MSIP prediction.

Key Words: Acute pancreatitis; Prediction model; Pregnancy; Severity; Nomogram; Random forest

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Core Tip: The severity of acute pancreatitis in pregnancy (APIP) is correlated with higher risks of maternal and fetal death. Few studies have focused on APIP severity prediction. We identified four predictors developed and established a prediction nomogram model for pregnant patients with moderate and severe acute pancreatitis. This model achieved good concordance indexes and may help guide doctors in the managementof APIP.

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INTRODUCTION

Acute pancreatitis (AP) is the most common gastrointestinal disease requiring acute admission to the hospital[1]. The incidence of acute pancreatitis in pregnancy (APIP) varies from 1/10000 to 11.3/10000 [2,3]. Geng *et al*[4] showed that APIP contributes to increased maternal death and fetal loss. Previous studies have shown that the maternal and perinatal mortality rates of APIP are as high as 3.3% and 11.6%-18.7%, respectively[4,5]. According to the revised Atlanta classification, AP was classified as mild acute pancreatitis (MAP), moderately severe acute pancreatitis (MSAP), and severe acute pancreatitis (SAP)[6]. MSAP and SAP develop in 20% of AP patients. Although, management strategies such as fluid resuscitation, early enteral nutrition, and organ supportive care are usually performed in the clinical setting, the mortality rate of MSAP and SAP can be as high as 35%, which is significantly higher than that of MAP[7,8]. Furthermore, some studies have shown that APIP severity is significantly associated with a higher risk of maternal and fetal death[5,9]. The first week after AP onset is usually defined as the early phase[6]. It would be useful in clinical management if the severity of APIP could be predicted in the early phase. Currently, several prediction systems, including the Acute Physiology and Chronic Health Evaluation, Ranson score, and Bedside Index for Severity in AP, are usually used for AP patients. However, the sensitivity and specificity of these prediction systems are not high enough, and cumbersome items limit their clinical use[10]. At present, few scoring systems have been designed for patients with APIP[11]. Therefore, this study aimed to develop a simple and useful prediction model to predict moderately severe and severe acute pancreatitis in pregnancy (MSIP).

MATERIALS AND METHODS

Study design and patients

We retrospectively collected the medical records of patients who were diagnosed with AP during pregnancy at West China Hospital from January 2012 to December 2018. Patients meeting the following criteria were excluded: (1) Were readmitted (only included first-time record); (2) Received a cesarean section before admission to West China Hospital; (3) Had a length of more than 7 d from AP onset to admission; (4) Had chronic kidney dysfunction; and (5) Had any missing data of candidate variables. The Ethics Committee of West China Hospital approved the study, and it was conducted according to the Declaration of Helsinki.

Data collection

The following clinical variables were collected: age, etiology (hypertriglyceridemia, gallstones, other), comorbidities (hypertension, diabetes, fatty liver), smoking, drinking, length of time from onset to admission, gestational weeks on admission, trimester of pregnancy on admission, blood infection, length of hospital stay (LOS), fetal death, and maternal hospital mortality. All laboratory variables were tested in the hospital, including hematocrit, platelet, white blood cell (WBC), and neutrophil levels. Laboratory variables were collected within 48 h of admission. The average levels of retested laboratory

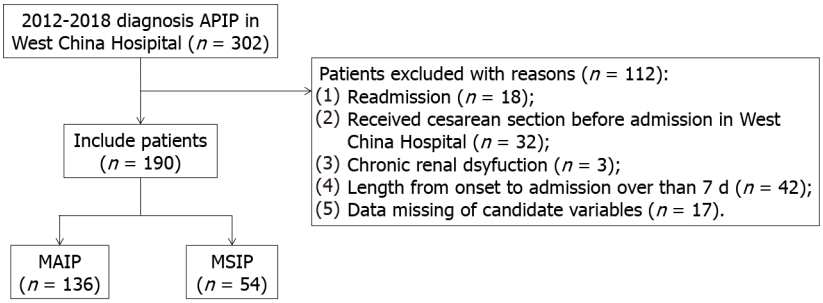


Figure 1 Flow chart of the study. APIP: Acute pancreatitis in pregnancy; MAIP: Mild acute pancreatitis in pregnancy; MSIP: Moderately severe and severe acute pancreatitis in pregnancy.

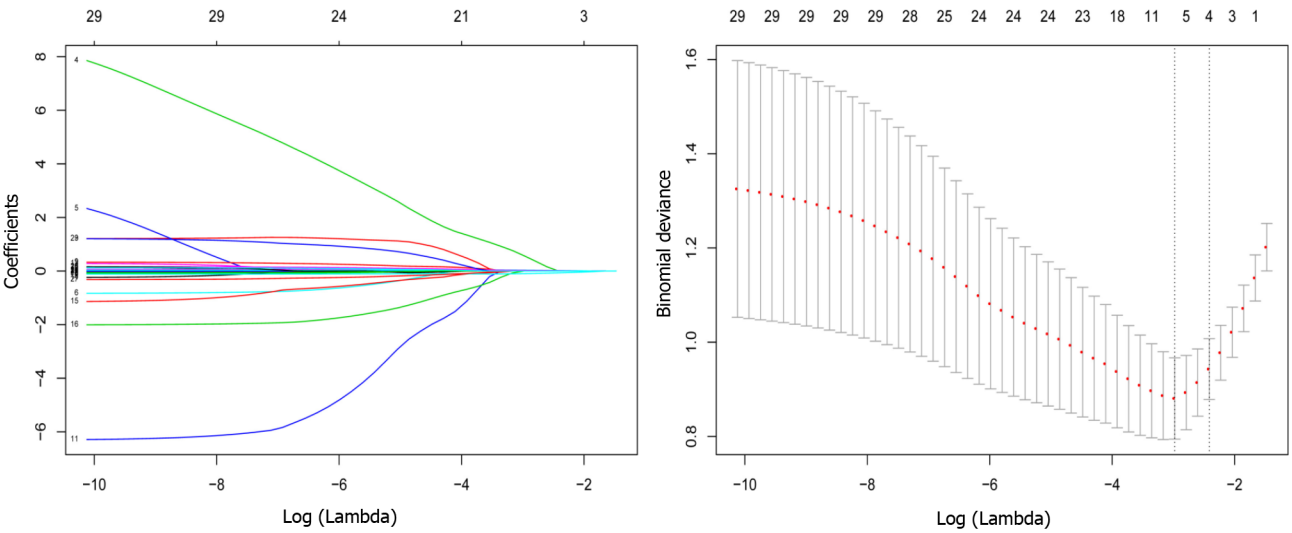


Figure 2 Selection of risk factors of moderately severe and severe acute pancreatitis in pregnancy using the least absolute shrinkage and selection operator logistic regression algorithm. Least absolute shrinkage and selection operator coefficient profiles of the 29 candidate variables. For the optimal lambda, 4 features with a non-0 coefficient were selected.

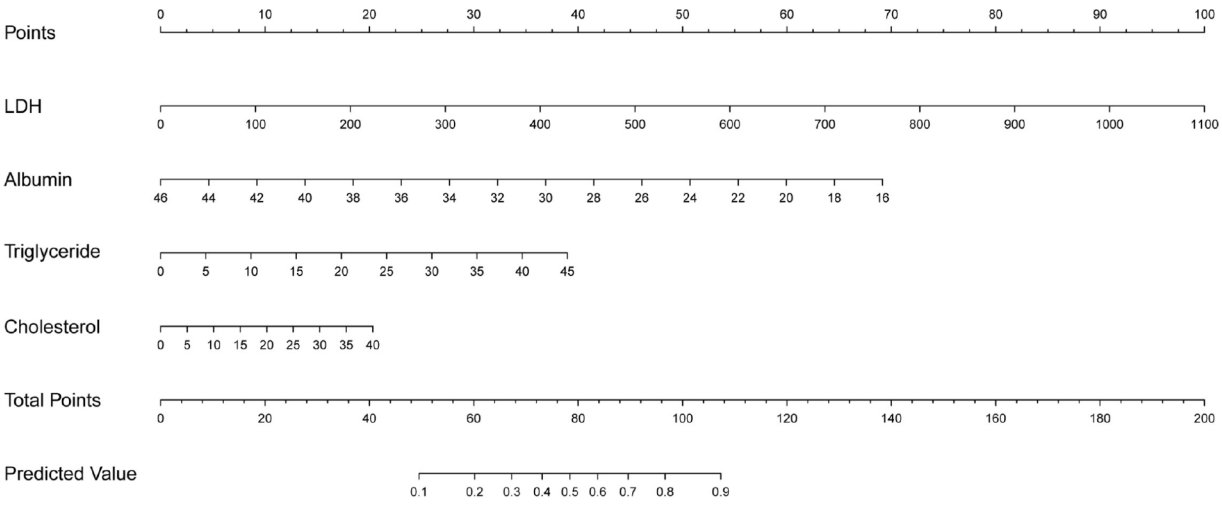


Figure 3 Nomogram for predicting moderately severe and severe acute pancreatitis in pregnancy. Nomogram including four risk factors (lactate dehydrogenase, triglyceride, cholesterol, and albumin were identified as risk factors) to predict moderately severe and severe acute pancreatitis in pregnancy. LDH: Lactate dehydrogenase.

variables are shown.

Candidate variables were age, etiology, comorbidity, smoking, drinking, gestational weeks on admission, trimester of pregnancy on admission, length of time from onset to admission, blood

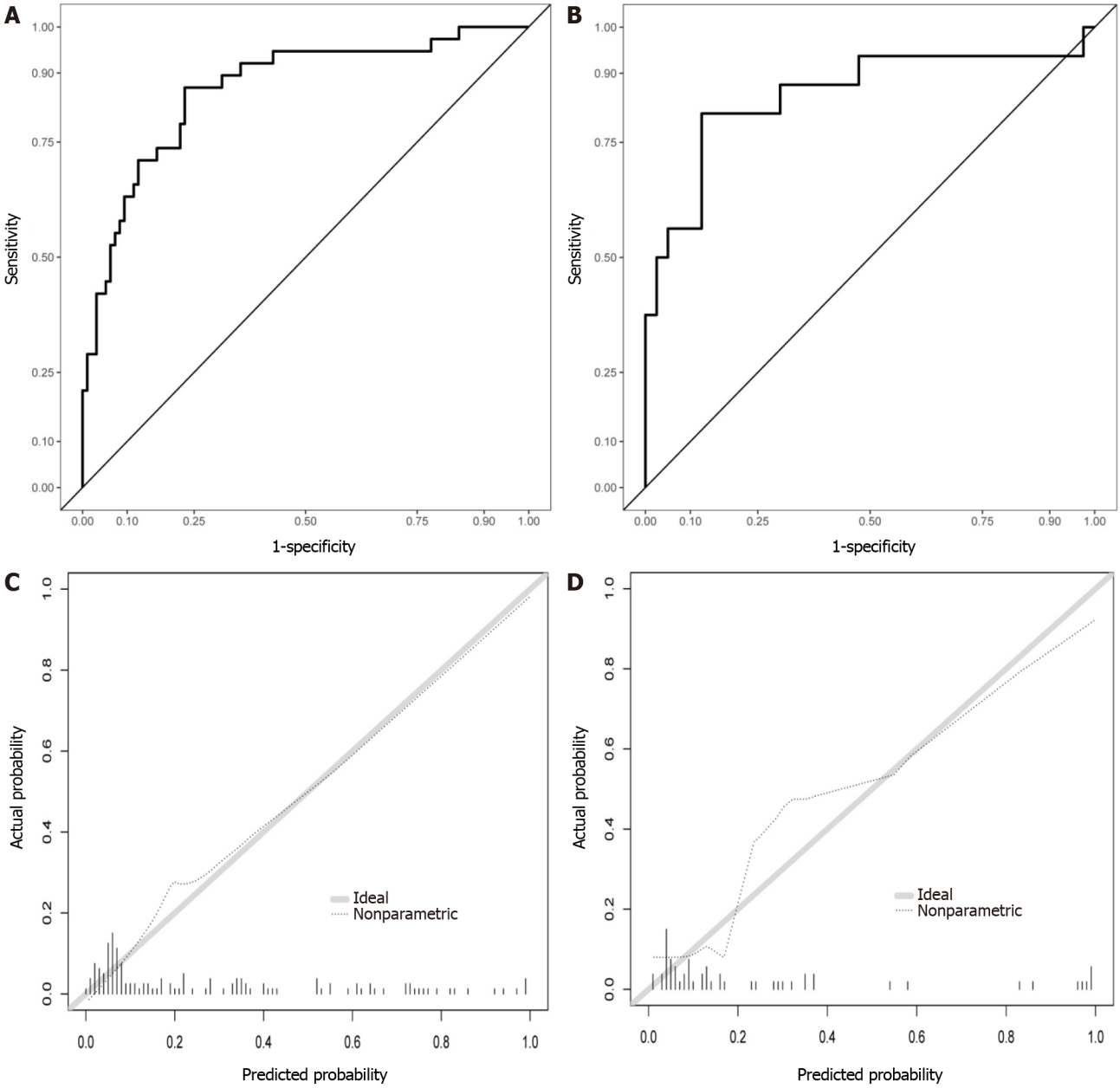


Figure 4 Performance of the nomogram in moderately severe and severe acute pancreatitis in pregnancy prediction. A: Receiver operating characteristic curves in the training set; B: Receiver operating characteristic curves in test set; C: Calibration curves of training set; D: Calibration curves of the test set.

infection, and hematocrit, platelet, WBC, neutrophil, lymphocyte, monocyte, alanine aminotransferase, albumin, creatinine, aspartate aminotransferase, alkaline phosphatase, creatine kinase, lactate dehydrogenase (LDH), triglyceride, cholesterol, high-density lipoprotein, low-density lipoprotein, sodium, potassium, and chlorine levels..

Definitions

According to the revised Atlanta Classification of Acute Pancreatitis[6], a diagnosis of acute pancreatitis requires two of the following three features: (1) abdominal pain consistent with acute pancreatitis (acute onset of a persistent, severe, epigastric pain often radiating to the back); (2) serum lipase activity (or amylase activity) at least three times greater than the upper limit of normal; and (3) characteristic findings of acute pancreatitis on contrast-enhanced computed tomography, and less commonly on magnetic resonance imaging or transabdominal ultrasonography. The grades of severity were also based on the revised Atlanta Classification of Acute Pancreatitis[6]. Patients with persistent organ failure (> 48 h) were classified as having severe acute pancreatitis. Patients with transient organ failure (< 48 h) and/or local or systemic complications without persistent organ failure were classified as having moderately severe acute pancreatitis. Organ failure was classified according to the Modified Marshall scoring system for organ dysfunction[6]. Patients who needed mechanical ventilation or had a

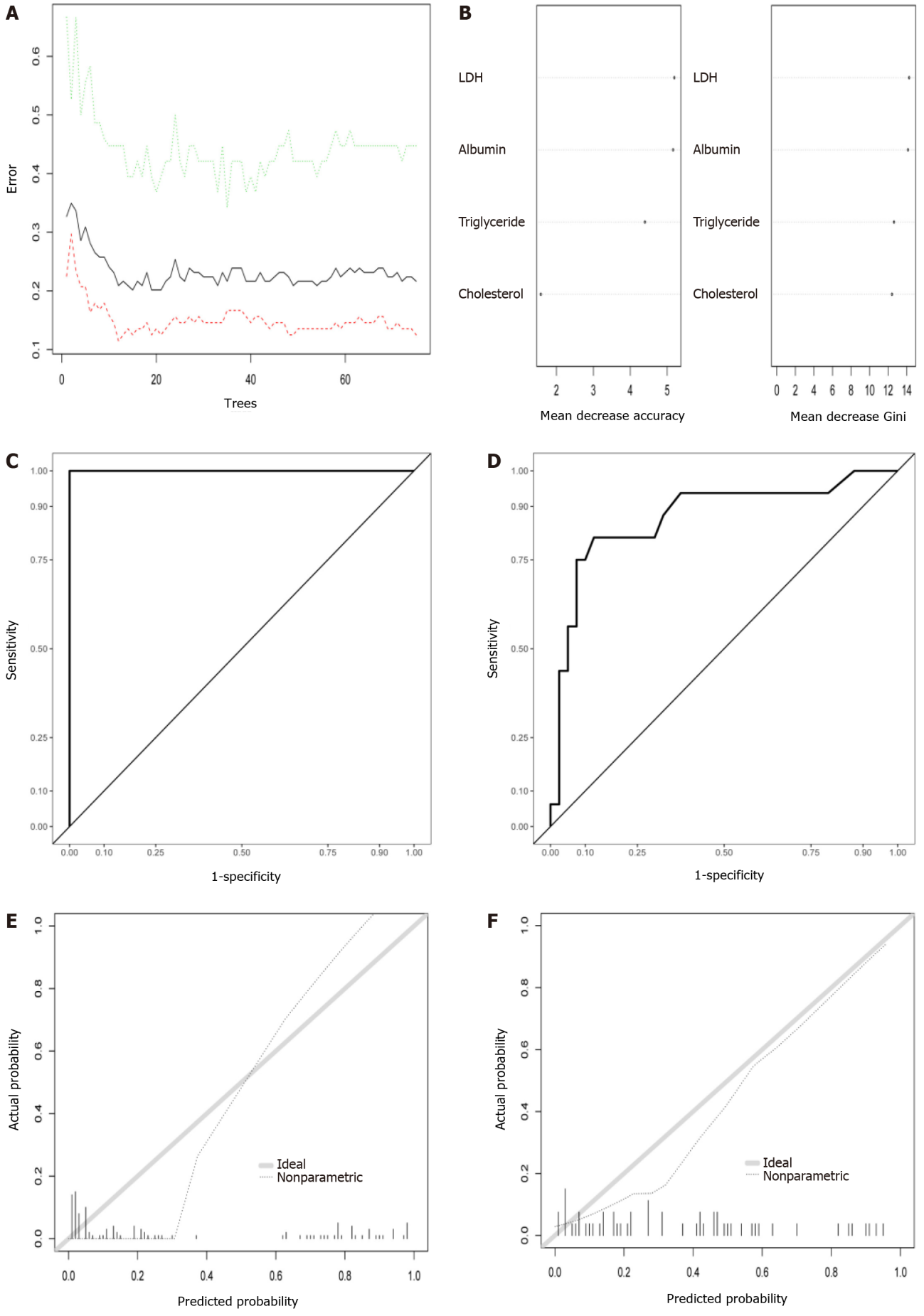


Figure 5 Development and assessment of the random forest algorithm in moderately severe and severe acute pancreatitis in pregnancy

prediction. A: Relationship between out-of-bag error and number of trees. In total, 75 trees are selected to establish a random forest model; B: Feature importance; C: Receiver operating characteristic curves in the training set; D: Receiver operating characteristic curves in test set; E: Calibration curves of training set; F: Calibration curves of the test set.

PaO_2/FiO_2 ratio less than 300 were diagnosed with respiratory failure. Patient need for vasopressor support was thought to indicate cardiovascular failure. When the serum creatinine level was over 170 μmol/L, renal failure was diagnosed. Blood infection was defined as described in a previous study[12].

Statistical analysis

Data are expressed as the mean ± SD for normally distributed continuous variables and as the median (interquartile range) for nonnormally distributed variables. Categorical data are expressed as numbers (percentages). Student's t-test was used to compare normally distributed continuous variables, and the Wilcoxon rank-sum test was used to compare nonnormally distributed continuous variables. The χ^2 -test or Fisher's exact test was used to compare categorical variables. Statistical analysis was performed using R software. (Version 3.6.1) A 2-sided P value < 0.05 was considered statistically significant.

First, least absolute shrinkage and selection operator (LASSO) regression was used to select potential prognostic factors from the candidate variables. Logistic regression was used to develop a nomogram. The random forest model further validated the predictive performance of the selected factors. To reduce the risk of overfitting, the whole dataset was randomly divided into the training set and validation set at a ratio of 7:3. The model's development was based on the training set, and the model's performance assessment was based on the validation set. Finally, a new nomogram based on the selected predictors was established. Receiver operating characteristic (ROC) curves and calibration curves were used to evaluate the model's predictive performance. ROC curves were calculated to estimate the discrimination of the prediction model. Calibration curves were plotted to evaluate the consistency between the predicted MSIP probability and actual MSIP proportion. Values of 1 and 0.5 indicated perfect discrimination and no discrimination, respectively.

RESULTS

Basic characteristics of the participants

Figure 1 shows the flow chart of the study. During the 7 years, 302 patients with APIP were admitted to West China Hospital. A total of 112 patients were excluded for various reasons, such as readmission, having a cesarean section before admission, and missing data. Finally, a total of 190 patients with APIP were included in this study. Among them, 134 patients (70.5%) were classified as having MAIP, and 56 patients (29.5%) were classified as having MSIP. The overall characteristics of the patients are presented in Table 1.

The mean ages of the MAIP and MSIP groups were 27.61 ± 5.25 years and 29.46 ± 5.57 years, respectively. Patients in the MSIP group were significantly older than those in the MAIP group (P = 0.032). The most common cause of APIP in both groups was hypertriglyceridemia. Biliary disease was the second most common cause of APIP, which was found in 45 (33.1%) and 19 (35.2%) patients in the MAIP and MSIP groups, respectively. The number of patients with diabetes in the MSIP group was significantly higher than that in the MAIP group (P = 0.001). The rate of blood infections (P < 0.001) in the MSIP group was significantly higher than that in the MAIP group. The LOS (P < 0.001) in the MSIP group was significantly longer than that in the MAIP group, and the rate of fetal deaths (P < 0.001) in the MSIP group was significantly higher than that in the MAIP group. Other clinical indicators were not different between the two groups.

Laboratory indices such as WBC (P = 0.035), neutrophil (P = 0.019), alanine aminotransferase (P = 0.006), albumin (P < 0.001), creatinine (P < 0.001), alkaline phosphatase (P = 0.020), creatine kinase (P < 0.001), LDH (P < 0.001), triglyceride (P < 0.001), cholesterol (P < 0.001), high density lipoprotein (P = 0.001), and sodium (P = 0.004) levels were significantly different between the two groups (P < 0.05).

Identification and validation of predictive factors for patients with MSIP

Variable selection using the LASSO regression model: The data were randomly divided into the training set and test set at a ratio of 7:3. The characteristics of the patients in the training and test sets are displayed in Table 2. Most of the included variables were well balanced between the two groups. Four variables (albumin, lactate dehydrogenase, triglyceride, and cholesterol levels) had nonzero coefficients in the LASSO regression model based on the analysis of the whole dataset (Figure 2).

Logistic regression development and validation prediction model: Four selected variables albumin, lactate dehydrogenase, triglyceride, and cholesterol levels, were incorporated into the nomogram model (Figure 3). The ROC curves and calibration curves of the training set and test set are shown in Figure 4. The parameters of the ROC curve at the optimal cutoff point are displayed in Table 3. The areas under

Table 1 Demographics and clinical characteristics of acute pancreatitis patients in pregnancy

Parameters	MAIP (*n* = 136)	MSIP (*n* = 54)	*P* value
Age	27.61 ± 5.25	29.46 ± 5.57	0.032
Etiology			0.514
Hypertriglyceridemia	50 (36.8)	24 (44.4)	
Gallstone	45 (33.1)	19 (35.2)	
Other	41 (30.1)	11 (20.4)	
Comorbidity			
Hypertension	0 (0.0)	2 (3.7)	0.080
Diabetes	8 (5.9)	13 (24.1)	0.001
Fatty liver disease	32 (23.5)	16 (29.6)	0.492
Smoking	3 (2.2)	2 (3.7)	0.937
Drinking	4 (2.9)	1 (1.9)	1.000
Trimester of pregnancy on admission			
Early (1–12 wk)	9 (6.6)	3(5.6)	
Mid (12–24 wk)	31 (22.8)	10(18.5)	
Late (24–40 wk)	96 (70.6)	41(75.9)	
Gestational weeks on admission	28.04 ± 7.72	28.80 ± 6.64	0.520
Onset to admission (days)	1.59 ± 1.37	1.88 ± 1.65	0.220
Blood infection	0 (0.0)	8 (14.8)	< 0.001
LOS	7.25 ± 4.27	11.88 ± 7.42	< 0.001
Fetal death	3 (2.2)	13(24.1)	< 0.001
Maternal hospital mortality	0 (0.0)	1(2.9)	0.284
Hematocrit	0.33 ± 0.05	0.32 ± 0.06	0.155
Platelet	164.42 ± 55.01	147.55 ± 65.17	0.072
WBC	12.59 ± 4.71	14.15 ± 4.15	0.035
Neutrophils	10.86 ± 4.40	12.49 ± 3.97	0.019
Lymphocytes	1.01 ± 0.40	0.88 ± 0.47	0.068
Monocytes	0.55 ± 0.23	0.48 ± 0.27	0.064
Alanine aminotransferase	50.94 ± 78.74	19.57 ± 37.40	0.006
Albumin	34.22 ± 3.70	29.36 ± 5.17	< 0.001
Creatinine	42.57 ± 9.30	75.87 ± 100.15	< 0.001
Aspartate aminotransferase	51.17 ± 67.61	35.07 ± 50.13	0.115
Alkaline phosphatase	113.56 ± 52.19	95.38 ± 36.87	0.020
Creatine kinase	36.20 ± 25.87	126.36 ± 213.49	< 0.001
LDH	185.32 ± 66.39	346.93 ± 208.95	< 0.001
Triglyceride	5.87 ± 6.72	12.57 ± 7.34	< 0.001
Cholesterol	7.34 ± 5.63	12.80 ± 6.64	< 0.001
High density lipoprotein	1.40 ± 0.48	1.16 ± 0.39	0.001
Low density lipoprotein	2.24 ± 1.23	1.94 ± 1.59	0.158
Sodium	135.62 ± 3.82	133.57 ± 5.43	0.004
Potassium	3.76 ± 0.34	3.83 ± 0.46	0.294
Chlorine	102.17 ± 4.46	102.44 ± 6.40	0.735

MAIP: Mild acute pancreatitis in pregnancy; MSIP: Moderately severe and severe acute pancreatitis in pregnancy; LOS: Length of hospital stay; WBC: White blood cell; LDH: Lactate dehydrogenase.

the curve in the training and validation sets were 0.865 and 0.853, respectively. The calibration curves showed that the nomogram has good consistency. The positive predictive value was 0.8750, and the negative predictive value was 0.8125.

Random forest model development and validation prediction model: The relationship between out-of-bag error and the number of trees is shown in Figure 5A. In total, 100 trees were selected to establish a random forest model. Two methods were used to rank the importance of the variables (Figure 5B). The ROC curves are shown in Figure 5C and D, and the optimal cutoff point is displayed in Table 3. In addition, the calibration curves indicated good agreement between the predicted probability and observed probability for MSIP in the training and test sets (Figure 5E and F).

DISCUSSION

APIP was thought to be associated with high rates of maternal death and fetal loss. The early and accurate prediction of APIP severity is of great importance for effective therapy. Previous studies have not only focused on the treatments of APIP[13,14] but have also shown interest in the prediction factors for APIP[15]. A single prediction factor cannot achieve the expected predictive power. Therefore, it is necessary to establish a multifactor model to predict the severity of APIP to help with risk stratification and management. In the present study, a new prediction model consisting of four risk factors (albumin, lactate dehydrogenase, triglyceride, and cholesterol levels) with good predictive value was built and verified.

Hypertriglyceridemia (HTG) induced APIP has received continuous attention[16-18]. HTG-induced AP is defined as AP patients with a triglyceride level above 1000 mg/dL (11.3 mmol/L) alone, or 500 mg/dL (5.65 mmol/L) accompanied by lipemic or lactescent blood, after excluding other etiologies[19]. In a recent study by Olesen *et al*[20], the mean incidence rate of HTG associated pancreatitis was 1.4 (95%CI, 1.1-1.7) per 100000 person-years and it has increased year by year. In addition, AP patients with severe HTG are not rare in Asia[21]. High-fat diets are common among pregnant women in China. In some studies, HTG was the second leading cause of AP in China[22,23]. In our study, HTG (38.9%) was the leading cause of APIP. A higher level of triglycerides not only contributes to more severe pancreatitis[21,24-26] but is also associated with more severe complications[27]. Thus, the detection of HTG is very important in APIP prediction.

As a cytoplasmic enzyme, LDH is widely expressed in tissues. It converts pyruvate to lactate when oxygen is in short supply[28]. In some disease conditions, such as tissue injury, hypoxia, or necrosis, elevated LDH levels are observed[29,30]. As a systemic inflammatory disease, AP can lead to organ dysfunction and pancreatic or peripancreatic necrosis when the disease progresses. Thus, LDH was recognized as a prognostic factor for severe AP in the 1992 Atlanta criteria[31]. More studies have shown that LDH is a useful predictor of AP severity[32,33]. Furthermore, LDH is used not only for the prediction of severity but also for the prediction of organ failure in AP patients[34]. A recent study displayed the high prediction ability of LDH in SAP prediction when levels were over 273.04 U/L[35]. In a study by Cui, an LDH level over 647 U/L showed a good ability to predict persistent organ failure in patients with AP[36]. In this study, LDH was the most important factor in the accuracy and Gini rank of the random forest model. Additionally, LDH accounted for the highest score in the final nomogram model. Moreover, convenient laboratory tests for LDH could be routinely utilized in the clinical setting.

Although hypercholesterolemia is a known risk factor for cardiovascular diseases, with further investigation of AP, the relationship between AP and hypercholesterolemia has been revealed. Hypercholesterolemia may lead to inflammatory responses, lysosomal damage, and proinflammatory cytokine secretion[37,38]. In particular, it promotes the augmentation of toll-like receptor signaling, which plays a significant proinflammatory role in the progression of AP[39]. Clinical studies also found a relationship between cholesterol and AP. Cholesterol is not only associated with AP occurrence[40] but is also thought to be an early predictor of persistent organ failure and mortality in AP patients[41, 42]. Some studies have produced inconsistent conclusions. Some reported that cholesterol was not identified as an independent risk factor for SAP[43,44]. However, cholesterol was thought to be a predictor of SAP development in the study by Hong *et al*[45]. Thus, it is unclear whether the relationship between AP severity and cholesterol is linear. A recent study suggested that cholesterol levels have a U-shaped association with AP severity[46]. This may explain the different conclusions in previous studies.

Some studies have shown that decreases in albumin levels predict the severity of AP[47,48]. An albumin level less than 30 g/L was an independent risk factor for acute respiratory distress syndrome in SAP patients[49]. In the present study, the albumin levels of patients in the MSIP group were less than

Table 2 Demographic and clinical characteristics of patients in training group

	Training set			Test set		
Parameters	**MAIP (96)**	**MSIP (38)**	***P* value**	**MAIP (40)**	**MSIP (16)**	***P* value**
Age	27.16 ± 5.46	30.13 ± 6.09	0.007	28.70 ± 4.60	27.88 ± 3.79	0.528
Etiology			0.620			0.804
Hypertriglyceridemia	32 (33.3)	18 (47.4)		18 (45.0)	7 (43.8)	
Gallstone	36 (37.5)	13 (34.2)		9 (22.5)	5 (31.2)	
Other	28 (29.2)	7 (18.4)		13 (32.5)	4 (25.0)	
Comorbidity						
Hypertension	0 (0.0)	2 (5.3)	0.079	0 (0.0)	0 (0.0)	-
Diabetes	6 (6.3)	9 (23.7)	0.012	2 (5.0)	4 (25.0)	0.049
Fatty liver disease	20 (20.8)	11 (28.9)	0.437	12 (30.0)	5 (31.2)	1.000
Smoking	1 (1.0)	2 (5.3)	0.400	2 (5.0)	0 (0.0)	0.909
Drinking	3 (3.1)	1 (2.6)	1.000	1 (2.5)	0 (0.0)	1.000
Trimester of pregnancy on admission						
Early (1–12 wk)	6 (6.3)	2 (5.3)		3 (7.5)	1 (6.3)	
Mid (12–24 wk)	22 (22.9)	8 (21.1)		9 (22.5)	2 (12.5)	
Late (24–40 wk)	68 (70.8)	28 (73.7)		28 (70.0)	13 (81.3)	
Gestational weeks on admission	27.53 ± 7.52	29.74 ± 6.34	0.113	29.25 ± 8.15	26.56 ± 7.01	0.252
Onset to admission (d)	1.63 ± 1.35	2.12 ± 1.87	0.090	1.50 ± 1.44	1.29 ± 0.63	0.586
Blood infection	0 (0.0)	6 (15.8)	< 0.001	0 (0.0)	2 (12.5)	0.139
LOS	6.99 ± 4.43	23.11 ± 48.52	< 0.001	7.90 ± 3.63	18.25 ± 12.96	0.001
Fetal death	1 (1.0)	9 (23.7)	< 0.001	2 (5.0)	4 (25.0)	0.049
Maternal hospital mortality	0 (0.0)	1 (2.6)	0.284	0 (0.0)	0 (0.0)	-
Hematocrit	0.33 ± 0.05	0.32 ± 0.06	0.734	0.33 ± 0.05	0.30 ± 0.05	0.040
Platelet	169.57 ± 56.91	141.97 ± 61.66	0.015	152.07 ± 48.61	160.80 ± 73.24	0.604
WBC	12.89 ± 4.82	13.94 ± 4.10	0.239	11.87 ± 4.42	14.64 ± 4.37	0.038
Neutrophils	11.19 ± 4.52	12.34 ± 3.83	0.166	10.09 ± 4.06	12.85 ± 4.39	0.029
Lymphocytes	1.00 ± 0.38	0.91 ± 0.49	0.246	1.01 ± 0.44	0.81 ± 0.41	0.129
Monocytes	0.55 ± 0.23	0.48 ± 0.29	0.147	0.56 ± 0.25	0.47 ± 0.22	0.249
Alanine aminotransferase	55.20 ± 82.18	22.91 ± 44.14	0.024	40.73 ± 69.69	11.62 ± 6.40	0.103
Albumin	34.44 ± 3.72	29.68 ± 5.49	< 0.001	33.71 ± 3.62	28.62 ± 4.41	< 0.001
Creatinine	42.90 ± 9.16	86.27 ± 117.45	< 0.001	41.78 ± 9.69	51.17 ± 22.10	0.030
Aspartate aminotransferase	54.45 ± 69.25	40.67 ± 58.67	0.281	43.33 ± 63.66	21.77 ± 10.97	0.186
Alkaline phosphatase	114.05 ± 51.61	100.65 ± 37.29	0.148	112.39 ± 54.23	82.86 ± 33.70	0.048
creatine kinase	36.46 ± 26.55	129.89 ± 242.89	< 0.001	35.58 ± 24.47	117.98 ± 124.10	< 0.001
LDH	183.85 ± 63.85	356.97 ± 234.19	< 0.001	188.86 ± 72.86	323.09 ± 134.59	< 0.001
Triglyceride	5.44 ± 6.86	12.62 ± 8.01	< 0.001	6.91 ± 6.33	12.46 ± 5.66	0.004
cholesterol	6.79 ± 4.43	12.19 ± 6.18	< 0.001	8.68 ± 7.70	14.24 ± 7.63	0.018
High density lipoprotein	1.42 ± 0.49	1.18 ± 0.39	0.007	1.34 ± 0.43	1.12 ± 0.40	0.078
Low density lipoprotein	2.27 ± 1.18	2.00 ± 1.58	0.282	2.17 ± 1.35	1.78 ± 1.67	0.364
Sodium	135.91 ± 3.19	133.94 ± 5.71	0.012	134.93 ± 5.01	132.68 ± 4.72	0.129
Potassium	3.76 ± 0.30	3.88 ± 0.47	0.075	3.78 ± 0.44	3.71 ± 0.45	0.588

Chlorine	102.63 ± 4.22	103.14 ± 6.80	0.597	101.06 ± 4.85	100.78 ± 5.14	0.849

MAIP: Mild acute pancreatitis in pregnancy; MSIP: Moderately severe and severe acute pancreatitis in pregnancy; LOS: Length of hospital stay; WBC: White blood cell; LDH: Lactate dehydrogenase.

Table 3 Receiver operating characteristic curves at the optimal cut-off point according to different models

Models	AUC	Sensitivity	Specificity
Training set			
Logistic model	0.865	0.868	0.771
Random forest model	1.000	1.000	1.000
Validation set			
Logistic model	0.853	0.812	0.875
Random forest model	0.870	0.812	0.875

AUC: Area under the receiver operating characteristic curve.

30 g/L and significantly lower than those of patients in the MAIP group. This was in accordance with previous studies.

Lactate dehydrogenase, triglyceride, albumin, and cholesterol are routine test items in clinical practice. They can be easily detected from blood samples at a low cost. Therefore, this nomogram will be easy to use and function for MSIP prediction in the clinical setting.

There are some limitations to this study. First, the sample size of 190 patients with APIP was greater than those of most previous studies, but the sample size of this study was still small. Second, this was a retrospective study, so some data were missing. Thus, some variables were not included in this study. Third, the prediction model has a good prediction ability of MSIP (consisting of MSAP and SAP), but further differentiation of MSAP and SAP cannot be achieved. The prognosis of MSAP is not as poor as that of SAP. Thus, separate predictions of MSAP and SAP should be considered in future studies. Moreover, this study only collected data from our institution. If validation can be performed in external institutions, the conclusion of this study would be more substantial.

CONCLUSION

We developed and validated a nomogram with good accordance for the prediction of MSIP. Incorporating blood indices for albumin, lactate dehydrogenase, triglyceride, and cholesterol levels into the nomogram facilitates the early individualized prediction of APIP severity.

ARTICLE HIGHLIGHTS

Research background

The severity of acute pancreatitis in pregnancy is correlated with higher risks of maternal and fetal death.

Research motivation

There is a lack of a scoring model for predicting the moderately severe and severe acute pancreatitis in pregnancy (MSIP).

Research objectives

We aimed to develop a prediction model for moderately severe and severe acute pancreatitis in pregnancy.

Research methods

The training set and test set were randomly divided at a ratio of 7:3. Least absolute shrinkage and selection operator regression was used to select potential prognostic factors. A nomogram was developed by logistic regression. A random forest model was used to validate the stability of the of

prediction factors. Receiver operating characteristic curves and calibration curves were used to evaluate the model's predictive performance.

Research results

A total of 190 patients were included in this study. Four predictors including lactate dehydrogenase, triglyceride, cholesterol, and albumin levels constitute the prediction model. The model had areas under the curve of 0.865 and 0.853 in the training and validation sets, respectively. The calibration curves showed that the prediction model has a good consistency.

Research conclusions

An effective prediction model that can predict MSIP was constructed.

Research perspectives

Our model could help to predict moderately severe and severe acute pancreatitis in pregnancy. Usability of the model needs validation by other center data.

FOOTNOTES

Author contributions: Yang DJ and Zhou ZG conception and design; Lu HM, Liu Y, Li M, and WH collection data; Yang DJ, Lu HM, and Zhou ZG analysis data; Yang DJ write the manuscript; Hu WM and Zhou ZG revised the manuscript.

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Country/Territory of origin: China

ORCID number: Du-Jiang Yang 0000-0002-0597-1143; Hui-Min Lu 0000-0002-5759-1919; Yong Liu 0000-0003-2558-7015; Mao Li 0000-0003-1728-9026; Wei-Ming Hu 0000-0003-1605-5084; Zong-Guang Zhou 0000-0002-7616-1199.

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LETTER TO THE EDITOR

Role of magnifying narrow-band imaging endoscopy for diagnosis of *Helicobacter pylori* infection and gastric precancerous conditions: Few issues

Saroj Kanta Sahu, Ayaskanta Singh

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Saroj Kanta Sahu, Department of Gastroenterology, Sum Ultimate Medicare, Bhubaneswar, Odisha, India 751003, Odisha, India

Ayaskanta Singh, Department of Gastroenterology, IMS and SUM Hospital, SOA University, Bhubaneswar 751003, Odisha, India

Corresponding author: Ayaskanta Singh, MBBS, MD, Professor, Department of Gastroenterology, IMS and SUM Hospital, SOA University, Kalinga Nagar, Bhubaneswar 751003, Odisha, India. ayaskant1ce@gmail.com

Abstract

Standard endoscopy with biopsy and narrow-band imaging with guided biopsy are techniques for the detection of *Helicobacter pylori (H. pylori)*-related gastritis and precancerous lesions. In this study, the authors compared standard endoscopy and magnified narrow-band imaging (commonly known as NBI-M) in the diagnosis of *H. pylori* infections, atrophic gastritis, and intestinal metaplasia. Although the sensitivity of NBI-M is better than standard endoscopy, the diagnostic accuracy did not differ substantially between the diagnostic modalities. Future prospective studies may guide endoscopists in difficult cases regarding which modality is more useful and cost-effective for the diagnosis of *H. pylori*-related gastritis and precancerous conditions.

Key Words: Standard endoscopy; Magnified narrow band imaging; *Helicobacter pylori*; Atrophic gastritis; Intestinal metaplasia; Pepsinogen

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Core Tip: Magnified narrow band imaging (NBI-M) is used for diagnosis of dysplastic and cancerous lesions. The study is the first of its kind to evaluate this modality for detection of *Helicobacter pylori* related gastritis and precancerous lesions. A procedure to be widely accepted should be cost effective and less time consuming. Whereas white light endoscopy is commonly used by endoscopist to detect any cancer or precancerous lesions, formal endoscopic training regarding use of NBI-M enhances feasibility and detection rate. Whether the combination of NBI-M and artificial intelligence can replace biopsy remains a million dollar question.

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TO THE EDITOR

We read with avid interest the study by Cho *et al*[1]. The authors compared standard endoscopy and magnified narrow-band imaging (NBI-M) in the diagnosis of *Helicobacter pylori* (*H. pylori*) infections, atrophic gastritis (AG) and intestinal metaplasia (IM). The authors have done excellent work comparing the role of NBI-M and standard endoscopy in the diagnosis of these entities.

Although several studies show the benefits of NBI-M in the diagnosis and characterization of AG and IM, this is perhaps one of the first studies that evaluated the role of NBI-M in *H. pylori* infections[2,3]. We want to raise a few minor points for discussion.

The financial implications and procedure time of NBI-M in relation to standard endoscopy should have been compared. The authors discussed that routine normal white light endoscopy combined with mucosal biopsies is time-consuming and costly. However, we feel that NBI-M evaluation for *H. pylori* infections, IM, and AG may increase procedure time (especially for an inexperienced endoscopist) and have an additional economic impact on the patients. The present study showed that there is no substantial disparity between standard endoscopy and NBI-M with respect to the diagnostic accuracy for detection of *H. pylori* gastritis, severe atrophy, and IM. The economic and time aspect will decide the utility of this procedure. Hence, future prospective studies should focus on this. Moreover, diagnostic accuracy was demonstrably low for the detection of precancerous lesions (72.6% and 61.1% for severe atrophy and IM in the corpus, respectively) even with NBI-M.

The experience of the endoscopist plays a significant role when the question of a definitive diagnosis of *H. pylori* gastritis and precancerous lesions using NBI arises. The diagnostic probability of AG and IM increases when the endoscopist has formal training regarding the diagnosis of AG and IM[4]. The authors have rightly pointed out this limitation in their study that a single experienced endoscopist was used. Artificial intelligence with NBI-M will likely increase the diagnostic yield. However, future robust prospective studies are required to confirm this hypothesis.

FOOTNOTES

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Country/Territory of origin: India

ORCID number: Saroj Kanta Sahu 0000-0001-9091-1657; Ayaskanta Singh 0000-0003-0616-8257.

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LETTER TO THE EDITOR

Therapeutic drug monitoring in inflammatory bowel disease treatments

Meng-Yao Wang, Jing-Wen Zhao, Chang-Qing Zheng, Li-Xuan Sang

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Meng-Yao Wang, Jing-Wen Zhao, Chang-Qing Zheng, Li-Xuan Sang, Department of Gastroenterology, Shengjing Hospital of China Medical University, Shenyang 110022, Liaoning Province, China

Corresponding author: Li-Xuan Sang, MD, PhD, Associate Professor, Department of Gastroenterology, Shengjing Hospital of China Medical University, No. 39 Gliding Road, Tiexi District, Shenyang 110022, Liaoning Province, China. sanglixuan2008@163.com

Abstract

Recently, biological drugs have played a leading role in the treatment of inflammatory bowel disease, and therapeutic drug monitoring (TDM) may be useful in maximizing their effectiveness. TDM involves the measurement of serum drug and anti-drug antibodies concentrations as the basis for dosage adjustments or drug conversions to achieve a higher response rate. We believe that concentration thresholds should be individualized based on patients' disease severity, extent and phenotype, and therapeutic purposes should also be considered, with higher cut-offs mainly needed for endoscopic and fistula healing than for symptomatic remission. Proactive and reactive TDM can help optimize treatment, especially in patients receiving anti-tumour necrosis factor, and guide dose adjustment or drug conversion with lower cost. TDM is a promising approach to achieve precision medicine and targeted medicine in the future.

Key Words: Therapeutic drug monitoring; Inflammatory bowel disease; Biologic therapies; Reactive; Proactive; Cost-effective

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Core Tip: Therapeutic drug monitoring (TDM) has proven to be useful in the management of patients with inflammatory bowel disease (IBD). The therapeutic value, feasibility and application prospect of TDM in the treatment of IBD were discussed.

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TO THE EDITOR

We read with interest the review by Albader *et al*[1] on the application of therapeutic drug monitoring (TDM) in patients with inflammatory bowel disease (IBD). The authors provided a comprehensive overview of the relationship between proactive or reactive TDM and clinical outcomes.

The treatment of IBD has progressed from the original mesalamine to glucocorticoids and immunosuppressants to biologics. Currently, biologic therapy is required by many patients to achieve and maintain clinical and endoscopic remission. However, up to one-third of patients receiving this treatment are primary non-responders, and some patients who show an initial response can also lose response over time[2]. TDM is a useful tool for managing patients on biologic therapy, especially those receiving anti-tumor necrosis factor (anti-TNF) therapy, and it can be used to monitor dose escalation, de-escalation or drug conversion by measuring serum drug concentrations and anti-drug antibodies (ADAs).

However, there does not seem to be a universal optimal cut-off for drug serum concentrations, and the majority of studies have shown that higher serum concentrations are associated with an increased likelihood of clinical response. In 2021, a prospective study of 32 pediatric patients demonstrated that children who achieved endoscopic remission at six months had significantly higher infliximab (IFX) concentrations at different time points during induction (at weeks 4, 6, and 12 as the start of maintenance therapy), and the IFX concentration ≥ 5.0 μg/mL at week 12 was a minimal target to achieve endoscopic remission at six months (area under the receiver operating characteristic curve: 0.796)[3]. A retrospective observational case–control study found that IFX levels below 6.8 μg/mL and antibodies to IFX levels above 4.3 μg/mL before the second infusion were associated with primary nonresponse, especially among patients with Crohn's disease (CD)[4]. A prospective observational study by Kennedy *et al*[5]showed that in multivariable analysis, the only factor independently associated with primary nonresponse was low drug concentration at week 14 [IFX: OR 0.35, $P = 0.00038$; adalimumab (ADA): OR 0.13, $P < 0.0001$], the optimal week 14 drug concentrations associated with remission at both week 14 and week 54 were 7 mg/L for IFX and 12 mg/L for ADA. Importantly, we believe that concentration thresholds should be individualized based on patients' disease severity, extent and phenotype, and therapeutic purposes should also be considered, with higher cut-offs mainly needed for endoscopic and fistula healing than for symptomatic remission.

TDM has not been widely recommended for non-anti-TNF biologicals. A study published in 2018 concluded that potential target vedolizumab (VDZ) concentrations at weeks 6 and 14 and steady state during treatment were proposed to be > 37.1, > 18.4 and > 12.7 μg/mL, respectively[6]. There are no definitive conclusions to guide practitioners regarding the target VDZ concentration for achieving endoscopic remission. A review published in 2020 noted that data from registration trials and real-world cohorts suggested an exposure-efficacy relationship of VDZ in patients with IBD, but future studies need to define an upper limit beyond which dose optimization is very unlikely to further increase response rates[7]. Ustekinumab (UST) is a monoclonal antibody against IgG that affects the immunity of IBD patients by binding to the P40 subunit common to interleukin 12 and interleukin 23. According to a review published in 2021, serum UST concentrations are associated with clinical, biochemical, and histological remissions in most clinical trials[8]. A multicenter crosssectional observational trial based on 110 CD patients concluded that there was no association between short-term clinical outcomes and UST concentrations[9]. We can assume that there is an exposure-efficacy association with UST based on current studies. Further study is required to identify the threshold below which dose optimization may be useful. The Janus kinase inhibitor tofacitinib is not impacted by enzyme polymorphisms or disease activity and is not expected to stimulate the formation of neutralizing ADAs. In addition, the drug concentration is not a meaningful determinant of efficacy, and no loss of efficacy due to low plasma concentration was identified in clinical trials; therefore, TDM is unlikely to be provided during treatment with tofacitinib, according to a review published in 2021[10].

TDM can be implemented in two forms, "Proactive" TDM refers to routine monitoring of serum concentrations, whereas "Reactive" TDM is defined as a measurement taken following treatment failure. This allows doctors to then choose to adjust the dose or change to another drug. Cost-effectiveness is an important factor in the choice of proactive and reactive TDM. According to Assa *et al*[11], proactive monitoring and ADA dose intensification to serum concentrations > 5 μg/mL resulted in a higher rate of clinical remission than reactive monitoring in cases of "loss of response". Fernandes *et al*[12] and Papamichael *et al*[13] concluded that patients in the proactive TDM group had greater clinical outcomes than those in the control group. However, guidelines or consensus in different countries and regions differ on the application of TDM. Guidelines published in 2020 by the European Colitis & Crohn's Organization pointed out that there is insufficient evidence to recommend for or against TDM [14]. The 2017 guidelines of the American Gastroenterology Association only recommend reactive TDM [15]. McNeill *et al*[16] found that reactive TDM of IFX optimizes dosing and reduces expenditure by over 50%, without affecting clinical outcomes. Proactive IFX TDM may confer long-term clinical benefit but is only modestly cost-effective. A systematic review published in 2020 noted that compared with standard treatment without TDM, TDM-guided strategies were consistently reported to be cost saving or cost effective, with no emphasis on proactive or reactive TDM[17]. There are no high-quality studies comparing the cost-effectiveness of proactive and reactive TDM; however, TDM is cost-effective

compared to empirical treatment. The problems to be solved in the implementation of TDM include high price, delivery and transportation difficulties. A survey of over 242 participants in India suggested that significant barriers to TDM use were availability, cost and time lag for results. If these barriers were removed, almost all clinicians would use TDM at least reactively, and 25% would use it proactively[18].

It should be emphasized that the therapeutic goal is to achieve clinical and endoscopic remission and not to target TDM to specific drug concentration levels. Different guidelines recommend different trough concentrations, different departments have different measurement methods, and individuals have different systems, so even if the same threshold is reached, some people will respond and some will not; thus, we recommend dynamic detection of blood drug concentrations. The future medical trend is precision medicine and targeted medicine. We hope that in the future, there will be a tool as convenient and fast as a glucose meter that can measure blood drug concentrations, perform real-time monitoring, combine clinical symptoms and endoscopic manifestations, and then adjust drugs to achieve targeted treatment. We wish to draw readers' attention to the fact that TDM is a promising approach for clinicians to optimize treatment.

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Country/Territory of origin: China

ORCID number: Meng-Yao Wang 0000-0002-6580-9324; Jing-Wen Zhao 0000-0002-1587-2501; Chang-Qing Zheng 0000-0003-4782-5715; Li-Xuan Sang 0000-0002-4562-0022.

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THERAPEUTIC AND DIAGNOSTIC GUIDELINES

Guidelines to diagnose and treat peri-levator high-5 anal fistulas: Supralevator, suprasphincteric, extrasphincteric, high outersphincteric, and high intrarectal fistulas

Pankaj Garg, Vipul D Yagnik, Sushil Dawka, Baljit Kaur, Geetha R Menon

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Pankaj Garg, Department of Colorectal Surgery, Garg Fistula Research Institute,Panchkula 134113, Haryana, India

Pankaj Garg, Department of Colorectal Surgery, Indus International Hospital,Mohali 140201, Punjab, India

Vipul D Yagnik, Department of Surgical Gastroenterology, Nishtha Surgical Hospital and Research Center, Patan 384265, Gujarat, India

Sushil Dawka, Department of Surgery, SSR Medical College, Belle Rive 744101,Mauritius

Baljit Kaur, Department of Radiology, SSRD Magnetic Resonance Imaging Institute, Chandigarh 160011, India

Geetha R Menon, Department of Statistics, Indian Council of Medical Research,New Delhi 110029, India

Corresponding author: Pankaj Garg, MD, MS, Chief Surgeon, Department of Colorectal Surgery, Garg Fistula Research Institute, 1042/15, Panchkula 134113, Haryana, India.
drgargpankaj@yahoo.com

Abstract

Supralevator, suprasphincteric, extrasphincteric, and high intrarectal fistulas (high fistulas in muscle layers of the rectal wall) are well-known high anal fistulas which are considered the most complex and extremely challenging fistulas to manage. Magnetic resonance imaging has brought more clarity to the patho-physiology of these fistulas. Along with these fistulas, a new type of complex fistula in high outersphincteric space, a fistula at the roof of ischiorectal fossa inside the levator ani muscle (RIFIL), has been described. The diagnosis, management, and prognosis of RIFIL fistulas is reported to be even worse than supralevator and suprasphincteric fistulas. There is a lot of confusion regarding the anatomy, diagnosis, and management of these five types of fistulas. The main reason for this is the paucity of literature about these fistulas. The common feature of all these fistulas is their complete involvement of the external anal sphincter. Therefore, fistulotomy, the simplest and most commonly performed procedure, is practically ruled out in these fistulas and a sphincter-saving procedure needs to be performed. Recent advances have provided new insights into the anatomy,

radiological modalities, diagnosis, and management of these five types of high fistulas. These have been discussed and guidelines formulated for the diagnosis and treatment of these fistulas for the first time in this paper.

