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REVIEW

Innate and adaptive immune escape mechanisms of hepatitis B virus

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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

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].

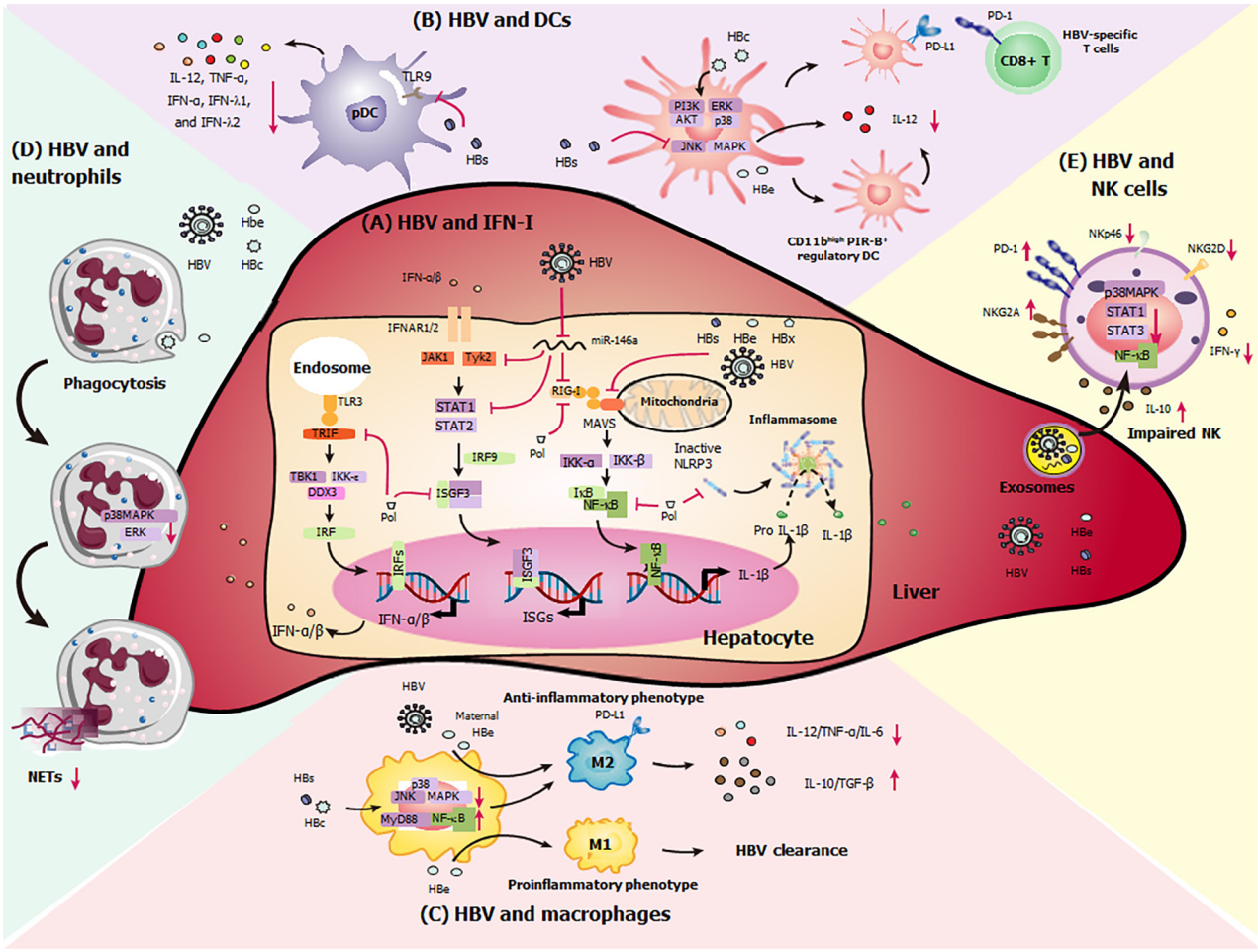

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Figure 1 The interaction between hepatitis B virus and innate immunity. A: Hepatitis B virus (HBV) suppression of the type I interferon (IFN-I) response. HBV infection inhibits IFN-I transcription and signal transduction and IFN-β synthesis; B: HBV 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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MINIREVIEWS

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

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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

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.