Key Words: Anal fistula; Supralevator; Suprasphincteric; Extrasphincteric; Magnetic resonance imaging

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Core Tip: These are the first published guidelines to manage the five types of peri-levator anal fistulas that involve almost the complete external anal sphincter and have, therefore, been grouped together as high-5 fistulas. These are supralevator, suprasphincteric, extrasphincteric, and high intrarectal and fistulas at the roof of ischiorectal fossa inside the levator ani muscle. The diagnosis and management of these five fistulas is quite challenging. Magnetic resonance imaging is the best modality to accurately delineate these fistulas. Once diagnosed, care should be exercised to avoid sphincter-cutting procedures (fistulotomy) in these fistulas, as the risk of incontinence would be very high. Sphincter-sparing procedures should be done. However, there is little literature available on satisfactory management of these fistulas.

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INTRODUCTION

Anal fistulas are feared by patients and surgeons alike[1]. The prime reasons for this are difficulty in understanding the pathophysiology, risk of debilitating incontinence, and high recurrence rates, especially in complex fistulas[2-5]. Amongst the category of complex fistulas, the three well-known notorious fistulas are supralevator, suprasphincteric and extrasphincteric fistulas[6,7]. Another less known fistula is the high intra-rectal fistula which occurs by cephalad extension of the fistula between the internal anal sphincter (IAS) and conjoint longitudinal muscle (CLM). This fistula was first described by Parks *et al*[8] in 1976. Recently, another fistula, fistula at the roof of ischiorectal fossa inside the levator ani muscle (RIFIL) has also joined the category of these high fistulas[9]. RIFIL fistulas are also highly complex and involve the complete external anal sphincter (EAS). The common feature of these five fistulas is that they are quite high as these reach up to the levator muscle and involve almost the complete EAS (Figure 1). Hence, these five fistulas have been grouped together as high-5 fistulas and are discussed in detail below.

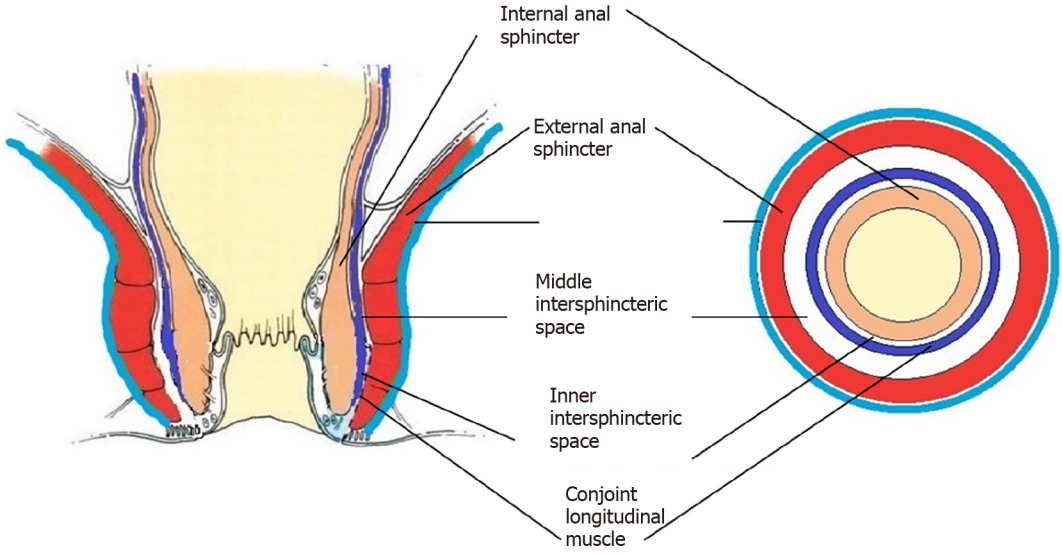
The high-five anal fistulas include: (1) Supralevator (Figures 2 and 3); (2) Suprasphincteric (Figures 4-6); (3) Extrasphincteric (Figure 7); (4) High intra-rectal (Figures 3,8 and 9)[8]; and (5) RIFIL (Figures 10-12)[9]. These fistulas are considered most dreaded because understanding their anatomy and pathophysiology, proper diagnosis, and successful safe treatment are quite challenging. All these five fistulas involve almost the complete EAS[10,11] (Figures 2-12). As the EAS plays a major role in continence, muscle-dividing procedures like fistulotomy or cutting setons are strictly contraindicated in these fistulas. In fact, fistulotomy with primary sphincter reconstruction or fistulectomy with primary sphincter reconstruction are also best avoided in these patients[1,12-14]. Therefore, the choice of procedures becomes limited. Of these, the extrasphincteric is the only type which is trans-levator, whereas the other four fistulas (supralevator, sphincteric, high intrarectal, and RIFIL) are peri-levator fistulas which do not traverse through the levator muscle.

This article will address the major concerns of these fistulas, including the challenges in managing and diagnosing the high-5 fistulas, as well as methods to formulate management guidelines..

CHALLENGES IN MANAGING THESE FISTULAS

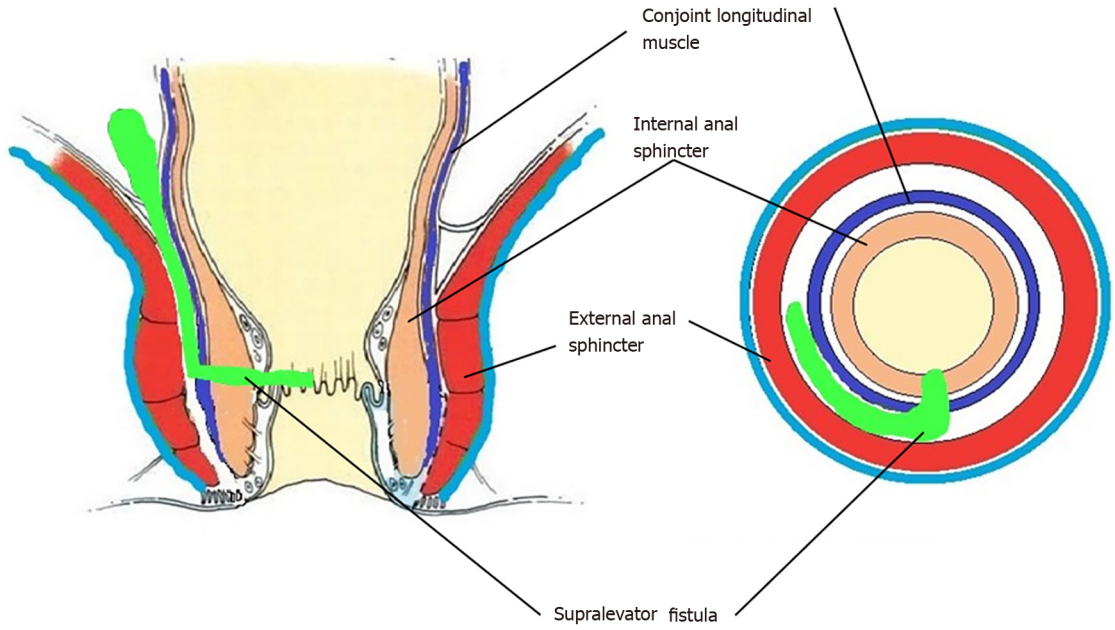
Confusion about pathophysiology of High-5 fistulas

There is a lot of confusion amongst surgeons about the exact anatomy, pathways of spread, and extent of these fistulas[2]. Not uncommonly, a high transsphincteric fistula is labelled as an extrasphincteric fistula and a suprasphincteric fistula is reported as a high transsphincteric fistula[2].



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Figure 1 Normal anatomy.



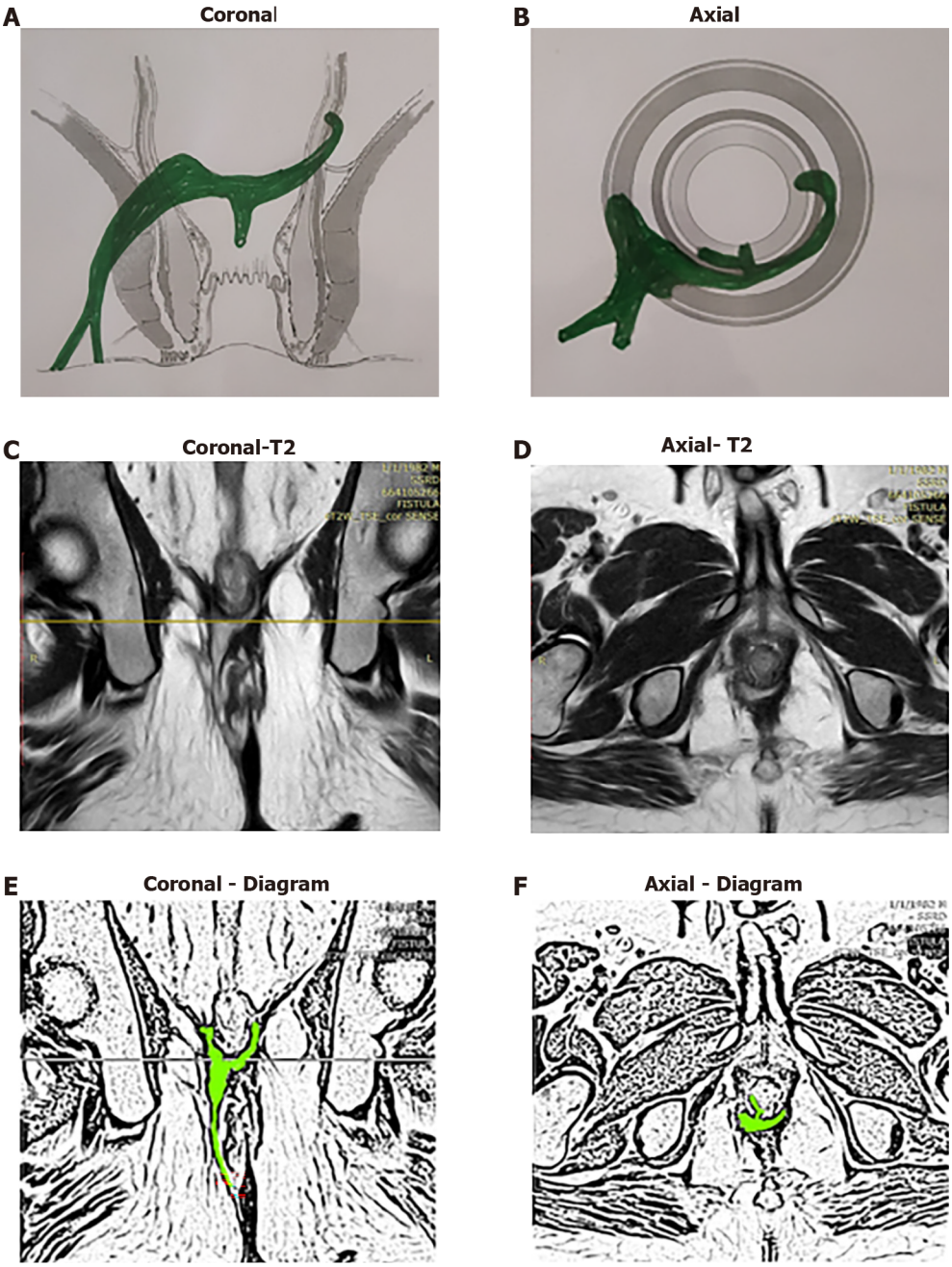
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Figure 2 Supralevator fistula.

In order to understand the pathways of extension of these fistulas, it is important to understand the anatomy of the anal canal. There are three muscle layers in the sphincter-complex in the anorectum which are downward extensions of the muscle layers of the gut. There are potential spaces between these muscle layers in which the fistula and pus can spread[9] (Figures 1, 2, 4, 7, 8 and 10).

The three muscle layers in the sphincter-complex[9] are (1) Inner circular muscle layer of gut continues downwards in the anal sphincter complex as IAS; (2) Outer longitudinal muscle layer of gut continues downwards in the anal sphincter complex as CLM; and (3) Puborectalis component of levator ani muscle continues downwards in the anal sphincter complex as EAS (Figure 1). Conventionally, it was assumed that inside the sphincter-complex, there is only one space where the fistula extends and this space between the IAS and EAS was labelled as the 'intersphincteric space'. However, with the availability of higher resolution magnetic resonance imaging (MRI) and increasing experience, it has been demonstrated that there are three potential spaces associated with in these muscle layers through which the fistula can extend[9].

The three potential spaces in the anal sphincter-complex are (1) Inner intersphincteric space (inner space): Between IAS and CLM; (2) Middle intersphincteric space (middle space): Between CLM and EAS



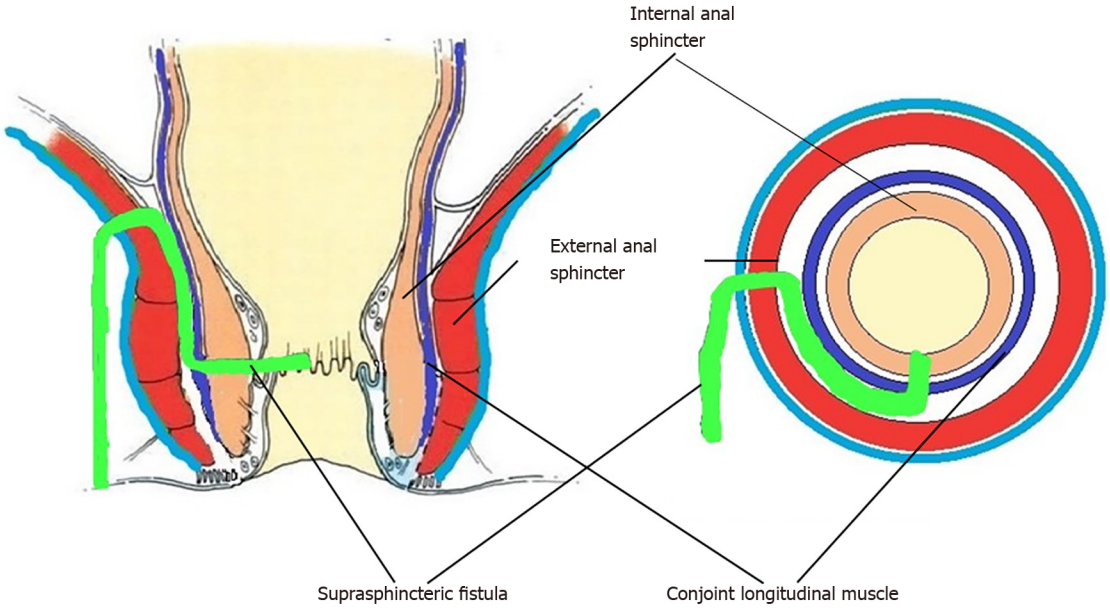
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Figure 3 A 39-year-old male patient with a high transsphincteric fistula with supralevator extension at 3 o'clock. There is a high intrarectal tract from 6 to 7 o'clock. The internal opening is at 6 o'clock position. A: Coronal section (schematic diagram); B: Axial section (schematic diagram); C: T2-weighted magnetic resonance imaging (MRI) Coronal section showing supralevator tract; D: T2-weighted MRI Axial section; E: Sketch of MRI Coronal section highlighting fistula tract (light green color); F: Sketch of MRI Axial section highlighting fistula tract (light green color).

(conventional intersphincteric space); and (3) Outer-sphincteric space (outer space): Between the lateral muscle fibers of EAS and its covering fascia[9] (Figure 1).

The anal fistula, usually initiating at crypt glands at the level of the dentate line, can extend superiorly (cephalad) in any of these three spaces.

The fistulas traversing superiorly in (1) Inner intersphincteric space (inner space) between IAS and CLM inside the rectal wall to become a high intrarectal fistula as these fistulas are present between the muscle layers of the rectal wall[8]. At times, these are erroneously labelled as submucous fistula[8] (Figures 8 and 9); (2) Middle intersphincteric space (middle space) between CLM and EAS become a supralevator fistula (if it continues into the supralevator space) (Figures 2 and 3) or a suprasphincteric fistula (if pus extending superiorly in the middle space reaches up to the top of the EAS and then traverses through the junction between the EAS and puborectalis to enter the ischiorectal fossa[15]) (Figures 4-6); and (3) Outer-sphincteric space (outer space) between the EAS and its lateral fascia becomes a RIFIL fistula[9] (Figures 10-12).



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Figure 4 Suprasphincteric fistula.

Thus, the definition of these five fistulas is: (1) Supralevator (Figures 2 and 3): These fistulas traverse in the middle intersphincteric space to enter the supralevator space. The fistula may open into the rectum through an additional high rectal opening in the supralevator space (Figures 3 and 6); (2) Suprasphincteric (Figures 4-6): These fistulas extend superiorly in the middle intersphincteric space to reach up to the top of the EAS and then traverses through the junction between the EAS and puborectalis to enter the ischiorectal fossa; (3) Extrasphincteric (Figure 7): These fistulas traverse through the ischiorectal fossa and enter the supralevator space penetrating the levator muscle. They generally do not traverse through the sphincter-complex (EAS and IAS), which is why they are labelled as 'extra-sphincteric' fistulas; (4) High intra-rectal (Figures 8 and 9): These fistulas traverse cephalad between IAS and CLM in the rectal wall and is thus present between the muscle layers of the rectal wall; and (5) RIFIL (Figures 10-12): These fistulas traverse the EAS but do not enter the ischiorectal fossa. They extend cephalad between the EAS and its lateral fascia to continue on the undersurface of puborectalis and levator ani muscle[9].

Incidence: In recently published large cohorts, the prevalence of these high-5 fistulas has been highlighted[9,16]. The incidence of these fistulas in a cohort of 419 consecutively operated patients over a 2-year period were RIFIL–10% (42/419), supralevator-9.5% (40/419), suprasphincteric-5.5% (23/419) and extrasphincteric-0%[9,16].

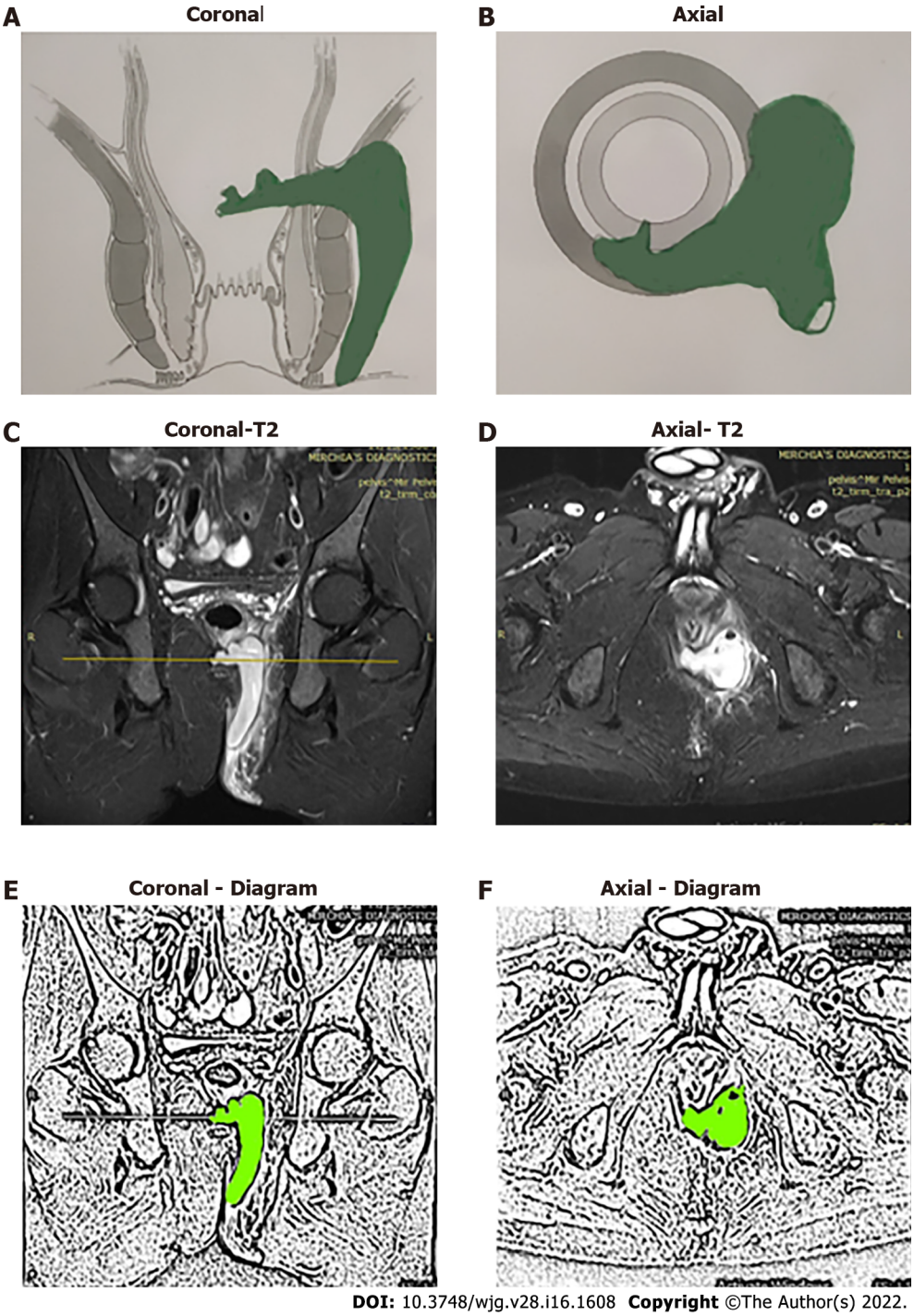
Difficulty in diagnosis

As these fistulas are deep and high, they are extremely difficult to diagnose on clinical examination. It usually requires advanced imaging modalities, MRI, or transrectal ultrasound (TRUS), to diagnose these fistulas[17-21]. Both MRI and 3D-TRUS are very effective in delineating various parameters of these fistulas, including the internal opening, primary tracts, and secondary extensions[17-20,22], though MRI has a slight edge over TRUS. It is really impressive that in the pre-MRI era, doyens in the field like Parks *et al*[8] could describe these deep fistulas with a reasonably high level of accuracy.

MRI helps to clearly differentiate between supralevator, suprasphincteric, high intrarectal, and RIFIL fistulas[23]. Rather it would not be wrong to say that MRI is mandatory to properly diagnose these fistulas. Therefore, whenever a fistula has recurred several times, or a tract or side branch of the main fistula is seen extending superiorly during surgery, an MRI should be done to rule out these fistulas[23].

Status of extrasphincteric fistulas

Increasing experience with MRI in fistulas has highlighted that extrasphincteric fistulas do not occur or are extremely rare[24]. It stands to reason that it is almost impossible for a fistula in the ischiorectal fossa to penetrate through the strong levator plate when it can extend with ease in the surrounding fat of the ischiorectal fossa[24]. The only likely possibility for an extrasphincteric fistula to occur is iatrogenic when an artery forceps in a high transsphincteric fistula is pushed through the levator muscle. Even this seems improbable as it would require a lot of force by the operating surgeon. Moreover, due to increased awareness amongst surgeons of iatrogenic injuries, better understanding of regional anatomy, and easy availability of advanced radiological modalities, these iatrogenic blunders seem exceedingly



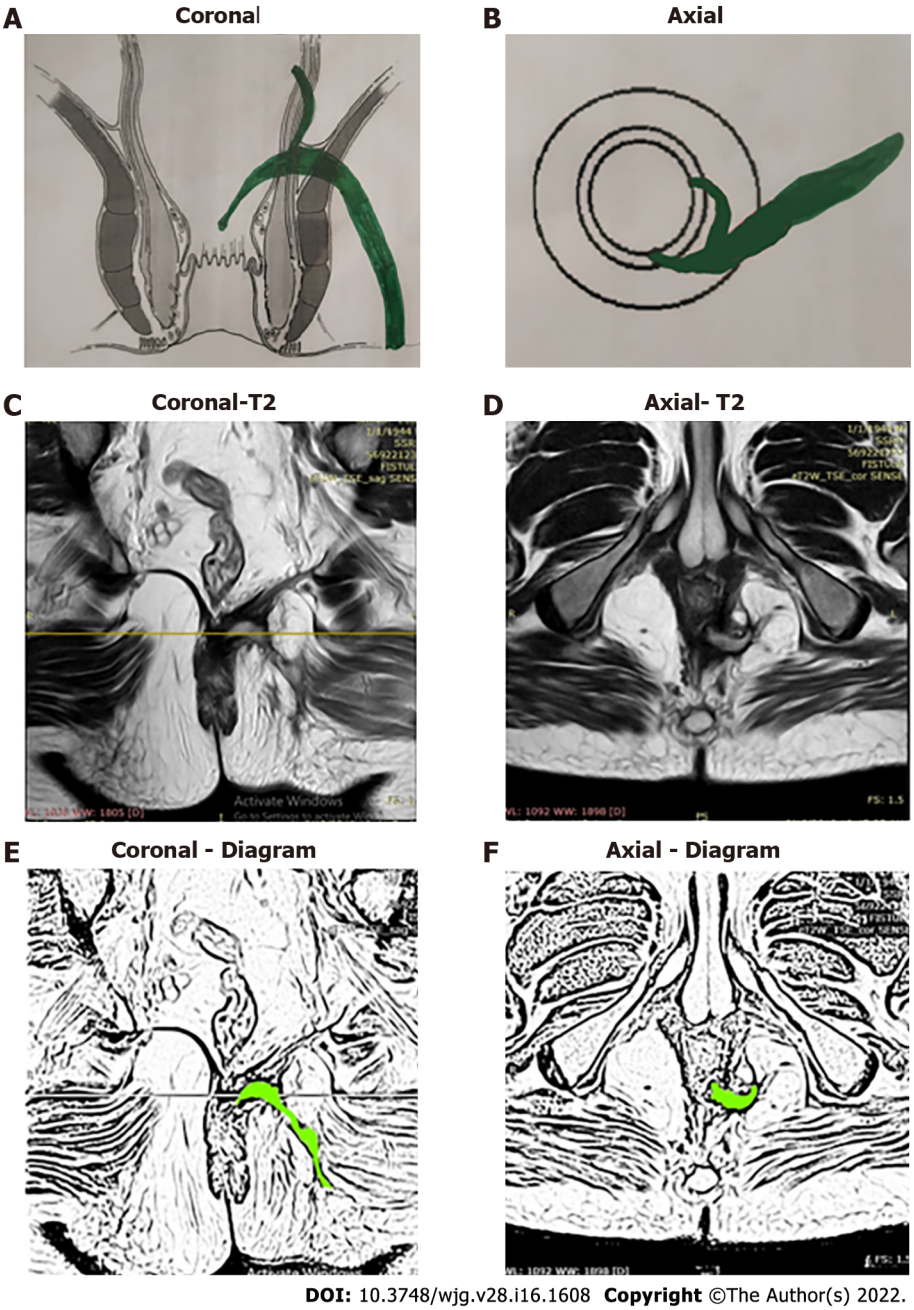
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Figure 5 A 40-year-old male patient with a suprasphincteric fistula and abscess on left side. The internal opening is at 6:30 o'clock position. A: Coronal section (schematic diagram); B: Axial section (schematic diagram); C: Short Tau Inversion Recovery (STIR) magnetic resonance imaging (MRI) Coronal section showing suprasphincteric tract and abscess; D: STIR MRI Axial section; E: Sketch of MRI Coronal section highlighting suprasphincteric fistula tract and abscess (light green color); F: Sketch of MRI Axial section highlighting fistula tract and abscess (light green color).

unlikely. Due to this, extrasphincteric fistulas are not seen (or are extremely rare) these days[24]. It is quite possible that the fistulas diagnosed as extrasphincteric fistulas in the pre-MRI era were perhaps supralevator or RIFIL fistulas. Understandably, in the absence of MRI, it is extremely difficult to differentiate between an extrasphincteric fistula and other deep fistulas. So, a diagnosis of extrasphincteric fistula should be made only after due deliberation and detailed analysis of MRI[24].

Complete involvement of EAS

The main challenge in these five fistulas is that they involve almost the complete EAS (Figures 2, 4, 7, 8 and 10). The EAS plays a major role in maintaining continence and risk of damage to the EAS is very high in these fistulas. Because this risk of incontinence is so high, these fistulas are extra concerning[4,5,



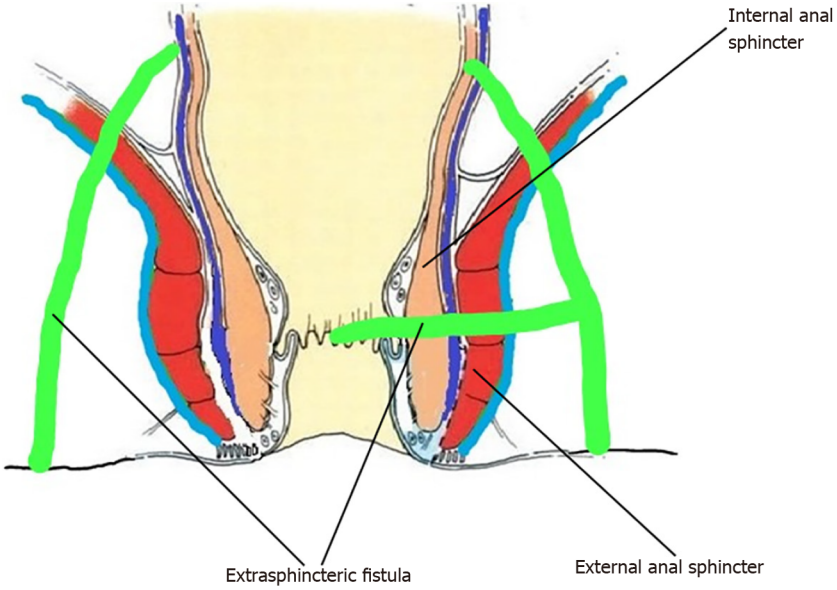
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Figure 6 A 47-year-old male patient with a suprasphincteric fistula on left side and supralevator tract and high rectal opening at 3 o'clock. The internal opening is at 6 o'clock position. A: Coronal section (schematic diagram); B: Axial section (schematic diagram); C: T2-weighted magnetic resonance imaging (MRI) Coronal section showing suprasphincteric tract; D: T2-weighted MRI Axial section; E: Sketch of MRI Coronal section highlighting supralevator fistula tract (light green color); F: Sketch of MRI Axial section highlighting fistula tract (light green color).

25]. The most commonly performed procedure, fistulotomy, is contraindicated in these fistulas[25]. There is little published data and there are no standard guidelines to manage these fistulas. Therefore, the fear of managing these fistulas amongst surgeons in easy to understand.

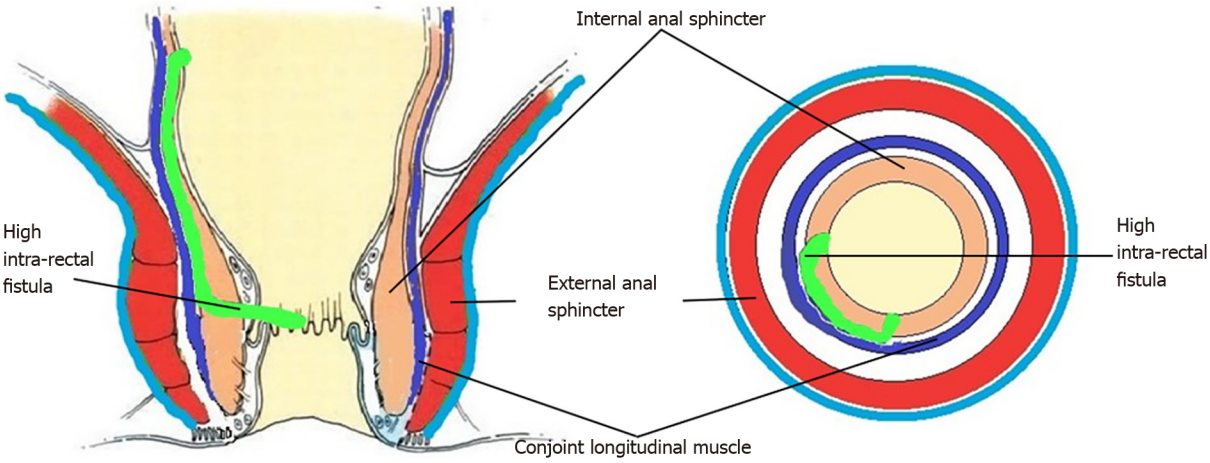
Presence of ASRO (additional supralevator rectal opening)

One of the features that make these fistulas more challenging is the presence of an ASRO along with a primary internal opening at the dentate line[6]. In a large cohort, ASRO was present in 2.8% (23/836) anal fistulas, but in supralevator fistulas, the incidence was 16.6% (23/138)[6]. At times, the presence of ASRO is detected accidentally during surgery (intraoperatively) when a colored solution injected into the external opening comes out through the primary internal opening at the dentate line as well as through the ASRO[6]. In many cases, a granulation polyp or a papilla can be seen at the site of the ASRO. However, ASRO can be diagnosed preoperatively with the help of MRI in almost every case[6]. MRI is an excellent tool to diagnose ASRO with a high level of accuracy[6].



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Figure 7 Extrasphincteric fistula.

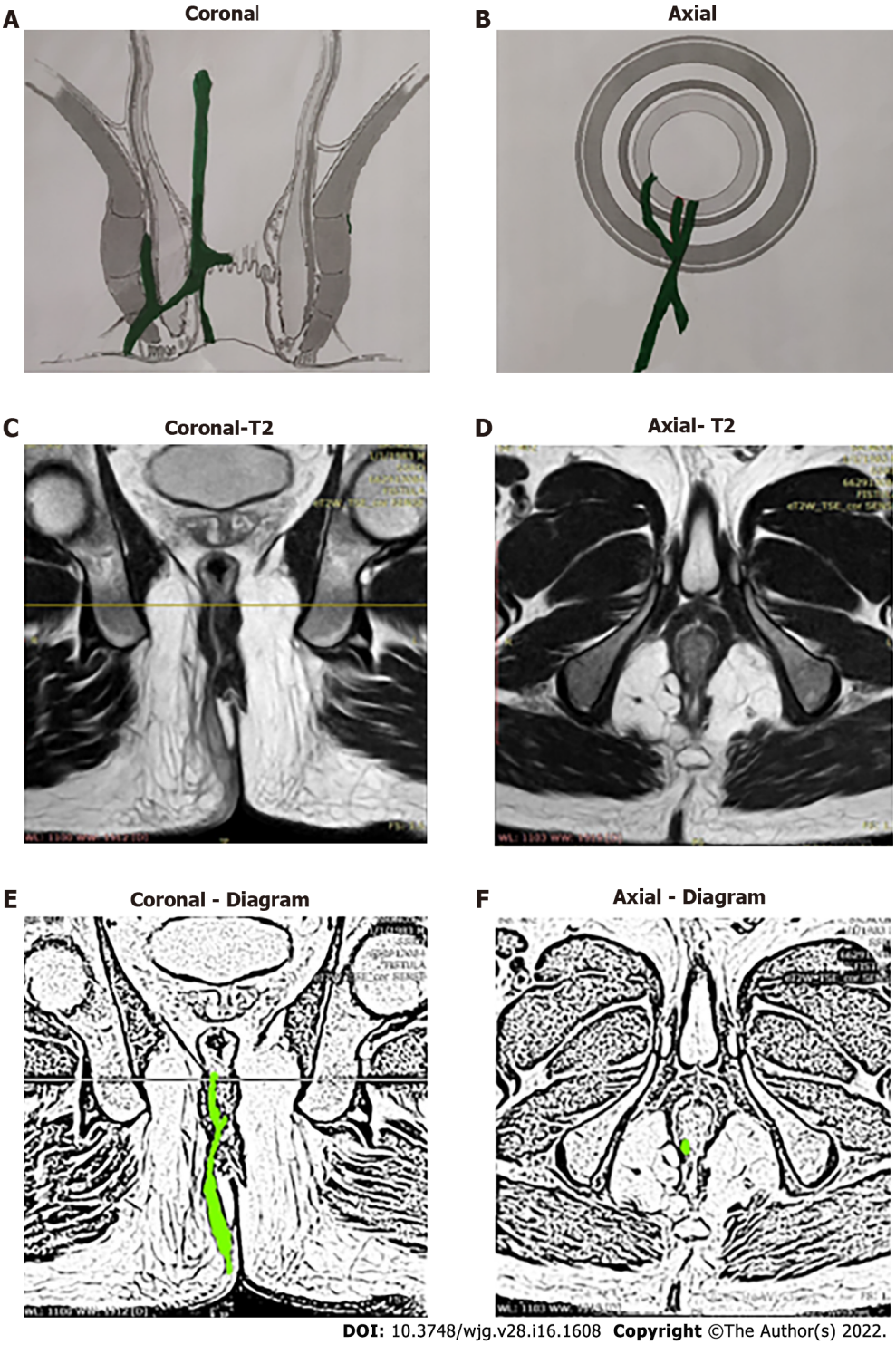


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Figure 8 High Intrarectal fistula.

The management of ASRO seems challenging. Closure of an opening high up in the rectum which, at times, is even difficult to reach through the transanal route is not easy. However, there is a sliver of relief in these otherwise complex fistulas. A recent study has demonstrated that even if nothing is done to ASRO, there is no impact on ultimate fistula healing[6].

There is an explanation for this surprising finding. Anal fistulas usually initiate in crypt glands located at the dentate line. The fistula then can extend in different directions from here. Some fistulas or abscesses extend superiorly and enter the supralevator space[6]. In a small subset (16%) of these supralevator abscesses, the pressure generated by the supralevator abscess is so high that the abscess ruptures into the rectum, thus creating an ASRO. In such situations, the flow of infection (and bacteria) in ASRO is from the supralevator space into the rectum[6]. This continues in the same manner because, unlike in the anal canal where intraluminal pressure rises during defecation, pressure in the mid-rectum is quite low (as the rectum is primarily a storage organ). Therefore, the bacteria usually enter the fistula tract from the primary internal opening at the dentate line, bacterial multiplication leads to suppuration, and then the pus egresses through the external opening and the ASRO (if present)[6]. Hence, ultimate healing of the fistula depends primarily on successful closure of the primary internal opening at the dentate line. Once the primary internal opening at the dentate line heals, the fistula heals irrespective of whether the ASRO is closed or open[6]. Therefore, it is a reassuring fact that ASRO left untreated does not affect the ultimate fistula outcome[6].



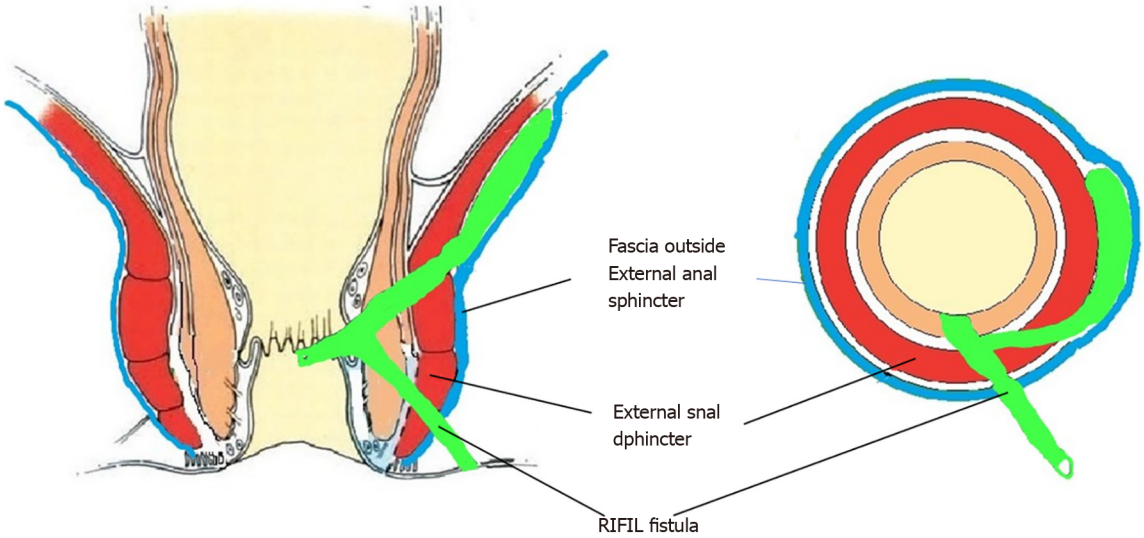
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Figure 9 A 36-year-old male patient with a high intrarectal fistula at 7 o'clock. A: Coronal section (schematic diagram); B: Axial section (schematic diagram); C: T2-weighted magnetic resonance imaging (MRI) Coronal section showing high intrarectal fistula tract; D: T2-weighted MRI Axial section; E: Sketch of MRI Coronal section highlighting high intrarectal fistula tract (light green color); F: Sketch of MRI Axial section highlighting fistula tract (light green color).

Detection of underlying secondary pathology

The high-5 fistulas are more complex and refractory to treatment as compared to other fistulas. The incidence of associated complicating pathology like Crohn's disease and tuberculosis is higher in these fistulas[24,26,27]. Therefore, it is important that these diseases should be ruled out whenever any of these high fistulas are diagnosed or suspected.

In regions where Crohn's disease is common, a colonoscopy should be done in all cases of high fistulas[28]. On the other hand, in regions where tuberculosis is common, polymerase chain-reaction (PCR) should be done in samples from the fistula (pus or fistula tract wall or tract lining)[24,29]. In a large series published from a TB-endemic region (India), tuberculosis was detected in 10% (133/1336) of



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Figure 10 Roof of ischiorectal fossa inside the levator ani muscle fistula. RIFIL: Roof of ischiorectal fossa inside the levator ani muscle.

tested samples (fistula tract lining or pus)[29]. The detection rate of tuberculosis was 12.5% (129/1034) by PCR, 1.5% (3/197) by histopathology, and 0.9% (1/105) by GeneXpert tests[29]. Therefore, PCR is significantly more sensitive than histopathology or GeneXpert to detect tuberculosis[29]. However, in spite of high sensitivity, it is not uncommon that tuberculosis is missed and goes undetected in the first sample. Therefore, in regions where tuberculosis is endemic, it is recommended that repeated samples should be sent for testing, especially in more complex refractory fistulas[29].

METHODS USED TO FORMULATE MANAGEMENT GUIDELINES

A search was performed on MEDLINE, PubMed, EMBASE, and the Cochrane Database of Collected Reviews from January 1975 to September 2021. Keyword combinations using MeSH terms included supralevator, suprasphincteric, extrasphincteric, intrarectal, abscess, fistula, fistula-in-ano, anal, rectal, perianal, perineal, seton, fistula plug, fibrin glue, advancement flap, tuberculosis, Crohn's disease, ligation of intersphincteric tract, ligation of intersphincteric fistula tract (LIFT), fistulectomy with primary sphincter reconstruction (FPR), fistulectomy with primary sphincter repair, transanal opening of the intersphincteric space (TROPIS) and stem cells.

Various guidelines, such as GRADE[30,31], RIGHT[32] AGREE[33] were evaluated, but considering the rarity of the disease condition (Hi-5 fistulas) in the study, the Levels of Evidence for Therapeutic Studies developed by Centre for Evidence-Based Medicine, http://www.cebm.net. (Oxford, United Kingdom) (Table 1) and Grade Practice Recommendations recommended by American Society of Plastic Surgeons (Table 2) were utilized[34]. Each diagnostic and therapeutic intervention was assigned a 'level of evidence' from 1A to 5 (1A being the strongest evidence and 5 being the weakest) (Table 1) and then a 'grade of recommendation' was awarded ranging from 'A' to 'D' ('A' being a strong recommendation and 'D' being a weak option) (Table 2).

Authors (PG, VDY) reviewed all English language articles and tabulated all the evidence available and allotted the level of evidence. After that, the grade of recommendation was decided with consensus of all the authors.

MANAGEMENT GUIDELINES

Diagnostic evaluation of Hi-5 fistulas

MRI or TRUS are preferred modalities to evaluate Hi-5 fistulas. MRI is better than TRUS to evaluate high secondary extensions. Level of Evidence-2B. Grade of recommendation-B.

MRI and TRUS are the diagnostic modalities of choice to evaluate complex anal fistulas[17,19-21]. MRI and 3D-TRUS have comparable efficacy in outlining the internal opening and primary tracts; however, MRI is significantly more effective than 3D-TRUS in detecting deep secondary extensions[35]. The sensitivity of 3D-TRUS to detect deep secondary extensions was 73.9% while MRI had sensitivity of 97% (P = 0.041)[35]. Therefore, MRI is the preferred modality to diagnose high fistulas especially the high-5 fistulas[10,36].

Table 1 Levels of evidence for therapeutic studies[49]

Level		Type of evidence
1	A	Systematic review (with homogeneity) of RCTs
	B	Individual RCT (with narrow confidence intervals)
	C	All or none study
2	A	Systematic review (with homogeneity) of cohort studies
	B	Individual Cohort study (including low quality RCT, *e.g.*, < 80% follow-up)
	C	"Outcomes" research; Ecological studies
3	A	Systematic review (with homogeneity) of case-control studies
	B	Individual Case-control study
4		Case series (and poor-quality cohort and case-control study
5		Expert opinion without explicit critical appraisal or based on physiology bench research or "first principles"

Citation: CEBM. OCEBM levels of evidence. [cited 11 January 2022]. Available from: https://www.cebm.ox.ac.uk/resources/levels-of-evidence/ocebm-levels-of-evidence. Copyright© 2022 University of Oxford.
RCTs: Randomized controlled trials.

Table 2 Grade practice recommendations[1]

Grade	Level of Recommendation	Qualifying evidence	Implications for clinicians
A	Strong recommendation	Level I evidence or consistent findings from several studies of levels II, III, or IV	These recommendations should be followed unless an alternative approach with clear and compelling rationale is present
B	Recommendation	Consistent evidence of levels II, III, or IV	These recommendations should be followed but clinicians should understand that there is scope of improvement and these may be slightly modified as *per* patient preferences
C	Option	Levels II, III, or IV evidence, but findings are inconsistent	These guidelines are broad guidelines and clinicians should be flexible in their decision-making regarding appropriate practice keeping patient preferences in mind
D	Option	Level V evidence: Little or no substantial evidence	These recommendations may be referred but clinicians should consider all other available options while making decisions

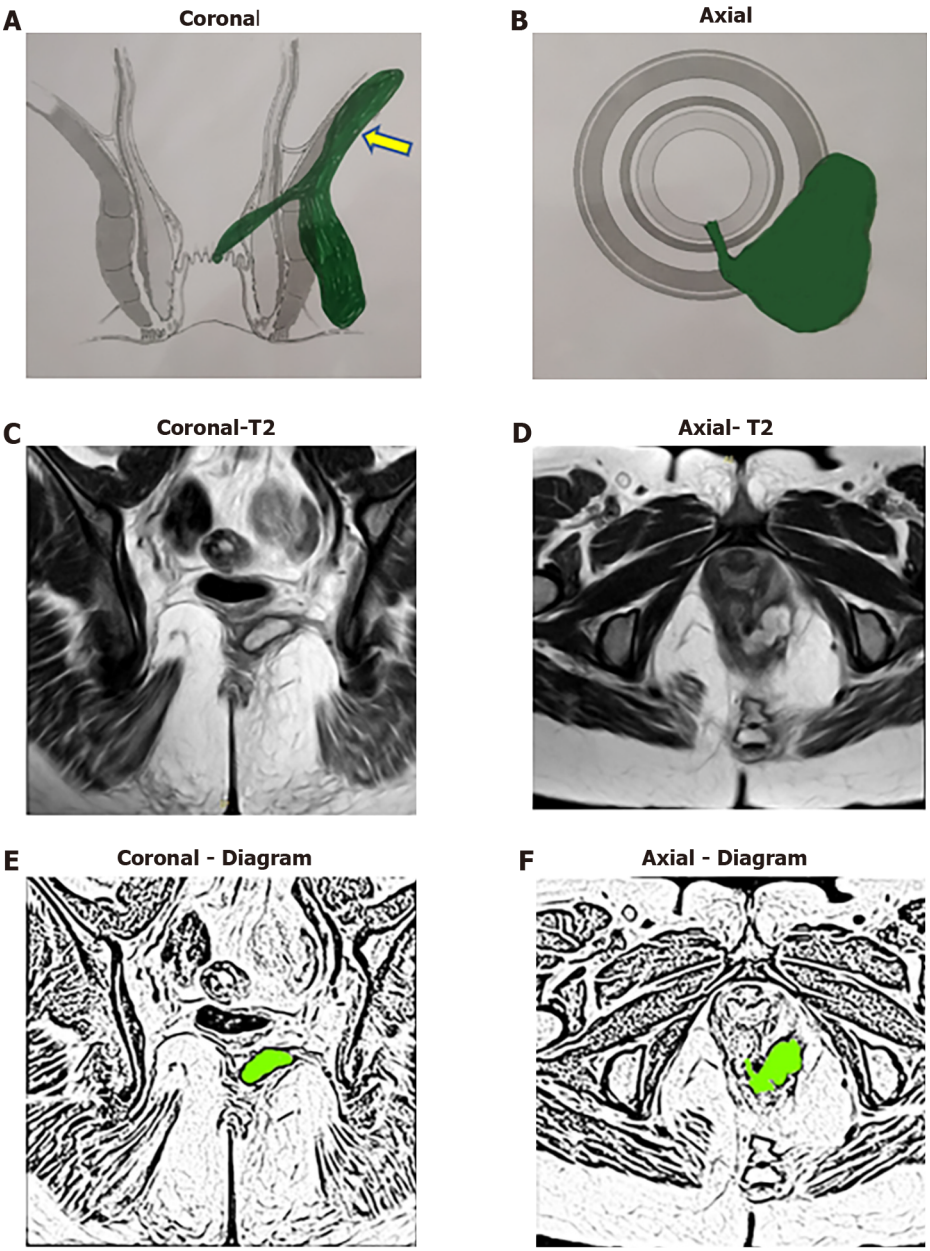
[1]Based on the American Society of Plastic Surgeons[34].

Surgical procedure

Due to the challenging factors discussed above, the management of high-5 fistulas is quite difficult and far from satisfactory. Apart from the associated complex parameters, the additional problem is minimal experience or published data on the management of these fistulas. Due to this, the quality of data is of a low evidence level.

As discussed earlier, due to complete involvement of EAS, the most commonly performed procedure, fistulotomy, is contraindicated in these fistulas[25] unless it is coupled with primary sphincter repair. The newer sphincter-sparing procedures, like video-assisted anal fistula treatment[24], fistula laser closure[24], over-the scope clip (OTSC)[37-39], anal fistula plug (AFP)[40], stem cells[28] *etc,* have dismal success rates in complex fistulas. In fact, there are hardly any studies that have analyzed the success rates of these newer procedures in high-5 fistulas. Therefore, the sphincter-preserving procedures, such as LIFT, FPR, advancement flaps, and TROPIS, seem more suitable procedures for high-5 fistulas.

TROPIS provides a ray of hope, as it has been shown to have satisfactory results in these fistulas[2,3,16,24,41]. In the TROPIS procedure, the intersphincteric space is laid open in the anal canal and the resultant wound is allowed to heal by secondary intention[16,24]. The transsphincteric tracts (tracts lateral to the EAS in the ischiorectal fossa) are thoroughly curetted and cleaned[3,41]. Thus, the tracts on both sides of the EAS are managed separately (tracts inner to the EAS are laid open into the anal canal and tracts outside the EAS in the ischiorectal fossa are curetted and cleaned) and the EAS is not cut or damaged at all[16,24]. Due to this, it has been shown that there is no deterioration in continence after the TROPIS procedure[16,24].



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Figure 11 A 33-year-old female patient with a roof of ischiorectal fossa inside the levator ani muscle fistula on left side at 5 o'clock and internal opening at 6 o'clock position. A: Coronal section showing roof of ischiorectal fossa inside the levator ani muscle (RIFIL) fistula (yellow arrow) (schematic diagram); B: Axial section (schematic diagram); C: T2-weighted magnetic resonance imaging (MRI) Coronal section showing RIFIL fistula tract; D: T2-weighted MRI Axial section; E: Sketch of MRI Coronal section highlighting RIFIL fistula tract (light green color); F: Sketch of MRI Axial section highlighting fistula tract (light green color).

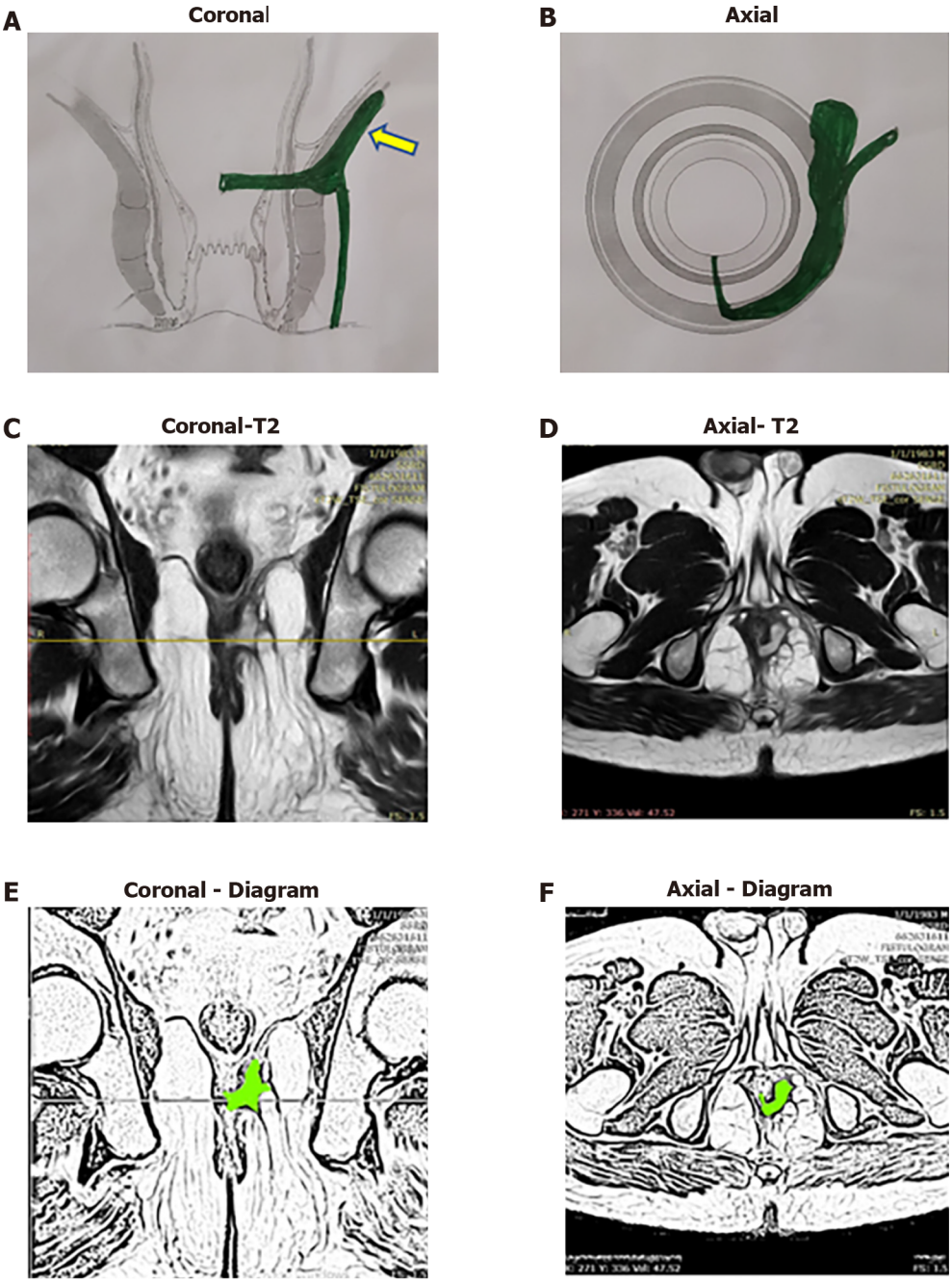
The reason for the higher success rate of the TROPIS procedure could be that, unlike other procedures, TROPIS adequately tackles the sepsis in the intersphincteric space[3,41]. It is now understood that the pus/sepsis in the intersphincteric part of the fistula tract is like an abscess in a closed space[24]. Therefore, this sepsis is best managed in the manner an abscess anywhere else in the body is managed-deroofing the abscess cavity and allowing it to heal by secondary intention[24].

Supralevator fistulas

A supralevator abscesses can be drained into the rectum with a moderate success rate[7,42-44]. The level of evidence for this is 2B with a grade of recommendation of B. For example, Garcia-Granero *et al*[7] drained the supralevator abscess into the rectum in four patients and all of them healed.

This not only leads to resolution of the acute symptoms, but also leads to fistula healing in many cases[7,44].

Fistulectomy with or without advancement flap may be done in selected cases with a moderate success rate[44]. The level of evidence is a -4, with the grade of recommendation a-D. Van Onkelen *et al*



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Figure 12 A 38-year-old male patient with a roof of ischiorectal fossa inside the levator ani muscle fistula on left side at 3 o'clock and internal opening at 6 o'clock position. A: Coronal section showing roof of ischiorectal fossa inside the levator ani muscle (RIFIL) fistula (yellow arrow) (schematic diagram); B: Axial section (schematic diagram); C: T2-weighted magnetic resonance imaging (MRI) Coronal section showing RIFIL fistula tract; D: T2-weighted MRI Axial section; E: Sketch of MRI Coronal section highlighting RIFIL fistula tract (light green color); F: Sketch of MRI Axial section highlighting fistula tract (light green color).

[44] performed advancement flap in three patients after draining the supralevator abscess. All patients healed but multiple surgeries were required in order to achieve fistula healing[44].

Supralevator fistula can be managed with laying open of the supralevator extension into the rectum through the transanal route (TROPIS) procedure with a satisfactory healing rate[6,16]. The level of evidence for this is 2B with a grade of recommendation of B. The success rate was 84.6% (11/13) in the initial series and 82.1% (92/112) on long-term follow-up (median 30 mo) in the same series[16].

LIFT and FPR can also be done in selected cases with good success rates (80%-91%) in high complex fistulas (the studies included supralevator fistulas)[45,46]. The level of evidence for this is 5 with a grade of recommendation of D. However, data for these two procedures specific to supralevator fistulas is not available.

Suprasphincteric fistulas

Advancement flap, fistulotomy/fistulectomy with primary sphincter repair and stem cells have moderate success rates, with a success rate range from 70%-85%, though the sample size was quite small [47]. The level of evidence for this is 2B with a grade of recommendation of D. Perez *et al*[47] compared advancement flap *vs* fistulotomy with sphincter reconstruction in suprasphincteric fistulas and the healing rate was 80% (4/5) and 83.3% (5/6) respectively[47]. However, the risk of cutting the entire EAS and then repairing the same seems a difficult option to most surgeons. Also, in the rare eventuality of suture dehiscence, the risk of incontinence would be very high.

Fibrin glue has a poor success rate in suprasphincteric fistulas. The level of evidence for this is 2B with a grade of recommendation of C. Garcia-Olmo *et al*[48] compared the healing rate of fibrin glue *vs* adipose stem cells in suprasphincteric fistulas. The healing rate in the fibrin glue group was 8% (2/16) while it was 71% (10/14) in the stem cells group[48].

The TROPIS procedure has had promising results with a good success rate with minimal impact on continence The level of evidence for this is 4 with a grade of recommendation of C. Out of 14 supras-phincteric fistulas, 78.6% (11/14) patients were cured with no deterioration in continence levels after surgery on long-term follow-up (median 30 mo)[16].

LIFT can be done in selected cases but is technically difficult. The level of evidence for this is 2B with a grade of recommendation of C.

There are studies in which LFT has shown a good success rate (80%-91%) in high complex fistulas[45, 46]. but data for LIFT exclusive to supralevator fistulas is not available. It is also expected that performing the LIFT procedure in suprasphincteric fistulas (ligating the intersphincteric tract high up in the intersphincteric plane) would be a technically demanding procedure.

Extrasphincteric fistulas

Temporary colostomy with management of the primary pathology can be used for extrasphincteric fisutlas. The level of evidence for this is 5 with a grade of recommendation of D.

Extrasphincteric fistulas are extremely rare these days, which means there is little data available on the management of these fistulas. As most of these fistulas are caused by secondary pelvic pathology or iatrogenic factors, a diverting stoma along with management of the underlying cause would be the mainstay of treatment[8].

High intra-rectal fistulas

Intra-anal fistulotomy (TROPIS procedure) has a high success rate with minimal impact on continence. The level of evidence for this is 4 with a grade of recommendation of B. These fistulas are perhaps the easiest to treat. Simple intra-rectal fistulotomy (laying open the fistula tract into the anorectum) would cure these fistulas with a high success rate (> 90%)[8,16].

RIFIL fistulas (fistula at the roof of ischiorectal fossa inside the levator ani muscle)

TROPIS has a moderate success rate with minimal impact on continence in RIFIL fistulas. The level of evidence for this is 2B with a grade of recommendation of B. RIFIL fistulas are challenging to manage because access to the RIFIL component might become difficult. TROPIS has shown a moderate success rate in RIFIL fistulas[9]. The healing rate in RIFIL fistulas by the TROPIS procedure was 69.4% (25/36) with a follow-up (median) of 12 mo[9]. There was no negative impact on continence following surgery.

LIFT and FPR are expected to have a moderate success rate; however, since RIFIL fistulas have been recently described[9] and till now, there are no studies of LIFT or FPR procedure in RIFIL fistulas. Conceptually it is expected that these procedures would also prove at least moderately effective for these fistulas. The level of evidence for this is 5 with a grade of recommendation of D.

CONCLUSION

Anal fistulas that reach up to the levator muscle and involve almost the complete EAS have been grouped together as high-5 fistulas. These comprise supralevator, supraphincteric, extrasphincteric, high intrarectal, and RIFIL fistulas. The diagnosis as well as the management of these five fistulas is quite challenging. Advanced radiological modalities, preferably MRI, are needed to accurately delineate these fistulas. Once diagnosed, care should be exercised to avoid sphincter-cutting procedures like fistulotomy or cutting seton in these fistulas as the risk of incontinence would be very high. Sphincter-sparing procedures like TROPIS or LIFT should be carried out in these fistulas. These guidelines may help improve understanding and outcomes in the management of these complex fistulas.

FOOTNOTES

Author contributions: Garg P conceived and designed the study, collected and analyzed the data, revised the data,

approved and submitted the final manuscript (guarantor of the review); Dawka S critically analyzed the data, reviewed and edited the manuscript, and approved and submitted the final manuscript; Kaur B and Yagnik VD collected and analyzed the data, revised the data, and approved and submitted the final manuscript; Menon GR analyzed the data, revised the data, and approved and submitted the final manuscript.

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Country/Territory of origin: India

ORCID number: Pankaj Garg 0000-0002-0800-3578; Vipul D Yagnik 0000-0003-4008-6040; Sushil Dawka 0000-0002-9372-3683; Baljit Kaur 0000-0002-3882-7578; Geetha R Menon 0000-0003-2491-0650.

Corresponding Author's Membership in Professional Societies: American Society of Colon Rectum Surgeons; American Society of Gastrointestinal Endoscopic Surgeons; Association of Surgeons of India.

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REVIEW

Noninvasive imaging of hepatic dysfunction: A state-of-the-art review

Ting Duan, Han-Yu Jiang, Wen-Wu Ling, Bin Song

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Ting Duan, Han-Yu Jiang, Bin Song, Department of Radiology, West China Hospital, Sichuan University, Chengdu 610041, Sichuan Province, China

Wen-Wu Ling, Department of Medical Ultrasound, West China Hospital of Sichuan University, Chengdu 610041, Sichuan Province, China

Corresponding author: Bin Song, MD, Chief Doctor, Professor, Department of Radiology, West China Hospital, Sichuan University, No. 37 Guoxue Alley, Chengdu 610041, Sichuan Province, China. anicesong@vip.sina.com

Abstract

Hepatic dysfunction represents a wide spectrum of pathological changes, which can be frequently found in hepatitis, cholestasis, metabolic diseases, and focal liver lesions. As hepatic dysfunction is often clinically silent until advanced stages, there remains an unmet need to identify affected patients at early stages to enable individualized intervention which can improve prognosis. Passive liver function tests include biochemical parameters and clinical grading systems (*e.g.*, the Child-Pugh score and Model for End-Stage Liver Disease score). Despite widely used and readily available, these approaches provide indirect and limited information regarding hepatic function. Dynamic quantitative tests of liver function are based on clearance capacity tests such as the indocyanine green (ICG) clearance test. However, controversial results have been reported for the ICG clearance test in relation with clinical outcome and the accuracy is easily affected by various factors. Imaging techniques, including ultrasound, computed tomography, and magnetic resonance imaging, allow morphological and functional assessment of the entire hepatobiliary system, hence demonstrating great potential in evaluating hepatic dysfunction noninvasively. In this article, we provide a state-of-the-art summary of noninvasive imaging modalities for hepatic dysfunction assessment along the pathophysiological track, with special emphasis on the imaging modality comparison and selection for each clinical scenario.

Key Words: Hepatic dysfunction; Ultrasound; Computed tomography; Magnetic resonance imaging

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Core Tip: Hepatic dysfunction can be frequently found in hepatitis, cholestasis, metabolic diseases, and focal liver lesions. It remains clinically silent until advanced stages, so there remains an unmet need to identify affected individuals at early stages. Imaging techniques, including ultrasound, computed tomography, and magnetic resonance imaging, allow morphological and functional assessment of the entire hepatobiliary system. In this article, we provide a state-of-the-art summary of noninvasive imaging modalities for assessing hepatic dysfunction in various clinical situations.

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INTRODUCTION

Hepatic dysfunction is a common result of a wide variety of diseases, including hepatobiliary disorders and systemic diseases. The clinical symptoms of hepatic dysfunction (*e.g.*, jaundice, anorexia, and abdominal pain) are varied and nonspecific[1]. Liver biopsy is the gold standard for hepatic dysfunction currently. Accurate as it is, liver biopsy is invasive, and susceptible to sampling errors and inter-observer variation. Besides, liver biopsy is limited by various complications and operator expertise. Therefore, the introduction of noninvasive diagnostic approaches is pivotal to addressing the above limitations of liver biopsy. Hepatic dysfunction usually manifests as biochemical abnormalities of serum markers, typically involving hepatocyte damage, cholestasis, bilirubin, synthesis function, and liver fibrosis[2,3]. Nevertheless, it is worth noting that not all patients with abnormalities in the above markers have primary liver disease, highlighting the wide differential diagnosis spectrum of abnormal liver chemistry and metabolic functions[2]. Considering the limited value of single serum markers in hepatic dysfunction evaluation, clinical grading systems integrating biochemical parameters and clinical symptoms have been developed to reveal impaired liver function. Among them, the Child-Pugh score is a widely adopted clinical scoring system that is particularly useful in selecting surgical candidates with hepatocellular carcinoma (HCC) and cirrhosis[4]. The Model for End-Stage Liver Disease score was initially developed to predict short-term survival in patients undergoing transcutaneous intrahepatic portosystemic shunt procedures and has been later expanded to stratify patients with end-stage liver disease awaiting transplantation[5]. Nevertheless, the performances of these clinical grading systems are suboptimal in mild liver injuries. Furthermore, despite widely used and readily available, biochemical parameters and clinical grading systems only provide indirect information about the hepatic function [6]. In contrast, dynamic quantitative tests, such as the indocyanine green (ICG) clearance test[7], allows direct measurements of liver clearance capacity and hence has become a routine test in preoperative liver function evaluation. However, discrepancies have been reported on the performances of ICG clearance test in clinical outcome prediction[8]. In addition, the accuracy of ICG clearance is affected by operator's proficiency and the concentration of blood oxygen and other competitive agents[9].

Noninvasive imaging techniques, including ultrasound (US), computed tomography (CT), and magnetic resonance imaging (MRI), allow morphological and functional assessment of the entire hepatobiliary system (Table 1). These techniques permit qualitative and quantitative evaluation of hepatocyte quantity and function, fibrosis degree, type and severity of metabolic disorders, and excretory function of the biliary system. Therefore, through accurate hepatic dysfunction measurement and identification of affected individuals at early diseases stages, noninvasive imaging modalities offer appeal in individualized clinical decision-making and improving patient prognosis. Therefore, this review provides a state-of-the-art summary of noninvasive imaging modalities for assessing hepatic dysfunction along the pathophysiological track in various clinical situations.

HEPATITIS-INCLUDED HEPATIC DYSFUNCTION

Hepatitis is a major global public health problem affecting hundreds of millions of people. The common causes are the virus, bacteria, amoeba, and other infections. Other relatively rare causes include drug and food poisoning. Most deaths from viral hepatitis are due to hepatitis B and hepatitis C. An estimated 257 million people were living with hepatitis B and 71 million people were living with hepatitis C[10].

Acute hepatitis

In mild hepatitis, edema of hepatocytes and inflammatory cells gather in the portal area at pathology. At

Table 1 Noninvasive imaging modalities for hepatic dysfunction

Imaging modality		Target changes
US	B-mode ultrasonography	Echo intensity
		Morphological changes
	Color Doppler US	Phase and velocity of blood flow
	Contrast-enhanced US	Hemodynamic changes with better contrast than Doppler US
	Transient elastography	Liver stiffness
		Steatosis
	Point shear wave elastography	Liver stiffness
	2D-shear wave elastography	Liver stiffness
CT	Conventional CT	CT value
		Morphological changes
		Steatosis
	Dynamic enhanced CT	Portal hypertension
		Hemodynamic changes
	CT perfusion	Quantitative measurement hemodynamic changes
	Liver extracellular volume on CT	Fibrosis
MR	Conventional MRI	Morphological changes
	MR elastography	Liver stiffness
	Diffusion-weighted MRI	Brownian motion of water molecules
	Gadoxetate-enhanced MRI	Number and function of hepatocytes
	MR perfusion	Quantitative measurement hemodynamic changes
	Chemical-shift-encoded MRI	Steatosis
		Iron overload
	MR cholangiopancreatography	Biliary system
	Quantitative susceptibility mapping	Iron overload
	Liver extracellular volume on MRI	Fibrosis

US: Ultrasound; CT: Computed tomography; MR: Magnetic resonance; MRI: MR imaging.

this stage, the imaging findings are generally nonspecific, such as enhanced echo on US, slightly decreased density on CT, or increased signals on T2-weighted imaging.

With the aggravation of inflammation, histologic changes become more pronounced, including lobular disarray, acidophilic degeneration of hepatocytes, focal lobular necrosis, disruption of bile canaliculi with cholestasis, and portal and parenchymal infiltration of inflammatory cells (predominantly lymphocytes and macrophages)[11], as well as hypertrophy and hyperplasia of Kupffer cells and macrophages. These changes can lead to heterogeneous appearances of the liver parenchyma on pre-contrast imaging. Meanwhile, the microcirculation in the liver deteriorates, causing patchy enhancement or wedge-shaped enhancement pattern of the liver parenchyma on contrast-enhanced imaging. In addition, the "halo-ring sign" or "track sign" appears around the portal vein as a result of increased lymph inflow and blocked lymph backflow[12]. The transient portal hypertension (PH) leads to increased pressure in the gallbladder vein, causing subsequent subserosal edema of the gallbladder wall. With the gallbladder wall thickening and protruding into the cavity, a typical sign of "centripetal edema" appears[13]. Enlarged lymph node can be detected on US, CT, or MRI[14].

A high proportion of severe acute hepatitis cases can result in significant liver failure[15,16]. In these cases, extensive hepatocyte necrosis can lead to substantial bridging. Irregular necrosis is depicted as map-like low density on CT images. On contrast-enhanced images in the portal venous phase, the necrotic areas usually become hyper-attenuating compared with adjacent liver parenchyma due to infiltrates of inflammatory cells, increased arterial blood supply, and widened intercellular space. This sign is called "reverse enhancement", which is a characteristic manifestation of severe hepatitis. In addition, ascites can be detected frequently[15] (Figure 1). Grillet *et al*[15] reported that heterogeneous



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Figure 1 Ultrasound and computed tomography images of a 19-year-old man with severe drug-induced hepatitis. A: High frequency ultrasound image showing increased and heterogenous echo intensity of the liver parenchyma; B: Pre-contrast computed tomography image showing map-like hypodense area in the liver parenchyma and moderate ascites; C: The hypodense areas on (B) became hyperattenuating on portal venous phase image, showing "reverse enhancement".

liver parenchyma on CT would be particularly beneficial for patients with acute severe autoimmune hepatitis as histological examinations could be technically challenging due to complications. They also reported heterogeneous CT features of severe alcoholic hepatitis, indicating that these imaging features were mainly associated with transient heterogeneous steatosis and liver perfusion disorders[15]. Furthermore, Tana *et al*[17] used texture analysis to quantify the heterogeneity of the liver parenchyma, and showed that texture features of the liver could provide important quantitative information in predicting the severity and outcomes of patients with acute alcohol-associated hepatitis.

In summary, ultrasound is recommended as the first-line imaging modality for morphologic evaluation in patients with acute hepatitis. Contrast-enhanced CT or MRI should be considered when intrahepatic necrosis is suspected.

Chronic hepatitis

Chronic hepatitis refers to a morphologic pattern that is usually observed in patients with chronic viral hepatitis, autoimmune hepatitis, drug-induced hepatitis, and alcoholic hepatitis. Chronic hepatitis is characterized by several pathologic changes. These include inflammations of the portal veins and sometimes of the bile ducts; periportal injury and inflammation; several degeneration and apoptosis of intra-acinar hepatocytes secondary to inflammatory response; and different forms of fibrosis[18]. The end-stage progression is cirrhosis. The image findings of liver fibrosis and cirrhosis are described in later sections.

Typical imaging characteristics of chronic hepatitis include unsmooth liver margin, blunt edge, widened portal vein, enlarged spleen, and thickened gallbladder wall[19] (Figure 2). Unfortunately, when the above signs appear, liver injury has usually occurred for a long time and become irreversible.

Many efforts have been devoted to capturing the early hepatic microcirculation and perfusion changes of chronic hepatitis using imaging techniques. The deposition of collagen in the space of Disse and sinusoidal capillarization result in increased resistance to incoming sinusoidal blood flow, leading to a decrease in portal venous flow to the liver and an increase in hepatic arterial flow, and subsequently the formation of intrahepatic and portosystemic shunts. Cao *et al*[20] reported a significant correlation between the ICG clearance rate and MR-based portal venous perfusion, suggesting that MR-based portal venous perfusion could be used as a surrogate for liver function assessment.

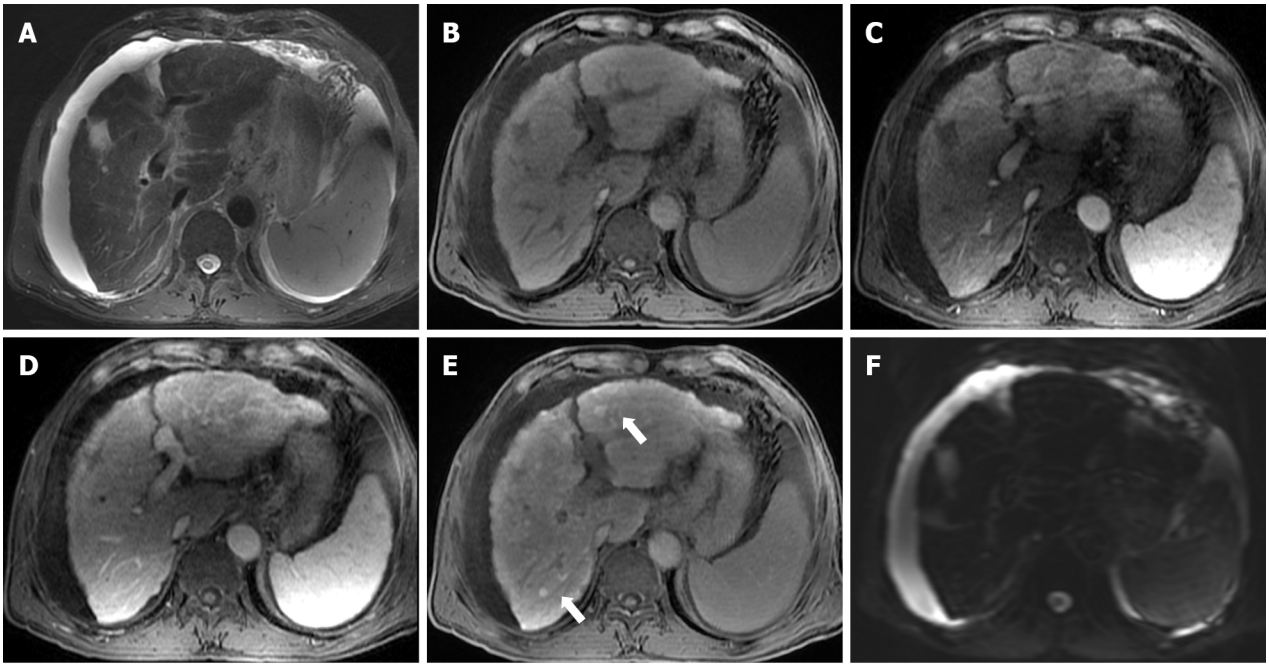
Another important cause for hepatic dysfunction in chronic hepatitis is the impaired hepatocytes. Active transport of MR hepatobiliary contrast agents (*e.g.*, gadoxetate and gadobenate dimeglumine) into the hepatocytes can reflect hepatocyte functions. Hepatobiliary phase (HBP) images can be acquired at about 20 min after contrast administration for gadoxetate and 1-2 h for gadobenate dimeglumine, with signal intensity on HBP images providing important information regarding liver function[21,22]. On this basis, studies further showed that T1 mapping could eliminate signal deviation and allow accurate liver function quantification[23-25].

Without proper and timely intervention, chronic hepatitis may progress to liver fibrosis (LF) and PH, which would be discussed in later sections.

CHOLESTASIS

Acute cholestasis

Acute cholestasis is characterized with mechanical biliary obstruction of any cause, such as choledocholithiasis, strictures (*e.g.*, neoplastic, inflammatory, or postoperative), pancreatitis, choledochal cysts,



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Figure 2 Gadoxetate-enhanced magnetic resonance images of a 70-year-old man with chronic hepatitis B. T2-weighted image (A) shows signal loss of the liver parenchyma, suggesting iron overload. T1-weighted pre-contrast (B), arterial phase (C), and portal venous phase (D) images show nodular contour and patchy enhancement of the liver parenchyma. Hepatobiliary phase image demonstrates diffuse hyperintense nodules (E, black arrows) without diffusion restriction on diffusion-weighted imaging (F), indicating regenerative nodules. Moderate ascites was also noted.

parasitic diseases (*e.g.*, ascariasis and fascioliasis), or even extrinsic pressure from enlarged lymph nodes [26]. US is promising for diagnosing early-stage acute cholestasis. However, magnetic resonance cholangiopancreatography (MRCP) is more sensitive in assessing the location, severity, cause, and extent of biliary obstruction[27]. MRCP images of a patient with suspected acute cholestasis can help: (1) Confirm the obstruction; (2) exclude other causes of jaundice; (3) determine the location of obstruction (intra- or extrahepatic ducts); (4) measure the approximate length of the biliary stricture; and (5) reveal the status of proximal bile ducts[28] (Figure 3).

Apart from MRCP, gadoxetate-enhanced MRI can also aid in evaluating acute cholestasis. Although less widely available than MRCP, it has a unique role in detecting bile leaks after biliary surgery or liver trauma[29].

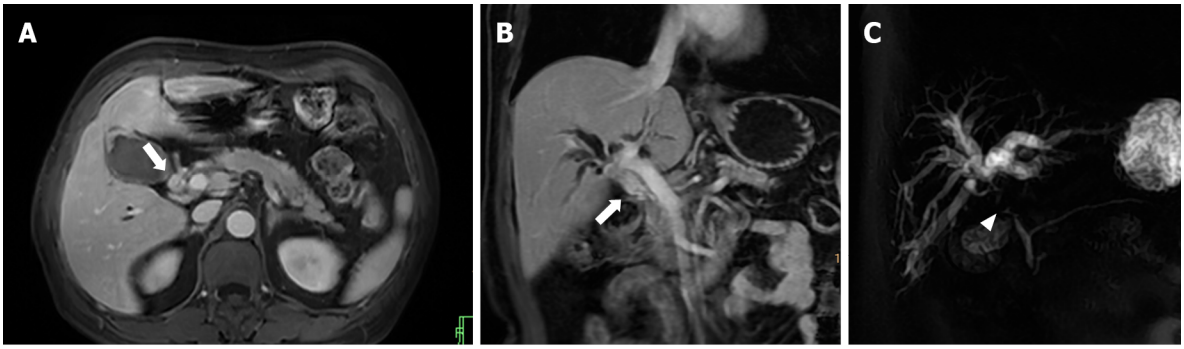
Recently, elastography has also been applied in acute cholestasis. Kim *et al*[30] reported that liver stiffness measured by MRI elastography (MRE) is elevated with the increase of cholestasis, and can be predictive for the sufficiency of biliary decompression after biliary drainage.

Chronic cholestasis

Most chronic cholestatic disorders are insidious in onset, and chronic cholestasis progresses slowly over the course of years before it becomes clinically apparent. The most frequent causes of chronic cholestasis are primary sclerosing cholangitis (PSC) and primary biliary cirrhosis (PBC). Furthermore, allograft rejection can lead to bile duct damage and subsequent chronic cholestasis in patients who have undergone liver transplantation.

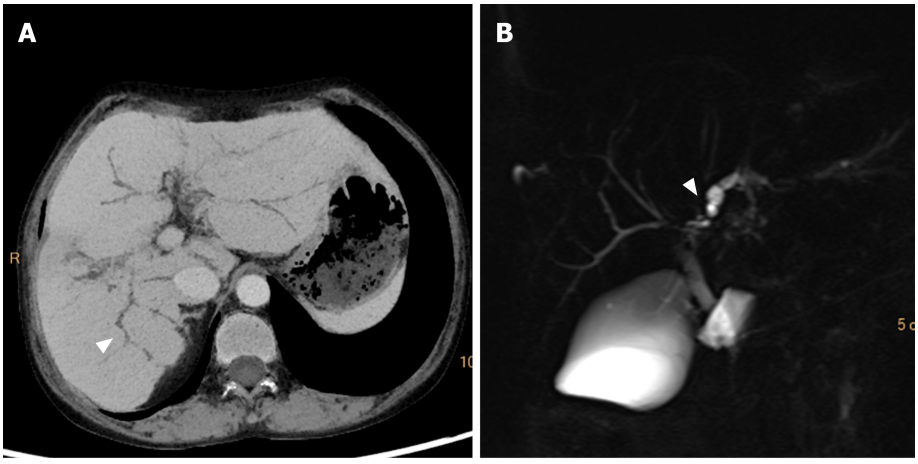
Characteristic imaging features of PSC include thickened concentric mural wall involving the extrahepatic biliary duct, with segmental intrahepatic biliary duct dilatation, preferentially affecting the left hepatic lobe. Gallbladder luminal sludge or stones and inflammatory polyps can also be depicted [31]. On MRCP, PSC can have typical features of biliary ductal changes, such as intrahepatic and extrahepatic short segmental bile duct strictures alternating with normal or mildly dilated bile ducts, giving rise to a beading appearance. At times, mild diffuse dilatation of the entire intrahepatic biliary system with a branching-tree appearance can be observed[32] (Figure 4).

On the other hand, PBC is characterized by chronic, non-suppurative lymphocytic cholangitis that predominantly affects small and interlobular bile ducts in the portal triads, leading to vanishing bile duct syndrome[33]. Diffuse hepatomegaly is the most pronounced morphological change. Patients usually develop micronodular or liver fibrosis. Most early PBCs had normal appearances on MRCP. As disease progresses, intrahepatic bile ducts become irregular. Thereafter, most peripheral branches of the intrahepatic bile ducts gradually become invisible, while medium-sized bile ducts present with reduced caliber and irregularity. These findings could be explained pathologically by destruction and disappearance of small intrahepatic bile ducts in PBC[34]. The assessments of liver function in PSC is



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Figure 3 Magnetic resonance images of a 63-year-old man with hilar cholangiocarcinoma. Axial (A) and coronal (B) portal venous phase images demonstrate thickened hilar bile duct wall (white arrows). The extrahepatic bile duct is absent on magnetic resonance cholangiopancreatography image (C, white arrowhead), and the intrahepatic bile ducts are dilated and distorted ("vine-sign").



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Figure 4 Computed tomography and magnetic resonance cholangiopancreatography images of a-42-year-old woman with primary sclerosing cholangitis. Minimum density projection computed tomography image of portal venous phase (A) and magnetic resonance cholangiopancreatography image (B) show a "beading appearance" of the intrahepatic bile ducts (white arrowheads).

similar to those in cirrhosis caused by chronic hepatitis[35].

In summary, when cholestasis is suspected, ultrasound is recommended for screening. When biliary obstruction or stricture is confirmed, MRI (MRCP in particular) is the preferred modality for further examinations.

METABOLIC DISEASES

Nonalcoholic fatty liver disease

Nonalcoholic fatty liver disease (NAFLD) is defined as liver fat exceeding 5%-10% by weight and exists as a spectrum from steatosis (usually stable) to nonalcoholic steatohepatitis (NASH) (characterized by cellular ballooning, necroapoptosis, inflammation, and fibrosis)[36]. Early detection and treatment of NAFLD can help prevent its progression to NASH and cirrhosis[37].

Among the imaging methods which enable liver fat quantification, transient elastography (TE) is the most widely studied US approach. A recent meta-analysis revealed that in NAFLD patients, the areas under the curve (AUC) of TE were 0.819 for S0 *vs* S1-S3 and 0·754 for S0-S1 *vs* S2-S3[38]. Another meta-analysis reported superior result of TE in the diagnosis of mild steatosis (AUC, 0.96) compared with severe steatosis (AUC, 0.70)[39]. Thus, an insufficient performance for TE in the diagnosis of moderate to severe steatosis should be noted.

The sensitivity and specificity of CT in detecting hepatic steatosis were reported ranging from 46% to 72% and from 88 to 95%, respectively[40]. However, given the potential additive radiation exposure, CT is not typically utilized as a screening test for NAFLD.

In addition, chemical-shift-encoded MRI-based proton density fat-fraction (PDFF) is increasingly accepted as an effective imaging modality in evaluating liver steatosis. A recent meta-analysis which included 2979 patients showed that MRI-PDFF offered pooled sensitivities of 0.71-0.91 and specificities of 0.88-0.93 for staging liver steatosis[41], with the optimal diagnostic performance achieved for detecting ≥ S1 (sensitivity, 0.92; specificity, 0.93) steatosis. Choi *et al*[42] compared the performance of MRI-PDFF and TE-based controlled attenuation parameter (CAP) in staging liver steatosis, and they found that MRI-PDFF correlated far better with hepatic fat measured (r = 0.978) than with CAP (r = 0.727). Besides, several clinical randomized controlled trials have shown that PDFF can be used to monitor and predict the therapeutic effect of NAFLD[43-45].

NASH is also characterized with a distinctive increase in liver extracellular fluid, which can be measured by an increase in T1 relaxation time. However, the accumulation of excess iron in liver tissue can be a confounding factor for T1 relaxation time. In this context, iron-corrected T1 can be generated to correct for this potential bias[46,47]. In a study of 50 patients undergoing standard-of-care liver biopsy for NAFLD, iron-corrected T1 has been demonstrated to correlate with ballooning and could accurately distinguish between steatosis and NASH patients[48].

Collectively, given the costs, availability, and diagnostic performances, US may be an appropriate modality to detect NAFLD. If accurate quantification of liver fat or monitoring of efficacy is needed, MRI PDFF should be a better choice.

Iron storage disorders

Iron storage disorders are characterized by unregulated iron increase or decrease in the liver[49]. An increase in systemic iron can be a consequence of: (1) Hereditary hemochromatosis; (2) ineffective erythropoiesis or chronic liver disease; and (3) parenteral iron administration. Excessive intracellular deposition of iron ultimately results in tissue and organ damage. The diagnosis of iron overload relies on serum iron studies (elevated transferrin saturation and elevated serum ferritin levels), genetic testing, and sometimes liver biopsy to assess the hepatic iron concentration and degree of liver injury [50].

The paramagnetic effect of liver iron on the neighborhood protons affects T2 and T2* relaxation times by accelerating the signal decay. Therefore, the presence of iron results in tissue signal loss on T2 and T2 * weighted images that is proportional to iron content, which is the basic principle of MRI in evaluating liver iron overload[51]. The MRI methods for liver iron quantification can be divided into signal intensity ratio methods and relaxometry methods.

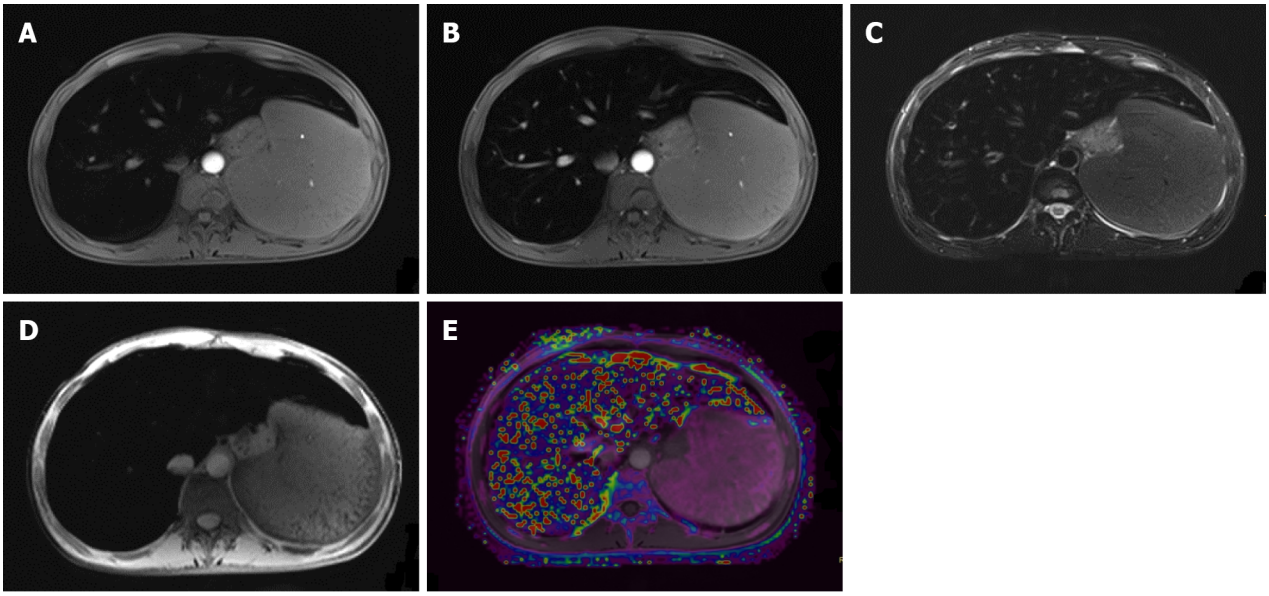
With signal intensity ratios, studies showed that although these methods tended to overestimate mild to moderate hepatic iron overload, it might be more precise in severe iron overload, particularly on 3T MRI[52,53]. On the other hand, relaxometry techniques measure the MR signal decay resulting from the shortening of T2 or T2* relaxation times. For practical purposes, the inverse of T2 or T2* (the relaxation rates, R2 or R2*) is generally used instead, because the elevation in liver iron concentration directly increases the R2 and R2*[54]. The most known R2 relaxometry method is commercially available as FerriScan and is FDA-approved for 1.5T machines[55]. Well validated across different sites and platforms, liver R2 has an excellent correlation with liver iron concentration, with low inter-exam variability and good inter-machine reproducibility[56]. However, major limitations of this technique include long acquisition times and high cost. In contrast, R2* relaxometry is performed with fast, single breath-hold spoiled GRE multi-echo sequences in most MR scanners. Several studies demonstrated an excellent linear relationship between R2* and liver iron concentration[57,58] (Figure 5). However, R2* measurements may be affected by liver fibrosis and the coexistence of fat[59].

Quantitative susceptibility mapping (QSM) was first used in the nervous system. It is based on the concept of transforming hypointense blooming artifacts into precise quantitative measurements of spatial biodistributions. Therefore, it is not affected by liver fibrosis and the coexistence of fat[59]. Tipirneni-Sajja *et al*[60] applied a multispectral autoregressive moving average model in QSM to liver iron concentration. They found that autoregressive moving average-QSM showed a good association with an iron concentration in both phantom study and *in vivo* cohort, indicating that autoregressive moving average-QSM could provide a potentially confounder-free assessment of hepatic iron overload [60].

Therefore, the influence of iron on MRI signal makes MRI the most appropriate imaging modality for quantifying liver iron concentration, and QSM may be the most potential sequence to serve this purpose.

PROGRESSION OF DIFFUSE LIVER DISEASE

Liver fibrosis is a scarring response that occurs in almost all chronic liver injuries mentioned above. Ultimately, liver fibrosis can lead to cirrhosis, in which PH is a common and lethal complication. Early diagnosis and accurate staging of these conditions can facilitate timely patient care and optimize prognoses.



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Figure 5 Magnetic resonance images of a 26-year-old man with hemochromatosis. Pre-contrast T1-weighted image (A), portal venous phase T1-weighted image (B), T2-weighted image (C), and SWI image (D) showed signal intensity in liver parenchyma, while $R2^*$ mapping (E) shows increased signal intensity in the liver, demonstrating severe iron overload. R2: Relaxation rate.

Liver fibrosis

With the deposition of collagen in the extracellular space, liver parenchyma stiffness increases as the disease progresses. These alterations can be measured by elastography techniques.

Among all elastography techniques, TE is the most widely used method to determine liver stiffness and may serve as a potential surrogate to assess liver fibrosis. The pooled AUC of TE for diagnosing liver fibrosis was 0.859 for NAFLD, 0.860 for chronic hepatitis B, and 0.830 for alcohol-related liver disease in previous meta-analyses[61-63]. In addition, shear wave elastography (SWE) was also reported with a high diagnostic accuracy for detecting early-stage liver fibrosis[64-66]. Petzold *et al*[67] found that a cutoff value of 8.05 kPa could differentiate patients with advanced fibrosis (F ≥ 3) from those with no or mild fibrosis (F0-F2) with AUCs ranging between 0.995 and 1.000. A meta-analysis revealed no significant difference between TE and SWE in the diagnosis of significant fibrosis, advanced fibrosis, and cirrhosis, but the proportion of failed measurements was over ten-fold greater with TE than SWE [68].

In addition to ultrasound-based elastography techniques, the MR-based elastography technique MRE is another promising noninvasive modality to assess liver fibrosis[69,70]. A prospective study of 67 PSC patients revealed a high sensitivity (87.5%) and specificity (96%) of MRE in detecting cirrhosis[71]. In another study, a significant discriminatory ability of MRE was confirmed when distinguishing between early to moderate and advanced liver fibrosis, shedding light on the incremental values of liver stiffness measurements on MRE in prognosis stratification[72]. Fu *et al*[73] found that the efficacy of MRE was superior compared with TE in detecting significant fibrosis (AUC: 0.965 *vs* 0.906) and advanced fibrosis (AUC: 0.957 *vs* 0.913). These results were confirmed by a meta-analysis in which the pooled AUC of MRE (0.97) was significantly higher than that of SWE (0.88) in detecting significant fibrosis[74].

As fibrosis progresses, the deposition of fibroglia can lead to enlarged extracellular space. Therefore, liver extracellular volume (LECV) measured by CT or MR T1 mapping can also be used to assess liver fibrosis[75-77]. In a cynomolgus monkey model of NASH, Lyu *et al*[78] found that LECV was significantly correlated with the fibrosis score (r = 0.949), and demonstrated an AUC of 0.945 in diagnosing liver fibrosis.

Diffusion-weighted imaging is a noninvasive technique based on the Brownian motion of water molecules in biological tissue and has shown potential in assessing liver fibrosis[79]. Studies showed that in chronic liver diseases, apparent diffusion coefficients in diffusion-weighted imaging decreased as the degree of fibrosis increased, but this relationship was not statistically significant due to confounding factor of blood microcirculation in the capillaries[80,81]. Recent studies have explored various diffusion models to avoid this influence. Lefebvre *et al*[82] reported that intravoxel incoherent motion parameter with 10 *b*-values was reproducible for liver tissue characterization and that perfusion fraction (f) provided good diagnostic performance for distinguishing dichotomized grades of inflammation. Park *et al*[83] showed that the distributed diffusion coefficient from the stretched exponential model was the most accurate diffusion-weighted imaging parameter for staging liver fibrosis as it could avoid the confounding effect by steatosis.

Besides, liver fibrosis can result in changes in hepatic microcirculation and perfusion. Fan *et al*[84] found that MR perfusion parameters, time to peak, and mean transit time in particular could reflect the degree of liver fibrosis. Similarly, Yoon *et al*[85] also found that portal blood flow was significantly lower in clinically significant hepatic fibrosis and that mean transit time and extracellular volume increased in cirrhosis.

In general, TE is the modality preferred for LF. SWE can be considered in patients who fail in TE examination. As a modality which is gaining increasingly popularity, MRE is preferred over sonographic elastography in patients with ascites and obesity, or requiring more comprehensive liver workup.

PH

PH is defined by values of hepatic venous pressure gradient (HVPG) > 5 mmHg, whereas clinically significant PH could be diagnosed if HVPG ≥ 10 mmHg. HVPG has been widely-validated as associated with variceal bleeding, hepatic decompensation, and mortality. However, its measurement is invasive and requires extensive expertise[86].

Characteristic imaging features of PH include portosystemic shunts, splenomegaly, ascites, and widening of the portal vein. However, these findings are often detectable at end stages of the disease, thus demonstrating limited sensitivities for diagnosing PH.

For quantitative methods, similar to liver fibrosis, elastography techniques have gained increasing attention in the assessment of PH[87]. Among ultrasound-based elastography techniques, TE was the most validated method for PH assessment. A meta-analysis involving 12 studies showed that liver stiffness measured on TE was well correlated with HVPG and demonstrated a sensitivity of 91.2% and specificity of 81.3% in diagnosing clinically significant PH (cut-off values 13.6-18.6 kPa)[88]. In contrast, despite much less applied than TE, SWE also exhibited encouraging profiles in predicting PH and esophageal varices (AUC: 0.86-0.89)[89-93].

Liver and spleen stiffness measured by MRE also showed promising performances in predicting PH and esophageal varices. A recent meta-analysis found that liver and spleen stiffness on MRE could serve as supplemental noninvasive assessment tools for detecting clinically significant PH and that spleen stiffness might be more specific and accurate than liver stiffness (AUC: 0.88 *vs* 0.92)[94].

Hemodynamic alteration is another distinct feature in PH. In patients with cirrhosis, decreased portal and total hepatic perfusion were observed[95,96]. Studies showed that mean portal vein velocity in cirrhosis was lower than that in normal subjects and decreased with the severity of liver cirrhosis and gastroesophageal varices[97,98]. The portal vein velocity measured by doppler US could be used as noninvasive triage tests before referral to endoscopy (sensitivity, 84%-97%), but the cutoff value varied from 16-19 cm/s[99,100]. Several MR techniques have also been proposed for liver hemodynamic assessment in PH. Chouhan *et al*[101] used phase-contrast MR to assess the portal vein and the infrahepatic and suprahepatic inferior vena cava. The hepatic artery flow was estimated by subtracting infrahepatic from suprahepatic inferior vena cava flow and portal vein flow, which showed significant positive correlations with HVPG[101]. Additionally, 4D flow MRI also demonstrated promising capacity in quantifying blood flow in the hepatic and splanchnic vasculature[102,103]. Motosugi *et al*[104] found that azygos flow > 0.1 L/min and portal venous flow less than the sum of splenic and superior mesenteric vein flow were useful markers to stratify the risk of gastroesophageal varices bleeding in patients with cirrhosis. Another study revealed that the combination of liver stiffness measured by MRE and perfusion metrics measured by contrast-enhanced-MRI had an AUC of 0.903 for diagnosing PH, and an AUC of 0.785 for detecting clinically significant PH[105].

In summary, TE and SWE are promising noninvasive approaches for preliminary PH screening. Nevertheless, for patients with increased risk for esophageal and gastric varices, multiparametric MRI may be a more accurate and comprehensive modality.

FOCAL LIVER LESIONS

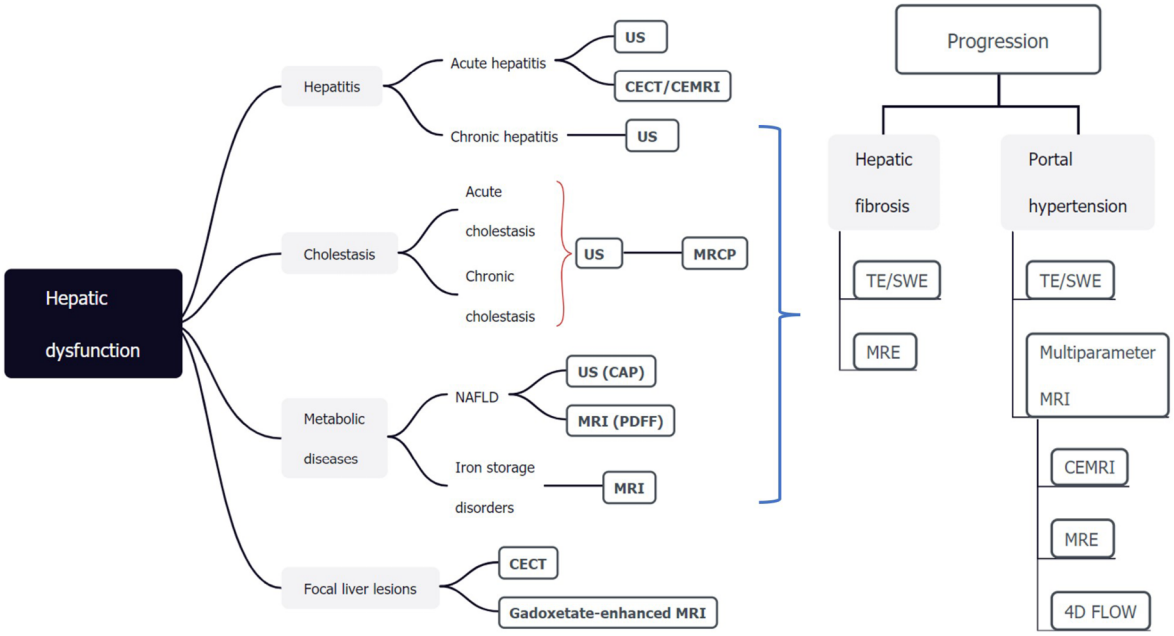
Focal liver lesions include benign tumors, malignant tumors, and hepatic echinococcosis. The impact of focal liver lesions on liver function includes the decrease of normal liver volume and the reduced hepatocyte function, especially in surgical candidates with malignant liver tumors. Previous studies have shown that a high residual to total liver volume ratio (≥ 40%) was required for patients with an impaired liver function to tolerate resection[106-108]. Gadoxetate-enhanced MRI is also used to evaluate the hepatic function of patients with focal liver lesions. Yoon *et al*[109] reported that T1 mapping on gadoxetate-enhanced MRI provided information on global liver function and demonstrated functional heterogeneity in patients with HCC. Other studies have combined liver volume with hepatocyte function, and their results showed that combined T1 mapping and residual liver volume on gadoxetate-enhanced MRI could assess liver function with good diagnostic accuracy in patients with liver tumors [110-112]. Kim *et al*[113] and Wang *et al*[114] reported that the combination could predict post hepatectomy liver failure better than the ICG clearance test in patients with HCC who underwent hepatectomy.

To sum up, CT can be used to calculate the residual liver volume for surgical candidates. Gadoxetate-enhanced MRI can not only reflect residual liver volume, but also reveal the functional information of hepatocytes.

CONCLUSION

In this article, we provide a summary of noninvasive imaging modalities for assessing hepatic dysfunction in various clinical situations and case scenarios (Figure 6). Several challenges still exist in noninvasive imaging of hepatic dysfunction. First, many imaging parameters have inconsistencies on the device. Therefore, a unified threshold cannot be adopted. Second, quantification of sensitivity and specificity usually requires an effective reference standard (*e.g.*, liver biopsy) which may not be readily available. Furthermore, most of the current studies focus on the role of a single method or sequence, with limited multiparametric, multimodal, and multidisciplinary approaches to evaluate liver dysfunction.

The long-term goal in hepatic dysfunction imaging is to develop reliable, noninvasive, and comprehensive methods which could reveal not only the disease severities but also etiologies using safe and clinically available techniques. However, to accomplish this goal will require advances in imaging sciences (improved image modalities standardization and quantitation, further exploration of US, CT, and MR imaging methods, and combination of multiparametric and multimodal imaging techniques). On this basis, radiomics and artificial intelligence may provide further assistance in quantifying high-level imaging features beyond human eyes and help in constructing effective predictive models. A better understanding of the human genetic variation underlying differences in the liver will further contribute to this field. Furthermore, the potential value of combining imaging and serum biomarkers should also be explored.



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Figure 6 Noninvasive imaging modalities for assessing hepatic dysfunction. The TextTitle modalities are recommended and should be the first-line methods. US: Ultrasound; CT: Computed tomography; MRI: Magnetic resonance imaging; TE: Transient elastography; SWE: Shear wave elastography; MRE: MRI elastography; MRCP: Magnetic resonance cholangiopancreatography; NAFLD: Nonalcoholic fatty liver disease; PDFF: Proton density fat-fraction; CAP: Controlled attenuation parameter; CECT: Contrast-enhanced CT; CEMRI: Contrast-enhanced MRI.

FOOTNOTES

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Country/Territory of origin: China

ORCID number: Ting Duan 0000-0001-6694-4520; Han-Yu Jiang 0000-0002-7726-1618; Wen-Wu Ling 0000-0002-6449-3831; Bin Song 0000-0001-7007-6367.

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REVIEW

Small nucleolar RNA host gene 3 functions as a novel biomarker in liver cancer and other tumour progression

Dan-Dan Shan, Qiu-Xian Zheng, Jing Wang, Zhi Chen

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Dan-Dan Shan, Qiu-Xian Zheng, Jing Wang, Zhi Chen, State Key Laboratory for Diagnosis and Treatment of Infectious Diseases, National Clinical Research Center for Infectious Diseases, National Medical Center for Infectious Diseases, Collaborative Innovation Center for Diagnosis and Treatment of Infectious Diseases, The First Affiliated Hospital, Zhejiang University School of Medicine, Hangzhou 310003, Zhejiang Province, China

Corresponding author: Zhi Chen, MD, PhD, Professor, State Key Laboratory for Diagnosis and Treatment of Infectious Diseases, National Clinical Research Center for Infectious Diseases, National Medical Center for Infectious Diseases, Collaborative Innovation Center for Diagnosis and Treatment of Infectious Diseases, The First Affiliated Hospital, Zhejiang University School of Medicine, No. 79 Qingchun Road, Shangcheng District, Hangzhou 310003, Zhejiang Province, China. zjuchenzhi@zju.edu.cn

Abstract

Cancer has become the most life-threatening disease in the world. Mutations in and aberrant expression of genes encoding proteins and mutations in noncoding RNAs, especially long noncoding RNAs (lncRNAs), have significant effects in human cancers. LncRNAs have no protein-coding ability but function extensively in numerous physiological and pathological processes. Small nucleolar RNA host gene 3 (SNHG3) is a novel lncRNA and has been reported to be differentially expressed in various tumors, such as liver cancer, gastric cancer, and glioma. However, the interaction mechanisms for the regulation between SNHG3 and tumor progression are poorly understood. In this review, we summarize the results of SNHG3 studies in humans, animal models, and cells to underline the expression and role of SNHG3 in cancer. SNHG3 expression is upregulated in most tumors and is detrimental to patient prognosis. SNHG3 expression in lung adenocarcinoma remains controversial. Concurrently, SNHG3 affects oncogenes and tumor suppressor genes through various mechanisms, including competing endogenous RNA effects. A deeper understanding of the contribution of SNHG3 in clinical applications and tumor development may provide a new target for cancer diagnosis and treatment.

Key Words: Small nucleolar RNA host gene 3; Long noncoding RNAs, Biomarker; Clinical characters; Molecular mechanism; Competing endogenous RNA; Liver cancer

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Core Tip: This review explores the differential expression of small nucleolar RNA host gene 3 (SNHG3) as a novel lncRNA in hepatocellular carcinoma as well as other tumours. SNHG3 is upregulated in most tumours and can influence tumourigenesis and progression through competing endogenous RNA effects and signalling pathways, thereby adversely affecting patient prognosis. Therefore, SNHG3 may become a new target for the diagnosis and treatment of many cancers, including hepatocellular carcinoma.

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INTRODUCTION

Cancer has become one of the significant sources of mortality worldwide. Approximately 14.1 million individuals are newly diagnosed each year, and approximately 8.2 million deaths occurred in 2021 based on GLOBOCAN[1]. Despite considerable achievements and advances in clinical practices, cancer remains a dreadful illness with a poor prognosis and high health burden, which presents a global threat to health[2]. Cancer is a heterogeneous disease with substantial genotypic and phenotypic diversity[3]. Genomic regulation of the cancer genome plays a vital role in cancer initiation, progression, and metastasis[4,5]. It is of most importance to explore the intricate link underlying cancer development and progression.

Long noncoding RNAs (lncRNAs) are a class of noncoding RNAs (ncRNAs) with no protein-coding ability[6]. Evidence has shown that lncRNAs participate in genomic regulation at the transcriptional, translational, and epigenetic levels[6,7]. The regulatory functions of lncRNAs include activation and silencing of genes[8,9], recruitment of epigenetic regulator[10], modification of RNA interactions[11], transcriptional and posttranscriptional processes[12], mRNA decay[13], and protein recruitment[14]. LncRNA-associated regulatory functions are dynamically regulated in a cell-, tissue-, development- and distribution-specific manner[15]. LncRNAs in the cytoplasm may act as sponges, stabilizing mRNAs and regulating the mRNA translation process, thus modifying downstream target gene expression[16]. LncRNAs located in the nucleus may play "cis-acting" or "trans-acting" functions[17-19]. Recently, growing evidence demonstrates that lncRNAs are involved in various cancer functions and pathological processes, particularly in the initiation and progression of tumors[20]. LncRNAs regulate various malignant activities, including tumor progression, proliferation, apoptosis, migration, invasion, chromatin remodeling, metabolism[21-23].

More recently, small nucleolar RNAs can be encoded by several lncRNAs called small RNA host genes, which are significantly differentially expressed in many diseases, including cancers[24]. Studies have unveiled that small nucleolar RNA host gene 3 (SNHG3), which is affiliated with the lncRNA class, plays an influential regulatory role in cancer initiation, development, progression, and tumor-associated microenvironment formation. LncRNA SNHG3 is located in 1p35.3, as shown in Figure 1 (GeneCards, http://www.genecards.org). SNHG3 is mainly localized to the nucleus, mitochondrion, and extracellular space. Although many studies on SNHG3 and tumors have been published, clinical studies and research remains limited. This paper reviews the upregulation of SNHG3 expression in most human tumors and its relationship with negative prognosis as well as the role of SNHG3 in the mechanism of tumorigenesis. Finally, it was concluded that SNHG3 can function as a biomarker in the diagnosis and prognosis of different cancers.

CLINICAL CHARACTERS OF LNCRNA SNHG3

Growing evidence has demonstrated that SNHG3 acts as an oncogene and plays a critical regulatory role in the initiation and development of various cancers. SNHG3 exhibits characteristic oncogenic properties in multiple cancers. Studies have indicated that SNHG3 is significantly upregulated in hepatocellular carcinoma (HCC)[25,26] and other cancers, such as cervical cancer (CC)[27,28], and breast cancer (BC)[29-34]. Clear cell renal cell carcinoma (ccRCC)[35], colorectal cancer (CRC)[36-38], glioma [39,40], HCC[25,41,42], non-small cell lung cancer (NSCLC)[43-47], ovarian cancer (OC)[48-51], and papillary thyroid carcinoma (PTC)[52]. Different views exist on SNHG3 expression in lung adenocarcinoma, as shown in Table 1. Aberrant SNHG3 expression might represent a prognostic prediction value and therapeutic target in various cancers.

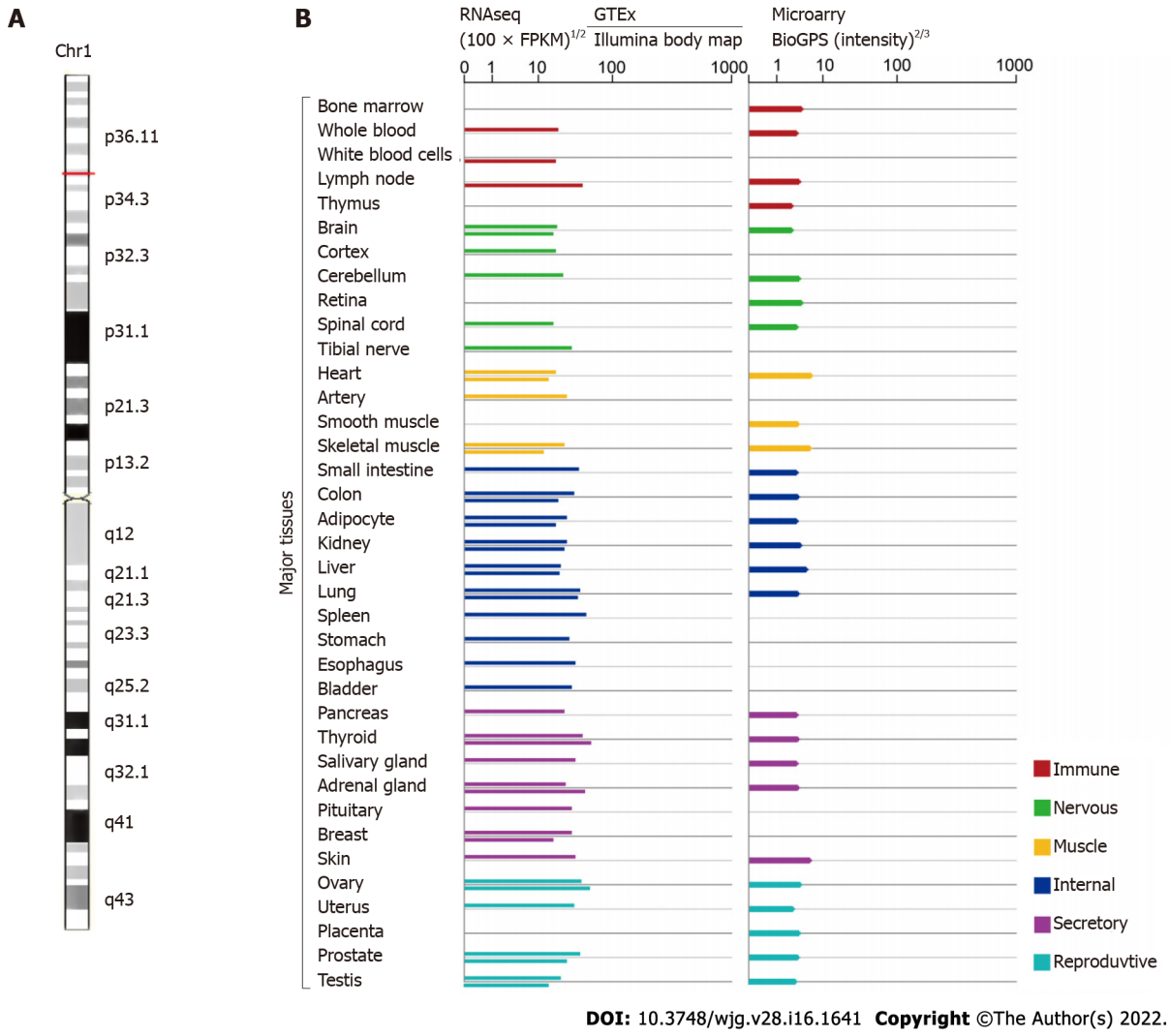
Table 1 The expression of small nucleolar RNA host gene 3 and its clinical significance in liver cancer and other cancers

Cancer types	Cases	Expression	Clinicopathologic parameters	PMID
Hepatocellular carcinoma	47 pairs	Upregulated	Tumor stage, lymph node metastasis, distant metastasis	31548493
Hepatocellular carcinoma	195 pairs	Upregulated	Tumor size, PVTT, relapse	26373735
Breast cancer	30 pairs	Upregulated		32495883
Breast cancer	60 pairs	Upregulated	Histological grade, TNM stage, lymph node metastasis, ER, Her-2	31586299
Breast cancer	60 pairs	Upregulated		32883233
Breast cancer	48 pairs	Upregulated		32945476
Cervical cancer	88 pairs	Upregulated	FIGO stage, metastasis	34816392
Clear cell renal cell carcinoma	36 pairs	Upregulated	Distant metastasis, T stage, pathological TNM stage, histologic grade	31505165
Colorectal cancer	58 pairs	Upregulated	Tumor stage, distant metastasis	32187965
	50 pairs	Upregulated		34661273
Esophageal cancer	384 pairs	Upregulated		33596916
Gastric cancer	60 pairs	Upregulated		32930970
Glioma	42 pairs	Upregulated	Tumor size, WHO grade	33817254
Glioma	8 low-grade and 8 high-grade and 8 normal tissues	Upregulated		34153159
Laryngeal carcinoma	18 pairs	Upregulated		31238052
Laryngeal carcinoma	25 pairs	Upregulated		32538668
Lung Adenocarcinoma	65 pairs	Downregulated	TNM stage, lymph node metastasis	32538668
Non-small-cell lung cancer	35 pairs	Upregulated	TNM stage, lymph node metastasis, tumor size	34132359
Non-small-cell lung cancer	32 pairs	Upregulated	TNM stage, lymph node metastasis, tumor size	31602642
Non-small-cell lung cancer	42 pairs	Upregulated	Stage, lymph node metastasis	33177840
Non-small-cell lung cancer	15 pairs	Upregulated		34032148
Oral squamous cell carcinoma	30 pairs	Upregulated		32989886
Osteosarcoma	54 pairs	Upregulated		30797154
Osteosarcoma	127 pairs	Upregulated		30791797
Ovarian cancer	18 pairs	Upregulated		29921511
Ovarian cancer	96 pairs	Upregulated	FIGO stage, histological grade, lymph node metastasis	33149611
Ovarian cancer	76 pairs	Upregulated	FIGO stage, lymph node metastasis	29758922
Ovarian cancer	40 patients with OC and 19 patients with benign OC	Upregulated		33552243
Papillary thyroid carcinoma	72 pairs	Upregulated	TNM stage, lymph node metastasis	32048306
Prostate cancer	50 pairs	Upregulated		33416420

PVTT: Portal vein tumor thrombosis; TNM: Tumor-node-metastasis; FIGO: International Federation of Gynecology and Obstetrics; WHO: World Health Organization.

HCC

HCC is one of the most widespread cancerous malignancies, ranking third in cancer-related deaths[53]. Cures for HCC tend to be more effective when used at an early stage[54]. However, patients at this stage



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Figure 1 Small nucleolar RNA host gene 3 genomic information (GeneCards, http://www.genecards.org). A: The chromosomal localization of small nucleolar RNA host gene 3 (SNHG3); B: The expression level of SNHG3 in normal human tissues.

do not have specific symptoms or lack biomarkers for early diagnosis, which usually delays diagnosis. Accordingly, the development of biomarkers for early diagnosis and prognosis is urgent. Recent studies have shown that SNHG3 expression is upregulated in HCC tissues compared with normal tissues and positively correlates with tumor stage ($P < 0.001$), tumor size ($P = 0.003$), lymph node metastasis ($P < 0.001$), distant metastasis ($P < 0.001$), portal vein tumor thrombosis ($P = 0.014$), and relapse ($P = 0.038$) [25,26]. Based on the above evidence that SNHG3 influences HCC metastasis and growth, SNHG3 has the potential to be a reliable biomarker for the diagnosis and treatment of HCC.

BC

BC accounts for 30% of all female tumors and is the second leading contributor to cancer deaths in women[55]. Early detection of BC can reduce mortality and improve patient survival[56-58]. The discovery of a new biomarker appears critical. Ma *et al*[31] reported elevated SNHG3 expression in BC tissues and correlated it with tumor malignancy. They also reported that SNHG3 expression was associated with clinicopathological features, such as histological grade ($P = 0.016$), tumor-node-metastasis (TNM) stage ($P = 0.001$), lymph node metastasis ($P < 0.001$), estrogen receptor ($P = 0.009$), and Her-2 ($P = 0.001$)[31]. ER and Her-2 are major molecular targets in the pathogenesis of BC and are associated with prognosis and treatment options for BC[59]. Therefore, SNHG3 plays a carcinogenic role in the progression and prognosis of BC. Therefore, SNHG3 may play an oncogenic role in the progression and prognosis of BC.

CC

CC is the fourth most prevalent cancer among women worldwide in terms of incidence and mortality [53]. The survivability of patients with advanced CC is not desirable, and early diagnosis and treatment of CC improve its prognosis[60]. It is important to further explore the mechanisms of CC development

to develop new therapeutic approaches[61]. The function of SNHG3 in CC has been recognized in several studies. Zhu *et al*[27] published that SNHG3 expression was obviously upregulated in CC tissues, and high SNHG3 expression affected International Federation of Gynecology and Obstetrics (FIGO) stage (P = 0.011) and metastasis (P = 0.018), implying that SNHG3 is a possible prognostic biomarker and a target for treatment in CC.

ccRCC

With high morbidity and mortality, ccRCC is the most common and serious type of renal cell carcinoma [62]. Using TCGA and GEO databases, Zhang *et al*[35] found that lncRNA SNHG3 expression was elevated in ccRCC and positively correlated with many clinicopathological parameters. They further quantified the relative expression of SNHG3 in ccRCC tissues compared with normal tissues using qRT–PCR and discovered that the relative expression of SNHG3 was increased in ccRCC tissues[35]. These results revealed that upregulated SNHG3 expression may play an essential role in the ccRCC pathway.

CRC

CRC is a dominant reason for morbidity and mortality worldwide, yet greater than half of patients have progressed to stage II/III at the time of diagnosis. The treatment of CRC typically consists of curative resection *via* surgery followed by adjuvant chemotherapy to reduce the risk of recurrence[63]. Therefore, it is critical to further elucidate the mechanisms of CRC and to pursue the development of effective targeted therapies. SNHG3 expression was remarkably upregulated in CRC tissues compared to nearby normal tissues and correlated with poor outcomes in CRC[36-38]. Moreover, Wen *et al*[36] explored the connection between SNHG3 and clinicopathological parameters, and the results reflected a close association with tumor stage (P = 0.006) and distant metastasis (P = 0.0033). Overall, SNHG3 promotes the progression of CRC, leading to an unfavorable prognosis.

Glioma

Glioma is a fatal brain tumor that can be treated with radiation and surgery, but the median survival of patients is only approximately 14-17 mo[64]. Effective curative treatments for glioma are lacking. Thus, it is especially vital to determine the molecular mechanisms of glioma and identify novel strategies for treatment[65]. SNHG3 is highly expressed in glioma tissues compared to normal tissues[39,40]. Zhang *et al*[39] explored the relationship between SNHG3 expression and the clinicopathological features of GM patients, including tumor size (P = 0.0300) and World Health Organization classification (P = 0.0278). These studies indicated that SNHG3 offer new insights for the future treatment of glioma.

Lung cancer

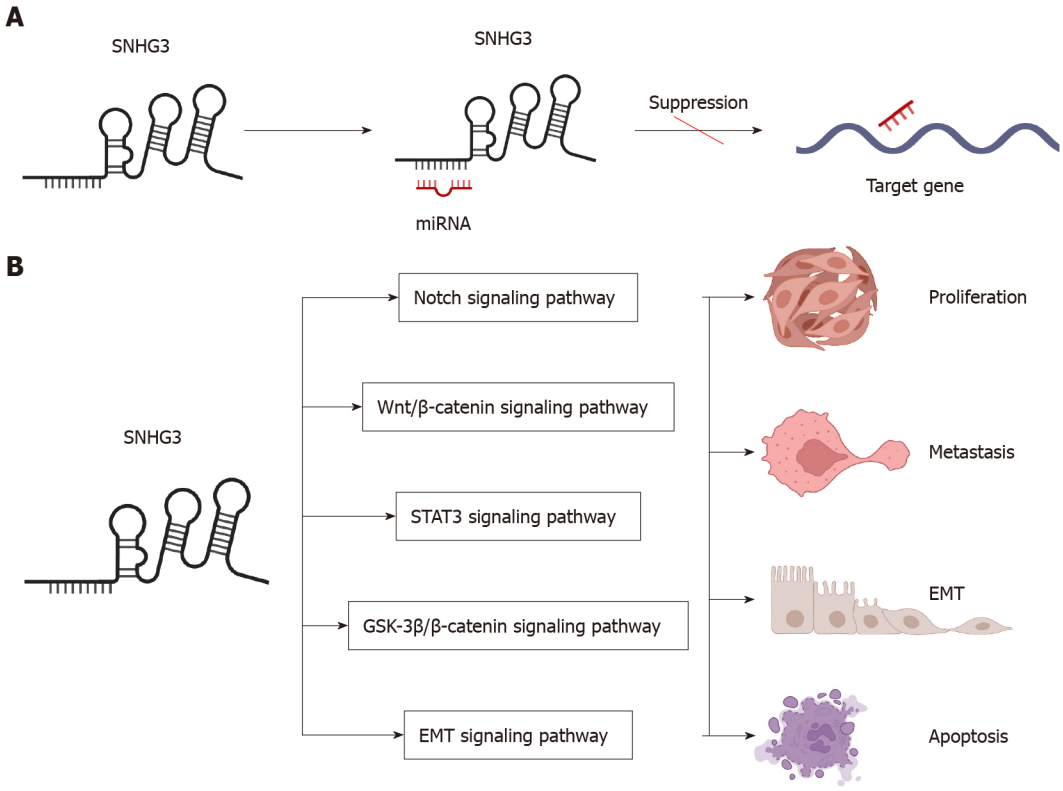
Lung cancer is one of the cancers with the highest mortality and morbidity rates worldwide[66], with NSCLC accounting for 85% of lung cancer cases[67]. Shi *et al*[44] revealed that high expression of lncRNA SNHG3 in NSCLC tissues was associated with TNM stage (P = 0.0053), lymph node metastasis (P = 0.0006), and tumor size (P = 0.0005). Thus, elevated SNHG3 expression is relevant to the lower overall survival (OS) rate of NSCLC patients. Liu *et al*[67] analyzed the TCGA database as well as the GSE19804 public database and found high SNHG3 expression in lung adenocarcinoma. These researchers used Kaplan–Meier plotter database analysis to determine that high SNHG3 expression decreases the OS time of lung adenocarcinoma patients. However, Kang *et al*[68] used quantitative polymerase chain reaction to show that SNHG3 expression levels were decreased in lung adenocarcinoma tissues, and Kaplan–Meier analysis demonstrated that patients with low SNHG3 expression levels had a short OS. In conclusion, SNHG3 is associated with unfavorable prognosis in lung cancer patients, but the expression and function of SNHG3 in lung adenocarcinoma still requires further research.

Ovarian cancer

OC claims the lives of 151900 women worldwide each year and is one of the leading contributors to death in women[69]. Because there are no specific symptoms in the early stages, greater than 79% of OC patients reach stage III or IV[70]. The lack of effective biomarkers translates to a poor prognosis in OC patients. Recently, Hong *et al*[50] described high SNHG3 expression in OC tissues compared with normal tissues adjacent to cancer, and a high SNHG3 expression level was positively correlated with the FIGO stage (P = 0.007) and lymph node metastasis (P = 0.001) of OC patients. This finding suggests that high SNHG3 expression may be an individual prognostic factor affecting OS in OC patients.

Papillary thyroid carcinoma

PTC is the most common type of thyroid cancer, and the incidence of PTC is increasing[71]. The high rate of metastases in the cervical lymph nodes of PTC patients is closely related to the recurrence of PTC [72,73]. It seems relevant to study the mechanism of metastasis in PTC. Sui *et al*[52] analyzed 42 pairs of tissues and determined that SNHG3 expression was higher in PTC tissues compared with controls. High SNHG3 expression was positively correlated with TNM stage (P = 0.014) and lymph node metastasis (P



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Figure 2 Small nucleolar RNA host gene 3-mediated mechanisms regulating cancer progression. A: Small nucleolar RNA host gene 3 (SNHG3) as a ceRNA; B: SNHG3 is involved in signaling pathways. SNHG3: Small nucleolar RNA host gene 3; EMT: Epithelial–mesenchymal transition.

= 0.0292) in advanced PTC. The above results suggest that SNHG3 could be a carcinogenic lncRNA in PTC.

Researchers have elucidated the high expression of SNHG3 in acute myeloid leukemia (AML)[74], esophageal cancer[75], gastric cancer (GC)[76-78], laryngeal carcinoma[79,80], oral squamous cell carcinoma[81,82], osteosarcoma[83,84], and prostate cancer[85,86], but have not yet explored the relationship between SNHG3 and clinicopathological features. Subsequent investigations into the biological mechanisms of SNHG3 in other diseases will further support the potential of SNHG3 as a new diagnostic and therapeutic biomarker.

SNHG3 *IN VIVO* STUDIES

SNHG3 may function as an oncogene and has the potential to become a novel potential therapeutic target for many cancers. Several studies have used animal models to explore the effect of SNHG3 *in vivo*. Zhao *et al*[25] assessed the role of SNHG3 in HCC growth *in vivo*. They injected stable SNHG3-depleted HepG2 cells subcutaneously into the right dorsum of female BALB/c nude mice and measured the volume of tumor. The results showed that SNHG3depleted tumors grew significantly slower than that of controls. HCC growth was inhibited *in vivo* due to the knockdown of the SNHG3 gene[25]. Similarly, SNHG3 gene knockdown inhibited the growth of other tumors, such as BC[34], CC[27], ccRCC[35], CRC [36,37], GC[77], glioma[40], laryngeal carcinoma[80], NSCLC[45,46], OC[49], PTC[52], prostate cancer [85]. Overall, SNHG3 promoted the progression of the abovementioned tumors.

BIOLOGICAL FUNCTION OF SNHG3 IN CANCER

In the following section, we will explore the biological function of SNHG3 in different cell lines. Many works have shown that SNHG3 affects cancer cell proliferation, epithelial-mesenchymal transition (EMT)[25,41,44,47,85-87], apoptosis, invasion, migration, and metabolism[29,48] specifically through the action of different molecular mechanisms. In HCC, SNHG3 affects cell proliferation, apoptosis, metastasis and invasion mainly by means of competing endogenous RNA (ceRNA) and EMT signaling pathway, as shown in Figure 2 and Table 2.

Table 2 Function and mechanism of small nucleolar RNA host gene 3 in liver cancer and other cancers

Cancer types	Assessed cancer cell lines	Expression	Related genes and pathways	Biological significance	PMID
Hepatocellular carcinoma	PLC/PRF/5, Hep3B, HepG2, MHCC97L, Huh7, SMMC-7721, HCCLM3	Up	miR-128, CD151	Invasion, EMT, sorafenib resistance	30132868
Hepatocellular carcinoma	HepG2, HCCLM3	Up	miR-326, SMAD3, ZEB1	Proliferation, migration, EMT, anti-apoptosis	31548493
Hepatocellular carcinoma	HepG2, HuH-7	Up	miR-214-3p, ASF1B		34336642
Acute myeloid leukemia	HL-60,THP-1,KG-1, NB4, MOLM-14	Up	miR-758-3p, SRGN	Proliferation, anti-apoptosis	31452272
Bladder cancer	5637,T24	Up	miR-515-5p, GINS2	Proliferation, migration, invasion, EMT	32596993
Breast cancer	MCF-7, MD-MBA-453	Up	miR-330, PKM	Proliferation, mitochondrial metabolism	31956955
Breast cancer	MDA-MB-231, BT-549, MDA-MB-468	Up	miR-326, ITGA5 Vav2/Rac1	Viability, migration, invasion, anti-apoptosis	32495883
Breast cancer	MDA-MB-231, MCF-7	Up	miR-384, HDGF	Proliferation, migration, invasion	31586299
Breast cancer	MCF-7, MDA-MB-231, HCC1937, BT474, SKBr-3	Up	miR-154-3p, Notch	Proliferation, migration, invasion	32883233
Breast cancer	MDA-MB-231, MCF-7	Up	miR-186-5p, ZEB1	Proliferation, migration, invasion	33594311
Breast cancer	MCF-7, MDA-MB-231, MDA-MB-468, BT-474	Up	miR-326	Proliferation, migration, invasion	32945476
Cervical cancer	C33A, HeLa, SiHa, CaSki and HCC94	Up	YAP1	Proliferation, migration, invasion	34816392
Cervical cancer	SiHa	Up		Proliferation, migration, invasion	34238747
Cholangiocarcinoma	HCCC9810, QBC939, RBE, HUCCT1	Up	miR-3173–5p, ERG	Proliferation, migration, invasion	34647226
Clear cell renal cell carcinoma	ACHN, A498, Caki-1	Up	miR-139-5p, TOP2A	Proliferation, migration, invasion	31505165
Colorectal cancer	HCT116, LoVo, SW480, and SW620	Up	miR-539, RUNX2	Proliferation, migration, invasion	32187965
Colorectal cancer	HT29, HCT116, SW480, and LoVo	Up	miR-182-5p, c-Myc	Proliferation, migration, invasion	28731158
Colorectal cancer	SW480, SW620, HCT8, HT29	Up	miR-370-5p, EZH1	Proliferation, migration, invasion	34661273
Esophageal cancer	KYSE-150, Eca-9706	Up	miR-186-5p, METTL3, m6A		33596916
Gastric cancer	MGC-803, AGS, BGC-823, SGC-7901, MKN-45, HGC-27	Up	EZH2, MED18	Proliferation, migration, invasion	31534128
Gastric cancer	SGC7901, BGC823	Up	IL-6, STAT3, SNHG3, miR-3619-5p, ARL2	Proliferation	32930970
Gastric cancer	HGC-27, GC9811-P	Up	miR-326, TWIST	Proliferation, migration, invasion	34257656
Glioma	A172, SHG44	Up	miR-384, HDGF	Proliferation, migration, invasion, anti-apoptosis	33817254
Glioma	U-87-MG, U-251-MG	Up	miR-485-5p, LMX1B	Proliferation, migration, invasion	34153159
Laryngeal carcinoma	16HBE, TU212, TU686	Up	miR-384, WEE1	Proliferation, migration, invasion	31238052
Laryngeal carcinoma	TU177, AMC-HN-8	Up	Wnt/β-catenin, miR-340-5p, YAP1	Proliferation, migration, anti-apoptosis, mitochondrial metabolism	32538668
Lung adenocarcinoma	A549, H1299	Up		Proliferation, cell cycle,anti-apoptosis	30154938
Lung adenocarcinoma	A549, H1299, H1975	Down	miR-890	Anti-proliferation, anti-migration, anti-invasion, apoptosis	34306585
Non-small-cell lung cancer	H1299, H358, A549, H1975	Up	miR-13433p, NFIX	Proliferation, migration, invasion	34132359

Non-small-cell lung cancer	CMT-167, LLC, CMT-170, CMT-181	Up	TGF-β pathway, IL-6, JAK2, STAT3 pathway	Proliferation, migration, EMT	31602642
Non-small-cell lung cancer	SPC-A1, A549, NCI-H23, NCI-H460	Up	miR-340-5p	Lymph node infiltration, distant metastases	33225676
Non-small-cell lung cancer	A549, H322, H1299, GLC-82, SPC-A1	Up	miR-216a, ZEB1	Proliferation, migration, invasion	33177840
Non-small-cell lung cancer	A549, HCC827, H2170, H520	Up	miR-515-5p, SUMO2	Proliferation, migration, invasion, EMT	34032148
Oral squamous cell carcinoma	SCC4, CAL-27	Up	miR-2682-5p, HOXB8	Proliferation, migration	32989886
Oral squamous cell carcinoma	SCC-15, SCC-9, CAL-27, HN5	Up	Wnt/β-catenin, NFYC	Proliferation, migration	31762107
Osteosarcoma	Saos2, MG63, U2OS, HOS	Up	miR-151a-3p, RAB22A	Proliferation, migration, invasion	30797154
Osteosarcoma	MG-63, 143B, HOS, SW-1353, Saos-2, U-2OS	Up	miR-196a-5p	Proliferation, colony formation	30791797
Ovarian cancer	SK-OV3, TOV-21G, and OVCAR-3	Up		Energy metabolism	29921511
Ovarian cancer	SKOV3, HeyA8, A2780	Up	miR-339-5p, TRPC3	Proliferation, anti-apoptosis	33149611
Ovarian cancer	SKOV3, 60OVCAR3, A2780, ES2	Up	GSK3β/β-catenin	Proliferation, migration	29758922
Ovarian cancer	A2780, SKOV3, OVCAR3, OV90	Up	miR-139-5p, Notch1	Proliferation, migration	33552243
Papillary thyroid carcinoma	BCPAP, TPC-1	Up	miR-214-3p, PSMD10	Migration, invasion, proliferation, and colony formation	32048306
Prostate cancer	PC3, DU145, 22RV1, NCaP	Up	miR-577, SMURF1	Proliferation, migration, EMT, anti-apoptosis	32248648
Prostate cancer	PC3, 22RV1, DU145, LNCaP	Up	miR-487a-3p,TRIM25	Viability, migration, invasion, EMT	33416420

EMT: Epithelial–mesenchymal transition.

SNHG3 function as a ceRNAs in cancers

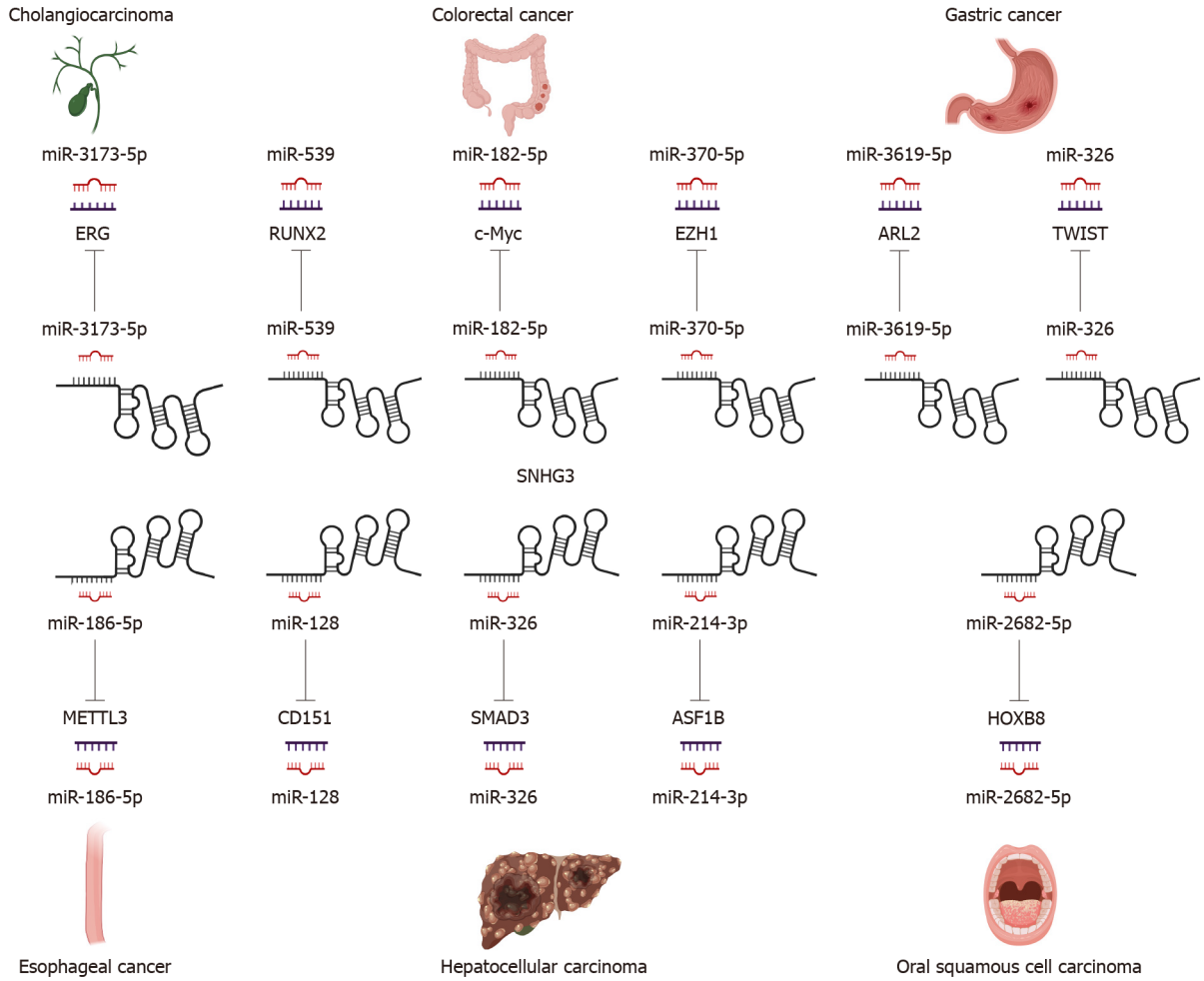
LncRNAs and microRNAs (miRNAs) are ncRNAs. LncRNAs compete with miRNAs by acting as sponges for miRNAs to reduce the activity or expression of miRNAs[88]. These lncRNAs are called ceRNAs. Numerous studies have found that SNHG3 can function as a ceRNA by competitively binding to various miRNAs, including miR-758-3p[74], miR-515-5p[87], miR-330[29], miR-326[25,30,34,78], miR-384[31,39,79], miR-154-3p[32], miR-186-5p[33,75], miR3173–5p[89], miR-139-5p[35], miR-539[36], miR-182-5p[37], miR-370-5p[38], miR-3619-5p[76], miR-485-5p[40], miR-128[41], miR-214-3p[42,52], miR-340-5p[45,80], miR-890[68], miR-1343-3p[43], miR-216a[46], miR-515-5p[47], miR-2682-5p[81], miRNA-151a-3p[83], miR-196a-5p[84], miR-339-5p[49], miR-139-5p[51], miR-577[85], and miR-487a-3p[86]. In HCC SNHG3 plays its role as a ceRNA mainly by binding to miR-128[41], miR-326[25] and miR-214-3p[42]. SNHG3 acts as a sponge to bind to miRNAs, subsequently blocking the effects of miRNAs on their downstream target mRNAs. Thus, SNHG3 regulates the expression of oncogenes or tumor suppressor genes, such as *SRGN*[74], *PKM*[29], *HDGF*[31], and *c-Myc*[37], ultimately affecting cancer cell proliferation, apoptosis, metastasis, metabolism and EMT, as shown in Figures 3 and 4.

EMT signaling pathway

The EMT process has been shown to be critical in cancer[90,91]. Shi *et al*[44] showed that human lung cancer cells overexpressing the SNHG3 gene exhibited increased expression of mesenchymal markers (N-calmodulin and waveform protein) and reduced expression of epithelial cell markers (E-calmodulin) while also promoting cancer cell proliferation and metastasis through the TGF-β pathway. In addition, Zhao *et al*[25] learned that SNHG3 promotes cancer cell migration by upregulating the expression of ZEB1, a key transcription factor of EMT in HCC cells. The same conclusion was reached in BC[33] and NSCLC[46]. The above achievements point to the potential application of SNHG3 in the tumor EMT signaling pathway.

Notch signaling pathway

The Notch signaling pathway is a strongly conserved cellular signaling system in most multicellular organisms and is required for a variety of cellular processes, including stem cell functions, cell proliferation, differentiation, and cell death. Several lncRNAs have been proven to participate in the Notch signaling pathway. Zhang *et al*[51] demonstrated that SNHG3 promotes OC proliferation and migration by regulating Notch1. In addition, Jiang *et al*[32], suggested that SNHG3 promotes BC cell proliferation



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Figure 3 Small nucleolar RNA host gene 3 functions as a competing endogenous RNA in digestive system tumors.

and metastasis through upregulation of the Notch signaling pathway. These studies indicate that SNHG3 plays a crucial part in Notch pathways, which have the potential to develop novel therapeutic targets for cancer therapy.

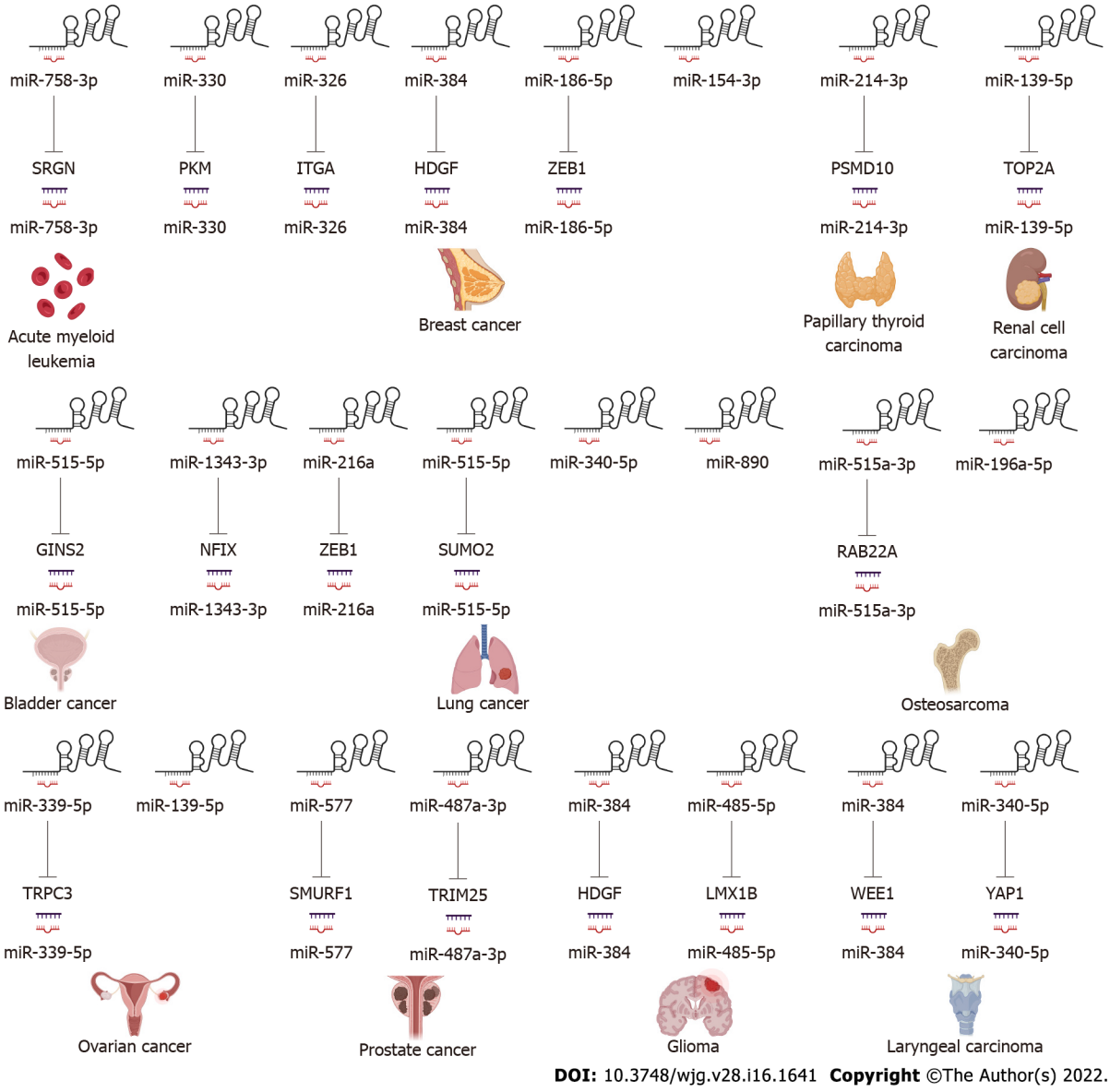
Wnt/β-catenin signaling pathway

Aberrant activation of Wnt/β-catenin signaling was found in some tumors, and Wnt/β-catenin signaling can regulate oncogenes, such as *c-Myc* and *Bcl-2*, leading to tumorigenesis and cell proliferation. SNHG3 may upregulate the Wnt/β-catenin pathway by regulating miR-340-5p and YAP1 in laryngeal cancer cells[80]. In addition, SNHG3 may also promote the proliferation and metastasis of oral squamous carcinoma cells by upregulating the NFYC and Wnt/β-catenin pathways[82]. SNHG3 is involved in the Wnt/β-catenin signaling pathway that mediates tumor development and could represent a new tool for tumor therapy.

STAT3 signaling pathway

Tyrosine kinase signaling delivered by STAT3 is frequently activated in cancer cells, and the STAT3 signaling pathway plays an important role in cancer progression, where it can be activated by cytokines, such as IL-6[92], and growth factors[93,94]. STAT3 is phosphorylated by receptor-associated JAK and thus enters the nucleus to act as a transcriptional activator, regulating oncogene expression[95]. The impacts of STAT3 signaling in suppressing tumor immune surveillance have also been reported[96].

Sun *et al*[76] demonstrated that SNHG3 was overexpressed in GC tissues, and cellular experiments revealed that IL-6-activated STAT3 positively regulates SNHG3 and that SNHG3 promotes stem cell-like properties in GC cells. Shi *et al*[44] found that the IL-6/JAK2/STAT3 pathway activated SNHG3 in NSCLC and promoted cell proliferation and migration. This finding leads to the conclusion that the STAT3 signaling pathway involved in SNHG3 is a novel mechanism of carcinogenesis, suggesting that SNHG3 may represent a biomarker for the treatment of these carcinomas.



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Figure 4 Small nucleolar RNA host gene 3 functions as a competing endogenous RNA in other systemic tumors.

GSK-3β/β-catenin signaling pathway

GSK-3β can negatively regulate β-catenin signaling, which is also implicated in cell proliferation[97]. Research has illustrated that GSK-3β/β-catenin signaling is, for example, one of the downstream drivers of SNHG3 in OC[50], and high expression of SNHG3 fosters cell proliferation and invasion *via* GSK-3β/β-catenin.

SNHG3 can revitalize multiple signaling pathways to promote human tumorigenesis, such as the Notch signaling pathway, Wnt/β-catenin signaling pathway, STAT3 signaling pathway, GSK-3β/β-catenin signaling pathway, and EMT signaling pathway. Thus, SNHG3 may become a target in the treatment of tumors.

CONCLUSION

LncRNAs function as multifunctional signaling modulators, facilitating tumor initiation, progression, and metastasis by regulating tumor cell proliferation, migration, apoptosis, cell cycle, drug resistance, epithelial-mesenchymal transition, metabolic reprogramming, and immune response[98-100]. Compelling studies have suggested that lncRNAs can act as diagnostic indices, prognostic biomarkers, and therapeutic targets for diseases[101,102]. These reports indicate that SNHG3 expression is upregulated in tumor tissues, such as HCC, GC, CC, PTC, and AML, compared to adjacent normal tissues. However, the expression of SNHG3 in lung adenocarcinoma has shown different results and

needs to be further explored. At the same time, SNHG3 has been associated with various clinicopathological parameters such as staging and distant metastasis. In addition, SNHG3 has been shown to promote tumour development in *in vivo* experiments, and at the cellular level, SNHG3 plays a significant role in promoting tumour cell proliferation, migration and metastasis, disrupting the cell cycle and inhibiting apoptosis in a variety of cancers through a variety of signalling pathways, resulting in a poor prognosis for patients.In short, these findings implied that SNHG3 might function as a new target in the diagnosis and treatment of tumours.

In summary, a better understanding of the function of SNHG3 in the clinicopathological features and mechanisms of tumor development may help to improve the efficiency and targeting of treatment. Further studies on SNHG3 and its regulatory mechanism may pave the way to improve prevention, cancer diagnosis, and treatment based on patients' biological and pathological characteristics.

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FOOTNOTES

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Country/Territory of origin: China

ORCID number: Dan-Dan Shan 0000-0003-4761-7385; Qiu-Xian Zheng 0000-0001-5609-7380; Jing Wang 0000-0001-6652-2854; Zhi Chen 0000-0002-0848-1502.

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ORIGINAL ARTICLE

Basic Study

LncRNA cancer susceptibility 20 regulates the metastasis of human gastric cancer cells *via* the miR-143-5p/MEMO1 molecular axis

Ke-Shu Shan, Wei-Wei Li, Wang Ren, Shuai Kong, Li-Pan Peng, Hong-Qing Zhuo, Shu-Bo Tian

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Ke-Shu Shan, Shuai Kong, Li-Pan Peng, Hong-Qing Zhuo, Shu-Bo Tian, Department of Gastrointestinal Surgery, Shandong Provincial Hospital Affiliated to Shandong First Medical University, Jinan 250021, Shandong Province, China

Ke-Shu Shan, Shuai Kong, Li-Pan Peng, Hong-Qing Zhuo, Shu-Bo Tian, Department of Gastrointestinal Surgery, Shandong Provincial Hospital Affiliated to Shandong University, Jinan 250021, Shandong Province, China

Wei-Wei Li, Department of Critical Care Medicine, The 960th Hospital of the People's Liberation Army Joint Logistics Support Force, Jinan 250031, Shandong Province, China

Wang Ren, Department of General Surgery, People's Hospital of Sishui County, Jining 273200, Shandong Province, China

Corresponding author: Shu-Bo Tian, PhD, Chief Doctor, Department of Gastrointestinal Surgery, Shandong Provincial Hospital Affiliated to Shandong First Medical University, Department of Gastrointestinal Surgery, Shandong Provincial Hospital Affiliated to Shandong University, Jinan 250021, Shandong Province, China. ttkl_bo@126.com

Abstract

BACKGROUND

Gastric cancer (GC) is considered as one of the most widespread malignancies. Emerging evidence has shown that lncRNAs can function as important oncogenes or tumor suppressors during GC progression.

AIM

To investigate the effect and mechanism of lncRNA cancer susceptibility 20 (CASC20) in the proliferation and metastasis of GC cells.

METHODS

Data mining and clinical samples were used to evaluate the expression of CASC20 in GC and adjacent tissues. CASC20 was down-regulated in GC cells by short-interfering RNA. Cell proliferation was evaluated by CCK-8 assay, and cell migration and invasion were detected by wound healing and Transwell assays. The expressions of proteins related to epithelial-mesenchymal transition were detected by western blot assay.

RESULTS

The expression of CASC20 was increased in GC tumor tissues and various GC cell lines. High CASC20 expression was correlated with a high risk of lymphatic metastasis and poor prognosis in GC patients. In vitro assays showed that silencing CASC20 reduced cell proliferation, migration, and invasion in GC cells. Mechanistic studies revealed that CASC20 exhibits oncogenic functions by regulating MEMO1 expression through competitive endogenous binding to miR-143-5p, leading to induction of epithelial-mesenchymal transition.

CONCLUSION

Our findings indicate that CASC20 serves as a tumor promoter by regulating metastasis in GC *via* the miR-143-5p/MEMO1 axis. CASC20 may be a potential therapeutic target for GC.

Key Words: Gastric cancer; LncRNA; Cancer susceptibility 20; miR-143-5p; MEMO1; Epithelial-mesenchymal transition

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Core Tip: LncRNA cancer susceptibility 20 (CASC20) is upregulated in gastric cancer (GC) and associated with a higher risk of lymphatic metastasis and poor prognosis of patients. CASC20 can promote the proliferation, invasion and metastasis of GC cells by absorbing miR-143-5p to upregulate MEMO1, thereby induced epithelial-mesenchymal transition *in vitro*.

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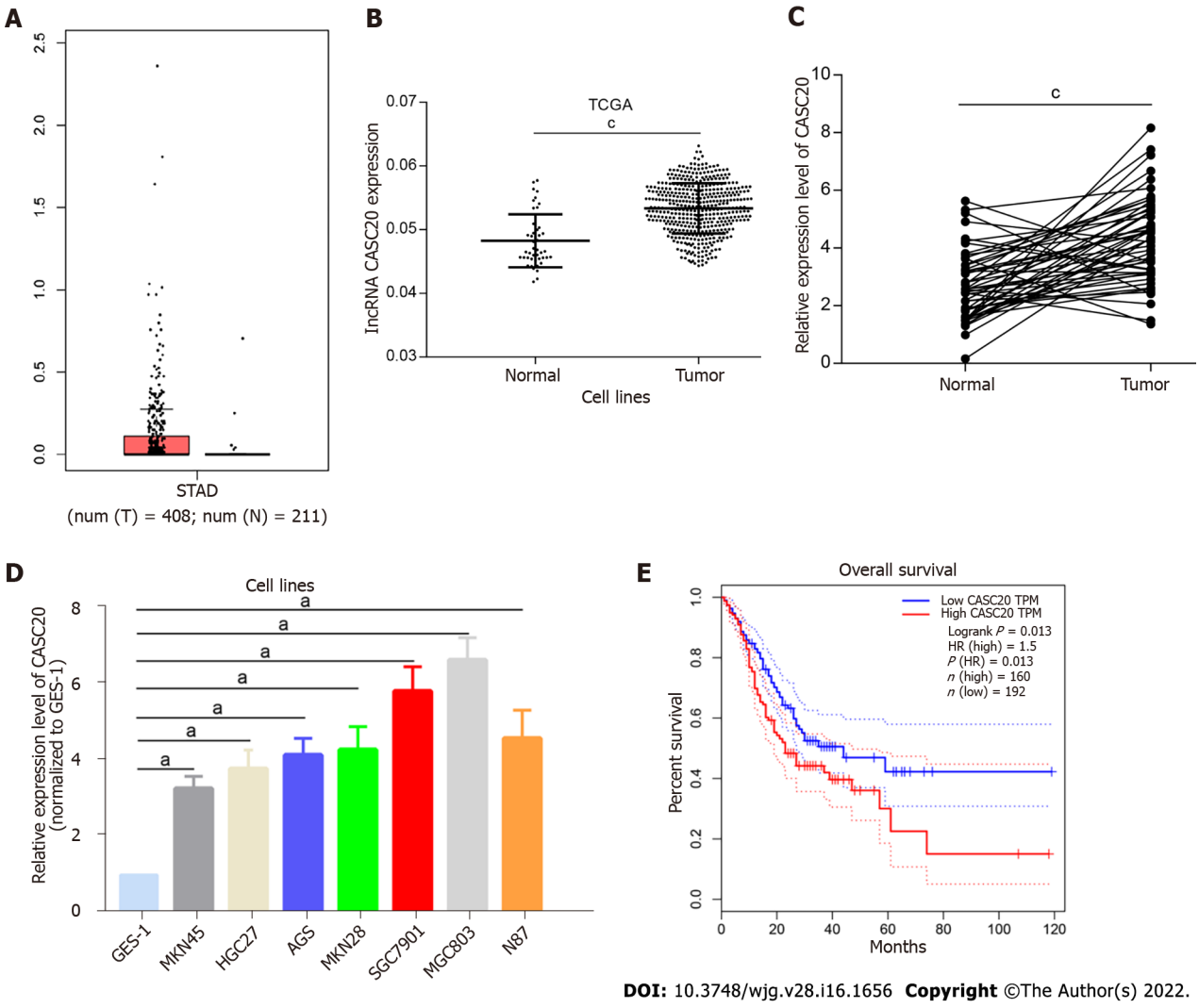
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INTRODUCTION

In 2020, gastric cancer (GC) was the fifth most common cancer and the fourth for mortality of cancer-related in the world[1]. Many patients are already at an advanced stage when diagnosed with GC. Surgical resection is the main treatment for GC. However, postoperative metastasis is the main cause of death from GC[2]. In 2011, Robert A. Weinberg published an article classifying metastasis as one of the top ten characteristics of cancer[3]. Therefore, finding effective molecular markers and therapeutic targets for metastasis is important for developing treatments for GC. The mechanisms underlying metastasis in GC have not yet been fully clarified and require further elucidation.

LncRNAs are RNA molecules more than 200 nucleotides in length that are located in the nucleus or cytoplasm and lack any protein-coding ability. Increasing studies have shown that lncRNAs play important roles in chromosome silencing, chromatin epigenetic modification, gene transcription, protein translation and protein localization[4,5]. In addition, lncRNAs have been shown to function in tumor progression[6,7]. Some lncRNAs function as competing endogenous RNA (ceRNA) and regulate the expression of target genes by binding miRNA elements in target miRNAs and "sponging" miRNAs[8]. MiRNAs can specifically target the 3'UTR of target mRNAs, thereby inhibiting mRNA translation. LncRNA 00473 is highly expressed in GC tissues and is closely related to lymph node metastasis and late TNM staging. LncRNA 00473 regulates the expression of CCND2 by adsorbing miR-16-5p to promote the proliferation and metastasis of GC cells[9]. LncRNAs also play emerging roles in endothelial-mesenchymal transition (EMT) process of GC[10]. The process of EMT involves downregulation of the epithelial cell specific proteins and upregulation of the mesenchymal cell specific proteins and it is an important cause of tumor metastasis[11]. LncRNA CASC19 is highly expressed in colorectal cancer, and CASC19 overexpression increased the invasion and migration ability of colorectal cancer cells. CASC19 regulates cell migration inducing hyaluronidase 1 (CEMIP) by targeting miR-140-5p and affecting the EMT process[12].

Several studies have revealed key roles for lncRNAs in GC progression, and multiple lncRNAs have been identified as diagnostic and prognostic markers or treatment targets for GC[4]. A recent study identified the HOXC-AS3 lncRNA as highly expressed in GC tissues. HOXC-AS3 binds YBX1 protein to regulate downstream gene transcription and participates in GC progression[13]. The lncRNA CASC9 is highly expressed in GC tissues and cells. CASC9 negatively regulates miR-370 and promotes the progression of GC through the EGFR/AKT pathway[14]. However, the precise mechanisms and roles of lncRNAs in GC progression are still unclear.



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Figure 1 High level of cancer susceptibility 20 in gastric cancer tissues and cell lines. A and B: Cancer susceptibility 20 (CASC20) expression in gastric cancer (GC) samples and normal samples from TCGA GC database; C: CASC20 expression in 50 pairs of clinically collected gastric cancer tissues and normal tissue samples; D: CASC20 expression in GC cell lines and GES-1 cell line was analyzed by quantitative real-time polymerase chain reaction; E: Overall survival curves of TCGA GC patients with different CASC20 expression. $^{a}P < 0.05$ and $^{c}P < 0.001$ *vs* control.

Cancer susceptibility 20 (CASC20) is a 1484 bp lncRNA mapped to chromosome 20p12.3. In this study, we explored the role and possible mechanism of the lncRNA CASC20 in GC metastasis to gain more insights into the mechanism of GC metastasis. We found that the CASC20 lncRNA is highly expressed in GC and closely associated with lymph node metastasis and poor prognosis of GC patients. Through *in vitro* and *in vivo* experiments, we found that CASC20 promotes the proliferation, migration and invasion of GC cells. Mechanistic studies revealed that CASC20 "sponges" miR-143-5p and promotes EMT by regulating MEMO1. This function of CASC20 plays an important role in the metastasis of GC.

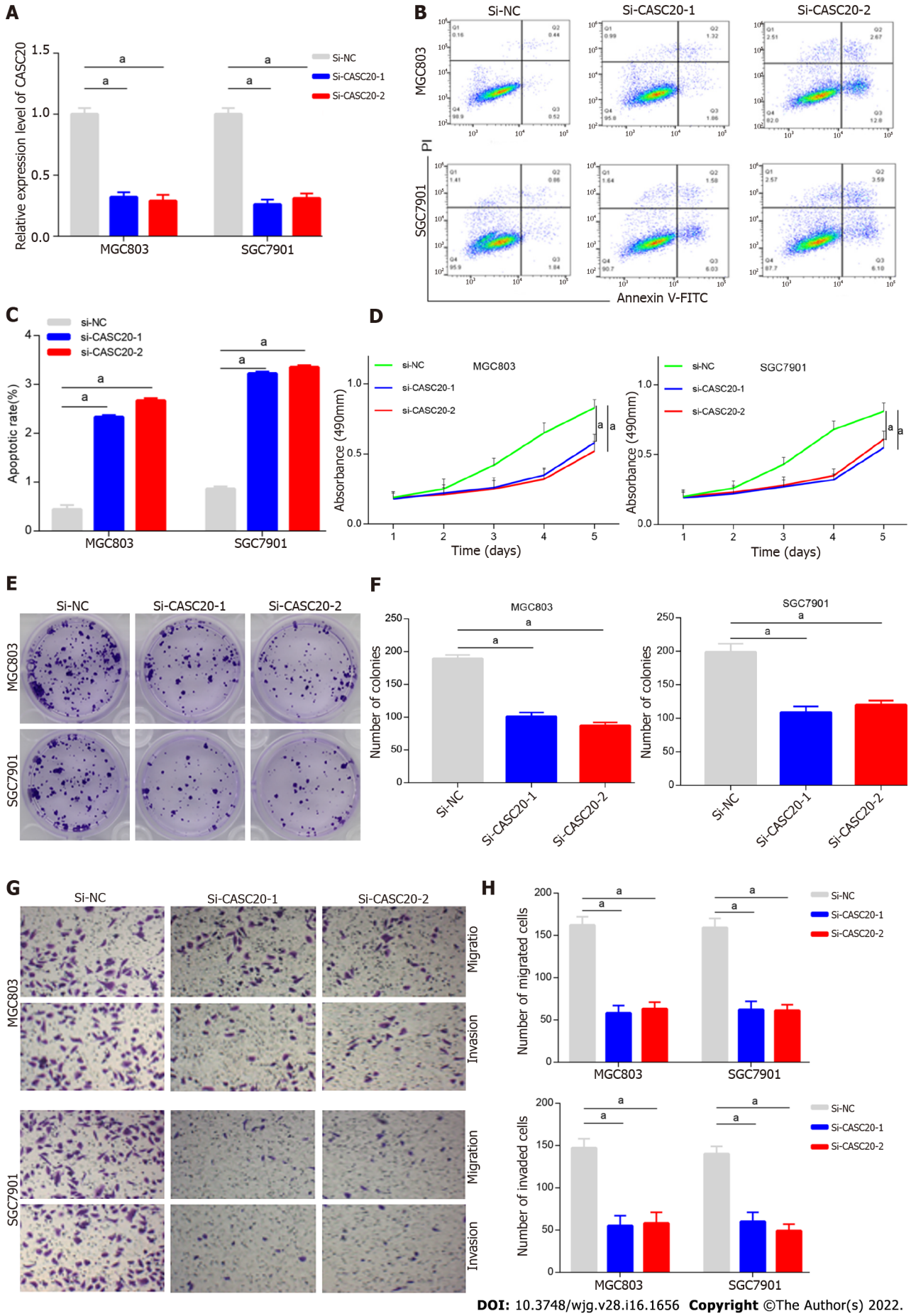
MATERIALS AND METHODS

Tissue specimens

All clinical samples and information in this study were from the Department of Gastrointestinal Surgery, Provincial Hospital Affiliated to Shandong First Medical University. Fresh pathological specimens (GC and adjacent normal tissues) were from patients undergoing radical GC surgery at our hospital. All patients provided informed consent and were approved by the Medical Ethics Committee.

Cell culture and transfection

Human GC cell lines (MKN45, MKN28, HGC27, MGC803, N87, AGS and SGC7901) and the immortalized gastric mucosa cell line (GES-1) were provided by the Cell Center of the Chinese Academy of Medical Sciences. All cell lines were cultured in RPMI-1640 medium supplemented with



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Figure 2 Konckdown of cancer susceptibility 20 inhibits the apoptosis, proliferation and metastasis of gastric cancer cells. A: Efficacy assessment of cancer susceptibility 20 (CASC20) silence through quantitative real-time polymerase chain reaction method in MGC803 and SGC7901 cells; B and C: Cell apoptosis evaluation in transfected cells through flow cytometry; D: Cell viability was verified by CCK-8 experiments after transfection with si-CASC20; E and F: Colony formation assay was used to detect the cell proliferation ability after transfection with si-CASC20; G and H: Transwell assay was employed for examining the effect of CASC20 low-expression on gastric cancer cell migration and invasion. $^{a}P < 0.05$ *vs* control.

10% fetal bovine serum (FBS, Gibco, United States). Cells were cultured in incubator at 37 °C and 5% CO_2. Cells were passaged when they were 70% confluent.

The miR-143-5p mimics and inhibitor were purchased from RiboBio (Guangzhou, China). siRNAs targeting CASC20 (si-CASC20) and MEMO1 (si-MEMO1) were designed and synthesized by RiboBio (Guangzhou, China). Lipofectamine2000 (Invitrogen, United States) was used to transfect siRNA or miRNA mimics/inhibitor into cells following the manufacturer's instructions. GC cells in logarithmic phase were collected and inoculated in a 6-well plate. Cells were transfected with siRNAs when cell confluence reached approximately 50%.

RNA extraction and quantitative real-time polymerase chain reaction

Trizol reagent (Invitrogen, United States) was used to extract total RNA from GC tissues and cells. RNA integrity was determined by evaluating the absorbance at $OD_{260}/280$ using a NanoDrop analyzer and confirmed at 1.8–2.0. Total RNA was reverse transcribed into cDNA using a reverse transcription kit (Takara, Dalian, China) in accordance with the manufacturer's instructions. The quantitative real-time polymerase chain reaction (qRT-PCR) three-step method was used, and the $2^{-\Delta\Delta Ct}$ method was used to calculate the relative expression levels of CASC20 and miR-143-5p. The primer sequences are as follows: CASC20, Forward 5′-ATAACCACCCTCCCCTCCTC-3′, Reverse 5′-GCTCCTCCCACTTTAACCCC-3′; miR-143-5p, Forward 5′-ATGGTTCGTGGGGTCCAGTTTTCCCAG-3′, Reverse 5′-GTGTCGTG-GAGTCGGCAATTC-3′; MEMO1, Forward, 5′-GCTTCGGCAGCACATATACTAAAAT-3′, Reverse 5'-CGCTTCACGAATTTGCGTGTCAT-3′; and β-actin, Forward 5′-TGGCACCCAGCACAATGAA-3′, Reverse 5′-CTAAGTCATAGTCCGCCTAGAAGCA-3′. U6, Forward 5′-CAGCACATATACTAAAAT-TGGAACG-3′ Reverse 5′-ACGAATTTGCGTGTCATCC-3′. β-actin (cytoplasm control) and U6 (nucleus control) were used for normalization.

CCK-8 assay

GC cells were inoculated into 96-well plate at 5×10^3 cells *per* well. At 24 h, 48 h, 72 h, 96 h and 120 h after transfection, 90 μL of fresh culture medium containing 10% FBS and 10 μL of CCK-8 reagent (Dojindo, Mashikimachi, Japan) were simultaneously added to each well. Cells were cultured for another 4 h. The OD value of each well at 450 nm was measured and proliferation curves were drawn.

Flow cytometric analysis

GC cells were collected after trypsin digestion and centrifuged at 1000 rpm/min for 5 min. The cells were washed twice with phosphate buffer solution, and 500 μL binding buffer was added to adjust the cell concentration to 1×10^6 cells/mL. Annexin V-FITC (5 μL) and propidium iodide solution (10 μL) (NeoBioscience, China) were added to the cell suspension, and cells were analyzed on a flow cytometer (BD Accuri C6).

Colony formation assay

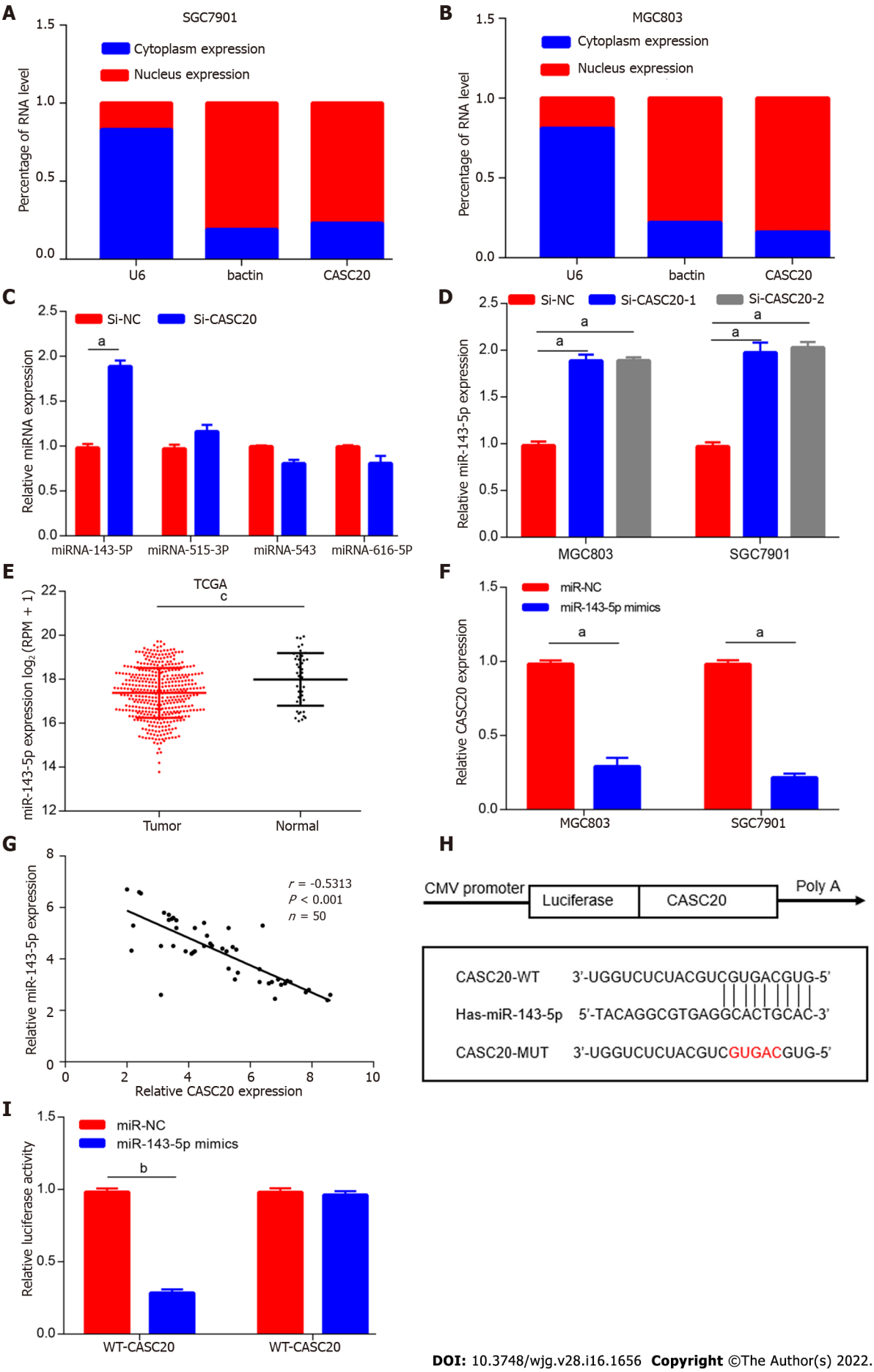
After 48 h of transfection, cells were collected and the cell density was adjusted to 1×10^5 cells/mL. The cell suspension was inoculated into a 6-well plate at 100 μL/well and the plates were incubated for 14 d. Cells were washed twice with phosphate buffered saline and fixed with 4% paraformaldehyde for 15 min; the cells were then stained with 0.1% crystal violet staining solution for 10 min. We counted the number of cell clones in five fields under the microscope and determined the clone formation rate as follows: Clone formation rate (%) = (number of clones/number of inoculated cells) × 100%. The experiment was repeated three times.

Wound healing assay

GC cells were cultured in a 6-well plate at 5×10^5 cells *per* well. Linear scratch wounds were created in the cell monolayer with a sterile 100 μL pipette tip. Cells were cultured for 24 h, and the wound widths were photographed and measured.

Transwell experiment

Assays using a Transwell chamber (8 μm, Corning, United States) were used to evaluate cell migration and invasion abilities. GC cells were collected and resuspended in serum-free RPMI 1640 medium at 1.5×10^4 cells/mL, and 200 μL of the cell suspension was inoculated into the upper chambers. Complete medium containing 10% FBS was added to the lower chamber. After culturing for 24 h, the cells were fixed with methanol. Cells in the upper chamber were removed with cotton swabs, and cells in the



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Figure 3 miR-143-5p served as a target of cancer susceptibility 20 in gastric cancer cells. A and B: Relative cancer susceptibility 20 (CASC20) expression levels in nucleus and cytoplasm of SGC7901 and MGC803 cells; C: The relative expressions of miRNAs in gastric cancer (GC) cells after downregulation of CASC20; D: The expression of miR-143-5p was detected by quantitative real-time polymerase chain reaction in GC cells after transfection with si-CASC20; E: The expression levels of miR-143-5p in GC from TCGA database; F: The detection of CASC20 expression after transfection of miR-143-5p mimics; G: The correlation analysis between miR-143-5p expression and CASC20 expression in 50 GC tissues; H: Bioinformatics presentation of CASC20 and miR-143-5p binding sites; I: The verification of CASC20 and miR-143-5p interaction by luciferase report assay. $^{a}P < 0.05$, $^{b}P < 0.01$ and $^{c}P < 0.001$ *vs* control.

lower chambers were stained with 0.1% crystal violet solution for 30 min. The cells were observed under a microscope and photographed; five random fields were obtained to count the number of migrated cells. Each experimental group was performed in triplicate, and the average value was taken. For the invasion experiment, the upper chamber was coated with Matrigel; the assay was then conducted following the methods for the migration assay.

Bioinformatics analysis

The online tool lncRNASNP2 was used to predict miRNAs that potentially bind to CASC20. We used miRDB, Targetscan, and miRTarbase to predict putative target genes of miR-143-5p.

Dual-luciferase reporter assay

Luciferase reporter pGL3 vectors with wild-type CASC20 (termed WT-CASC20), wild-type MEMO1 (MEMO1-WT), mutant CASC20 (MUT-CASC20) and mutant MEMO1 (MEMO1-MUT) were constructed (Promega, Madison, United States). The reporter plasmids were transfected into MGC803 cells along with miR-143-5p mimics. At 48 h after transfection, the luciferase activity was measured using the Dual Luciferase Reporter Assay system (Promega, United States) in accordance with the manufacturer's instructions.

Western blot analysis

Cells were collected and lysed with RIPA lysis buffer (Beyotime, Shanghai, China). After lysates were centrifuged, the supernatant was collected and protein concentration was evaluated by the BCA method. The samples (60 μg/well) were separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis and electro-transferred to a polyvinylidene difluoride membrane (Millipore, Billerica, MA, United States). After blocking in 5% skim milk at room temperature for 2 h, the membrane was incubated with primary antibody overnight at 4 °C. The membrane was then washed three times with Tris buffered saline tween and incubated with the secondary antibody at room temperature for 2 h. ECL luminescent solution was used for chemiluminescence and Image J software was used to analyze the protein expression level. MEMO1 and β-actin monoclonal antibodies were obtained from Sigma-Aldrich (St Louis, MO, United States). The other antibodies were purchased from Abcam (Cambridge, England).

Statistical analysis

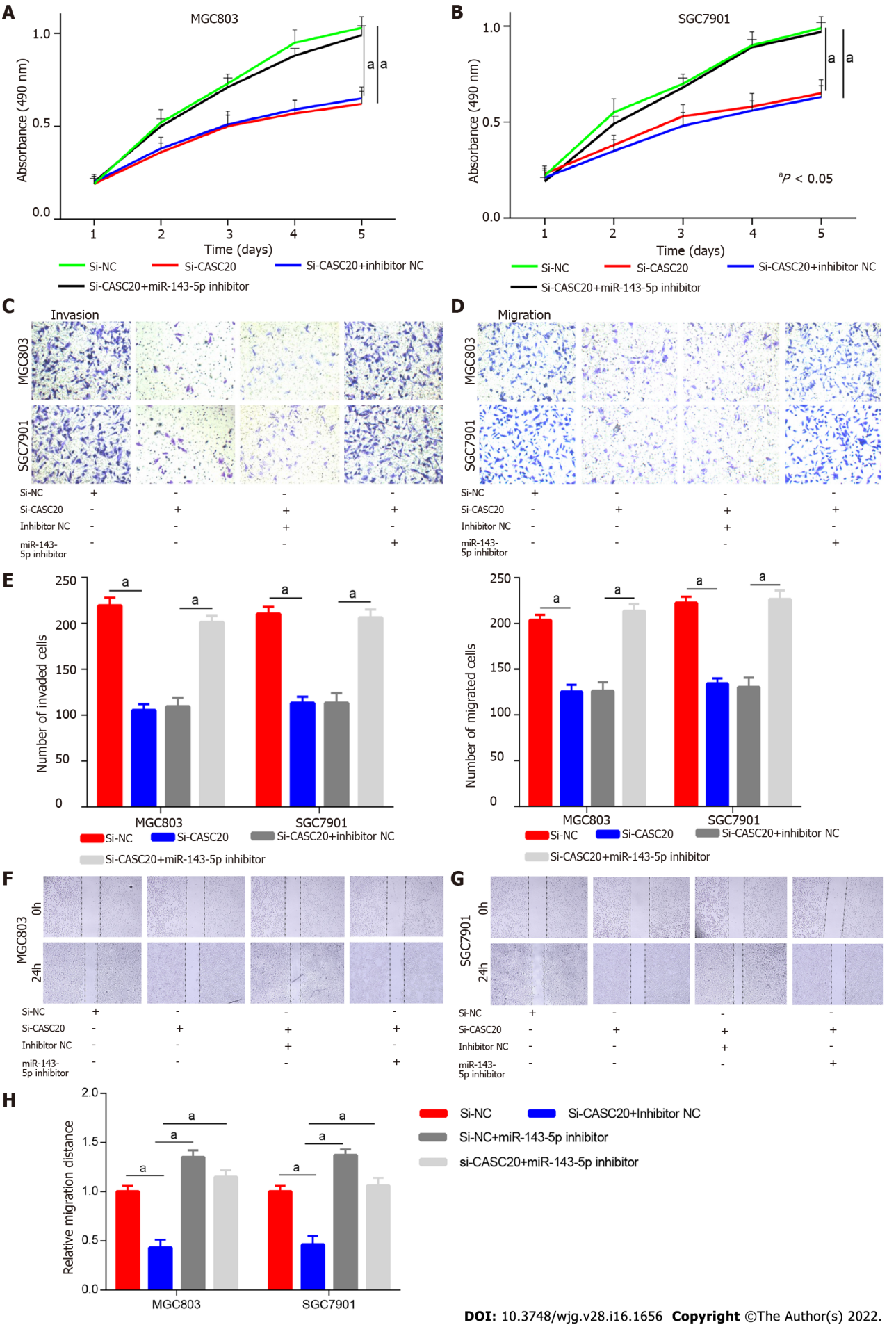
Statistical analyses were performed using GraphPad Prism (version 7.0; GraphPad Prism software, San Diego, CA, United States). Data are shown as mean ± SD. For measurement data, the difference between two or more groups was calculated using the Student's t-test or one-way ANOVA analysis. Chi-square test was used to analyze the correlation between CASC20 and clinicopathological parameters of GC patients. The survival curve was determined by the Kaplan–Meier method and log-rank test. $P < 0.05$ was considered statistically significant.

RESULTS

LncRNA CASC20 is highly expressed in GC tissues and cells

Analysis of TCGA database revealed that CASC20 expression was significantly higher in 408 GC tumor tissues than that in 211 normal gastric tissues ($P < 0.01$, Figure 1A and B). We performed qRT-PCR to assess the expression of CASC20 in 50 cases of GC tissues and paired normal gastric mucosa tissues adjacent to tumors. The results showed that the expression of CASC20 in GC tissues was significantly higher than that in normal adjacent gastric mucosa tissues ($P < 0.01$, Figure 1C). We further found that the expression of CASC20 in seven GC cell lines was significantly higher than that of GES-1 normal gastric mucosal epithelial cells (Figure 1D). We next analyzed the relationship between CASC20 expression and the survival rate of GC patients in TCGA database. The results indicated that the survival rate of GC patients with high expression of CASC20 was significantly lower than that of patients with low expression (Figure 1E). Together these data indicate that CASC20 is highly expressed in GC and may play an important role in the development of GC.

Next, we examined the correlation between CASC20 expression and the pathological characteristics of 50 GC patients. GC patients were divided into high and low CASC20 expression groups based on the



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Figure 4 The regulation of cancer susceptibility 20 on gastric cancer cells is mediated by miR-143-5p. A and B: Gastric cancer (GC) cells were treated with si-NC, si-cancer susceptibility 20 (CASC20), si-CASC20 + inhibitor NC, si-CASC20 + miR-143-5p inhibitor, CCK-8 assays evaluated the proliferation; C-E: Transwell assays tested invasion and migration abilities of transfected cells; F-H: GC cells were treated with si-NC, si-CASC20 + inhibitor NC, si-NC + miR-143-5p inhibitor, si-CASC20 + miR-143-5p inhibitor, wound healing assay was used to detect migration ability. $^{a}P < 0.05$ *vs* control.

median ratio of relative CASC20 expression. High CASC20 expression in GC patients was significantly correlated with a higher risk of lymphatic metastasis ($P = 0.011$), but did not show associations with age, gender, TNM stage and differentiation degree (Table 1). These results suggest that CASC20 is closely related to the malignant biological behavior of GC.

Knockdown of CASC20 inhibits GC cell proliferation, invasion and metastasis and induces apoptosis

We next evaluated the role and effects of CASC20 in GC cells. We transfected siRNA targeting CASC20 into GC cells and qRT-PCR confirmed that the expression of CASC20 was significantly down-regulated after siRNA transfection (Figure 2A). Flow cytometry results showed that knockdown of CASC20 significantly increased the apoptosis rate of SGC7901 and MGC803 GC cells compared with the control cells (Figure 2B and C). CCK8 assays demonstrated that knockdown of CASC20 significantly inhibited the proliferation of SGC7901 and MGC803 cells (Figure 2D). Colony formation experiments revealed that inhibiting CASC20 expression resulted in decreased clonogenic survival (Figure 2E and F). Transwell experiments showed that knocking down CASC20 significantly reduced the numbers of invading and migrating GC cells (Figure 2G and H). Together, these findings show that knockdown of CASC20 inhibits the malignant phenotype of GC cells, suggesting that CASC20 exhibits oncogenic functions in GC.

CASC20 adsorbs miR-143-5p in GC cells

To gain better insights into the function of CASC20, we first determined the subcellular location of CASC20 by GeneCards software (https://www.genecards.org). The results showed that CASC20 is mainly located in the cytoplasm. The results of nuclear cytoplasmic experiments further confirmed that CASC20 mostly localizes in the cytoplasm (Figure 3A and B), suggesting that CASC20 may function as a ceRNA to "sponge" miRNA.

We next used bioinformatics tools (lncRNASNP2) to predict miRNAs with potential binding sites for CASC20 and the results identified miR-143-5p, miR-515-3p, miR-543 and miR-616-5p. We evaluated the expressions of these miRNAs in cells transfected with si-CASC20 and found that the expression of miR-143-5p was up-regulated when CASC20 was knocked down, while miR-515-3p, miR-543 and miR-616-5p showed no significant changes (Figure 3C and D). We thus selected miR-143-5p for further analyses.

TCGA database analysis showed that miR-143-5p was expressed at low levels in GC tissues (Figure 3E), which was consistent with previous studies on miR-143-5p[15]. We transfected miR-143-5p mimics into GC cells and observed significantly decreased expression of CASC20 (Figure 3F). In addition, we identified a negative correlation between CASC20 and miR-143-5p in 50 GC tissues (Figure 3G).

To further determine the regulatory mechanism between CASC20 and miR-143-5p, we performed dual luciferase reporter assays. The results showed that transfection of miR-143-5p mimics decreased the luciferase activity of the WT-CASC20 reporter in MGC803 cells, while miR-143-5p mimics had no impact on the MUT-CASC20 reporter activity (Figure 3H and I). This result suggests that the regulatory relationship of CASC20 and miR-143-5p in GC cells involves interactions between CASC20 and miR-143-5p.

CASC20 inhibits miR-143-5p to regulate the malignant phenotype of GC cells

To determine whether miR-143-5p played a role in the effects of CASC20 in GC cells, we co-transfected si-CASC20 and miR-143-5p inhibitor into SGC7901 and MGC803 cells. CCK8 assays showed that si-CASC20 transfection inhibited GC cell proliferation, and co-transfection of miR-143-5p inhibitor restored proliferation (Figure 4A and B). Transwell experiments showed while transfection of si-CASC20 inhibited the invasion and migration of GC cells, co-transfection of miR-143-5p inhibitor restored the invasion and migration abilities (Figure 4C–E). Wound healing assays also confirmed that co-transfection of miR-143-5p inhibitor partly rescued the inhibitory effect of knockdown of CASC20 on GC cells (Figure 4F–H). These results indicate that CASC20 regulates GC cell activities through miR-143-5p.

MEMO1 mRNA is a target of miR-143-5p, and miR-143-5p regulates MEMO1 mRNA expression

To further explore the role of miR-143-5p in GC, we screened and identified the potential target mRNAs of miR-143-5p. Bioinformatics tools miRDB, Targetscan and miRTarbase predicted several mRNAs (HDAC7, ZNF85, ZNF347, MEMO1, ESCO1, MLXIP, MAP3K2 and DYRK1A mRNAs) that potentially interact with miR-143-5p (Figure 5A). Among them, MEMO1 was selected as the target gene of miR-143-5p because it had the highest score in the prediction analysis.

Table 1 The correlation between cancer susceptibility 20 expression and the clinicopathological factors of 50 gastric patients

Characteristics	Number of cases	CASC20 expression		*P* value
		High (*n* = 26)	Low (*n* = 24)	
Gender				0.382
Male	23	14	9	
Female	27	12	15	
Age				0.546
≤ 55	22	13	9	
> 55	28	13	15	
TNM stage				0.262
I + II	24	10	14	
III	26	16	10	
Lymphatic metastasis				0.011[a]
Positive	25	18	7	
Negative	25	8	17	
Differentiation				0.073
Moderate/well	30	12	18	
Poor	20	14	6	

[a]*P* < 0.05 was considered significant. CASC20: Cancer susceptibility 20.

TCGA database analysis showed that MEMO1 was highly expressed in GC tissues (Figure 5B). In addition, the mRNA expression of MEMO1 was significantly increased in 50 GC tissues compared with its expression in adjacent tissues (Figure 5C).

We next designed luciferase reporter vectors containing the wild-type 3′UTR of MEMO1 mRNA or the 3′UTR with mutated binding sites (Figure 5D). Dual luciferase reporter assay showed that miR-143-5p significantly reduced the luciferase activity of the reporter vector with the wild-type 3′UTR of MEMO1 mRNA but had no effects on the mutated reporter (Figure 5E), suggesting that MEMO1 mRNA may be a target gene of miR-143-5p. We then transfected miR-143-5p mimics into MGC803 cells and found that the expression of MEMO1 was significantly reduced at both gene and protein levels (Figure 5F). These data indicate that miR-143-5p regulates MEMO1 mRNA expression.

We also found that the expression of CASC20 was positively correlated to MEMO1 expression in GC tissues (Figure 5G). In cells with CASC20 knockdown, MEMO1 expression was downregulated, suggesting a positive regulatory relationship between CASC20 and MEMO1 (Figure 5H).

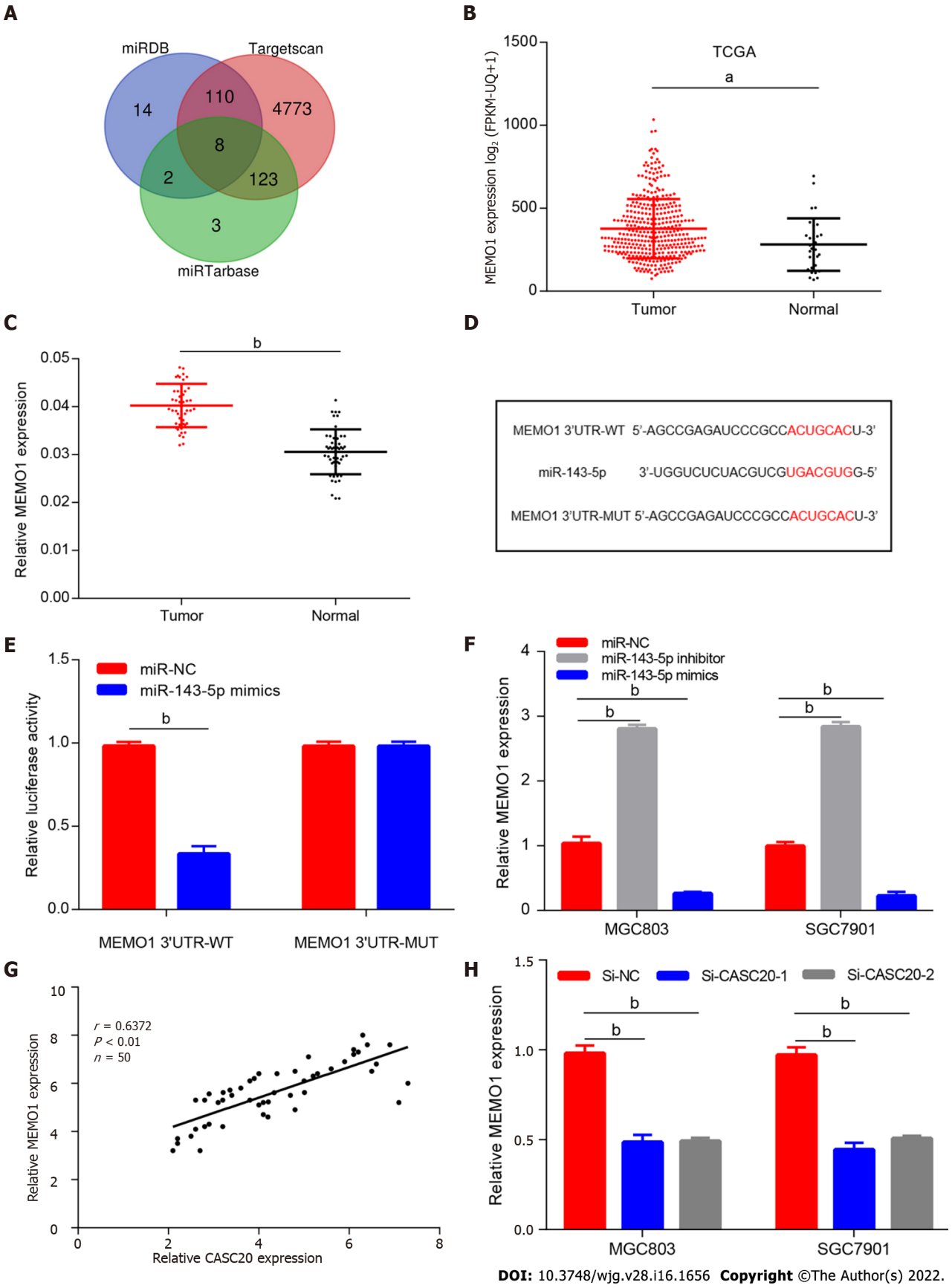
To explore the role of MEMO1 in GC, we transfected siRNA targeting MEMO1 into GC cells. Scratch experiments and Transwell experiments revealed that knockdown of MEMO1 inhibited the invasion and migration of GC cells. The migration and invasion abilities were partially rescued by co-transfection with miR-143-5p inhibitor (Figure 6A–G), indicating that miR-143-5p inhibition reversed the effects of MEMO1 knockdown.

CASC20 regulates EMT through miR-143-5p/MEMO1 to promote the invasion and migration of GC cells

To investigate whether CASC20 regulates MEMO1 through miR-143-5p, we transfected si-CASC20 and miR-143-5p inhibitor into MGC803 cells. Western blot results indicated that MEMO1 expression decreased in GC cells after transfection with si-CASC20, while MEMO1 expression was restored after co-transfection with si-CASC20 and miR-143-5p inhibitor (Figure 6H).

We next evaluated the effects of CASC20 on EMT. In MGC803 cells with CASC20 knockdown, the expression level of the epithelial protein marker E-cadherin was up-regulated, while the expression levels of the mesenchymal protein markers Vimentin and ZEB1 were down-regulated. After transfection with miR-143-5p inhibitor, the expressions of MEMO1, Vimentin and ZEB1 were up-regulated, and the expression of E-cadherin was down-regulated (Figure 6H and I).

Together these results indicated that CASC20 may sequester miR-143-5p to increase the MEMO1 expression, leading to the induction of EMT and metastasis of GC.



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Figure 5 Cancer susceptibility 20 regulates the miR-143-5p target MEMO1. A: Bioinformatic tools were used to predict the potential target mRNA of miR-143-5p; B: The expression levels of MEMO1 in gastric cancer (GC) from TCGA STAD database; C: MEMO1 expression in 50 pairs of gastric cancer tissues and normal tissue samples; D: Putative miR-143-5p binding sequence and mutation sequence of MEMO1 mRNA were as shown; E: Dual luciferase reporter assays were used to confirm the direct target between miR-143-5p and MEMO1; F: MEMO1 expression was decreased in GC cells transfected by miR-143-5p mimics; G: The correlation analysis between cancer susceptibility 20 (CASC20) and MEMO1 expression in 50 GC tissues; H: MEMO1 expression was decreased after knockdown of

CASC20. [a]*P* < 0.05, [b]*P* < 0.01 *vs* control.

DISCUSSION

Mounting evidence confirmed that lncRNAs play extensive regulatory roles in the initiation and progression of human cancers. A complex ceRNA crosstalk mechanism has been identified in GC[16]. However, only a small fraction of lncRNAs has been thoroughly studied, with no research on CASC20 in tumors. We analyzed the TCGA GC database and found that CASC20 is highly expressed in GC and associated with poor prognosis of GC patients. Functional experiments revealed that knock down of CASC20 significantly inhibited the proliferation, invasion, and migration of GC cells. These findings indicate a potential oncogenic function of CASC20 in GC.

miRNAs are 18–24 nt in length and negatively regulate gene expression by interacting with mRNA, thereby playing important physiological functions in several cellular processes. In tumors, the combination of miRNA and the 3'UTR of the target gene can function as an oncogene or tumor suppressor gene by regulating its expression[17,18]. miRNAs may thus become potential therapeutic targets for cancer metastasis, and the application of miRNA mimics and their antagonists for therapeutic purposes is expanding[19].

Our study showed that CASC20 was mainly distributed in the cytoplasm in GC cells, which suggested that CASC20 may function as a ceRNA for sponging miRNA. LncRNASNP2 was used to predict miRNAs that potentially bind CASC20, and our *in vitro* experiments indicated that CASC20 acts as a molecular sponge for miR-143-5p.

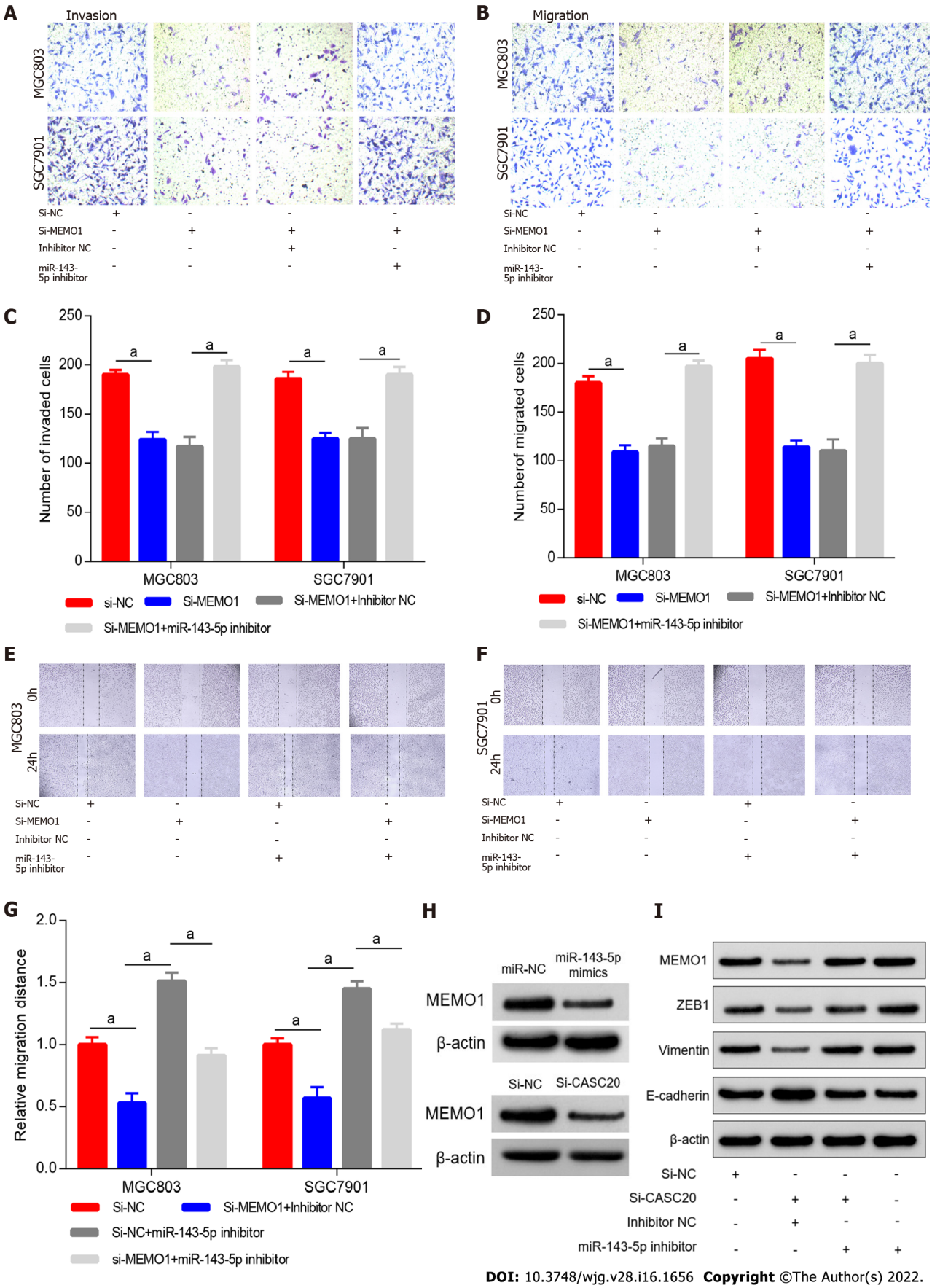
miR-143-5p is located in the human 5q32 chromosome region. Wu *et al*[15] found that miR-143-5p is expressed at low levels in GC and functions as tumor suppressor gene by targeting COX-2[15]. The lncRNA ZEB2-AS is highly expressed in GC tissues and cell lines, and ZEB2-AS was shown to promote the proliferation and metastasis of GC cells through the miR-143-5p/HIF-1α pathway[20]. These studies have shown that miR-143-5p is expressed at low levels in GC and exhibits tumor suppressor functions. Our qRT-PCR results showed that miR-143-5p was markedly down-regulated in GC tissues, and its expression level was negatively correlated with CASC20. Downregulation of miR-143-5p partially reversed the effects of CASC20 knockdown on the proliferation, invasion and migration of GC cells.

Bioinformatics analysis predicted that miR-143-5p interacts with the 3′UTR of MEMO1 (mediator of ErbB2-driven cell motility 1), a key mediator of receptor tyrosine kinase activation. MEMO1 plays an important role in breast cancer cell invasion and migration by activating the PI3K/Akt signaling pathway, leading to up-regulated Snail1 and induction of the EMT program[21]. MEMO1 is highly expressed in colon cancer tissues and cells, and miR-219a-1 regulates the proliferation, invasion and metastasis of colon cancer cells by targeting MEMO1, thereby functioning as a tumor suppressor gene [22]. The role of MEMO1 in the progression of GC has not yet been reported. In this study, we found that MEMO1 is highly expressed in GC in TCGA GC database and clinical samples. Overexpression of miR-143-5p reduced the expression of MEMO1 at the gene and protein level. Dual luciferase reporter assay was performed to reveal the regulatory relationship between miR-143-5p and MEMO1. Reduced migration and migration abilities of GC cells caused by MEMO1 knockdown were reversed by miR-143-5p inhibitor. EMT is a prerequisite for distant metastasis of malignant tumor cells, and many studies reported roles for lncRNAs in the tumor EMT process[23]. Our western blot results showed that CASC20 expression resulted in decreased expression of the epithelial cell marker E-cadherin and increased expression of the mesenchymal cell marker Vimentin. Together, our results indicate that CASC20 functions as ceRNA to sponge miR-143-5p, leading to up-regulated MEMO1 expression and initiation of the EMT process in GC.

This study has several limitations. Only 50 GC tissues were collected and analyzed in this study. The small clinical sample size may be one of the reasons why CASC20 expression was only related to lymph node metastasis, but showed no significant association with tumor size and clinical stage. In addition, *in vivo* experiments are required to further establish the biological function of CASC20 in GC.

CONCLUSION

In summary, this study identified the lncRNA CASC20/miR-143-5p/MEMO1 axis in GC and provided insights into the mechanistic relationships among CASC20, miR-143-5p and MEMO. These findings enrich the molecular regulatory network driving the occurrence and development of GC and deepen our understanding of the function and mechanism of CASC20 in cancer. Our results suggest that CASC20 may be a potential therapeutic target and clinical prognostic marker for GC.



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Figure 6 Inhibition of miR-143-5p blocked the function of MEMO1 knockdown in gastric cancer cells. A-D: MGC803 and SGC7901 cells were transfected with si-NC, si-MEMO1, si-MEMO1 + inhibitor NC, si-MEMO1 + miR-143-5p inhibitor, respectively. Transwell assay was employed for the detection of cell invasion and migration ability; E-G: MGC803 and SGC7901 cells were transfected with si-NC, si-MEMO1 + inhibitor NC, si-NC + miR-143-5p inhibitor, si-MEMO1 +

miR-143-5p inhibitor, respectively. Wound healing assay was used to detect migration ability; H: The protein expression of MEMO1 was detected by western blot assay after transfecting with miR-143-5p mimics or si-cancer susceptibility 20; I: Western blot was utilized to detect the expressions of EMT-related proteins (ZEB1, Vimentin and E-cadherin). $^{a}P < 0.05$ *vs* control.

ARTICLE HIGHLIGHTS

Research background

LncRNAs have been indicated to play critical roles in gastric cancer (GC) tumorigenesis and progression. However, their roles in GC remain to be further elucidated.

Research motivation

To discover promising diagnostic and therapeutic targets for GC.

Research objectives

To investigate the underlying mechanisms of the lncRNA cancer susceptibility 20 (CASC20) in GC.

Research methods

The expression of CASC20, miR-143-5p and MEMO1 genes were detected by quantitative real-time polymerase chain reaction. The oncogenic functions of CASC20 was investigated by experiments including cell counting kit-8, colony formation, wound-healing and Transwell assays in GC cells in vitro. Bioinformatics and dual-luciferase report assay were performed for determining the regulatory relationship of lncRNA-miRNA-mRNA.

Research results

We found that the expression of lncRNA CASC20 was significantly upregulated in GC tissues and cell lines, and increased expression of CASC20 was positively correlated with lymph node metastasis in patients with GC. Additionally, knockdown of CASC20 could inhibit GC cells growth, migration and invasion, whereas its overexpression could reverse these effects. Mechanistically, CASC20 specifically inhibited miR-143-5p expression, thus up-regulating MEMO1 expression.

Research conclusions

CASC20 promotes GC cell metastasis *via* adsorbing miR-143-5p and up-regulating MEMO1, it plays an important oncogenic function in GC.

Research perspectives

This study identified the lncRNA CASC20/miR-143-5p/MEMO1 axis in GC and provided insights into the mechanistic relationships among CASC20, miR-143-5p and MEMO. The results of our research suggest that CASC20 may act as a promising diagnostic marker for GC.

FOOTNOTES

Author contributions: Shan KS and Li WW contributed equally to this work; Tian SB and Shan KS designed the study; Shan KS, Li WW and Ren W performed all experiments; Kong S and Peng LP collected tissue samples and the clinical data; Shan KS and Li WW analyzed and interpreted the data; Tian SB and Zhuo HQ drafted the manuscript; all authors read and approved the final manuscript.

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Country/Territory of origin: China

ORCID number: Ke-Shu Shan 0000-0001-5907-2189; Wei-Wei Li 0000-0003-3201-6739; Wang Ren 0000-0001-6607-7519; Shuai Kong 0000-0003-3751-6679; Li-Pan Peng 0000-0003-1221-3524; Hong-Qing Zhuo 0000-0003-0829-5563; Shu-Bo Tian 0000-0001-6022-6288.

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ORIGINAL ARTICLE

Retrospective Cohort Study

Aspartate transferase-to-platelet ratio index-plus: A new simplified model for predicting the risk of mortality among patients with COVID-19

Ali Madian, Ahmed Eliwa, Hytham Abdalla, Haitham A Azeem Aly

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Ali Madian, Haitham A Azeem Aly, Department of Internal Medicine, Faculty of Medicine, Al-Azhar University-Assiut, Assiut 71524, Egypt

Ahmed Eliwa, Department of Internal Medicine, Faculty of Medicine, Al-Azhar University-Cairo, Cairo 11754, Egypt

Hytham Abdalla, Department of Chest Diseases, Faculty of Medicine, Al-Azhar University-Assiut, Assiut 71524, Egypt

Corresponding author: Ali Madian, MD, MSc, Lecturer, Department of Internal Medicine, Faculty of Medicine, Al-Azhar University-Assiut, Assiut 71524, Egypt.
a.madian@azhar.edu.eg

Abstract

BACKGROUND

Coronavirus disease 2019 (COVID-19) has a spectrum of clinical syndromes with serious involvement of the lung and frequent effection of the liver and hemostatic system. Blood biomarkers are affordable, rapid, objective, and useful in the evaluation and prognostication of COVID-19 patients.

AIM

To investigate the association between aspartate transferase-to-platelet ratio index (APRI) and in-hospital mortality to develop a COVID-19 mortality prediction model.

METHODS

A multicenter cohort study with a retrospective design was conducted. Medical records of all consecutive adult patients admitted to Al-Azhar University Hospital (Assiut, Egypt) and Chest Hospital (Assiut, Egypt) with confirmed COVID-19 from July 1, 2020 to October 1, 2020, were retrieved and analyzed. The patient cohort was classified into the following two categories based on the APRI: (1) COVID-19 presenting with APRI ≤ 0.5; and (2) COVID-19 presenting with APRI (> 0.5 and ≤ 1.5). The association between APRI and all-cause in-hospital mortality was analyzed, and the new model was developed through logistic regression analyses.

RESULTS

Of the 353 patients who satisfied the inclusion criteria, 10% were admitted to the intensive care unit (n = 36) and 7% died during the hospital stay (n = 25). The median age was 40 years and 50.7% were male. On admission, 49% had aspartate transferase-dominant liver injury. On admission, APRI (> 0.5 and ≤ 1.5) was independently associated with all-cause in-hospital mortality in unadjusted regression analysis and after adjustment for age and sex; after stepwise adjustment for several clinically relevant confounders, APRI was still significantly associated with all-cause in-hospital mortality. On admission, APRI (> 0.5 and ≤ 1.5) increased the odds of mortality by five-times (P < 0.006). From these results, we developed a new predictive model, the APRI-plus, which includes the four predictors of age, aspartate transferase, platelets, and serum ferritin. Performance for mortality was very good, with an area under the receiver operating curve of 0.90.

CONCLUSION

APRI-plus is an accurate and simplified prediction model for mortality among patients with COVID-19 and is associated with in-hospital mortality, independent of other relevant predictors.

Key Words: COVID-19; Aspartate transferase-to-platelet ratio index; Aspartate transferase; All-cause in-hospital mortality; Serum ferritin; SARS-CoV-2

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Core Tip: Aspartate transferase-to-platelet ratio index-plus can be used to predict the severity of coronavirus disease 2019. The performance of the model for mortality was very good, with an area under the receiver operating curve of 0.90. This new prediction model could help in estimating the risk of mortality and may, therefore, assist in triaging patients. Moreover, our study confirmed that an aspartate transferase-dominant pattern, diabetes mellites, leukocytosis, and increased ferritin levels are associated with fatal outcomes.

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INTRODUCTION

The coronavirus disease 2019 (COVID-19) pandemic has prompted a global race to develop a variety of vaccine platforms. As a result, the first vaccine had been approved for emergency use by approximately 1 year from the beginning of the outbreak[1]. However, the ongoing limited availability of vaccines, particularly in low-income countries, means that the approach will not be sufficient to achieve global herd immunity. Therefore, effective and efficient allocation of health care resources is essential during the management of COVID-19 patients.

Blood biomarkers are affordable, rapid, readily available, and objective. It has been proven that blood biomarkers are useful in the evaluation and prognostication of COVID-19 patients. Many studies have demonstrated lymphopenia and thrombocytopenia among COVID-19 patients on hospital admission[2,3]. Furthermore, both lymphopenia[3,4] and thrombocytopenia[5] have been shown as predictive of COVID-19 severity and mortality.

Previously, many reports have cited hepatocellular injury with an aspartate aminotransferase (AST)-dominant pattern at hospital admission[6,7]. In addition, it has been demonstrated that AST elevation at admission is associated with severe COVID-19 disease status and poor outcomes, including intensive care admission, need for mechanical ventilation[7], and all-cause in-hospital mortality[6]. It has also been reported that elevated serum ferritin on admission is associated with fatal outcomes in patients with COVID-19[8,9].

Patients with COVID-19 associated with thrombocytopenia and hepatocellular injury progress to severe disease and poor outcomes. Previously, a simple scoring system, the AST-to-platelet ratio index (APRI), was developed to predict fibrosis in patients with chronic hepatitis C, being based upon routine blood biomarkers, including AST and platelets[10]. Using this as a premise, we hypothesized that it is possible to build a similar prediction model for patients with COVID-19 using objective, inexpensive, and readily available items.

The aim of the current study was to investigate whether APRI is associated with all-cause in-hospital mortality among patients with COVID-19 and develop a predictive model using objective and readily available factors.

MATERIALS AND METHODS

We conducted a multicenter retrospective cohort study using medical records of all consecutive adult patients admitted to Al-Azhar University Hospital in Assiut, Egypt and Chest Hospital in Assiut, Egypt with confirmed COVID-19 from July 1, 2020 to October 1, 2020, as the Egyptian Ministry of Health had allocated both hospitals for the management of COVID-19 patients. The inclusion criteria for patients were complete medical record data, hospitalized adults of ages ≥ 18 years, and COVID-19 diagnosis established by examination of nasopharyngeal swab specimens using reverse-transcriptase PCR [*CerTest* Viasure® SARS-CoV-2 Real Time PCR Detection Kit (CerTest; Biotec, Spain)] for severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). All laboratory tests were operated in the accredited laboratories at the Clinical Pathology Departments (Microbiology and Immunology units) in Al-Azhar University Hospital and Chest Hospital. The exclusion criteria were pediatric patients of ages < 18 years, patients with known chronic liver disease or newly diagnosed with chronic liver disease at hospital admission, pregnant females, patients with incomplete data, patients with splenomegaly, splenectomy, chemotherapy, or radiotherapy within 1 mo of hospital admission, and patients on immunosuppressive therapy. In addition, we excluded any patient with marked fibrosis, as determined by both APRI and fibrosis-4 (FIB-4) scores (FIB-4 > 2.67 and APRI > 1.5). The institutional review boards approved the study protocol.

Cohort assignment

AST and platelets were measured within 24 h of hospital admission. The normal upper limit for AST was 40 U/L, and the normal upper limit and lower limit for platelets were 350 ´ 10^9/L and 150 ´ 10^9/L, respectively. APRI was calculated according to the following equation: "AST Level/ULN ÷ Platelet count × 100"[10]. We categorized our patient cohort into the following two categories based on APRI: (1) COVID-19 presenting with APRI ≤ 0.5; and (2) COVID-19 presenting with APRI (> 0.5 and ≤ 1.5).

Covariates

For every participant of our cohort, demographic criteria, including age, sex, and smoking status, comorbidities, including as diabetes mellites (DM), hypertension (HTN), chronic kidney disease (CKD), chronic obstructive pulmonary disease (COPD), and Deyo-Charlson index, and laboratory biomarkers, including complete blood count, liver enzymes, serum albumin, serum total bilirubin, blood urea, and serum creatinine and creatine kinase, were retrieved and analyzed. The FIB-4 was calculated by the following equation:

Age (year) × AST(U/L) ÷ Platelets (1000/μL) × √ALT(U/L)[11].

Outcome measurement

The primary outcome of interest was all-cause in-hospital mortality. The vital status of each patient in the study cohort was confirmed from hospital records. Follow-up of outcome was ended on October 15, 2020.

Statistical analysis

We summarized continuous variables as medians and interquartile ranges, and categorical variables were summarized as absolute numbers and percentages. We utilized unadjusted binary logistic regression for analyses of group differences. We performed a stepwise analysis adjusted for age and sex, as well as for relevant confounding factors, to investigate clinical confounders. Spearman's correlation coefficients were utilized to analyze the relationships between variables. All *P* values calculated were two-tailed; values less than 0.05 were considered as indicative of statistical significance. Prediction performance was evaluated by the area under the curve of the receiver operating curve (AUROC). Overall fit of the models was evaluated by the Hosmer-Lemeshow test and the Bayesian information criterion. We used Stata Software (Stata Statistical Software: Release 16. College Station, TX, United States: Stata Corp LP) for data visualization and analysis.

RESULTS

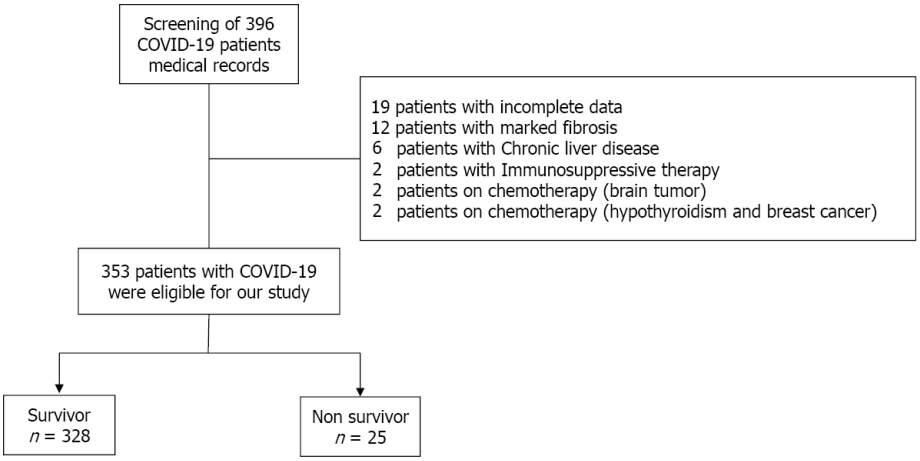
Out of the 396 patients considered, 353 satisfied the inclusion criteria. Among those, 10% of the study cohort was admitted to the intensive care unit (n = 36) and 7% of the study participants succumbed during hospitalization (n = 25) (Figure 1). The median age at hospital admission was 40 years [interquartile range (IQR): 28-55 years], and 50.7% were males (n = 179) (Table 1). The frequency of

Table 1 Baseline demographic, clinical and laboratory characteristics of survivors and non-survivors' groups

Characteristics	Survivors, *n* = 328	Non-survivors, *n* = 25	Unadjusted OR	*P* value
Age in yr, median (IQR)				
< 40	178 (98.89)	2 (1.11)	Ref.	
40-60	112 (94.92)	6 (5.08)	4.76 (0.94-24.03)	0.05
> 60	38 (69.09)	17 (30.91)	39.81 (8.82-179.59)	0.0001
Male sex, *n* (%)	167 (93.30)	12 (6.70)	0.88 (0.39-2.00)	0.77
Comorbidities				
Chronic kidney disease, *n* (%)	4 (44.44)	5 (55.56)	20.25 (5.04-81.31)	0.0001
Diabetes mellites, *n* (%)	55 (77.46)	16 (22.54)	8.82 (3.70-20.98)	0.0001
Chronic obstructive pulmonary disease, *n* (%)	55 (88.71)	7 (11.29)	1.93 (0.76-4.84)	0.16
Hypertension, *n* (%)	84 (84.00)	16 (16.00)	5.16 (2.19-12.12)	0.0001
Deyo-Charlson index, *n* (%)				
0-1	267 (97.45)	7 (2.55)	Ref.	
2-3	60 (80.00)	15 (20.00)	9.53 (3.72-24.40)	0.0001
> 3	1 (25.00)	3 (75.00)	114.42 (10.54-1241.77)	0.0001
Laboratory biomarkers				
Hemoglobin < 12 mg/dL, *n* (%)	125 (92.59)	10 (7.41)	1.08 (0.47-2.48)	0.85
Total leukocytic count > 11 × 10^9/L, *n* (%)	50 (80.65)	12 (19.35)	5.13 (2.21-11.89)	0.0001
Platelet				
150-350	248 (93.58)	17 (6.42)	Ref.	
< 150 × 10^9/L, *n* (%)	32 (94.12)	2 (5.88)	0.91 (0.20-4.13)	0.90
> 350 × 10^9/L, *n* (%)	48 (88.89)	6 (11.11)	1.82 (0.68-4.86)	0.23
Serum AST, *n* (%)				
< 40 U/L	239 (97.15)	7 (2.85)	Ref.	
40-80 U/L	81 (84.38)	15 (15.63)	6.32 (2.49-16.05)	0.0001
> 80 U/L	8 (72.73)	3 (27.27)	12.80 (2.78-58.83)	0.001
Serum ALT, *n* (%)				
< 40 U/L	217 (93.94)	14 (6.06)	Ref.	
40-80 U/L	90 (90.00)	10 (10.00)	1.72 (0.73-4.02)	0.20
> 80 U/L	21 (95.45)	1 (4.55)	0.73 (0.09-5.89)	0.77
Serum albumin < 3.5 g/dL, *n* (%)	112 (86.15)	18 (13.85)	4.95 (2.01-12.22)	0.001
Serum total bilirubin > 1.5 mg/dL, *n* (%)	2 (40.00)	3 (60.00)	22.22 (3.52-140.03)	0.001
Serum creatinine > 1.1 mg/dL for males; > 0.95 mg/dL for females, *n* (%)	64 (80.00)	16 (20.00)	7.33 (3.09-17.34)	0.0001
Serum ferritin > 400 μg/L for males; > 150 μg/L for females, *n* (%)	169 (87.56)	24 (12.44)	22.57 (3.01-168.86)	0.002
D-dimer > 0.5 μg/mL	319 (92.73)	25 (7.27)	1	-
C-reactive protein ≥ 1 mg/L, *n* (%)	330 (92.70)	26 (7.30)	1	-
Creatine kinase > 117 IU/L, *n* (%)	0.00 (100.00)	0.00 (0.00)	-	-
APRI, *n* (%)				
≤ 0.5	254 (95.85)	11 (4.15)	Ref.	
> 0.5	74 (84.09)	14 (15.19)	4.36 (1.90-10.02)	0.001

FIB-4, *n* (%)				
≤ 2	300 (96.15)	12 (3.85)	Ref.	
> 2	17 (73.91)	6 (26.09)	8.82 (2.95-26.37)	0.0001
> 2.67	11 (61.11)	7 (38.89)	15.90 (5.24-48.24)	0.0001

Odds ratios were calculated by univariate logistic regression. Univariate logistic regression was used to calculate *P* value for the characteristics' differences between survivors and survivors. ALT: Alanine transferase; APRI: AST-to-platelet ratio index; AST: Aspartate transferase; CRP: C-reactive protein; FIB-4: Fibrosis-4 score; IQR: Interquartile range; OR: Odds ratio.



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Figure 1 Flowchart of the studied cohort. COVID-19: Coronavirus disease 2019.

thrombocytopenia was 9.6% (*n* = 34). Thrombocytosis was reported among 15% (*n* = 54) of the study cohort. However, there was no statistically significant difference in platelet count between the survivor and death groups (Table 1). On admission, 49% of study participants had AST-dominant liver injury (*n* = 174). Study participants were grouped based on all-cause in-hospital death into survivor and nonsurvivor groups, and their features are provided in Table 1.

Predictors of the primary outcome

Variables associated with in-hospital mortality were first assessed by univariate analysis. Significant variables from univariate analysis ($P < 0.05$) or clinically relevant variables were then utilized for multivariate analysis by forward logistic regression to identify independent predictors associated with in-hospital death among patients with COVID-19.

Regression analysis: (1) Binary logistic regression analysis. Age, Deyo-Charlson index, CKD, DM, and HTN were the clinical predictors significantly associated with hospital mortality. Additionally, the unadjusted regression demonstrated that APRI, serum ferritin, total leukocytic count, serum total bilirubin, serum creatinine, serum AST, and serum albumin levels were significant biochemical markers associated with hospital death. There was no association between hospital mortality and sex, COPD, hemoglobin, platelets, serum alanine transferase (ALT), C-reactive protein (CRP), creatine kinase, or D-dimer levels (Table 1). (2) Multivariable logistic regression analysis. The APRI (> 0.5 - ≤ 1.5) at admission was significantly associated with hospital mortality in the unadjusted binary logistic regression analysis and remained a significant predictor of the odds of hospital mortality in model 1 adjusted for age and sex (Table 2). In addition, in model 2, after adjustment for several covariates, APRI was still a significant predictor of hospital mortality and associated with increased odds of hospital mortality by five times ($P < 0.005$) (Table 2).

Variables in the best model (full model) for the prediction of death among patients with COVID-19 included APRI, age, CKD, DM, total leukocytic count, and serum ferritin. The AUROC for the prediction of death among patients with COVID-19 was 0.94 [95% confidence interval (CI): 0.90-0.98]. When the prediction model comprised APRI alone (APRI model), the AUROC for the prediction of mortality was 0.66 (95%CI: 0.56-0.76). The model with APRI, age, and serum ferritin (APRI-plus model) had better accuracy than the APRI model, and the AUROC became 0.90 (95%CI: 0.86-0.95) ($P < 0.0001$) (Figure 2). In addition, the APRI-plus model showed good calibration (Hosmer-Lemeshow $\chi^2 = 1.7$, $P = 0.97$) and was a better-fitting model than the full model (Bayesian information criterion 148.4) (Table 3).

Table 2 Odds ratios of aspartate transferase-to-platelet ratio index associated mortality and 95% confidence intervals

	Unadjusted			Model I (Adjusted for age and sex)			Model II		
APRI	OR	CI	*P* value	OR	CI	*P* value	OR	CI	*P* value
≤ 0.5	Ref.			Ref.			Ref.		
> 0.5 - ≤ 1.5	4.36	1.90-10.02	0.001	3.23	1.29-8.12	0.01	5.03	1.63-15.52	0.005
						Covariates			
						Age in yr			
						< 40	Ref.		
						40-60	1.75	0.25-11.89	0.56
						> 60	10.45	1.51-72.09	0.01
						Sex	1.19	0.39-3.65	0.75
						CKD	8.24	1.37-49.53	0.02
						DM	7.77	1.82-33.04	0.006
						HTN	0.25	0.05-1.11	0.07
						WBC	4.20	1.32-13.32	0.01
						Albumin	1.17	0.33-4.09	0.81
						Bilirubin	10.10	0.97-104.45	0.05
						Ferritin	12.94	1.38-121.08	0.02

Adjusted odds ratios for hospital mortality. Model 1 adjusted for age and sex; Model 2 adjusted for age, sex, diabetes mellites, chronic kidney disease, hypertension, white blood cells, serum albumin, serum total bilirubin, and serum ferritin. APRI: Aspartate transferase-to-platelet ratio index; CKD: Chronic kidney disease; CI: Confidence interval; DM: Diabetes mellites; HTN: Hypertension; IQR: Interquartile range; WBCs: White blood cells.

Table 3 Comparison of full model and aspartate transferase-to-platelet ratio index-plus in prediction of mortality among patients with coronavirus disease 2019 in the study cohort

	AUROC	HL-χ^2	BIC
APRI-plus model	0.90 (95%CI: 0.86–0.95)	0.97	148.4
Full model	0.94 (95%CI: 0.90–0.98)	0.95	164.9

AUROC: Area under the receiver operating characteristic curve; BIC: Bayesian information criterion; CI: Confidence interval; HL-χ^2: Hosmer-Lemeshow χ^2 goodness-of-fit test.

The adjusted equation for the APRI-plus in patients with COVID-19 was *P* (in-hospital mortality) = -0.18 + 0.06 × (APRI > 0.5 and ≤ 1.5) (yes = 1, no = 0) + 0.004 × age + 0.00013 × ferritin.

Comparison with FIB-4

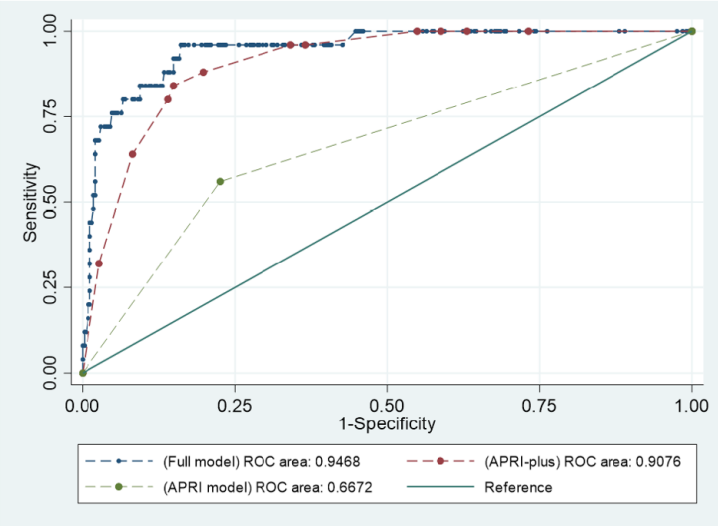
The AUROC for FIB-4 score was 0.72 (95%CI: 0.61-0.82). Upon comparison of the APRI-plus with FIB-4, the AUROC was significantly higher for APRI-plus, at 0.92 (95%CI: 0.86-0.98) ($P < 0.0001$) (Figure 3).

Association of D-dimer with inflammatory markers

D-dimer was examined as a marker of thrombosis. On admission, platelets, serum ferritin, CRP, total leukocytic count, and AST were correlated with D-dimer ($r = 0.1$; $P < 0.03$, $r = 0.4$; $P < 0.0001$, $r = 0.4$; $P < 0.0001$, $r = 0.1$; $P < 0.01$, and $r = 0.1$; $P < 0.03$, respectively).

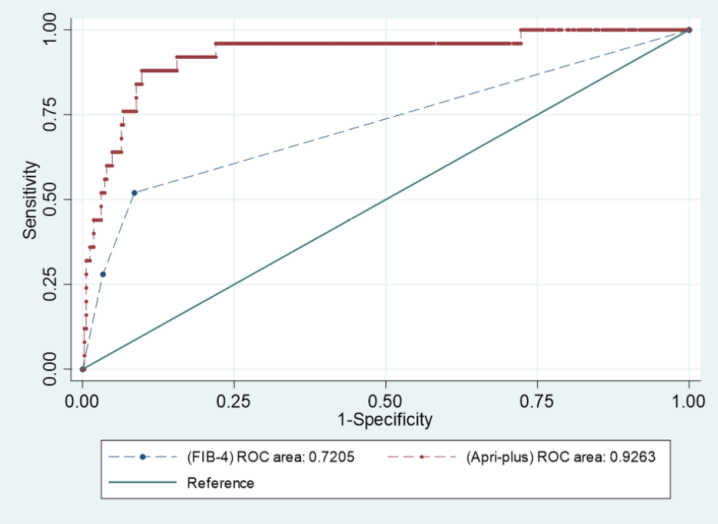
Association of APRI components with markers of inflammation

Serum ferritin, CRP, and total leukocytic count were investigated as inflammatory markers. Serum levels of AST were correlated with serum ferritin ($r = 0.2$; $P < 0.0001$), CRP ($r = 0.2$; $P < 0.0001$), and total leucocytic count ($r = 0.1$; $P < 0.007$). Platelets were correlated with serum ferritin ($r = 0.1$; $P = 0.04$), CRP ($r = 0.07$; $P = 0.1$), and total leukocytic count ($r = 0.1$; $P = 0.05$).



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Figure 2 Receiver operating characteristic curves comparing full model, aspartate transferase-to-platelet ratio index and aspartate transferase-to-platelet ratio index-plus in prediction of mortality among patients with coronavirus disease 2019 in the study cohort. APRI: Aspartate transferase-to-platelet ratio index; COVID-19: Coronavirus disease 2019.



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Figure 3 Receiver operating characteristic curves comparing aspartate transferase-to-platelet ratio index-plus with the fibrosis-4 score in prediction of mortality among patients with coronavirus disease 2019 in the study cohort. APRI: Aspartate transferase-to-platelet ratio index; COVID-19: Coronavirus disease 2019; FIB-4: Fibrosis-4.

DISCUSSION

In the current study, we developed APRI-plus, a new, simple, inexpensive, and objective clinical score that is significantly associated with mortality among patients with COVID-19. APRI (> 0.5 and ≤ 1.5) increased the odds of mortality by five-fold. Since patients with chronic liver disease were excluded from participation in our analysis, this association is likely due to inflammation related to COVID-19 and the direct impact of SARS-Cov-2 on the liver and hemostatic system.

We observed a significant difference in AST between survivors and nonsurvivors at hospital admission. The AST-dominant pattern of hepatocellular injury among patients with COVID-19 has been reported many times in previous studies[6,7]. Likewise, the levels of serum ferritin were significantly higher among nonsurvivors. Platelets have been reported to be one of the strongest indicators of adverse outcomes and mortality among patients with COVID-19[2,3]. However, in our cohort, we did not find any statistically significant difference in platelets between survivors and nonsurvivors. Thus, platelets alone are less ideal in predicting outcomes among patients with COVID-19.

APRI includes AST in the numerator and platelets in the denominator. These criteria make the APRI score a better predictor of COVID-19 outcomes than AST or platelets alone. Regarding its accuracy in predicting mortality among patients with COVID-19, the APRI has an AUROC of 0.66. However, when age and serum ferritin were incorporated in the regression model (APRI-plus model), the performance of the model had an AUROC of 0.90. In contrast, compared with another noninvasive score, FIB-4 (composed of AST, ALT, age, and platelets), we found that the AUROC was significantly lower for FIB-4 (0.72). In line with our findings, the reported AUROC in an earlier study for FIB-4 was 0.79[11]. Using APRI-plus, it is possible to predict mortality among patients with COVID-19 with good performance.

Liver stiffness among patients with COVID-19 is likely to be multifactorial. Hepatic congestion, hepatocellular injury, and systemic inflammation may all play a role in the development of liver stiffness among patients with COVID-19. It has been reported that COVID-19 is associated with increased pressure within the right ventricle[12]; thus, this may result in hepatic venous congestion and liver stiffness[11].

Severe liver injury caused by SARS-CoV-2 Likely results in mitochondrial damage and defective clearance of AST, leading to an AST-dominant pattern. At the onset of severe SARS-CoV-2 infection, severe liver injury is likely associated with extensive inflammation, hepatocyte swelling, and tissue edema, especially among patients with an AST-dominant pattern. Similar to acute viral hepatitis, extensive necroinflammatory activity among patients with COVID-19 increases liver stiffness[13].

Our cohort revealed that platelet count did not have a significant association with mortality. Interestingly, not all previous studies have found platelet counts to be a predictor of COVID-19 mortality[14]. Inflammatory mediators associated with SARS-CoV-2 infection may result in the activation of platelets. Activated platelets have two pathways. First, enhanced platelet clearance/sequestration from circulation by the spleen results in thrombocytopenia. Second, consumptive coagulopathy, such as inflammation-mediated endothelial damage/activation in addition to platelet activation, leads to the formation of platelet aggregates through systemic circulation and/or pulmonary circulation[15]. Therefore, we can speculate that the early phase of SARS-CoV-2 infection results in the activation of platelets followed by thrombocytopenia. Our results support this speculation, as inflammatory markers (CRP, serum ferritin, and leukocytosis) were correlated with D-dimer. This may suggest that the mechanism and progression of platelet activation rather than platelet number were associated with worse outcomes.

The rising question is why we did not find a significant difference in D-dimer between the survivor and nonsurvivor groups. This could be explained by COVID-19-associated coagulopathy. Coagulation changes associated with COVID-19 have the following three proposed stages: stage 1 presents with raised D-dimer; stage 2 presents with raised D-dimer together with modestly increased prothrombin time/international normalization ratio, activated partial thromboplastin time, and mild thrombocytopenia; and stage 3 presents with critical illness and laboratory markers, directing towards classic disseminated intravascular coagulopathy[14]. Therefore, our data may be retrieved early during platelet activation and before firing of stage 1 COVID-19-associated coagulopathy.

Limitations of the current study

The retrospective design of the study reflected the association between APRI-plus and risk of mortality among patients with COVID-19 but did not reflect causality. We used APRI at the time of admission for group categorization without knowing follow-up changes. Because of the lack of follow-up, we do not know whether these necroinflammatory changes were self-limiting or progressive. Moreover, we do not know the extent and duration of resolution. We did not evaluate platelet activity, fibrin degradation products, or coagulation profiles. Elastography should be evaluated in different age groups to dissect the impact of age from COVID-19 on liver stiffness. Validation of the APRI-plus is required.

CONCLUSION

The current study showed that, on admission, APRI-plus among patients with COVID-19 has good performance in predicting mortality. A prediction model could help stratify the risk of mortality. This association may be explained by the impact of SARS-CoV-2 infection on the liver and hemostatic system. Further studies are required to investigate this association.

ARTICLE HIGHLIGHTS

Research background

Coronavirus disease 2019 (COVID-19) has a spectrum of clinical syndromes with serious involvement of the lung and frequent effection of the liver and hemostatic system.

Research motivation

Development of a prediction model using objective, inexpensive, and readily available items is needed for patients with COVID-19.

Research objectives

To investigate whether the aspartate transferase-to-platelet ratio index (APRI) is associated with all-cause in-hospital mortality among patients with COVID-19 and develop a predictive model using objective and readily available factors.

Research methods

A retrospective cohort study was carried out with 353 consecutive adult patients admitted to Al-Azhar University Hospital (Assiut, Egypt) and Chest Hospital (Assiut, Egypt) with confirmed COVID-19 from July 1, 2020 to October 1, 2020.

Research results

The prediction model comprised APRI alone (APRI model), and the area under the receiver operating curve (AUROC) for the prediction of mortality was 0.66 [95% confidence interval (CI): 0.56-0.76]. A modified model of APRI that included age and serum ferritin (APRI-plus model) had better accuracy than the APRI model, as the AUROC became 0.90 (95%CI: 0.86-0.95) ($P < 0.0001$).

Research conclusions

APRI-plus among patients with COVID-19 showed good performance in predicting mortality. A prediction model could help stratify the risk of mortality.

Research perspectives

Further studies are required to investigate this association. Validation of the APRI-plus is required.

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FOOTNOTES

Author contributions: Madian A was responsible for the study conception and design, the data acquisition, analysis and interpretation, the statistical analysis, and writing of the first draft of the manuscript; Eliwa A and Abdalla H contributed to the data collection; Azeem HA reviewed the manuscript and provided critical scientific input; all authors approved the final manuscript.

Institutional review board statement: The study was approved by the Ethics Committee of Faculty of Medicine, Al-Azhar University, Assiut, Egypt.

Informed consent statement: No informed consent was required, as patient identity is not revealed in the retrospective analysis.

Conflict-of-interest statement: No benefits in any form have been received or will be received from a commercial party related directly or indirectly to the subject of this article.

Data sharing statement: The original anonymous dataset is available on request from the corresponding author at a.madian@azhar.edu.eg.

STROBE statement: The authors have read the STROBE Statement—checklist of items, and the manuscript was prepared and revised according to the STROBE Statement—checklist of items.

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Country/Territory of origin: Egypt

ORCID number: Ali Madian 0000-0002-8322-7504; Ahmed Eliwa 0000-0003-0818-3086; Hytham Abdalla 0000-0001-5377-6572; Haitham A Azeem Aly 0000-0001-5732-5277.

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ORIGINAL ARTICLE

Retrospective Study

Association of maternal obesity and gestational diabetes mellitus with overweight/obesity and fatty liver risk in offspring

Jing Zeng, Feng Shen, Zi-Yuan Zou, Rui-Xu Yang, Qian Jin, Jing Yang, Guang-Yu Chen, Jian-Gao Fan

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Jing Zeng, Feng Shen, Zi-Yuan Zou, Rui-Xu Yang, Qian Jin, Jing Yang, Guang-Yu Chen, Jian-Gao Fan, Department of Gastroenterology, Xinhua Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai 200092, China

Jian-Gao Fan, Shanghai Key Lab of Pediatric Gastroenterology and Nutrition, Shanghai 200092, China

Corresponding author: Jian-Gao Fan, MD, PhD, Professor, Department of Gastroenterology, Xinhua Hospital, Shanghai Jiao Tong University School of Medicine, No. 1665 Kongjiang Road, Shanghai 200092, China. fanjiangao@xinhuamed.com.cn

Abstract

BACKGROUND

Childhood obesity and fatty liver are associated with adverse outcomes such as diabetes, metabolic syndrome, and cardiovascular diseases in adulthood. It is very important to identify relevant risk factors and intervene as early as possible. At present, the relationship between maternal and offspring metabolic factors is conflicting.

AIM

To estimate the association of maternal obesity and gestational diabetes mellitus (GDM) with overweight/obesity and fatty liver risk in offspring at 8 years of age.

METHODS

The prospective study included mothers who all had a 75-g oral glucose tolerance test at 24-28 wk of gestation and whose offspring completed follow-up at 8 years of age. Offspring birth weight, sex, height, weight, and body mass index (BMI) were measured and calculated. FibroScan-502 examination with an M probe (Echosens, Paris, France) was prospectively conducted in offspring aged 8 years from the Shanghai Prenatal Cohort Study.

RESULTS

A total of 430 mother-child pairs were included in the analysis. A total of 62 (14.2%) mothers were classified as obese, and 48 (11.1%) were classified as having GDM. The mean age of the offspring at follow-up was 8 years old. Thirty-seven (8.6%) offspring were overweight, 14 (3.3%) had obesity, and 60 (14.0%) had fatty liver. The prevalence of overweight, obesity and fatty liver in offspring increased significantly across maternal BMI quartiles (all $P < 0.05$). Among offspring of

mothers with GDM, 12 (25.0%) were overweight, 4 (8.3%) were obese, and 12 (25.0%) had fatty liver vs. 25 (6.5%), 10 (2.6%) and 48 (12.6%), respectively, for offspring of mothers without GDM (all $P < 0.05$). In multiple logistic regression, after adjustment for variables, the OR for fatty liver in offspring was 8.26 (95%CI: 2.38-28.75) for maternal obesity and GDM.

CONCLUSION

This study showed that maternal obesity can increase the odds of overweight/obesity and fatty liver in offspring, and GDM status also increases the odds of overweight/obesity in offspring. Weight management and glycemic control before and during pregnancy need to be highlighted in primary prevention of pediatric obesity and fatty liver.

Key Words: Maternal obesity; Gestational diabetes mellitus; Offspring overweight/obesity; Offspring fatty liver; FibroScan

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Core Tip: It is very important to identify relevant risk factors for childhood obesity and fatty liver and intervene as early as possible, considering their adverse outcomes. In this work, we reported the association of maternal obesity and gestational diabetes mellitus (GDM) with overweight/obesity and fatty liver risk in offspring at 8 years of age. This study showed that maternal obesity can increase the odds of overweight/obesity and fatty liver in offspring, and GDM status also increases the odds of overweight/obesity in offspring. Weight management and glycemic control before and during pregnancy need to be highlighted in primary prevention of pediatric obesity and fatty liver.

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INTRODUCTION

With economic development and changing living and eating habits, the prevalence of obesity in children has been rapidly increasing in recent decades[1]. It is alarming because it is also associated with health consequences such as metabolic syndrome, diabetes, cardiovascular diseases, and even many types of cancers in adulthood[2,3]. The incidence of fatty liver in children is also rising, due, in part, to the increasing prevalence of childhood obesity. At present, there is still no effective noninvasive means for diagnosing fatty liver in children. Recently, some novel noninvasive techniques for the assessment of liver fat have been developed. Transient elastography (TE) is one of these new techniques based on inducing a shear wave to the liver and measuring the velocity of the wave. The device (FibroScan-502, Echosens, Paris, France) was developed using the TE technique, and controlled attenuation parameter (CAP) and liver stiffness measurement (LSM) can be obtained simultaneously by the device in a rapid, noninvasive, reproducible, and painless way. FibroScan-502 has also been used in the assessment of liver fat and fibrosis in pediatric individuals with liver diseases, and the reference values of CAP have been studied in our previous article[4].

Recent studies have suggested that maternal body mass index (BMI) is associated with the birth weight of offspring and is a risk factor for offspring obesity[5,6]. Gestational diabetes mellitus (GDM) is the occurrence of glucose intolerance during pregnancy and usually resolves after birth[7]. Meanwhile, many studies have shown that GDM can increase the incidence of impaired glucose tolerance in offspring and increase the risk of offspring obesity[8,9].

Therefore, in this article, we aimed to assess whether maternal BMI and in utero exposure to GDM are associated with a long-term risk of overweight/obese and fatty liver among offspring 8 years postpartum.

MATERIALS AND METHODS

Study population

The individuals included in the prospective study were 430 maternal-child pairs from the Shanghai Prenatal Cohort Study, which is a prospective study that enrolled 1043 Han maternal-child pairs between January 2012 and December 2013 at Xinhua Hospital and International Peace Maternity and Child Hospital in Shanghai. The offspring were followed up at the age of 8 years (94 to 98 mo) with medical examinations. The exclusion criteria for the study population were as follows: (1) Non-Shanghai residents; (2) lost to follow-up; (3) missing some of the mothers' clinical information on prepregnancy and the offspring's anthropometric data; (4) mothers' medical history of diabetes (diagnosed before the index pregnancy) and other participants whose fasting glucose was ≥ 7.0 mmol/L before 12 gestational weeks; and (5) failure of FibroScan-502 measurement with an M probe. Ethics approval was obtained by the Ethics Committees (XHEC-C-2012-023). The parents of all the participating children were required to give informed consent for study participation and sign the written documents.

Clinical and laboratory data collection

All mothers' heights and weights were measured in light indoor clothing and without shoes during early pregnancy. The oral glucose tolerance test (OGTT) was conducted between 24 and 28 wk gestation among those mothers. All followed-up offspring underwent annual medical examination at the health examination center in Xinhua Hospital. Stadiometers (Seca 416 Infantmeter, United States) were used to measure height to the nearest 0.1 cm. Digital scales (Detector 6745 Baby Scale, United States) were used to measure body weight to the nearest 0.1 kg. Participant characteristics and anthropometric indices, including age, sex, body weight, height, chest circumference, waist circumference, hip circumference and BMI, were obtained.

Following a fast of at least 6 h, all offspring underwent FibroScan-502 examination with an M-probe (3.5 MHz) (Echosens, Paris, France) by the same physician. The device estimates liver stiffness in kilopascals (kPa) and liver steatosis in decibels/meter (dB/m). CAP in dB/m and LSM in kPa were obtained simultaneously by each examination. A TE examination was considered successful when 10 valid measurements with a success rate of at least 60% were conducted and the interquartile range (IQR) was less than 30% of the median LSM value[10]. Subjects with unsuccessful examinations were excluded from the analyses.

Work definitions

Maternal obese, overweight and lean: A BMI during the early pregnancy greater than 25 kg/m^2 was used to define the obese population, and a BMI less than 25 kg/m^2 was used to define the nonobese population. The nonobese population was further divided into lean (< 23 kg/m^2) and overweight (23-25 kg/m^2) groups.

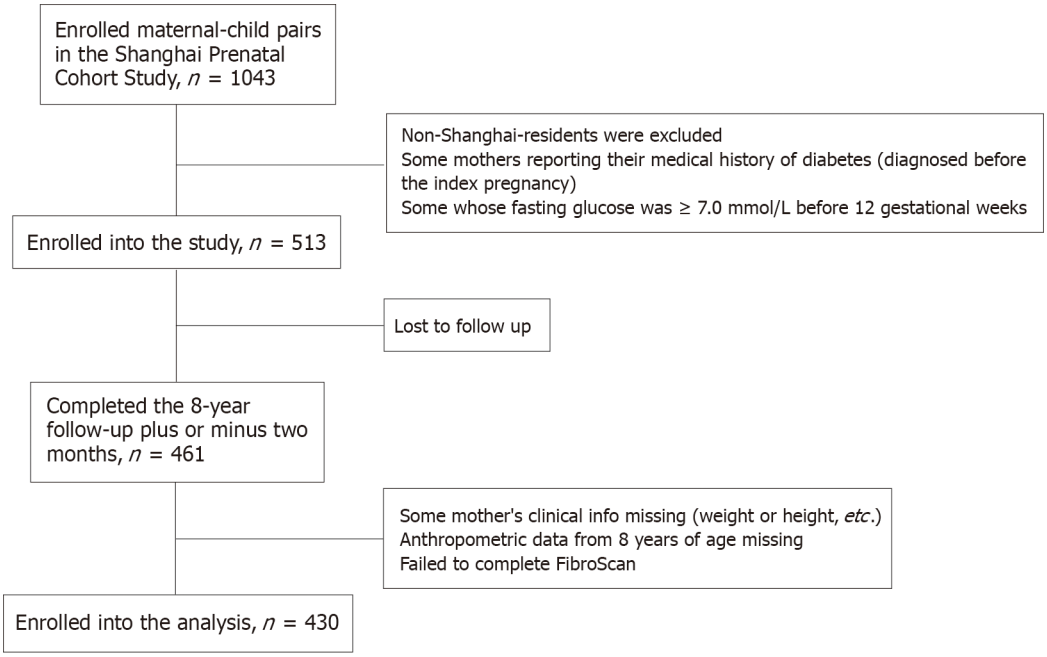
GDM: All mothers without diagnosed diabetes were screened for GDM by a one-step approach undergoing a 75g OGTT after fasting overnight between 24 and 28 wk gestation according to the guideline from Obstetrics and Gynecology Branch of Chinese Medical Association[11]. GDM was diagnosed when the glucose level that met or exceeded any of the following standards: a blood glucose value of 92, 180 or 153 mg/dL before or one or two hours after taking a 75 g glucose tolerance test, respectively.

Offspring overweight/obesity were defined by using the International Obesity Task Force age- and sex-specific cutoff points[12].

Offspring fatty liver: The offspring were considered to have fatty liver when the CAP value exceeded the normal value of 214.53 dB/m[4].

Statistical analysis

Continuous variables are expressed as the mean ± SD for a normal distribution and as the median ± IQR for a skewed distribution. General linear models for continuous variables were used to compare means of characteristics, and the χ^2 test for categorical variables was applied to compare offspring proportions across quartiles of maternal BMI during early pregnancy. We further explored the effects of maternal GDM status on such associations by a stratified analysis according to GDM status. Multivariate logistic regression models were used to examine the relationship between maternal BMI during early pregnancy and GDM status and offspring overweight/obesity status and fatty liver prevalence. Multiple logistic regression was used for continuous outcomes, and the results are reported as odds ratios (ORs) with 95%CIs. Three multivariate-adjusted models were included in these analyses. Significance tests were two tailed, and a P value < 0.05 was considered statistically significant. The data analysis for this article was generated using SAS Version 9.4.



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Figure 1 Flow diagram of participants included in this study.

RESULTS

Participant characteristics

A total of 513 maternal-child pairs from the Shanghai Prenatal Cohort Study were prospectively followed for 8 years. Of these individuals, 430 mothers and their offspring were included in the analysis (Figure 1).

The characteristics of participating mothers and offspring are shown in Table 1. The mean maternal age before pregnancy was 29 (4.9) years (20 to 42 years old). The mean maternal BMI was 21.55 (3.59) kg/m^2. A total of 62 (14.2%) mothers were classified as obese, and 48 (11.1%) were classified as having GDM. The mean birth weight of the offspring was 3.40 ± 0.48 kg and 210 (48.8%) were boys. A total of 37 (8.6%) offspring were classified as overweight, 14 (3.3%) offspring were classified as obese, and 60 (14.0%) had fatty liver (Table 1).

Maternal BMI and the characteristics of the mothers and offspring

Across maternal BMI quartiles, mothers in higher maternal BMI quartiles were more likely to have a greater weight and GDM. The offspring of mothers in higher maternal BMI quartiles were also more likely to have greater birth weight, BMI, waist circumference, hip circumference, chest circumference and CAP values and to be more prone to overweight/obese and fatty liver (Table 1).

The association of maternal BMI with outcomes for offspring

The fatty liver risk of these offspring increased progressively from the lowest to the highest quartiles of maternal BMI, with odds ratios (ORs) of 5.84 (95%CI: 0.67-50.67), 9.76 (95%CI: 1.21-78.83), and 26.3 (95%CI: 3.21-215.3), respectively, after controlling for the sex and age of the offspring (Model 1). Further adjusting for maternal age, nulliparity (Model 2), GDM status of the mothers and birth weight (Model 3) did not change the associations (Table 2).

The OR of the offspring overweight/obesity risk in the highest quartile of maternal BMI was 10.6 (95%CI: 2.17-51.76) after controlling for the sex and age of the children (Model 1) (Table 2).

Association of maternal GDM status with offspring overweight/obesity and CAP values

As shown in Table 3, mothers with GDM weighed more, had higher maternal BMIs and had a higher prevalence of maternal obesity than mothers without GDM (all $P < 0.05$). The offspring of mothers with GDM had higher BMI, chest circumference, hip circumference, and CAP values (all $P < 0.05$). Among offspring whose mothers had GDM, 4 (8.3%) were obese, compared with 10 (2.6%) offspring whose mothers did not have GDM ($P < 0.000$). However, there were no significant differences in birth weight, sex distribution, weight at follow-up, waist circumference, or LSM values between the offspring of mothers with and without GDM (Table 3).

Table 1 Characteristics of mothers and their offspring stratified into groups based on maternal body mass index quartiles at follow-up

Variables	All (*n* = 430)	Quartiles of maternal BMI (kg/m²)				
		Q1 (*n* = 108): 15.00-19.13	Q2 (*n* = 105): 19.14-20.76	Q3 (*n* = 115): 20.77-23.44	Q4 (*n* = 102): 23.45-42.00	*P* value
Maternal characteristics						
Age, yr	29.33 ± 4.89	29.21 ± 4.59	28.97 ± 3.14	29.59 ± 3.73	29.44 ± 7.36	0.805
Height, cm	162.97 ± 16.46	162.77 ± 5.09	161.84 ± 4.93	161.61 ± 4.29	162.26 ± 6.18	0.358
Weight, kg	56.73 ± 9.77	47.48 ± 3.45	52.21 ± 3.53	57.76 ± 3.53	69.9 ± 9.1	0.000
BMI, kg/m^2	21.55 ± 3.59	17.92 ± 0.97	19.91 ± 0.43	22.1 ± 0.79	26.57 ± 3.3	0.000
GDM, *n* (%)	48 (11.2)	6 (5.6)	8 (7.6)	11 (9.6)	23 (22.5)	0.000
Offspring characteristics						
Birth weight, kg	3.40 ± 0.48	3.33 ± 0.42	3.34 ± 0.39	3.42 ± 0.55	3.51 ± 0.51	0.021
Boy, *n* (%)	210 (48.8)	47 (43.5)	53 (50.5)	53 (46.1)	57 (55.9)	0.304
Height, cm	122.78 ± 29.93	121.59 ± 31.31	129.03 ± 4.91	118.81 ± 36.96	123.35 ± 31.01	0.242
Weight, kg	25.58 ± 10.57	22.98 ± 8.83	26.37 ± 7.8	25.18 ± 10.25	28.15 ± 13.12	0.070
BMI, kg/m^2	15.89 ± 4.42	14.58 ± 3.81	15.70 ± 4.00	16.20 ± 3.69	17.25 ± 5.81	0.025
Waist circumference, cm	58.70 ± 10.79	54.33 ± 12.76	58.33 ± 7.08	59.15 ± 9.32	63.13 ± 9.42	0.000
Hip circumference, cm	69.04 ± 13.73	65.3 ± 14.55	69.17 ± 13.47	69.39 ± 7.36	72.14 ± 13.39	0.041
Chest circumference, cm	56.19 ± 21.03	49.79 ± 22.55	53.44 ± 22.62	56.72 ± 16.15	63.41 ± 14.73	0.003
Waist-height ratio	0.46 ± 0.05	0.44 ± 0.05	0.45 ± 0.05	0.46 ± 0.04	0.48 ± 0.06	0.000
Waist-hip ratio	0.84 ± 0.08	0.83 ± 0.06	0.84 ± 0.07	0.84 ± 0.06	0.86 ± 0.12	0.292
Chest-height ratio	0.44 ± 0.14	0.4 ± 0.16	0.44 ± 0.12	0.41 ± 0.17	0.48 ± 0.11	0.023
CAP, dB/m	167.71 ± 46.09	151.76 ± 37.01	164.33 ± 41.45	169.97 ± 40.76	188.38 ± 58.89	0.001
LSM, kPa	3.36 ± 0.75	3.31 ± 0.8	3.52 ± 0.8	3.35 ± 0.73	3.4 ± 0.71	0.480
Overweight, *n* (%)	37 (8.6)	4 (3.7)	7 (6.7)	5 (4.4)	21 (20.6)	0.000
Obesity, *n* (%)	14 (3.3)	0 (0)	0 (0)	2 (1.7)	12 (11.8)	0.000
Fatty liver, *n* (%)	60 (14.0)	2 (1.9)	10 (9.5)	19 (16.5)	29 (28.4)	0.001

BMI: Body max index; GDM: Gestational diabetes mellitus; CAP: Controlled attenuation parameter; LSM: Liver stiffness measurement.

The CAP values of the offspring gradually increased in the mothers with neither obesity nor GDM (163.62 ± 44.41) dB/m, GDM but no obesity (173.43 ± 34.57) dB/m, obesity but no GDM (190.73 ± 49.74) dB/m to both obesity and GDM (202.15 ± 61.55) dB/m (all $P < 0.05$) (Figure 2).

Association of maternal obesity and GDM with outcomes for offspring

Maternal obesity was positively associated with childhood fatty liver with OR 4.57 (95%CI: 1.96-10.67) and childhood overweight/obesity with OR 5.73 (95%CI: 2.18-15.10) (Model 1). Further adjustment for maternal age, nulliparity (Model 2), GDM status of the mother and birth weight (Model 3) did not change the associations (Table 4).

Maternal GDM was also positively associated with childhood overweight/obesity, with an OR of 4.70 (95%CI: 1.72-12.81) (Model 1). Additionally, further adjustment for maternal age, nulliparity (Model 2), and offspring birth weight (Model 3) did not change the associations. In addition, the association of maternal GDM with childhood fatty liver was not statistically significant, with an OR of 2.39 (95%CI: 0.91-6.29) (Model 1) (Table 4).

Table 2 Adjusted odds ratios (95% confidence interval) of offspring overweight/obesity and fatty liver according to quartiles of maternal body max index

	Quartiles of BMI	Model 1		*P* value	Model 2		*P* value	Model 3		*P* value
		OR	95%CI		OR	95%CI		OR	95%CI	
Offspring fatty liver		1.21	1.1-1.33	0.000	1.21	1.1-1.34	0.000	1.23	1.11-1.36	0.000
	Q1	Reference			Reference			Reference		
	Q2	5.84	0.67-50.67	0.109	5.70	0.65-49.77	0.115	5.52	0.63-48.36	0.123
	Q3	9.76	1.21-78.83	0.033	9.60	1.18-77.79	0.034	9.50	1.17-77.33	0.035
	Q4	26.3	3.21-215.3	0.002	26.95	3.27-222.23	0.002	26.09	3.08-220.72	0.003
Offspring overweight/obesity		1.19	1.07-1.33	0.002	1.19	1.07-1.33	0.002	1.20	1.07-1.34	0.002
	Q1	Reference			Reference			Reference		
	Q2	2.09	0.37-11.60	0.401	2.23	0.40-12.57	0.364	2.17	0.38-12.5	0.385
	Q3	1.30	0.22-7.62	0.770	1.43	0.24-8.49	0.694	1.43	0.24-8.58	0.696
	Q4	10.6	2.17-51.76	0.004	11.66	2.34-58.14	0.003	10.75	2.05-56.28	0.005

Model 1: Adjusted for offspring age and sex; Model 2: Further adjusted for maternal age and nulliparous; Model 3: Further adjusted for gestational diabetes mellitus status of the mothers included in the model and offspring birth weight. OR: Odds ratio; CI: Confidence interval; BMI: Body mass index.

DISCUSSION

In this prospective birth cohort, we assessed the causal association of maternal metabolic disorders with offspring overweight/obesity and fatty liver in Han Chinese populations. This study demonstrated that high maternal BMI increased the odds of both childhood overweight/obesity and fatty liver, independent of maternal age, offspring birth weight, and childhood waist circumference at 8 years of age. Furthermore, maternal pregnancy glucose concentrations were positively correlated with offspring CAP values at school age. These two findings corroborated that the negative impacts of maternal obesity and impaired glucose metabolism on offspring livers are long-term and not merely limited to infancy.

More impressively, the negative effects of maternal obesity and impaired glucose metabolism might vary in degree. A recent cohort study based on magnetic resonance imaging found that maternal early-pregnancy glucose levels were associated with a 1.95-fold increase in odds of offspring non-alcoholic fatty liver disease (NAFLD) only among mothers of European ancestry[13]. In our study, maternal blood samples were collected in the second trimester. We observed that maternal mid-pregnancy glucose levels had only a weak relation with offspring fatty liver among Han Chinese populations, while maternal obesity was more strongly associated with offspring fatty liver than GDM.

Maternal obesity and impaired glucose metabolism have lasting impacts on offspring hepatic health through epigenetic, dietary, and metabolic factors[14,15]. A sibling comparison cohort reported that maternal weight gain was aligned with the odds of offspring obesity[16], suggesting that maternal overnutrition may be a predisposing factor for offspring metabolic dysbiosis. This conclusion was also validated in some animal models, such as macaques and mice, and investigators found that reversing the high-fat diet to a low-fat diet during the subsequent pregnancy alleviated offspring hepatic lipid accumulation[17-19]. In terms of mechanisms, one study demonstrated that maternal obesity might render innate immunity dysfunctional, and another study observed that maternal obesity accelerated the progression of offspring NAFLD through activation of lipogenesis and oxidative stress pathways[20,21].

Our observations were mutually verified with previous studies and have several differences as follows. Two studies focused on the relation between maternal factors and infant hepatic fat[22,23]. Modi *et al*[23] observed that increasing maternal BMI might initiate lipid accumulation in infant livers. Subsequently, Brumbaugh and colleagues reported that infants of GDM mothers had greater hepatic steatosis than infants of non-GDM mothers[22]. In contrast, our study revealed the relatively long-term health outcomes in school-age children to corroborate that such associations might predispose children to fatty liver later in life.

Another study in obese mothers observed a positive relation with offspring ultrasound-diagnosed NAFLD during adolescence. However, ultrasound has limited power to detect mild steatosis and cannot quantify histological characteristics such as hepatic lipid content and liver stiffness. In our studies, we assessed pediatric liver pathology through TE, which is a reliable noninvasive diagnostic tool for fibrosis assessment in NAFLD[24]. Meanwhile, a biopsy-confirmed study reported that an association

Table 3 Characteristics of mothers with and without gestational diabetes mellitus and their offspring at follow-up

Variables	Mother without GDM (*n* = 382)	Mother with GDM (*n* = 48)	*P* value
Maternal characteristics			
Age, yr	29.25 ± 5.1	29.98 ± 3.17	0.172
Height, cm	163.12 ± 17.48	162.02 ± 4.96	0.336
Weight, kg	56.16 ± 9.65	61 ± 9.47	0.001
BMI, kg/m^2	21.33 ± 3.59	23.22 ± 3.28	0.000
Obesity, *n* (%)	42 (11.0)	20 (41.7)	0.000
Children characteristics			
Birth weight, kg	3.41 ± 0.45	3.37 ± 0.61	0.653
Boy, *n* (%)	186 (49.1)	25 (52.1)	0.699
Height, cm	122.19 ± 30.67	125.07 ± 25.75	0.597
Weight, kg	25.13 ± 10.28	29.15 ± 10.38	0.058
BMI, kg/m^2	15.74 ± 4.43	17.72 ± 3.94	0.024
Waist circumference, cm	58.2 ± 10.79	61.39 ± 10.23	0.148
Hip circumference, cm	68.15 ± 13.73	73.83 ± 7.94	0.003
Chest circumference, cm	54.38 ± 21.03	64.48 ± 7.96	0.000
Waist-height ratio	0.46 ± 0.05	0.47 ± 0.07	0.202
Waist-hip ratio	0.85 ± 0.08	0.83 ± 0.07	0.265
Chest-height ratio	0.43 ± 0.15	0.49 ± 0.05	0.024
CAP, dB/m	166.49 ± 45.65	187.26 ± 50.59	0.029
LSM, kPa	3.36 ± 0.75	3.53 ± 0.77	0.303
Overweight, *n* (%)	25 (6.5)	12 (25.0)	0.000
Obesity, *n* (%)	10 (2.6)	4 (8.3)	0.000
Fatty liver, *n* (%)	48 (12.6)	12 (25.0)	0.073

BMI: Body max index; GDM: Gestational diabetes mellitus; CAP: Controlled attenuation parameter; LSM: Liver stiffness measurement.

between parental obesity and offspring liver fibrosis was found in Italians[25]. As maternal impaired glucose metabolism was only related to offspring NAFLD in Europeans[13], the association between maternal obesity and progression of NAFLD in offspring may also differ across ethnic groups and the possible mechanisms need to be explored[26,27].

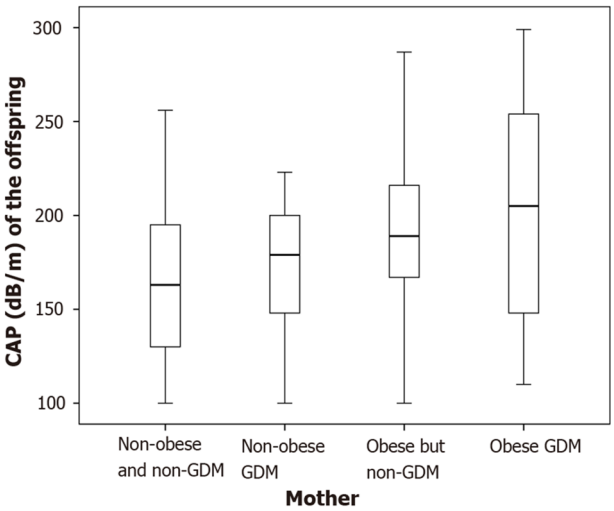
To the best of our knowledge, the present study is the first prospective birth cohort to assess the causal relationship between maternal metabolic dysbiosis and the odds of fatty liver in offspring. After adjustment for multiple regression models, the results were rigorous and trustworthy. Nonetheless, there are still several limitations that are worthy of discussion. First, to date, there is no widely accepted threshold of TE to detect childhood liver steatosis and fibrosis[28]. We used the 95th percentile cutoff values reported in a large health check-up cohort of preschool children as a surrogate threshold for this study[4]. Second, in this cohort, only 11.1% of mothers developed GDM during pregnancy, which reflected the true prevalence of GDM in the Chinese population. However, the small number of mothers with GDM might lead to type-2 statistical errors. Further nested case-control studies can address this issue and are recommended. Ultimately, single nucleotide polymorphisms of the patatin-like phospholipase domain containing 3, transmembrane 6 superfamily member 2, glucokinase regulatory protein, and several other susceptibility genes were not determined in this cohort. Further studies are needed to explore whether a predisposed genetic background mediates the influence of maternal metabolic dysbiosis on offspring NAFLD.

With the rapid spread of childhood fatty liver, it is urgent to develop preventive strategies against childhood fatty liver. In this regard, the current observations could be applied to the primary prevention of childhood obesity and fatty liver. The earliest timepoints of primary prevention of pediatric fatty liver could be before pregnancy. Weight management and glycemic control before and during pregnancy may help to promote liver and metabolic health status in children. Furthermore, lifestyle intervention before pregnancy is worth further investigation.

Table 4 Adjusted odds ratios for the association of maternal obesity and gestational diabetes mellitus with outcomes among children in a follow-up study

Outcomes	Risk factors	Model 1		*P* value	Model 2		*P* value	Model 3		*P* value
		OR	95%CI		OR	95%CI		OR	95%CI	
Fatty liver	Maternal obesity	4.57	1.96-10.67	0.000	4.79	2.03-11.31	0.000	4.64	1.82-11.87	0.001
	GDM	2.39	0.91-6.29	0.077	2.45	0.93-6.49	0.071	2.49	0.94-6.61	0.068
	Maternal obesity and GDM	7.19	2.15-24.13	0.001	7.72	2.25-26.46	0.001	8.26	2.38-28.75	0.001
Overweight/obesity	Maternal obesity	5.73	2.18-15.10	0.000	5.69	2.14-15.10	0.000	4.15	1.44-11.97	0.009
	GDM	4.70	1.72-12.81	0.003	4.85	1.76-13.37	0.002	4.84	1.76-13.36	0.002
	Maternal obesity and GDM	16.97	4.07-70.88	0.000	16.97	4.07-70.88	0.000	17.22	4.08-72.79	0.000

Model 1: Adjusted for offspring age and sex; Model 2: Further adjusted for maternal age and nulliparity; Model 3: Further adjusted for offspring birth weight. OR: Odds ratio; CI: Confidence interval; BMI: Body mass index; GDM: Gestational diabetes mellitus.



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Figure 2 Controlled attenuation parameter values of the children of mothers with or without gestational diabetes mellitus and/or obesity. CAP: Controlled attenuation parameter; GDM: Gestational diabetes mellitus.

CONCLUSION

In this study, maternal obesity increased the odds of both fatty liver and obesity in offspring, independent of maternal age, GDM status and offspring birth weight at 8 years of age. On another note, the association between maternal GDM and childhood fatty liver trended toward significance in the Chinese population, and this association needs to be confirmed in studies with larger sample sizes. To prevent these intergenerational predisposing factors, weight management and glycemic control before and during pregnancy need to be highlighted for primary prevention of pediatric fatty liver.

ARTICLE HIGHLIGHTS

Research background

Associations were found among childhood obesity, fatty liver and adverse outcomes such as diabetes, metabolic syndrome, and cardiovascular diseases in adulthood. It is important to identify relevant risk factors and intervene as early as possible.

Research motivation

We aimed to discover the possible relationship between metabolic factors in mothers and offspring.

Research objectives
We aimed to estimate the association of maternal obesity and gestational diabetes mellitus (GDM) with overweight/obesity and fatty liver risk in offspring.

Research methods
The mothers in the study all underwent a 75 g oral glucose tolerance test at 24-28 wk of gestation, and their offspring completed follow-up at 8 years of age. An examination was prospectively conducted in offspring using a FibroScan-502 with an M probe (Echosens, Paris, France).

Research results
A total of 430 mother-child pairs were included in the analysis. The prevalence of overweight, obesity and fatty liver in offspring increased significantly across maternal BMI quartiles and among mothers with GDM (all $P < 0.05$). In the multiple logistic regression analysis, after adjustment for variables, the OR for fatty liver in offspring was 8.26 (95%CI: 2.38-28.75) for participants with maternal obesity and GDM.

Research conclusions
Maternal obesity can increase the odds of overweight/obesity and fatty liver in offspring, and GDM status also increases the odds of overweight/obesity in offspring.

Research perspectives
To prevent these intergenerational predisposing factors, weight management and glycemic control before and during pregnancy need to be emphasized for primary prevention of pediatric fatty liver.

FOOTNOTES

Author contributions: Zeng J and Shen F contributed equally to this work; Zeng J, Shen F, and Yang RX carried out the experiments; Zeng J, Shen F, Yang RX, Jin Q, Yang J, and Chen GY contributed to acquisition, analysis, or interpretation of the data; Zeng J and Zou ZY wrote the paper; Fan JG made critical revision of the manuscript for important intellectual content.

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Country/Territory of origin: China

ORCID number: Jing Zeng 0000-0001-7764-155X; Feng Shen 0000-0001-7782-2211; Zi-Yuan Zou 0000-0001-9566-5045; Rui-Xu Yang 0000-0001-9384-6408; Qian Jin 0000-0002-6077-9437; Jing Yang 0000-0001-8255-1903; Guang-Yu Chen 0000-0002-9564-8971; Jian-Gao Fan 0000-0001-7443-5056.

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ORIGINAL ARTICLE

Retrospective Study

Evaluating the accuracy of American Society for Gastrointestinal Endoscopy guidelines in patients with acute gallstone pancreatitis with choledocholithiasis

Supisara Tintara, Ishani Shah, William Yakah, Awais Ahmed, Cristina S Sorrento, Cinthana Kandasamy, Steven D Freedman, Darshan J Kothari, Sunil G Sheth

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Supisara Tintara, Cristina S Sorrento, Cinthana Kandasamy, Department of Internal Medicine, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA 02215, United States

Ishani Shah, William Yakah, Awais Ahmed, Steven D Freedman, Sunil G Sheth, Division of Gastroenterology & Hepatology, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA 02215, United States

Darshan J Kothari, Division of Gastroenterology, Duke University Medical Center, Durham, NC 27710, United States

Darshan J Kothari, Division of Gastroenterology, Durham VA Medical Center, Durham, NC 27705, United States

Corresponding author: Sunil G Sheth, AGAF, FACG, FASGE, MBBS, MD, Associate Professor, Division of Gastroenterology & Hepatology, Beth Israel Deaconess Medical Center, Harvard Medical School, 330 Brookline Ave, Boston, MA 02215, United States.
ssheth@bidmc.harvard.edu

Abstract

BACKGROUND

Acute gallstone pancreatitis (AGP) is the most common cause of acute pancreatitis (AP) in the United States. Patients with AGP may also present with choledocholithiasis. In 2010, the American Society for Gastrointestinal Endoscopy (ASGE) suggested a management algorithm based on probability for choledocholithiasis, recommending additional imaging for patients at intermediate risk and endoscopic retrograde cholangiopancreatography (ERCP) for patients at high risk of choledocholithiasis. In 2019, the ASGE guidelines were updated using more specific criteria to categorize individuals at high risk for choledocholithiasis. Neither ASGE guideline has been studied in AGP to determine the probability of having choledocholithiasis.

AIM

To determine compliance with ASGE guidelines, assess outcomes, and compare

2019 *vs* 2010 ASGE criteria for suspected choledocholithiasis in AGP.

METHODS

We conducted a retrospective cohort study of 882 patients admitted with AP to a single tertiary care center from 2008-2018. AP was diagnosed using revised Atlanta criteria and AGP was defined as the presence of gallstones on imaging or with cholestatic pattern of liver injury in the absence of another cause. Patients with chronic pancreatitis and pancreatic malignancy were excluded as were those who went directly to cholecystectomy prior to assessment for choledocholithiasis. Patients were assigned low, intermediate or high risk based on ASGE guidelines. Our primary outcomes of interest were the proportion of patients in the intermediate risk group undergoing magnetic resonance cholangiopancreatography (MRCP) first and the proportion of patients in the high risk group undergoing ERCP directly without preceding imaging. Secondary outcomes of interest included outcome differences based on if guidelines were not adhered to. We then evaluated the diagnostic accuracy of 2019 in comparison to the 2010 ASGE criteria for patients with suspected choledocholithiasis. We performed the *t* test or Wilcoxon rank sum test, as appropriate, to analyze if there were outcome differences based on if guidelines were not adhered to. Kappa coefficients were calculated to measure the degree of agreement between pairs of variables.

RESULTS

In this cohort, we identified 235 patients with AGP of which 79 patients were excluded as they went directly to surgery for cholecystectomy without prior MRCP or ERCP. Of the remaining 156 patients, 79 patients were categorized as intermediate risk and 77 patients were high risk for choledocholithiasis according to the 2010 ASGE guidelines. Among 79 intermediate risk patients, 54 (68%) underwent MRCP first whereas 25 patients (32%) went directly to ERCP. For the 54 patients with intermediate risk who had MRCP first, 36 patients had no choledocholithiasis while 18 patients had evidence of choledocholithiasis prompting ERCP. Of these patients, ERCP confirmed stone disease in 11 patients. Of the 25 intermediate risk patients who directly underwent ERCP, 18 patients had stone disease. One patient with a normal ERCP developed post ERCP pancreatitis. Patients undergoing MRCP in this group had a significantly longer length of stay (5.0 *vs* 4.0 d, $P = 0.02$). In the high risk group, 64 patients (83%) had ERCP without preceding imaging, of which, 53 patients had findings consistent with choledocholithiasis, of which 13 patients (17%) underwent MRCP before ERCP, all of which showed evidence of stone disease. Furthermore, all of these patients ultimately had an ERCP, of which 8 patients had evidence of stones and 5 had normal examination.

RESULTS

Our cohort also demonstrated that 58% of all 156 patients with AGP had confirmed choledocholithiasis (79% of the high risk group and 37% of the intermediate group when risk was assigned based on the 2010 ASGE guidelines). When the updated 2019 ASGE guidelines were applied instead of the original 2010 guidelines, there was moderate agreement between the 2010 and 2019 guidelines (kappa = 0.46, 95%CI: 0.34-0.58). Forty-two of 77 patients were still deemed to be high risk and 35 patients were downgraded to intermediate risk. Thirty-five patients who were originally assigned high risk were reclassified as intermediate risk. For these 35 patients, 26 patients had ERCP findings consistent with choledocholithiasis and 9 patients had a normal examination. Based on the 2019 criteria, 9/35 patients who were downgraded to intermediate risk had an unnecessary ERCP with normal findings (without a preceding MRCP).

CONCLUSION

Two-thirds in intermediate risk and 83% in high risk group followed ASGE guidelines for choledocholithiasis. One intermediate-group patient with normal ERCP had post-ERCP AP, highlighting the risk of unnecessary procedures.

Key Words: American Society for Gastrointestinal Endoscopy guidelines; Choledocholithiasis; Acute gallstone pancreatitis; Endoscopic retrograde cholangiopancreatography; Magnetic resonance cholangiopancreatography

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Core Tip: We demonstrated that more than half of patients with acute gallstone pancreatitis (AGP) have choledocholithiasis. We also found that approximately 2/3 of patients in the intermediate group and 83% of patients in the high risk group followed American Society for Gastrointestinal Endoscopy guidelines for management of choledocholithiasis in the setting of AGP. There was associated longer length of stay for patients undergoing magnetic resonance cholangiopancreatography (MRCP) in both groups. Importantly, one patient who had a normal endoscopic retrograde cholangiopancreatography (ERCP) in the intermediate group without preceding MRCP suffered from post ERCP pancreatitis, highlighting the risk of unnecessary procedures.

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INTRODUCTION

Acute gallstone pancreatitis (AGP) is the most common cause of acute pancreatitis (AP) in the United States[1]. Patients with AGP may also present with choledocholithiasis as the occurrence of common bile duct (CBD) stones is present in up to 15% of patients with gallstones[2]. The diagnosis of choledocholithiasis is challenging and requires a high degree of suspicion as it often cannot be made conclusively without invasive procedures like endoscopic retrograde cholangiopancreatography (ERCP)[3]. In 2010, the American Society for Gastrointestinal Endoscopy (ASGE) suggested a management algorithm based on probability for choledocholithiasis to assist in risk stratifying patients[4]. These guidelines stratify patients into those with high, intermediate, and low risk based on clinical criteria, liver tests, and abdominal ultrasound. High risk (> 50% probability of choledocholithiasis) is defined as the presence of any very strong predictor (clinical ascending cholangitis, CBD stone seen on ultrasound, and/or total bilirubin > 4 mg/dL) or the presence of both strong predictors (CBD dilated more than 6 mm and bilirubin 1.8-4 mg/dL). Intermediate risk (10%-50% probability of choledocholithiasis) is defined as presence of age > 55, clinical gallstone pancreatitis, any other abnormal liver test. Finally, low risk patients do not have any of these features[4].

These guidelines aim to provide evidence-based recommendations with the goal of optimizing the efficacy and safety of patient care by minimizing the morbidity and cost from unnecessary invasive biliary evaluation. Furthermore, they recommend imaging [*i.e.* magnetic resonance cholangiopancreatography (MRCP) or endoscopic ultrasound (EUS)] for patients with intermediate risk and ERCP for patients with high probability of choledocholithiasis[4].

In 2019, the ASGE guidelines were updated using more specific criteria to categorize individuals at high-risk for choledocholithiasis[5]. These updated criteria propose that high-risk patients are those with elevated total bilirubin > 4 mg/dL and dilated CBD. The presence of only elevated total bilirubin > 4 or total bilirubin 1.8-4 with dilated CBD are no longer in the criteria for the high-risk group. These guidelines still recommend ERCP for those with high risk and imaging (MRCP or EUS) for those with intermediate risk for choledocholithiasis[6]. Additionally, clinical gallstone pancreatitis was removed as one of the criteria for assigning intermediate risk[6].

Neither ASGE guideline has been studied in AGP to determine the probability of having choledocholithiasis. Thus, our study aimed to determine the true incidence of choledocholithiasis in patients with AGP and determine compliance with the guidelines at our institution and assess outcomes when guidelines were not adhered to. In our patient population of AGP, we also evaluated the performance and diagnostic accuracy of 2019 *vs* 2010 ASGE criteria for suspected choledocholithiasis in patients with AGP.

MATERIALS AND METHODS

Data source, patient selection and study design

This retrospective observational cohort study was approved by Beth Israel Deaconess Medical Center institutional review board. This manuscript adheres to the applicable STROBE reporting guidelines for cohort studies.

We performed a review of all adult (age > 18) patients with a discharge diagnosis of AP who were admitted to our tertiary center between January 1, 2008 and December 31, 2018. The diagnosis of AP was confirmed by a review of the electronic medical record to ensure all patients met the 2012 Revised

Atlanta Criteria for the diagnosis of AP (at least two of the following three criteria: epigastric pain, elevation of serum lipase level > three times the upper limit of normal, and/or evidence of pancreatitis on cross-sectional imaging)[7]. Patients with chronic pancreatitis or known pancreatic malignancy were excluded from the study. AGP was defined as the presence of gallstones on imaging or with cholestatic pattern of liver injury in the absence of another cause. Patients who directly went to surgery for cholecystectomy without having either MRCP or ERCP were excluded.

Several parameters including demographic, clinical, laboratory, and radiologic data of the study population were collected. The severity of AP was defined based on the 2012 Revised Atlanta Criteria with mild defined as AP without any local or systemic complications, moderately severe as AP with transient (< 48 h) end-organ failure with or without local complications), and severe as AP with persistent (> 48 h) end-organ damage with or without local complications)[7]. The severity of AP in each patient was also characterized based on the bedside index for severity of AP (BISAP) score[8,9].

Study groups and outcomes of interest

Our cohort of interest was divided based on their risk for choledocholithiasis. These included: low, intermediate or high risk based on the 2010 ASGE guidelines. Since the guidelines defined AGP as at least intermediate risk, no patients were in the low risk category, thus leaving only two groups. We then compared demographic characteristics, comorbidities, and outcomes between the two groups.

Our primary outcomes of interest were the proportion of patients in the intermediate risk group undergoing MRCP first and the proportion of patients in the high risk group undergoing ERCP directly without preceding imaging. Secondary outcomes of interest included outcome differences based on if guidelines were not adhered to. We then evaluated the diagnostic accuracy of the 2019 in comparison to the 2010 ASGE criteria for patients with suspected choledocholithiasis. Additionally, we calculated the sensitivity and specificity of the 2010 and 2019 ASGE guidelines for patients categorized as high risk and intermediate risk for choledocholithiasis. Evidence of choledocholithiasis on ERCP was used as the gold standard for true positives.

Statistical analysis

All data analysis was performed using R software (version 3.6.1, R Core Team 2018a) within RStudio (version 1.1463, RStudio, Inc) *via* the tidyverse (Wickham, 2017) package. Continuous variables were presented as means with range and standard deviation. These were analyzed using the *t*-test or Wilcoxon rank sum test, as appropriate. Categorical variables were presented as frequencies (%) and analyzed using the Pearson χ^2 test or Fisher's exact test, as deemed appropriate. Univariate analyses comparing several characteristics and outcomes between the two groups were performed. A significant *P* value was assigned at 0.05. To determine concordance between the 2019 and 2010 guidelines, Kappa coefficients were calculated to measure the degree of agreement between pairs of variables.

RESULTS

Patient characteristics

In our cohort, 882 patients with AP were hospitalized of which 235 patients had AGP. Seventy-nine patients were excluded as they went directly to surgery for cholecystectomy without prior MRCP or ERCP. These 79 patients had mild AP with resolution of abdominal pain and rapid normalization of liver enzymes suggesting that CBD stones had likely already passed. Moreover, these patients had abdominal ultrasound that showed normal size CBD. Thus, these patients directly underwent cholecystectomy without preceding ERCP or MRCP.

Of the remaining 156 patients, 79 patients were categorized as intermediate risk (Tables 1 and 2) and 77 patients were high risk (Tables 3 and 4) for choledocholithiasis according to the 2010 ASGE guidelines.

Baseline characteristics and demographics are summarized in Table 5. When comparing demographics between the intermediate risk and high risk groups, there was no difference in age, gender, race, Charlson Comorbidity Index, severity of pancreatitis and baseline BISAP. Patients in the high risk group had a higher BMI than those in the intermediate group (29.0 *vs* 29.6, $P = 0.05$). While most of the baseline laboratory data were similar in both groups, total bilirubin was significantly higher in the high risk group (4.04 mg/dL *vs* 1.32 mg/dL, $P < 0.01$). Further, alanine aminotransferase and alkaline phosphatase were higher in the high risk groups and approached significance (335 IU/L *vs* 241 IU/L, $P = 0.06$ and 196 IU/L *vs* 156 IU/L, $P = 0.07$, respectively) (Table 5).

Intermediate risk

Seventy-nine patients were assigned to have intermediate risk of choledocholithiasis based on the 2010 ASGE criteria. In this group, 45 were older than 55 years-old, 73 had abnormal liver tests and 22 had dilated CBD or total bilirubin of 1.8-4.0mg/dL (Table 1). Thirty-three patients met all 4 criteria for intermediate risk group, while 45 patients met 3 criteria, 1 patient met 2 criteria and no patients met

Table 1 Patients categorized as intermediate risk for choledocholithiasis, *n* = 79

	Number of patients meeting criteria
Dilated common bile duct greater than 6 mm or total bilirubin 1.8-4.0 mg/dL (only one)	22
Abnormal liver biochemical other than bilirubin	73
Age greater than 55 yr	45
Acute gallstone pancreatitis	79

Table 2 Number of criteria met for patients categorized as intermediate risk for choledocholithiasis, *n* = 79

	Number of patients
1 criterion	0
2 criteria	1
3 criteria	45
4 criteria	33

Table 3 Patients categorized as high risk for choledocholithiasis, *n* = 77

	Numbers patients meeting criteria
Cholangitis	1
Common bile duct stone on imaging	12
Total Bilirubin greater than 4.0 mg/dL	38
Dilated common bile duct greater than 6 mm and total bilirubin 1.8-4.0 mg/dL	31

Table 4 Number of criteria met for patients categorized as high risk for choledocholithiasis, *n* = 77

	Number of patients
1 criterion	75
2 criteria	2
3 criteria	0
4 criteria	0

only 1 criterion (Table 2).

Among 79 intermediate risk patients, 54 (68%) underwent MRCP first whereas 25 patients (32%) went directly to ERCP (Figure 1). For the 54 patients with intermediate risk who had MRCP first, 18 patients had evidence of choledocholithiasis prompting ERCP, while 36 patients had imaging that did not show choledocholithiasis. Of the 18 patients undergoing ERCP after MRCP, 11 patients had choledocholithiasis, whereas 7 patients had normal examinations. There were no complications from ERCP noted in this group.

Of the 25 intermediate risk patients who directly underwent ERCP, 18 patients had choledocholithiasis whereas 7 patients had a normal ERCP (Figure 1). Notably, one of these patients with a normal ERCP developed post ERCP pancreatitis. Patients undergoing MRCP first in this group had a significantly longer length of stay (LOS, 5.0 *vs* 4.0 d, P = 0.02). They also had higher incidence of pancreatic necrosis (33% *vs* 8%, P = 0.03) and higher rate of 1-year readmission (52% *vs* 20%, P = 0.02) (Table 6). There were multiple causes of readmissions in our cohort such as diverticulitis, autonomic dysfunction, pneumonia and urinary tract infection. The 1-year readmission rates for recurrence of acute gallstone pancreatitis or choledocholithiasis were 9% for patients who had MRCP first and 8% for those who directly underwent ERCP, likely because some of them refused or did not undergo cholecystectomy.

High risk

Seventy-seven patients were assigned to have high risk of choledocholithiasis based on the 2010 ASGE

Table 5 Demographics of gallstone pancreatitis patients with intermediate and high risk for choledocholithiasis

	Intermediate risk (*n* = 79)	High risk (*n* = 77)	*P* value
Age (yr) mean ± SD	58.6 ± 19.2	58.1 ± 17.3	0.86
BMI (kg/m^2) mean ± SD	29.0 ± 6.83	29.6 ± 9.82	0.05
Female	44 (60%)	45 (58%)	0.95
Race			0.70
White	50 (72%)	51 (68%)	
Black	15 (22%)	15 (20%)	
Other	4 (6%)	9 (12%)	
Charlson Comorbidity Index			0.60
0	17 (23%)	19 (25%)	
1 to 2	24 (33%)	20 (26%)	
3 to 5	21 (29%)	29 (38%)	
6 and above	11 (15%)	9 (12%)	
Baseline laboratory			
Creatinine (mg/dL) mean ± SD	0.98 ± 0.55	0.94 ± 0.68	0.71
White blood cell count (K/L) mean ± SD	11.4 ± 4.64	12.1 ± 7.27	0.49
Lactate (mmol/L) mean ± SD	1.60 ± 1.33	1.75 ± 1.44	0.63
Lipase (IU/L) mean ± SD	4012 ± 5264	3576 ± 4699	0.60
ALT (IU/L) mean ± SD	241 ± 269	335 ± 324	0.06
AST (IU/L) mean ± SD	232 ± 280	294 ± 269	0.18
Alkaline phosphatase (IU/L) mean ± SD	156 ± 130	196 ± 140	0.07
Total bilirubin (mg/dL) mean ± SD	1.32 ± 0.78	4.04 ± 2.06	< 0.01
Presence of gallstone on imaging	79 (100%)	77 (100%)	0.91
Severity of pancreatitis			
Mean BISAP ± SD	0.90 ± 0.91	0.88 ± 0.88	0.90
Modified Atlanta Criteria			0.13
Mild	57 (72%)	63 (83%)	
Moderate	21 (27%)	10 (13%)	
Severe	1 (1%)	3 (4%)	

BMI: Body mass index; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; BISAP: Bedside index of severity in acute pancreatitis.

criteria. In this group, 1 patient was suspected to have cholangitis, 38 patients had total bilirubin > 4.0 mg/dL, 12 patients had CBD stone on imaging, and 31 patients had dilated CBD with total bilirubin 1.8-4.0 mg/dL (Table 3). Seventy-five patients met 1 criterion for high risk group and 2 patients met 2 criteria. No patient met 3 or more criteria (Table 4).

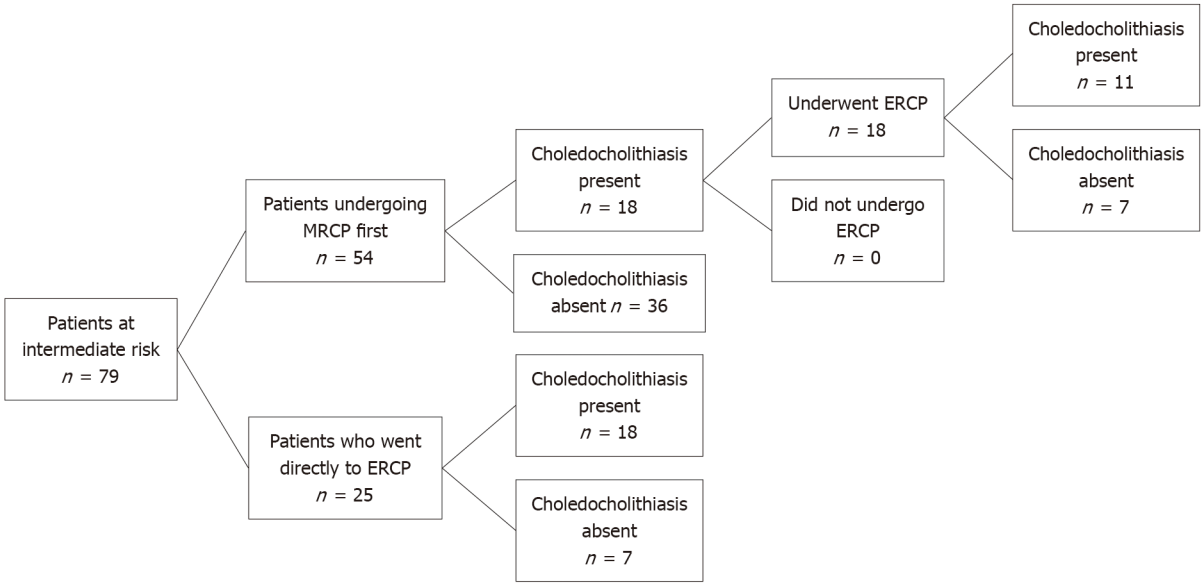
In this group, 64 patients (83%) had ERCP without preceding imaging, of which, 53 patients had findings consistent with choledocholithiasis (27 patients with stones and 26 patients with sludge) while 10 had a normal examination (Figure 2). In comparison, 13 patients in this group (17%) underwent MRCP before ERCP, all of which showed evidence of stone disease. Furthermore, all of these patients ultimately had an ERCP, of which 8 patients had evidence of choledocholithiasis and 5 had normal examination (Figure 2). Two of the 13 patients who underwent imaging first due to altered luminal anatomy and body habitus. For the remaining 11 patients, there was no identifiable reason for not directly proceeding to ERCP.

As with the intermediate group, for the high risk group, the MRCP-first group had longer LOS compared to ERCP-first group which approached significance (4.28 *vs* 3.00 days, $P = 0.08$). There were no significant differences in outcomes such as readmission rate, 1-year mortality between the two groups (Table 6).

Table 6 Outcomes of patients with intermediate and high risk for choledocholithiasis

	Intermediate risk (*n* = 79)			High risk (*n* = 77)		
Outcomes	**ERCP first (*n* = 25)**	**MRCP first (*n* = 54)**	***P* value**	**ERCP first (*n* = 64)**	**MRCP first (*n* = 13)**	***P* value**
Mean length of stay (Q1; Q3)	4.00 (2.74; 5.00)	5.00 (4.00; 8.00)	0.02	3.00 (2.54; 5.00)	4.38 (4.00; 6.00)	0.08
30-d readmission (%)	3 (12.0%)	11 (20.8%)	0.53	6 (9.52%)	2 (16.7%)	0.61
1-yr readmission (%)	5 (20.0%)	28 (51.9%)	0.02	19 (29.7%)	7 (53.8%)	0.11
1-yr mortality (%)	0 (0.00%)	1 (1.85%)	0.09	1 (1.56%)	0 (0.00%)	1.00
Pancreas necrosis (%)	2 (8.0%)	18 (33.3%)	0.03	6 (10.7%)	3 (35.0%)	0.19
Days to enteral nutrition (Q1; Q3)	2.00 (1.00; 2.50)	2.50 (1.00; 4.00)	0.16	2.00 (1.00; 2.00)	3.00 (2.00; 4.00)	0.10

ERCP: Endoscopic retrograde cholangiopancreatography; MRCP: Magnetic resonance cholangiopancreatography.



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Figure 1 Patients at intermediate risk for choledocholithiasis. MRCP: Magnetic resonance cholangiopancreatography; ERCP: Endoscopic retrograde cholangiopancreatography.

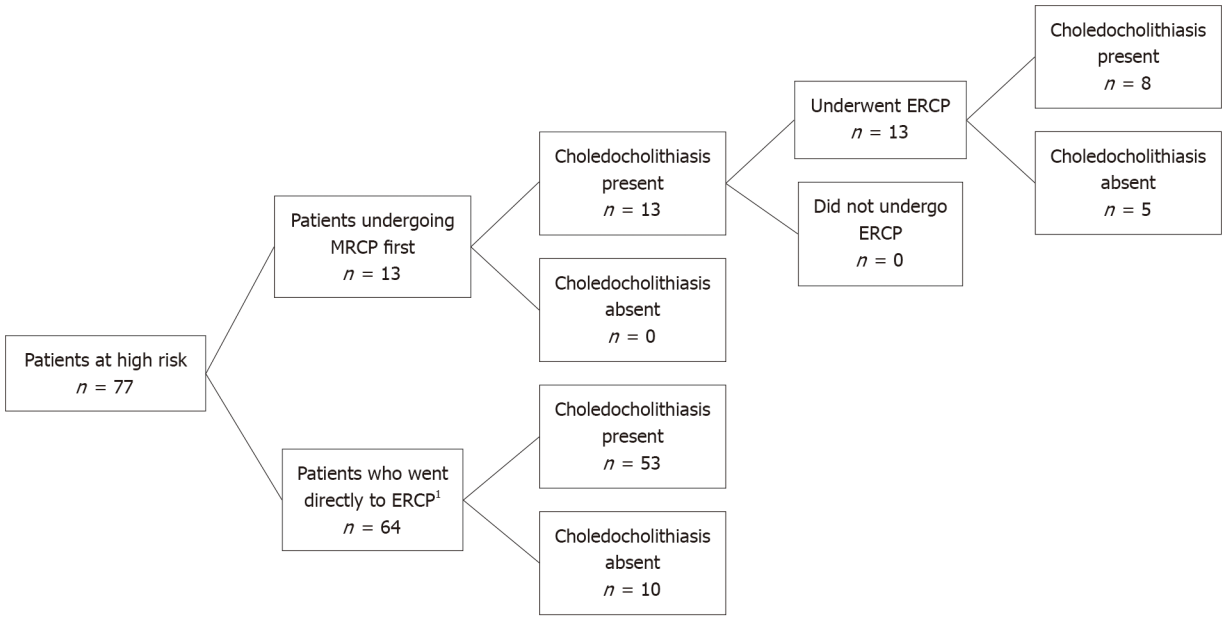
Incidence of confirmed choledocholithiasis in patients with AGP

Our cohort also demonstrated that of all 156 patients with AGP, 90 (58%) were found to have choledocholithiasis on ERCP. When stratified by assigned choledocholithiasis risk based on the 2010 ASGE guidelines, 61 of 77 patients in the high risk group (79%) and 29 of 79 patients in the intermediate risk group (37%) were found to have choledocholithiasis (Figure 3).

Comparison to the 2019 ASGE guidelines

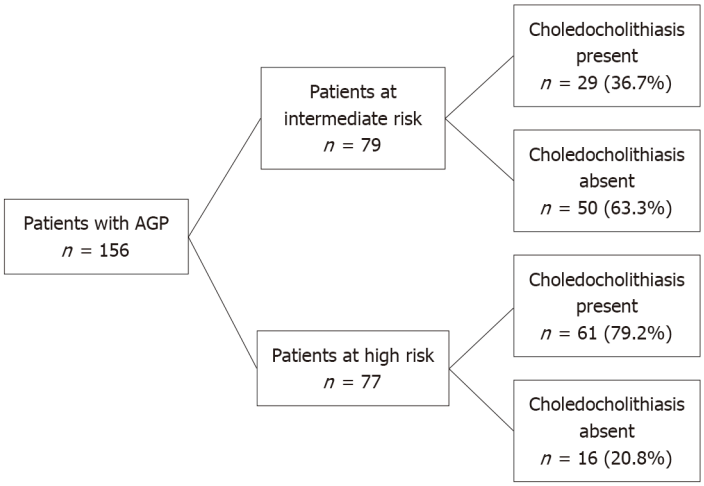
Our cohort of patients was admitted to the hospital between 2008 and 2018, thus the 2010 ASGE guideline were used in the above analysis. We then applied the 2019 criteria to this group to see how results may have changed. There was moderate agreement between the classifications by the 2010 and 2019 guidelines (kappa = 0.46, 95%CI: 0.34-0.58). When the 2019 ASGE guidelines were applied to our 79 patients with intermediate risk for choledocholithiasis, all of these patients were still assigned intermediate risk. Among the 77 patients in the high risk group, when the updated 2019 ASGE guidelines were applied instead of the original 2010 guidelines, 42 patients were still deemed to be high risk and 35 patients were downgraded to intermediate risk. In the high risk group, 34 of 42 who still remained high risk went directly to ERCP of which showed 28 patients had a confirmatory findings of choledocholithiasis and 5 patients had a normal examination (Figure 4).

Thirty-five patients who were originally assigned high risk were then reclassified as intermediate risk (Figure 4). For these 35 patients, 26 patients had ERCP findings consistent with choledocholithiasis and 9 patients had a normal examination. In further review of those patients with a normal ERCP, 5 patients had a bilirubin of > 4 mg/dL and 4 patients had a dilated CBD and a bilirubin of 1.8 to 4 mg/dL. Hence,



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Figure 2 Patients categorized as high risk by American Society for Gastrointestinal Endoscopy guidelines. MRCP: Magnetic resonance cholangiopancreatography; ERCP: Endoscopic retrograde cholangiopancreatography. [1]One patient's procedure could not be completed due to failure of bile duct cannulization.



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Figure 3 Patients with choledocholithiasis. AGP: Acute gallstone pancreatitis.

based on the 2019 criteria, 9 out of 35 patients who were downgraded to intermediate risk had an unnecessary ERCP with normal findings (without a preceding MRCP).

Finally, the sensitivity and specificity of the 2010 and 2019 ASGE guidelines in assessing choledocholithiasis were calculated (Tables 7-10). In our cohort of patients with AGP, the 2010 ASGE criteria for predicting high risk for choledocholithiasis had a sensitivity of 67.8% and specificity of 75.8%. On the other hand, the 2019 ASGE criteria for predicting high risk for choledocholithiasis had a sensitivity of 38.2% and specificity of 89.4%.

DISCUSSION

AGP is the most common cause of AP in the United States accounting for approximately one-third of all cases[1]. Furthermore, given the potential for comorbid choledocholithiasis, patients with AGP should be evaluated for CBD stone disease[2]. In our cohort of patients with AGP, 37% of patients with intermediate risk and 79.2% of patients with high risk for choledocholithiasis based on the 2010 ASGE

Table 7 Sensitivity and specificity of 2010 American Society for Gastrointestinal Endoscopy guidelines for predicting high risk of choledocholithiasis in patients with acute gallstone pancreatitis

	Choledocholithiasis present	Choledocholithiasis not present
Positive for high risk criteria	61	16
Negative for high risk criteria	29	50

Sensitivity = 67.8%; specificity = 75.8%.

Table 8 Sensitivity and specificity of 2010 American Society for Gastrointestinal Endoscopy guidelines for predicting intermediate risk of choledocholithiasis in patients with acute gallstone pancreatitis

	Choledocholithiasis present	Choledocholithiasis not present
Positive for intermediate risk criteria	29	50
Negative for intermediate risk criteria	61	16

Sensitivity = 82.9%; specificity = 24.2%.

Table 9 Sensitivity and specificity of 2019 American Society for Gastrointestinal Endoscopy guidelines for predicting high risk of choledocholithiasis in patients with acute gallstone pancreatitis

	Choledocholithiasis present	Choledocholithiasis not present
Positive for intermediate risk criteria	34	8
Negative for intermediate risk criteria	55	59

Sensitivity = 38.2%; specificity = 88.1%.

Table 10 Sensitivity and specificity of 2019 American Society for Gastrointestinal Endoscopy guidelines for predicting intermediate risk of choledocholithiasis in patients with acute gallstone pancreatitis

	Choledocholithiasis present	Choledocholithiasis not present
Positive for intermediate risk criteria	55	59
Negative for intermediate risk criteria	34	8

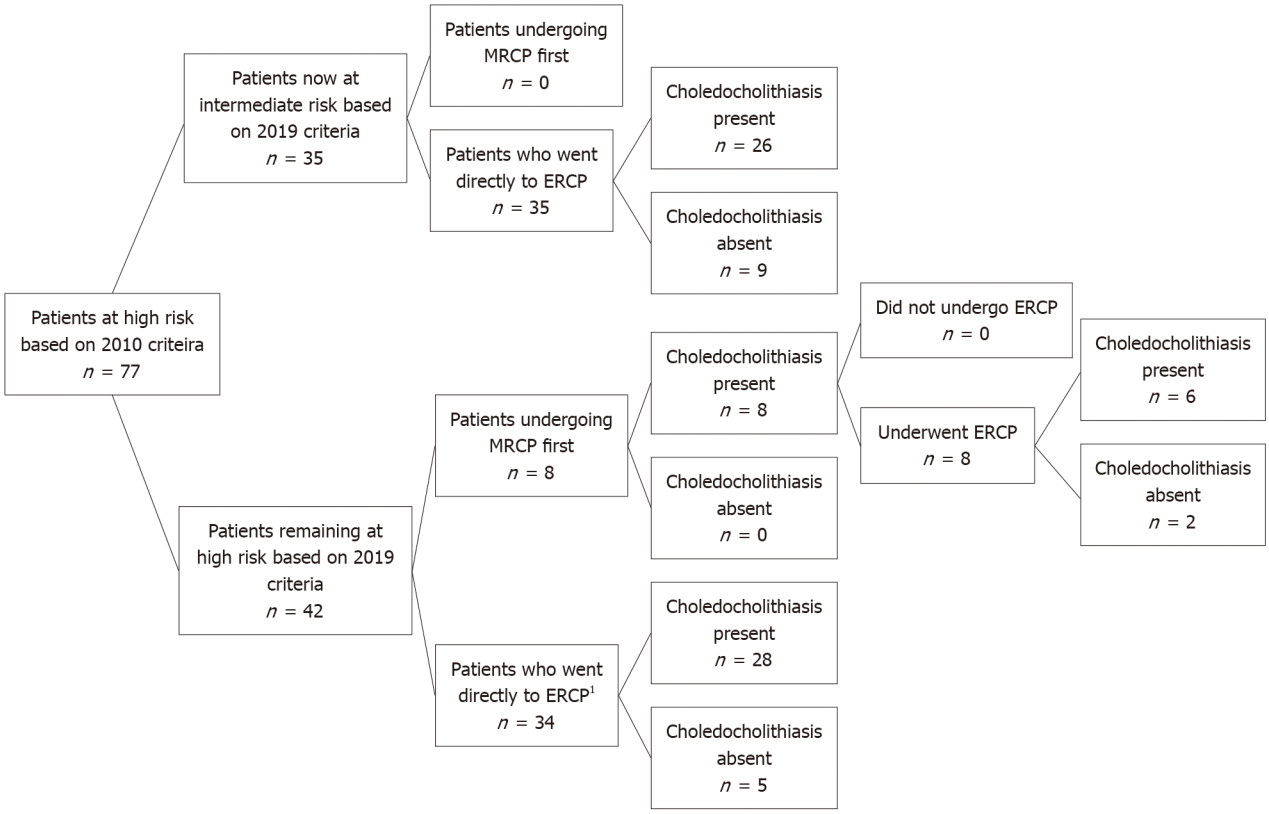
Sensitivity = 61.8%; specificity = 11.9%.

guidelines had documented CBD stone disease. When combined, our data demonstrated that the true incidence of choledocholithiasis in patients with AGP is 58%. The overall high prevalence of concurrent choledocholithiasis in patients with AGP may help explain the relatively high risk of recurrent pancreatitis in patients with AGP[10].

The ASGE published guidelines in 2010 which proposed a risk stratification system to clarify how to best manage patients with suspected choledocholithiasis, categorizing patients into low, intermediate and high risk[4].

In this scheme, patients at high risk are recommended to undergo ERCP directly without further imaging and those at intermediate risk should have additional imaging with MRCP or EUS. Those at low risk require no further evaluation. In this study, we aimed to apply these guidelines to patients admitted with AGP to a single tertiary care center over 10 years and determine what effect deviation had on outcomes.

In our cohort of 156 patients, approximately one-half of patients were either classified as intermediate or high risk. Since all patients in our cohort had AGP and the presence of AGP was a criteria for at least intermediate risk for choledocholithiasis according to the 2010 ASGE guidelines, no patients in our cohort were considered low risk for choledocholithiasis. After applying the guidelines to these groups, 76% of patients (54 patients in the intermediate group and 64 patients in the high risk group) were managed in accordance to the ASGE recommendations and 24% of patients (25 patients in the



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Figure 4 Patients at high risk based on 2010 American Society for Gastrointestinal Endoscopy guidelines now re-stratified based on 2019 guidelines. MRCP: Magnetic resonance cholangiopancreatography; ERCP: Endoscopic retrograde cholangiopancreatography. [1]One patient's procedure could not be completed due to failure of bile duct cannulization.

intermediate group and 13 patients in the high risk group) deviated from the guidelines. Specifically in the intermediate group, 7 patients who had an ERCP first had a normal examination suggesting that an inappropriate procedure was performed and importantly, one of these patients suffered from post-ERCP pancreatitis. These 7 intermediate risk patents who directly underwent ERCP and had normal findings did not receive EUS or MRCP. Thus, it may be prudent to consider EUS to evaluate for choledocholithiasis to prevent unnecessary ERCPs. Furthermore, all 13 of the patients in the high risk group who underwent MRCP prior to ERCP had a positive finding, suggesting an unnecessary test (*i.e.* MRCP) was performed first. Importantly, patients who underwent MRCP prior to ERCP were found to have longer hospital lengths of stay and delays in initiation of enteral feeding.

The ASGE guidelines provide evidence-based recommendations with the goal of optimizing the efficacy and safety of patient care. Additionally, a study has shown that adherence to ASGE guidelines also result in cost-saving from unnecessary imaging studies[3]. Accordingly, patients with intermediate risk (10%-50%) should undergo additional imaging because the risk of choledocholithiasis is too low to warrant proceeding directly to ERCP[6]. MRCP is often preferred in patients with pancreatitis who have intermediate risk for choledocholithiasis because it has high sensitivity in detecting CBD stones without procedural risks[11]. While ERCP is highly sensitive and specific in diagnosis and effective in therapeutic management of choledocholithiasis[12], the procedure also has a number of associated risks such as post-ERCP pancreatitis and post-endoscopic sphincterotomy bleeding[13,14]. One of the intermediate risk patients in our cohort who went directly to ERCP which showed normal findings developed worsening of pancreatitis thought to be related to post ERCP AP. This highlights that unnecessary invasive procedure and adverse outcome could have been prevented by adhering to the guidelines.

In 2019, the ASGE updated guidelines on management of choledocholithiasis, which further fine-tuned the criteria needed for the high risk group[6]. Specifically, patients now required the presence of a bilirubin greater than 4 mg/dL and imaging with a dilated CBD to qualify as high risk and thus those patients with a total bilirubin of 1.8-4.0 mg/dL with CBD dilation on imaging were downgraded to intermediate risk. The impetus for these changes were largely driven by studies that demonstrated that up to 30% of patients had ERCPs without evidence of CBD stones[15,16]. In our study, we found that approximately 26% (9/35) of patients categorized as high risk by 2019 criteria had a normal ERCP.

Since the cohort we studied was prior to the update, the 2010 guidelines were used in the initial analysis. In our subsequent analysis we aimed to determine concordance in categorization between the 2010 and 2019 guidelines. After applying the 2019 guidelines, we found that all patients in the intermediate group remained the intermediate group, whereas there was less concordance in the high risk group which was largely due to the group of patients who had lower total bilirubin levels. Importantly, using the newer guidelines, 9 patients may have been spared an ERCP. We also found that while the 2010 ASGE guidelines in predicting high risk for choledocholithiasis had a specificity of 75.8%, using the 2019 ASGE guidelines led to an improved specificity of 89.4%. This demonstrated that the use of the revised guidelines in assessing risk for choledocholithiasis in AGP patients can lead to a decrease in unnecessary invasive and costly procedures. Moreover, although AGP was removed from the intermediate risk criteria, all patients in our intermediate cohort remained intermediate suggesting that AGP implicitly increases the risk to the intermediate level for choledocholithiasis. This was verified in our cohort where 58% of patients with AGP were confirmed to have choledocholithiasis. Thus, clinicians should remain vigilant for concurrent choledocholithiasis in patients admitted with AGP.

We recognize that our study also has several limitations. First, this is a retrospective cohort study which has a potential for selection bias. The smaller size and retrospective nature of this study may limit the assessment of the degree of adherence to the ASGE guidelines. As the decision to proceed with MRCP or ERCP were at the discretion of the physician on service, the detailed reasoning behind selecting each modality was not always apparent in the medical record. Our results may not be generalizable to all medical settings as this study was done at a single tertiary care center at an academic institution located in a large metropolitan city. Further work is needed to determine the influencing factors driving deviation from the guidelines.

CONCLUSION

In the study cohort, we demonstrated that more than half of patients with AGP have choledocholithiasis. We also found that approximately two thirds of patients in the intermediate group and 83% of patients in the high risk group followed ASGE guidelines for management of choledocholithiasis in the setting of AGP. There was associated longer LOS for patients undergoing MRCP in both groups. Importantly, one patient who had a normal ERCP in the intermediate group without preceding MRCP suffered from post ERCP AP, highlighting the risk of unnecessary procedures. Further work is needed to determine the influencing factors driving deviation from the guidelines.

ARTICLE HIGHLIGHTS

Research background

Acute gallstone pancreatitis (AGP) is the most common cause of acute pancreatitis (AP) in the United States. Patients with AGP may also present with choledocholithiasis. In 2010, the American Society for Gastrointestinal Endoscopy (ASGE) suggested a management algorithm based on probability for choledocholithiasis. In 2019, the ASGE guidelines were updated.

Research motivation

Neither 2010 nor 2019 ASGE guidelines has been studied in AGP to determine the probability of having choledocholithiasis.

Research objectives

Our study aimed to determine compliance with ASGE guidelines, assess outcomes, and compare 2019 *vs* 2010 ASGE criteria for suspected choledocholithiasis in AGP.

Research methods

We conducted a retrospective cohort study of 882 patients admitted with AP to a single tertiary care center from 2008-2018. Patients with AGP were assigned low, intermediate or high risk for choledocholithiasis based on ASGE guidelines. Our primary outcomes of interest were the proportion of patients in the intermediate risk group undergoing magnetic resonance cholangiopancreatography (MRCP) first and the proportion of patients in the high risk group undergoing endoscopic retrograde cholangiopancreatography (ERCP) directly without preceding imaging. Secondary outcomes of interest included outcome differences based on if guidelines were not adhered to. We then evaluated the diagnostic accuracy of 2019 in comparison to the 2010 ASGE criteria for patients with suspected choledocholithiasis.

Research results

Among 79 intermediate risk patients according to the 2010 ASGE guidelines, 54 (68%) underwent MRCP first whereas 25 patients (32%) went directly to ERCP. Of the 25 intermediate risk patients who directly underwent ERCP, 18 patients had stone disease. One patient with a normal ERCP developed post ERCP pancreatitis. In the high risk group, 64 patients (83%) had ERCP without preceding imaging. When the updated 2019 ASGE guidelines were applied instead of the original 2010 guidelines, there was moderate agreement between the 2010 and 2019 guidelines (kappa = 0.46, 95%CI: 0.34-0.58). Based on the 2019 criteria, 9/35 patients who were downgraded to intermediate risk had an unnecessary ERCP with normal findings (without a preceding MRCP).

Research conclusions

In the study cohort, we demonstrated that more than half of patients with AGP have choledocholithiasis. We also found that approximately two thirds of patients in the intermediate group and 83% of patients in the high risk group followed ASGE guidelines for management of choledocholithiasis in the setting of AGP. Importantly, one patient who had a normal ERCP in the intermediate group without preceding MRCP suffered from post ERCP pancreatitis, highlighting the risk of unnecessary procedures. We also found that while the 2010 ASGE guidelines in predicting high risk for choledocholithiasis had a specificity of 75.8%, using the 2019 ASGE guidelines led to an improved specificity of 89.4%.

Research perspectives

Further work is needed to determine the influencing factors driving deviation from the guidelines.

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FOOTNOTES

Author contributions: Tintara S, Shah I, Ahmed A, Freedman SD, Kothari DJ and Sheth SG contributed to the study design and coordination; Tintara S contributed to the acquisition and interpretation of data, and primarily drafting the manuscript; Shah I assisted in drafting the manuscript; Yakah W contributed to the acquisition and interpretation of data and statistical analysis; Ahmed A, Sorrento CS and Kandasamy C contributed to the acquisition of data; Freedman SD contributed to revision of manuscript for intellectual content; Kothari DJ and Sheth SG contributed to the interpretation and analysis of data, revision of manuscript for intellectual content, and study supervision; All authors have approved the final draft submitted.

Institutional review board statement: This retrospective observational cohort study was approved by Beth Israel Deaconess Medical Center institutional review board.

Informed consent statement: Informed written consent was obtained fromthe patient for publication of this report and any accompanying images.

Conflict-of-interest statement: The authors of this study have no relevant conflict of interests to declare.

Data sharing statement: Statistical code, and dataset available from the corresponding author at ssheth@bidmc.harvard.edu. This retrospective observational cohort study was approved by the Beth Israel Deaconess Medical Center institutional review board which did not require individual patient consent for retrospective chart review.

STROBE statement: This manuscript adheres to the applicable Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) reporting guidelines for cohort studies.

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Country/Territory of origin: United States

ORCID number: Supisara Tintara 0000-0002-9427-2845; Ishani Shah 0000-0003-2916-1089; William Yakah 0000-0003-3264-5404; Awais Ahmed 0000-0001-7254-5921; Cristina S Sorrento 0000-0002-1111-5271; Cinthana Kandasamy 0000-0002-4235-

6932; Steven D Freedman 0000-0003-1255-9701; Darshan J Kothari 0000-0002-6835-218X; Sunil G Sheth 0000-0003-0602-8509.

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SYSTEMATIC REVIEWS

Risk of venous thromboembolism in children and adolescents with inflammatory bowel disease: A systematic review and meta-analysis

Xin-Yue Zhang, Hai-Cheng Dong, Wen-Fei Wang, Yao Zhang

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Xin-Yue Zhang, Hai-Cheng Dong, Wen-Fei Wang, Yao Zhang, Department of Traditional Chinese Medicine, The Children's Hospital, Zhejiang University School of Medicine, National Clinical Research Center for Child Health, Hangzhou 310005, Zhejiang Province, China

Corresponding author: Xin-Yue Zhang, MM, Attending Doctor, Department of Traditional Chinese Medicine, The Children's Hospital, Zhejiang University School of Medicine, National Clinical Research Center for Child Health, No. 57 Zhugan Lane, Xiancheng District, Hangzhou 310005, Zhejiang Province, China. cicelyxyz@zju.edu.cn

Abstract

BACKGROUND

A two- to three-fold increased risk of venous thrombotic events (VTE) has been demonstrated in patients with inflammatory bowel disease (IBD) compared to the general population, but less is known about the risk of VTE in child- and pediatric-onset IBD. In recent years, several studies have reported the rising incidence rate of VTE in juvenile patients with IBD, and the related risk factors have been explored.

AIM

To evaluate the risk of VTE in children and adolescents with IBD.

METHODS

Articles published up to April 2021 were retrieved from PubMed, Embase, Cochrane Library, Web of Science, SinoMed, CNKI, and WANFANG. Data from observational studies and clinical work were extracted. The outcome was the occurrence of VTE according to the type of IBD. The available odds ratio (OR) and the corresponding 95% confidence interval (CI) were extracted to compare the outcomes. Effect size (P), odds ratio (OR), and 95%CI were used to assess the association between VTE risk and IBD disease. Subgroup analyses stratified by subtypes of VTE and IBD were performed.

RESULTS

Twelve studies (7450272 IBD patients) were included in the meta-analysis. Child and adolescent IBD patients showed increased VTE risk (P = 0.02, 95%CI: 0.01-0.03). Subgroup analyses stratified by IBD (ulcerative colitis (UC): P = 0.05, 95%CI: 0.03-0.06; Crohn's disease (CD): P = 0.02, 95%CI: 0.00-0.04) and VTE subtypes (portal vein thrombosis: P = 0.04, 95%CI: 0.02-0.06; deep vein thrombosis: P = 0.03, 95%CI: 0.01-0.05; central venous catheter-related thrombosis: P = 0.23, 95%CI: 0.00-0.46; thromboembolic events: P = 0.02, 95%CI: 0.01-0.03) revealed a sign-

ificant correlation between VTE risk and IBD. Patients with IBD were more susceptible to VTE risk than those without IBD (OR = 2.99, 95%CI: 1.45-6.18). The funnel plot was asymmetric, suggesting the presence of significant publication bias. Pediatric and adolescent IBD patients have an increased VTE risk. UC and CD patients exhibited a high risk of VTE. The risk of VTE subtypes was increased in IBD patients.

CONCLUSION

The current meta-analysis showed that the incidence and risk of VTE are significantly increased in pediatric and adolescent IBD patients. Thus, IBD might be a risk factor for VTE in children and young adults. High-quality prospective cohort studies are necessary to confirm these findings.

Key Words: Thromboembolism; Children; Adolescents; Ulcerative colitis; Crohn's disease; Meta-analysis

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Core Tip: This study suggested that child and adolescent inflammatory bowel disease (IBD) patients showed an increased risk of venous thrombotic events (VTE) (P = 0.02, 95%CI: 0.01-0.03). Subgroup analysis stratified by IBD (ulcerative colitis: P = 0.04, 95%CI: 0.03-0.06; Crohn's disease: P = 0.02, 95%CI: 0.00-0.04) and VTE subtypes (portal vein thrombosis: P = 0.04, 95%CI: 0.02-0.06; deep vein thrombosis: P = 0.03, 95%CI: 0.01-0.05; central venous catheter-related thrombosis: P = 0.13, 95%CI: 0.02-0.23; thromboembolic events: P = 0.02, 95%CI: 0.01-0.03) revealed a significant correlation between VTE incidence and IBD.

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INTRODUCTION

Inflammatory bowel disease (IBD) is a digestive system disorder characterized by chronic inflammation of the gastrointestinal (GI) tract[1-5]. The two common manifestations of IBD are ulcerative colitis (UC) and Crohn's disease (CD)[1-5]. The pathogenesis of IBD involves a complex interaction between the host genetics, external environment, gut microbiota, and immune responses[6-8]. The incidence of pediatric and adult IBD is rising steadily worldwide, especially in developed countries[9-11]. Approximately 25% of patients with IBD are diagnosed before the age of 18 years[12,13]. Furthermore, 4% of pediatric IBD occur before the age of 5 years and 18% before the age of 10 years, peaking in adolescence[7].

Venous thromboembolism (VTE) is a potentially severe medical condition with a high recurrence rate [14,15]. IBD is a high-risk factor for the occurrence and development of VTE in adults[16,17]. Routine VTE prophylaxis is recommended for IBD patients during hospitalization[1,18-20]. Pediatric patients with IBD also have an increased predisposition to develop VTE, including deep venous thrombosis (DVT) and pulmonary embolism (PE), although the available evidence is of lower quality[21,22]. The etiology of VTE in IBD is multifactorial and not well defined. It involves the cross-talk between coagulation and inflammation[23,24]. IBD-associated inflammation causes a hypercoagulable state, leading to systemic thrombotic events and local microthrombi in the vessels of the inflamed intestinal mucosa[25,26]. Pediatric IBD patients exhibit various clinical features, including disease location and severity, endoscopic appearance, histology, comorbidities, complications, and response to treatment options[27]. The reported risk factors for VTE in children with IBD include inherent genetics, ethnicity, gender, infection, parenteral nutrition, surgery, specific therapies, disease history, and increased use of central venous catheters (CVCs; the most common factor)[28-30].

Nonetheless, whether the development of VTE complications in IBD treatment is age-dependent is yet to be clarified. The increased risk of VTE in adult patients with IBD is widely recognized[31]. A two- to three-fold increased risk of VTE has been demonstrated in patients with IBD compared to the general population[26]. However, less is known about the risk of VTE in child- and pediatric-onset IBD. In recent years, several studies have reported the rising incidence rate of VTE in juvenile patients with IBD, and the related risk factors have been explored[32-35]. Therefore, this meta-analysis evaluated the association between postoperative VTE risk and IBD in children and adolescent populations.

MATERIALS AND METHODS

According to the Preferred Reporting Items for Systematic Reviews (PRISMA, 2020) guidelines, we conducted a systematic review and meta-analysis. PubMed, Embase, Cochrane Library, Web of Science, SinoMed, CNKI, and WANFANG databases were systematically searched for studies published up to April 2021.

Eligibility criteria

Articles were included if they met the following criteria: (1) Investigating the incidence or risk of VTE in children and adolescents with IBD; (2) IBD and VTE (DVT, pulmonary embolism; CVC-related thrombosis, intracranial venous sinus thrombosis, portal vein thrombosis, thromboembolic events, intra-abdominal thrombus, and cerebrovascular thrombosis) were confirmed medically; (3) language was limited to English or Chinese; and (4) RCT, cohort, or database review design.

Publications were excluded if they were: (1) Letters, review articles, meta-analyses, case-control, case reports, and experimental animal studies; (2) Missing primary data; or (3) Full text unavailable.

Search strategy

PubMed, Embase, Cochrane Library, Web of Science, SinoMed, CNKI, and WANFANG databases were systematically searched for potentially eligible studies published up to April 2021. The MeSH terms used were as follows: "inflammatory bowel disease", "Crohn Disease", "Colitis, Ulcerative" and "Children", "pediatric*" and "Venous Thrombosis Pulmonary", "Embolism vein thrombosis", as well as relevant keywords. The search strategy for PubMed, Embase and Cocharane Central is listed in Supplementary Table 1.

Data extraction and quality assessment

The selection and inclusion of studies were performed by two independent reviewers in two stages, which included the analysis of the titles/abstracts, followed by a full-text assessment. Disagreements were resolved by a third reviewer. Data including the authors' names, publication year, study design, country, sample size, mean age, IBD therapy, patient, VTE type, and male percentage were extracted. The quality assessment was performed in duplicate by two researchers independently (xx and xx). The Newcastle-Ottawa Scale (NOS)[36] was used to assess the methodological quality of eligible observational studies.

Statistical analysis

Statistical analyses were performed using STATA SE 14.0 (StataCorp, College Station, TX, USA). The risk of VTE in IBD was estimated by ES (p) and the corresponding 95% confidence interval (CI). The available odds ratio (OR) and the corresponding 95%CI were extracted to compare the outcomes. In addition, the event incidence was relatively small ($0 < P < 0.2$), and the double arcsine method was also used for data conversion.

Cochran's Q statistic ($P < 0.10$ indicated evidence of heterogeneity) was used to assess heterogeneity among the included studies[37]. When significant heterogeneity ($P < 0.10$) was detected, the random-effects model was applied to combine the effect sizes of the included studies; otherwise, the fixed-effects model was adopted[38]. In addition, sensitivity analyses were performed to identify individual study effects on pooled results and test the reliability of the results.

RESULTS

Literature search and study characteristics

The initial literature database search retrieved 438 studies. The duplicate records and automatically marked ineligible records were filtered out. After screening, 100 reports were assessed for eligibility. Due to insufficient information on participants, exposure, outcomes, and different reports from the same participants, only 12 reports were finally included in this meta-analysis. The study screening flowchart is illustrated in Figure 1.

The characteristics of the 12 eligible studies, including nine cohort studies[21,27,30,32-35,39,40], one RCT[28], and two database reviews[25,41], are listed in Table 1. These studies encompassed 7450272 IBD patients. Eight studies were carried out in the United States, and the others were performed in Canada, Switzerland, Iran, and Korea. The IBD patients had a mean age of < 1-21 years. The proportion of male patients ranged from 42.9% to 71.2%. The IBD subtypes covered UC, CD, indeterminate colitis (IC), and undefined IBD type (IBD-U). Moreover, various VTE subtypes, including intra-abdominal thrombosis (IAT), DVT, venous thromboembolism (VTE), TE, pulmonary thromboembolism (PTE), intracranial venous sinus thrombosis (IVST), PVT, and cerebrovascular thrombosis (CVT), were investigated. In addition, the confounding factors, including age, sex, hypercoagulable status, CVC, parenteral nutrition, cancer, sickle cell anemia, tobacco use, race/ethnicity, payer status, urban/rural status, hospital region,

Table 1 Characteristics of the included studies: ulcerative colitis patients and Crohn's disease patients

Ref.	Country	Study design	Period	Sample size N	Age, years (mean or median)	Male, (%)	Patient	IBD therapy	VTE type
Antiel *et al* [31], 2013	United States	Cohort	1999.1-2011.12	366	≤ 21	No data	UC	Surgery	IAT
Bence *et al* [26], 2020	United States	Cohort	2020.1-2016.6	276	15 (13, 17)	46	CD, UC, IC	Corticosteroids, Biologic therapy, Immunologic therapy	DVT
Cairo *et al* [36], 2017	United States	RCS	2012-2015	410	< 1 (9.8%); 1-5 (15.7%); 6-10 (30%); 11-15 (34.6%); 16-18 (9.9%)	59.5	IBD	No data	VTE
Derderian *et al*[27], 2020	United States	Cohort	2008-2018	49	9.8 ± 4.4	65	UC/IBD-U	Surgery	DVT
Diamond *et al*[32], 2018	United States	Cohort	2015-2017	47	14	46.8	IBD	Systemic steroids; Anti-TNF therapy	CVC-related thrombosis
Diamond *et al*[33], 2010	Canada	Cohort	1999.11.1-2008.2.29	85	14.8 (2.8)	54.1	IBD	Surgery	DVT
Herzog *et al* [34], 2018	Switzerland	Cohort	2006-2017	63	< 17	54	CD; UC or IBD-U	5-ASA Systemic CS; Immunomodulators; Calcineurin inhibitors; TNFα inhibitors, Surgery	TE
Jarchin *et al* [29], 2019	United States	Cohort	2008.1-2017.12	58	0-5 (5%); 6-10 (10%); 11-18 (85%)	66	UC	Anti-TNF only, Vedolizumab only, Anti-TNF+ vedolizumab, Anti-TNF+ vedolizumab+ ustekinumab, Surgery	PVT (within 30 d, > 30 d)
Khosravi *et al*[28], 2020	Iran	Cohort	2019	21	11.12 ± 5.65	42.9	IBD	Surgery	DVT
Lee *et al* [35], 2016	Korea	Cohort	1995.7-2011.6	73	12.49 ± 2.47	71.2	CD	Systemic corticosteroids 5-ASA only, 5-ASA+; azathioprine; 5-ASA only or 5-ASA+ azathioprine	PTE
Nylund *et al*[19], 2013[1]	United States	Database review	1997, 2000, 2003, 2006, and 2009	61076/7318/7379898	15.72 ± 0.02/15.39 ± 0.06/13.57 ± 0.01	49.32/47.55/49.64	CD, UC	No data	TE, PE, DVT, IVST, PVT
Zitomersky *et al*[37], 2013	United States	Database review	2006-2011	532	No data	No data	CD, UC	No data	CVT

[1]Adjusted factors: Age category, sex, hypercoagulable status, central venous catheter, parenteral nutrition, any cancer, sickle cell anemia, tobacco use, race/ethnicity, payer status, urban/rural status, hospital region, and year of inpatient stay.

DVT: Deep venous thrombosis; CD: Crohn's disease; UC: Ulcerative colitis; IC: Indeterminate colitis; VTE: Venous thromboembolism; PE: Pulmonary embolism; RCS: Retrospective comparative study; CVC: Central venous catheter; PTE: Pulmonary thromboembolism; IVST: Intracranial venous sinus thrombosis; PVT: Portal vein thrombosis; TE: Thromboembolic events; IAT: Intra-abdominal thrombosis; CVT: Cerebrovascular thrombosis.

and length of inpatient stay, were adjusted in various studies. According to the NOS evaluation criteria, the methodological quality score was 6 for 10 studies and 8 and 9 for the other two studies, respectively, indicating low-to-moderate bias (Table 2).

Table 2 Quality assessment

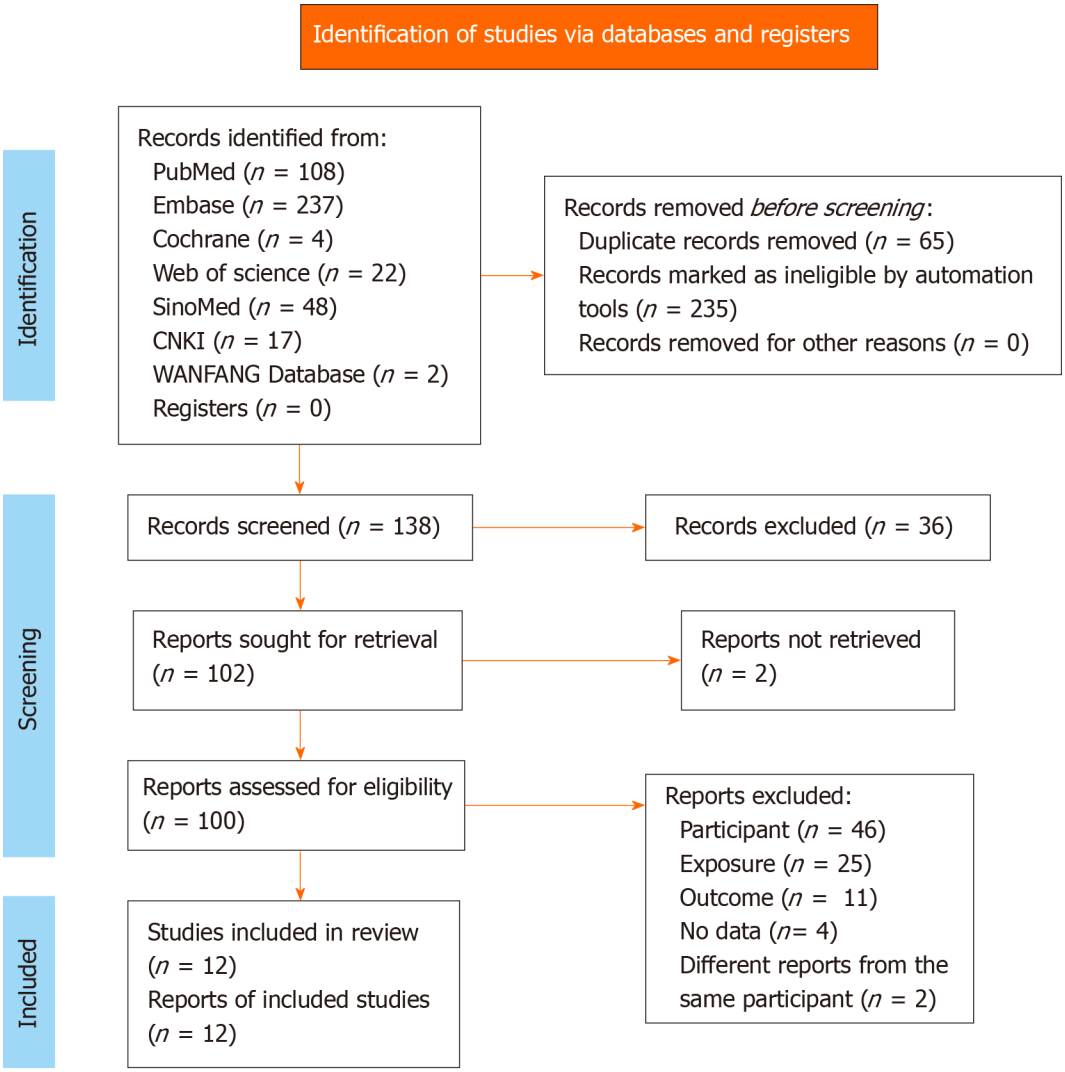
Ref.	Representativeness of the exposed cohort	Selection of the non-exposed cohort	Ascertainment of exposure	Demonstration that outcome of interest was not present at start of study	Comparability of cohorts on the basis of the design or analysis	Assessment of outcome	Was follow-up long enough for outcomes to occur	Adequacy of follow-up of cohorts	Total quality scores
Antiel *et al* [31], 2013	1	0	1	1	0	1	1	1	6
Bence *et al* [26], 2020	1	0	1	1	0	1	1	1	6
Cairo *et al* [36], 2017	1	1	1	1	1	1	1	1	8
Derderian *et al*[27], 2020	1	0	1	1	0	1	1	1	6
Diamond *et al*[32], 2018	1	0	1	1	0	1	1	1	6
Diamond *et al*[33], 2010	1	0	1	1	0	1	1	1	6
Herzog *et al*[34], 2018	1	0	1	1	0	1	1	1	6
Jarchin *et al* [29], 2019	1	0	1	1	0	1	1	1	6
Khosravi *et al*[28], 2020	1	0	1	1	0	1	1	1	6
Lee *et al* [35], 2016	1	0	1	1	0	1	1	1	6
Nylund *et al*[19], 2013	1	1	1	1	2	1	1	1	9
Zitomersky *et al*[37], 2013	1	0	1	1	0	1	1	1	6

Association between IBD and VTE risk

The association between IBD and the incidence of VTE was assessed in 12 studies. The pooled results showed that the risk of VTE was significantly increased in IBD patients compared to those without IBD (P = 0.02, 95%CI: 0.01-0.03). Moderate heterogeneity was detected across the included studies (I^2 = 54.7%, $P_{heterogeneity}$ = 0.012, Figure 2). In addition, double arcsine analysis showed similar results (P = 0.03, 95%CI: 0.02-0.04, Supplementary Figure 1).

The subgroup analyses stratified by IBD subtype were performed to evaluate the VTE risk in IBD. Five studies analyzed the UC subtype, and the pooled results suggested significantly increased VTE incidence in UC patients (P = 0.05, 95%CI: 0.03-0.06). No heterogeneity was detected among the studies (I^2 = 0.0%, $p_{heterogeneity}$ = 0.571, Figure 3A). Pooled analysis of four studies established a correlation between VTE risk and CD (P = 0.02, 95%CI: 0.00-0.04), without heterogeneity (I^2 = 0.0%, $p_{heterogeneity}$ = 0.709, Figure 3A). The IBD subtype was not defined in three studies, and a high rate of VTE was also detected in these patients (P = 0.02, 95%CI: 0.01-0.03), without obvious heterogeneity (I^2 = 0.0%, $p_{heterogeneity}$ = 0.702, Figure 3A). Based on one study, the VTE incidence was not increased in IC patients (P = 0.12, 95%CI: -0.04 to 0.27, Figure 3A).

Stratified by VTE subtype, two studies showed significantly increased PVT incidence in IBD patients (P = 0.04, 95%CI: 0.02-0.06; I^2 = 0.0%, $p_{heterogeneity}$ = 0.568, Figure 3B). The pooled analysis of four studies showed a high risk of DVT in IBD patients (P = 0.03, 95%CI: 0.01-0.05) with low heterogeneity (I^2 = 21.8%, $p_{heterogeneity}$ = 0.280, Figure 3B). In addition, one study revealed an increased risk of CVC-related thrombosis in IBD (P = 0.23, 95%CI: 0.00-0.46, Figure 3B). Furthermore, two studies showed a significant association between TE risk and IBD (P = 0.02, 95%CI: 0.01-0.03), without heterogeneity (I^2 = 0.0%, $p_{heterogeneity}$ = 0.862, Figure 3B). No association was found between PTE and IBD disease (P = 0.01, 95%CI: -0.01 to 0.04, Figure 3B) based on one study.



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Figure 1 Flowchart of the search process of our study.

Pooled analysis of two studies comparing IBD and non-IBD patients showed a significant correlation between VTE risk and IBD disease (OR = 2.99, 95%CI: 1.45-6.18). Moderate heterogeneity was observed among these studies (I^2 = 58.9%, $p_{heterogeneity}$ = 0.119, Figure 4).

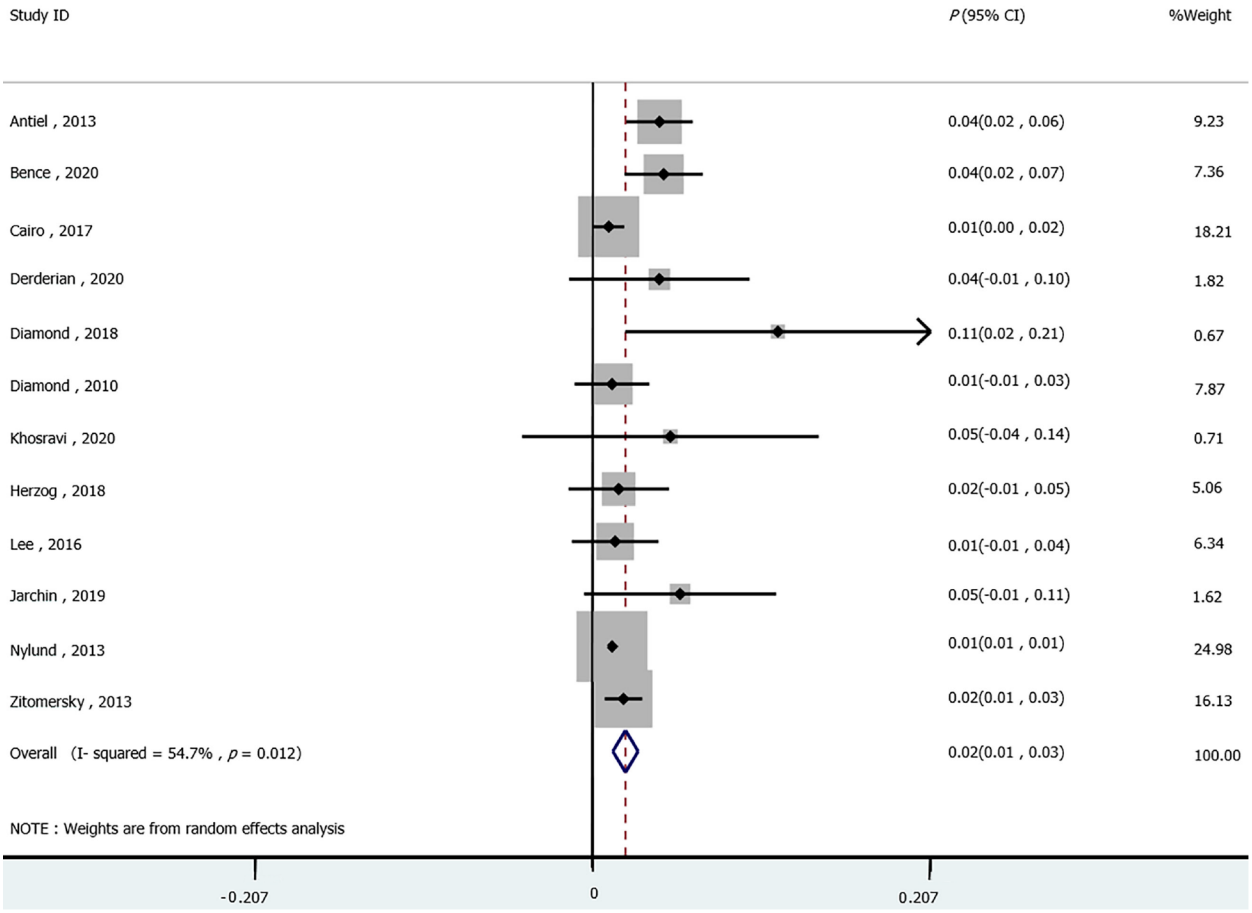
Publication bias

Publication bias was analyzed using a funnel plot. The asymmetrical distribution suggested significant publication bias (Figure 5).

DISCUSSION

The present meta-analysis found that the VTE risk was significantly increased in children and young adults with IBD. A high incidence of VTE was observed in the different subtypes of IBD, including UC, CD, and other undefined IBD diseases. Moreover, multiple VTE signs related to PVT, DVT, TE, and CVC-related thrombosis might be prone to developing during the progression of pediatric and adolescent IBD.

This study suggested that children and young adult IBD patients were susceptible to VTE risk after treatment, despite a low overall incidence. The increased risk of VTE is specific to IBD. Other chronic inflammatory diseases, such as rheumatoid arthritis, or chronic bowel diseases, such as celiac disease, are not found to be accompanied by a high incidence of VTE[26]. The association between VTE risk and IBD has been demonstrated in various IBD patient populations. Currently, there is a lack of systemic reviews on VTE risk in pediatric and adolescent patients. Female pediatric IBD patients were found to be at a high risk of developing VTE[28]. The meta-analysis by Kim *et al*[42] focused on pregnant women with IBD. The findings showed a significantly higher incidence of VTE during pregnancy and



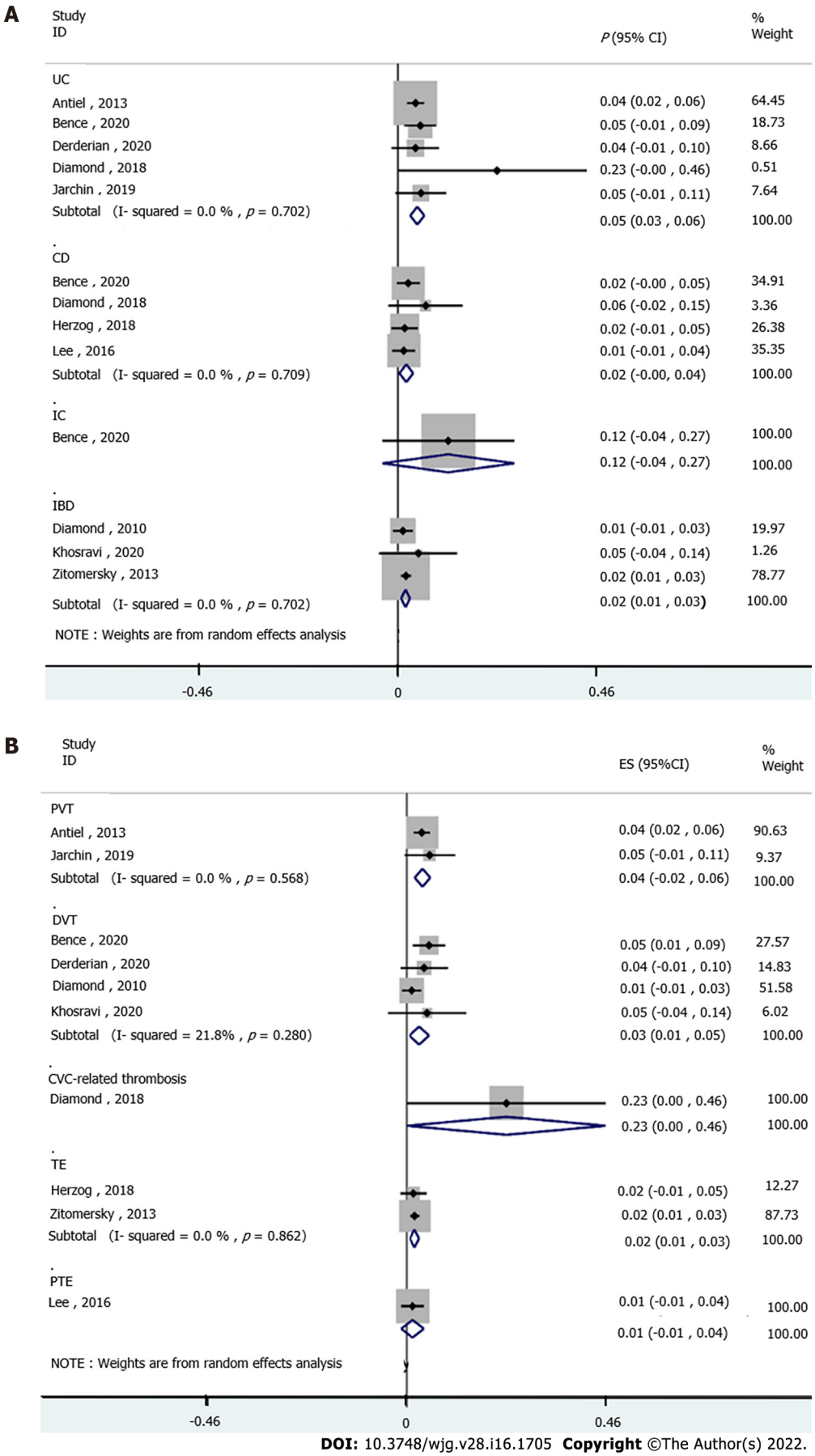
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Figure 2 Forest plots of the incidence of venous thromboembolism in inflammatory bowel diseases.

postpartum in female IBD patients. According to the VTE subtype, the risk of DVT increased significantly in pregnant women, whereas the increase in PE was not significant during pregnancy. In the subgroup analysis based on the location of IBD, patients with UC had significantly higher VTE risk during both pregnancy and the postpartum period, while CD patients exhibited increased VTE incidence only during pregnancy. Therefore, elevated VTE risk was found to be more apparent in UC patients than CD patients. A recent study showed a high postsurgical VTE risk in patients with UC but not in those with CD[43]. Similar results were published earlier[44,45]. However, those studies were performed in the postsurgical setting, which is a state known to be associated with an increased risk of VTE[46,47]. Similarly, the current study showed a higher risk of VTE in UC compared to CD in children and young adult IBD patients, and consistently suggested that children with UC might be more susceptible to VTE. The exact causes for this higher VTE risk in UC than CD are unknown, but severe UC is associated with anemia and reactive thrombocytosis, which is conducive to VTE[48,49].

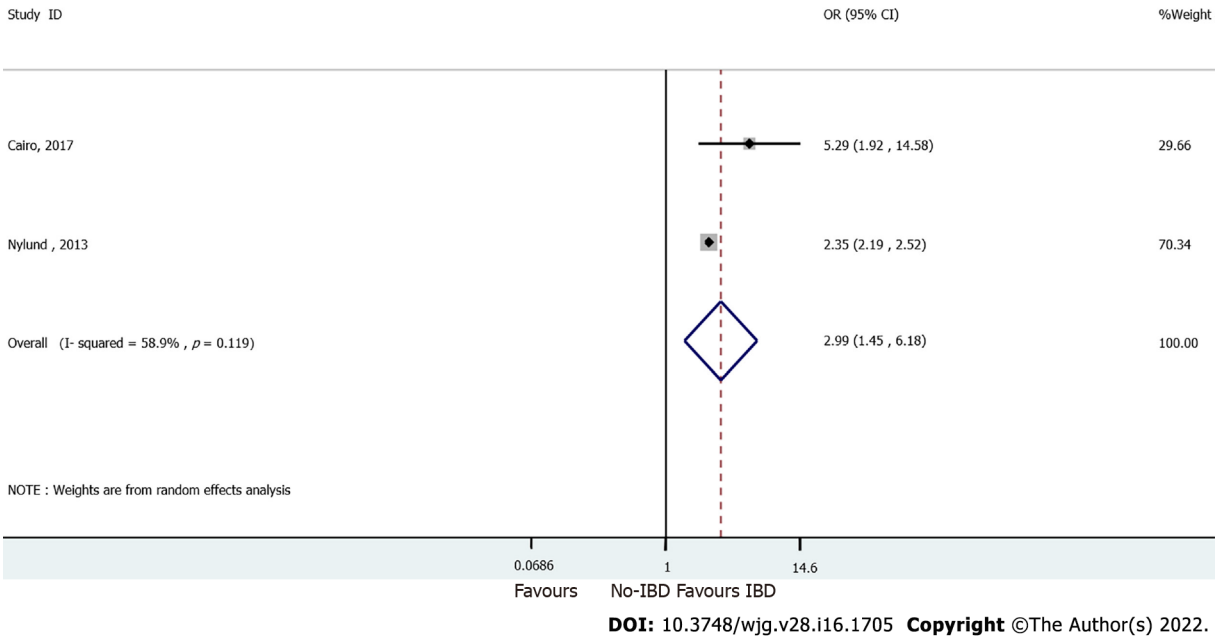
Another meta-analysis by Yuhara *et al*[50] demonstrated that adult patients with IBD (> 20-years-old) were at increased risk for both DVT and PE. The study also showed that confounding factors, such as smoking and body mass index (BMI), did not affect the correlation between IBD and DVT/PE. A large-population database study by Nylund *et al*[25] found that hospitalized children and adolescents with IBD had a high incidence of TE, including DVT and PE. In addition, the study identified older age and abdominal surgery as risk factors for TE in children and young adults with IBD. The Hispanics had a low risk of TE among all children and adolescents. Although tobacco use was a risk factor for TE in adults, it was a protective factor in children with IBD. Interestingly, an increasing trend of TE was observed through the years among children and young adults with IBD, which might be associated with increased hospital admission rates. However, this increasing trend was not clear after multivariable regression analysis. Similarly, the review by Lazzerini *et al*[51] suggested an increased risk of TE in pediatric IBD patients compared to the general population. Our meta-analysis included several updated publications and showed that the risk of PVT, DVT, TE, and CVC-related thrombosis was increased in IBD child and adolescent patients. Moreover, the underlying mechanism of location-related VTE occurrence and development needs further exploration.

Nevertheless, the present study has several limitations. First, moderate heterogeneity was detected in the overall analysis of the correlation between VTE and IBD. On the other hand, low or no heterogeneity was detected after stratifying the studies by subtypes of VTE and IBD. Patients' characteristics,



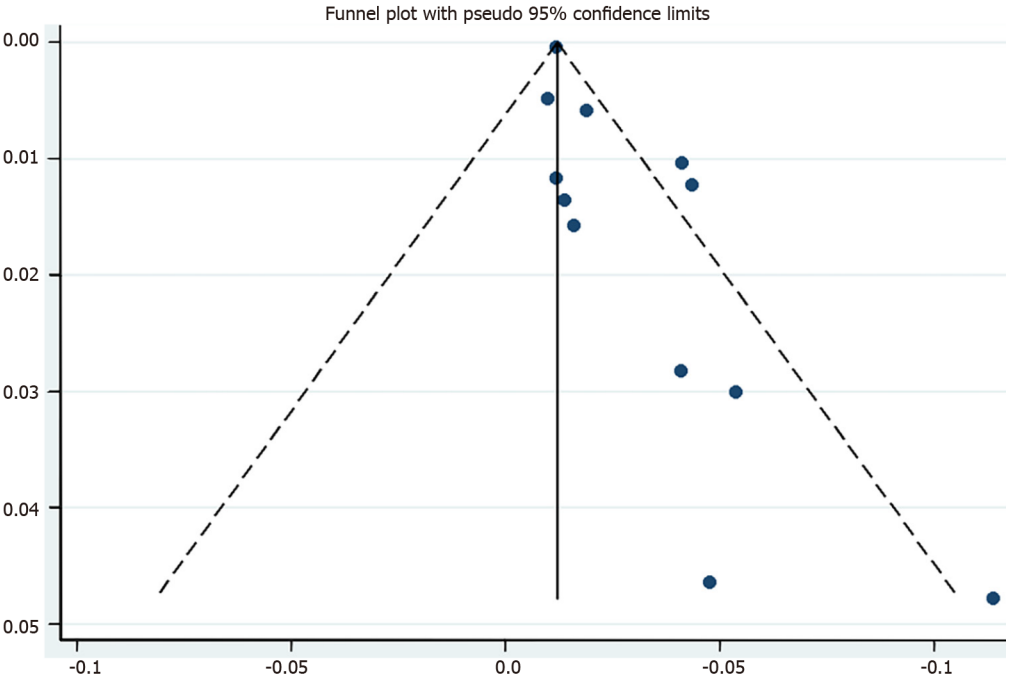
DOI: 10.3748/wjg.v28.i16.1705 **Copyright** ©The Author(s) 2022.

Figure 3 Forest plots. A: Subgroup analysis of the incidence of venous thromboembolism (VTE) in inflammatory bowel diseases (IBD) subtype; B: Subgroup analysis of the incidence of VTE subtype in IBD.



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Figure 4 Forest plots of the risk of venous thromboembolism in inflammatory bowel diseases and non-inflammatory bowel diseases.



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Figure 5 Publication bias; funnel plot of the incidence of venous thromboembolism in inflammatory bowel diseases.

diagnosis of VTE, and intervention therapies might contribute to the variations across the studies and the magnitude of association. Second, certain subgroup analyses only involved a small number of studies and patients, and hence, the findings should be interpreted with caution. Third, the included large-size database review study incorporated registry data of medical records with different populations and settings, which might generate a high selection bias. Fourth, VTE was challenging to diagnose, which might have led to the misclassification of VTE.

CONCLUSION

Pediatric and adolescent IBD patients have an increased risk of VTE. UC and CD patients exhibited a

high risk of VTE. The risk of VTE subtypes was increased in IBD patients.

ARTICLE HIGHLIGHTS

Research background

A two- to three-fold increased risk of venous thrombotic events (VTE) has been demonstrated in patients with inflammatory bowel disease (IBD) compared to the general population, but less is known about the risk of VTE in child- and pediatric-onset IBD. In recent years, several studies have reported the rising incidence rate of VTE in juvenile patients with IBD, and the related risk factors have been explored.

Research motivation

To evaluate the risk of VTE in children and adolescents with IBD.

Research objectives

To evaluate the risk of VTE in children and adolescents with IBD.

Research methods

Articles published up to April 2021 were retrieved from PubMed, Embase, Cochrane Library, Web of Science, SinoMed, CNKI, and WANFANG. Data from observational studies and clinical work were extracted. The outcome was the occurrence of VTE according to the type of IBD. The available odds ratio (OR) and the corresponding 95% confidence interval (CI) were extracted to compare the outcomes. Effect size (P), OR, and 95%CI were used to assess the association between VTE risk and IBD disease. Subgroup analyses stratified by subtypes of VTE and IBD were performed.

Research results

Twelve studies (7450272 IBD patients) were included in the meta-analysis. Child and adolescent IBD patients showed an increased risk of VTE (P = 0.02, 95%CI: 0.01-0.03). Subgroup analyses stratified by IBD (ulcerative colitis (UC): P = 0.05, 95%CI: 0.03-0.06; Crohn's disease (CD): P = 0.02, 95%CI: 0.00-0.04) and VTE subtypes (portal vein thrombosis (PVT): P = 0.04, 95%CI: 0.02-0.06; deep vein thrombosis: P = 0.03, 95%CI: 0.01-0.05; central venous catheter-related thrombosis: P = 0.23, 95%CI: 0.00-0.46; thromboembolic events: P = 0.02, 95%CI: 0.01-0.03) revealed a significant correlation between VTE risk and IBD. Patients with IBD were more susceptible to VTE risk than those without IBD (OR = 2.99, 95%CI: 1.45-6.18). The funnel plot was asymmetric, suggesting the presence of significant publication bias.

Research conclusions

Pediatric and adolescent IBD patients have an increased VTE risk. UC and CD patients exhibited a high risk of VTE. The risk of VTE subtypes was increased in IBD patients.

Research perspectives

Pediatric and adolescent IBD patients have an increased VTE risk. UC and CD patients exhibited a high risk of VTE. The risk of VTE subtypes was increased in IBD patients.

FOOTNOTES

Author contributions: Wang CL, Liang L, Fu JF, Zou CC, Hong F and Wu XM designed the research; Wang CL, Zou CC, Hong F and Wu XM performed the research; Xue JZ and Lu JR contributed new reagents/analytic tools; Wang CL, Liang L and Fu JF analyzed the data; Wang CL, Liang L and Fu JF wrote the paper.

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Country/Territory of origin: China

ORCID number: Xin-Yue Zhang 0000-0001-9934-1566; Hai-Cheng Dong 0000-0001-7830-2728; Wen-Fei Wang 0000-0001-7373-234X; Yao Zhang 0000-0003-2855-8690.

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LETTER TO THE EDITOR

Viral hepatitis: A global burden needs future directions for the management

Henu Kumar Verma, Kiran Prasad, Pramod Kumar, Bhaskar Lvks

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Henu Kumar Verma, Department of Immunopathology, Institute of Lungs Biology and Disease, Comprehensive Pneumology Center, Helmholtz Zentrum, Munich 80331, Bayren, Germany

Kiran Prasad, Bhaskar Lvks, Department of Zoology, Guru Ghasidas Vishwavidyalaya, Bilaspur 495001, Chhattisgarh, India

Pramod Kumar, Department of Drug Delivery, Institute of Lung Biology and Disease, Helmholtz Research Center, Munich 80331, Bayren, Germany

Corresponding author: Henu Kumar Verma, PhD, Research Scientist, Senior Researcher, Department of Immunopathology, Institute of Lungs Health and Immunity, Comprehensive Pneumology Center, Helmholtz Zentrum, 85764 Neuherberg, Munich 80331, Bayren, Germany. henu.verma@yahoo.com

Abstract

Viral hepatitis is an acute or chronic liver disease due to the infection from Hepatitis A, B, C, D and E viruses. It can cause severe liver damage such as cirrhosis, liver failure and liver cancer. To avoid such fatal complications, hepatitis patients must be diagnosed, pathologized and treated as soon as possible. Furthermore, these hepatitis viruses infect through different routes, resulting in distinct disease pathologies, severity and even the need for specific treatment strategies to combat the infection.

Key Words: Viral hepatitis; Vaccination; Chronic; Acute; Viral therapy

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Core Tip: Vaccination is the primary strategy for neutralizing several hepatitis viruses and it is highly effective against most hepatitis viruses. However, additional precautions must be taken for patients at a higher risk of infection such as those who take drugs, prisoners, the homeless or homosexuals. From interferon monotherapy and interferon combination therapy with direct-acting antiviral agents to interferon-free regimens which act by viral chain braking are among the measures to control hepatitis. These strategies can play a critical role in achieving World Health Organization's an ambitious but attainable goal of eliminating hepatitis infection by 2030.

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TO THE EDITOR

Viral hepatitis, particularly hepatitis B and C, are one of the biggest threats to human health contributing to nearly one-fourth of all deaths among overall infectious disease patients[1]. Despite the substantial evolvement in antiviral therapy and access to effective vaccines, the hepatitis virus elimination goal of the United Nations by the year 2030 is doubtful[2]. To this end, healthcare providers and physician assistants can reduce disease burden through infection prevention, early detection, medical management and collaborative care. At the same time, the development of interferon-based and interferon-free therapeutic approaches may help eradicate the hepatitis viral infection.

We recently read Dr. Persico's group paper entitled "Viral hepatitis: Milestones, unresolved issues and future goals" in your prestigious journal "*World Journal of Gastroenterology*[3]." We sincerely thank the author for providing details about the impact of various hepatitis viruses, current research, the gaps between effective management and currently applicable approaches, and finally, the plans that might effectively manage viral hepatitis.

Viral hepatitis is classified into several types: A, B, C, D, and E. Among these types, B and C are the most common types of viruses that can be transmitted through blood transfusions and are the most lethal due to the induction of chronic illness[4]. In the present review article, the authors mainly focused on the pathologies, clinical manifestations, and various advancements in therapeutic regimes of different hepatitis virus infections.

Furthermore, they elegantly demonstrated progression in hepatitis C virus (HCV) infection treatment regimens from interferon to direct-acting antiviral agents (DAAs) with a relative increase in sustained virological response (SVR) rate. Newer pan-genotypic antiviral therapies, such as sofosbuvir/velpatasvir and glecaprevir/pibrentasvir, have 98%-99% SVR in all genotypes of hepatitis C virus and low drug resistance. It was approved by the FDA in 2016. DAAs are now known to be effective in the treatment of HCV patients who do not have cirrhosis, have compensated cirrhosis or have extrahepatic manifestations and have a lower risk of hepatocellular carcinoma (HCC) recurrence[5]. Besides, various host targeting agents (HTAs) are under clinical studies that target molecules essential for hepatitis C virus entry and replication. Its main advantage is its low mutation rate. The primary targets of HTAs are microRNA-122, Cyclophilin A and HMG-CoA reductase[6].

Current hepatitis B virus (HBV) infection management protocols include the use of nucleoside/nucleotide analogues and interferons both of which reduce HBV replication but do not eradicate the virus. Current therapies' lack of direct impact on virus covalently closed circular DNA (cccDNA) is a major limiting factor for HBV virus elimination[7]. Thus, various gene-editing methods like transcription activator-like effector nucleases, CRISPR/Cas system and zinc finger nuclease are understudies to target cccDNA expression[8].

Several immunomodulatory agents that induce HBV-specific immune responses have recently been developed. Immunomodulatory therapies include agonists, immune checkpoint inhibitors, therapeutic vaccines and engineered HBV-specific T-cell transfer. Agonists activate Toll-like receptors, stimulator of IFN genes, and Retinoic Acid-Inducible Gene-1 to initiate the innate immune response. While immune checkpoint inhibitors such as programmed cell death-1 trigger an adaptive immune response. GS-4774 (vector-based vaccine) trials showed that the vaccine was safe but no significant reduction in HBsAg levels was observed. Other vaccines like INO-1800, TG-1050 and ABX-203 are under clinical investigation[9]. RNAi-based therapies are also evolving against HBV infection which exerts its antiviral activity by post-transcriptional silencing. ARC-520 and ARC-521 (RNAi-based drug) showed a reduction in HBsAg and HBV DNA levels but were discontinued due to rising safety concerns related to drug delivery. Both siRNA-based drug, JNJ-3989, earlier called ARO-HBV and VIR-218 has shown promising results against chronic HBV infection and are under ongoing clinical trials[10].

Hepatitis D virus infection occurs only in HBV-infected people. Pegylated interferon-alpha is the only effective therapy against HDV infection in clinical practice. However, HBV vaccination protects from both HBV and HDV infection. Other therapeutic drugs under clinical trials against HDV infection include Pegylated IFN-lambda, Myrcludex B that blocks hepatitis B and D virus entry in hepatocytes, Lonafarnib that inhibits farnesylation of L-HDAg and its subsequent interaction with HBsAg and REP 2139. Its mechanism is still unclear but it is known to be related to blocking HBsAg release[11].

For hepatitis A, no specific treatment is available. Both improving sanitary conditions and HAV vaccination is the most effective preventive strategy. Vaccination is recommended to high-risk people, patients having chronic liver disease, HIV-positive patients and pregnant women[4].

The authors describe various hepatitis E virus (HEV) genotypes and their transmission routes in detail. HEV1 and HEV2 are the only ones that infect humans and spread *via* the fecal-oral route. There is no effective HEV vaccine available to prevent infection. China developed the HEV-239 vaccine which is safe for pregnant women and provides longer protection. However, it is not permitted in other countries [12].

Other treatment strategies like liver transplantation and management of hepatitis in pregnant women can be included. Liver transplantation is the most effective therapy for HCC and cirrhosis due to HBV and HCV infections. Post-transplantation use of DAA reduces the risk of recurrence and increases the survival rate of patients[13].

Pregnant women are prone to acute and chronic hepatitis infection with a risk of developing fulminant hepatitis and vertical transmission, especially in hepatitis E. Seto *et al*[14] described various management strategies for different subtypes of hepatitis. Ribavirin is known to be teratogenic and thus should be avoided during pregnancy; however, supportive care is preferred. Breastfeeding is encouraged in hepatitis C, D, and A, while in hepatitis E, it is not recommended. HAV vaccination is opted to prevent fetal transmission. DAA treatment during pregnancy is still debatable[14]. Ledipasvir and sofosbuvir use during pregnancy in HCV infection has not been associated with safety concerns[15].

Even with advanced therapies like DAA, there are still challenges to cure and eradicate various subtypes of viral hepatitis. Thus, more investigations are required for multiple drugs under clinical trials to develop better preventive and management strategies. We genuinely appreciate Torre *et al*[3] and colleagues for providing relevant and detailed information on various subtypes of viral hepatitis along with their clinical manifestation and treatment methods.

FOOTNOTES

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Country/Territory of origin: Germany

ORCID number: Henu Kumar verma 0000-0003-1130-8783; Kiran Prasad 0000-0001-5817-5661; Pramod Kumar 0000-0002-7995-5613; Bhaskar Lvks 0000-0003-2977-6454.

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LETTER TO THE EDITOR

Comment on "Artificial intelligence in gastroenterology: A state-of-the-art review"

Thomas Bjørsum-Meyer, Anastasios Koulaouzidis, Gunnar Baatrup

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Thomas Bjørsum-Meyer, Gunnar Baatrup, Department of Surgery, Odense University Hospital, Svendborg 5700, Denmark

Thomas Bjørsum-Meyer, Anastasios Koulaouzidis, Gunnar Baatrup, Department of Clinical Research, University of Southern Denmark, Faculty of Health Science, Odense 5230, Denmark

Corresponding author: Thomas Bjørsum-Meyer, MD, PhD, Associate Professor, Surgeon, Department of Surgery, Odense University Hospital, Baagøes Alle 15, Svendborg 5700, Denmark. thomas.bjoersum-meyer@rsyd.dk

Abstract

Colon capsule endoscopy (CCE) was introduced nearly two decades ago. Initially, it was limited by poor image quality and short battery time, but due to technical improvements, it has become an equal diagnostic alternative to optical colonoscopy (OC). Hastened by the coronavirus disease 2019 pandemic, CCE has been introduced in clinical practice to relieve overburdened endoscopy units and move investigations to out-patient clinics. A wider adoption of CCE would be bolstered by positive patient experience, as it offers a diagnostic investigation that is not inferior to other modalities. The shortcomings of CCE include its inability to differentiate adenomatous polyps from hyperplastic polyps. Solving this issue would improve the stratification of patients for polyp removal. Artificial intelligence (AI) has shown promising results in polyp detection and characterization to minimize incomplete CCEs and avoid needless examinations. Onboard AI appears to be a needed application to enable near-real-time decision-making in order to diminish patient waiting times and avoid superfluous subsequent OCs. With this letter, we discuss the potential and role of AI in CCE as a diagnostic tool for the large bowel.

Key Words: Video capsule endoscopy; Wireless capsule endoscopy; Artificial intelligence; Colonic polyps; Endoscopic surgical procedures; Colon neoplasm

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Core Tip: Colon capsule endoscopy (CCE) generates a vast amount of image material-currently, this material must be assessed manually. Artificial intelligence (AI) as an adjunct to CCE has been reported as having high accuracy for detecting colonic lesions. Future studies need to evaluate AI algorithms for estimating the likelihood of neoplasia and predicting which patients are most likely to benefit from CCE. Onboard capsule intelligence has the potential to generate result reports immediately after completed examinations.

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TO THE EDITOR

We read with interest the in-depth review by Kröner *et al*[1]. The authors presented a thorough account of the status and knowledge of artificial intelligence (AI) in everyday practice in gastroenterology[1]. As we apply AI in picture analysis of colon capsule endoscopy (CCE), we feel obliged to offer some relevant insights. The coronavirus disease 2019 (COVID-19) pandemic has accelerated the clinical adoption of CCE as an alternative first-line diagnostic procedure to optical colonoscopy (OC)[2]. CCE is a painless, low-risk procedure that can be performed in the patient's home with minimal contact with healthcare workers and/or other patients, therefore abiding (when possible) by the distancing measures recommended to contain the spread of COVID-19.

Furthermore, CCE is seen as a measure to relieve the pressure on overburdened endoscopy units with long waiting times for OC. The Scottish Capsule Programme has implemented CCE in a large-scale roll-out to patients presenting with lower gastrointestinal symptoms to their general practitioners and found to be eligible for colonic investigation[3]. The programme enables effective upfront screening at the community level to address increasing demand and capacity pressure within the National Health Service.

Until recently, the clinical implementation of CCE has been hampered by low completion rates compared to OC. A significant drawback for CCE is its reliance on extensive orally administered bowel cleansing preparations to gain adequate visualization of the mucosa, as, unlike OC, water wash and suction are not possible. Although research into optimizing and standardizing bowel preparation regimens is ongoing, to date it has failed to meet the minimum standard of 90% for OC[4]. A recent systematic review and meta-analysis of 46 studies including 5000 patients showed that CCE's (pooled) adequate colon cleanliness rate (ACCR) was only 77%[5]. Therefore, further studies aiming to improve CCE's ACCR are mandatory. Another way to achieve more conclusive CCE examinations is to enhance our ability to predict patients at high risk of an insufficient colon cleanliness level. AI algorithms based on demographic data are one possible solution. We are currently running a large randomized trial in Denmark (CFC2015) in which 2015 citizens are allocated to CCE. After the study is completed in Summer 2022, we plan to create algorithms for just such a purpose. The cost-effectiveness of CCE needs to be improved before wider clinical adoption is considered. Hassan *et al*[6] found that improved compliance in the general population is mandatory to make CCE cost-effective compared to OC[6].

CCE produces many images, and reading them is very time-consuming and monotonous, increasing the risk of missing important lesions. Hence, the development of AI-based tools to assist readers is needed. Saraiva *et al*[7], in a recent article, developed a convolutional neural network based on AI. They found that it could detect protruding lesions in the colon with a sensitivity of 90.7% and a specificity of 92.6%[7]. To avoid unnecessary OC after CCE, it is essential to differentiate between hyperplastic polyps (HPs) and adenomatous polyps (APs). By applying flexible spectral imaging colour enhancement, Nakazawa *et al*[8] differentiated HPs from APs with a sensitivity of 91.2% and specificity of 88.2%[8]. Further research is needed to detect sessile serrated lesions using AI-assisted CCE.

The delay between capsule egestion and the completion of video reading and report writing is currently two days. Onboard capsule intelligence has the potential to make near real-time assessments of colonic pathology, creating a report almost immediately after CCE completion. The authors of this letter aspire to the development of onboard integrated AI to make CCE an expeditious diagnostic tool comparable to OC.

FOOTNOTES

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Country/Territory of origin: Denmark

ORCID number: Thomas Bjørsum-Meyer 0000-0001-5253-0802; Anastasios Koulaouzidis 0000-0002-2248-489X; Gunnar Baatrup 0000-0003-0300-5766.

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