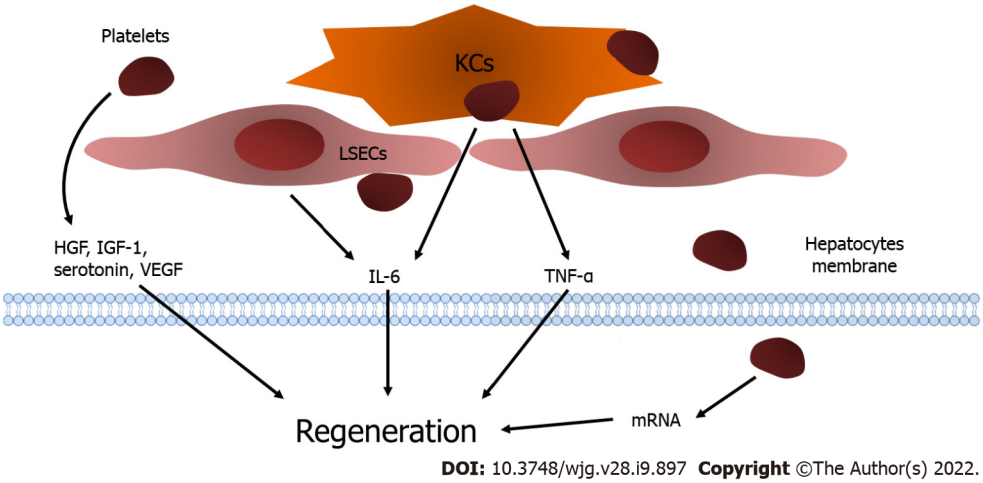

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Figure 1 Platelets and liver regeneration. Platelets translocate into the space of Disse and release insulin-like growth factor-1, hepatocyte growth factor, and vascular endothelial growth factor. The direct contact of platelets with liver sinusoidal 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.

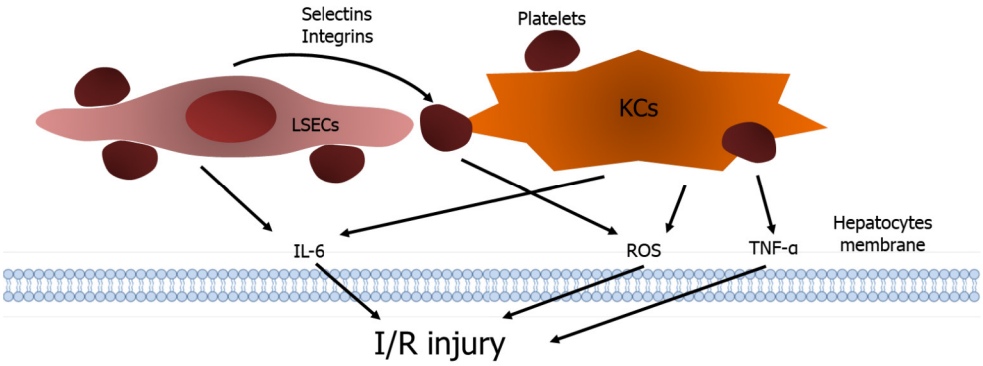

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Figure 2 Platelets and ischemia/reperfusion injury. Liver sinusoidal endothelial cells (LSECs) express selectins and integrins to stimulate the interaction between platelets and LSECs, and platelets result in the excretion of interleukin-6 (IL-6) from 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

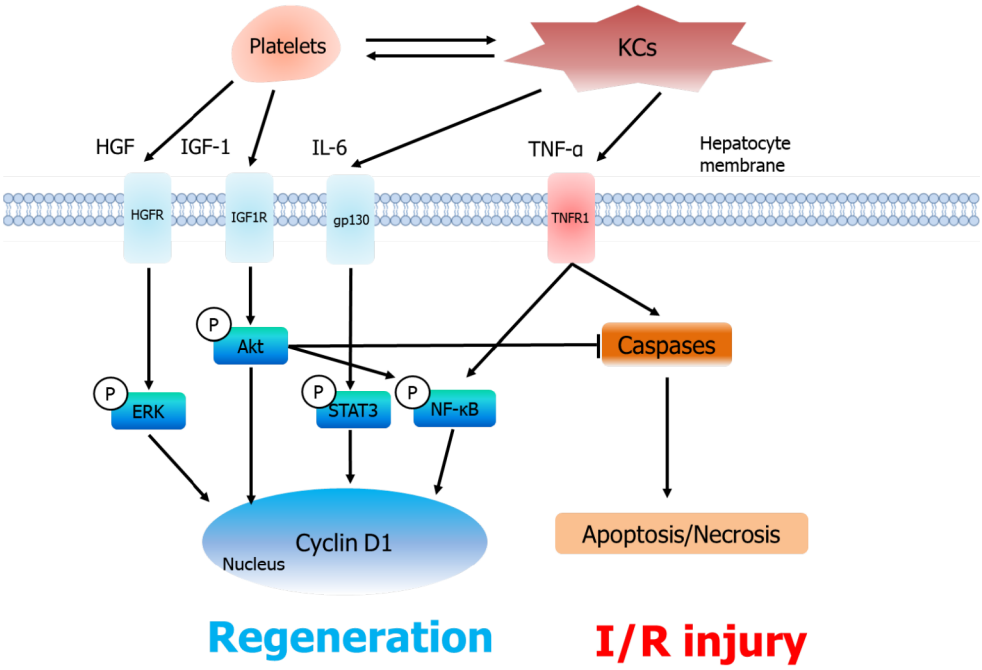

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Figure 3 Platelets, liver regeneration and ischemia/reperfusion injury after partial liver transplantation. After accumulating in the liver graft, platelets excrete hepatocyte growth factor (HGF) and insulin-like growth factor-1 (IGF-1) and collaborate 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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MINIREVIEWS

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

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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

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.

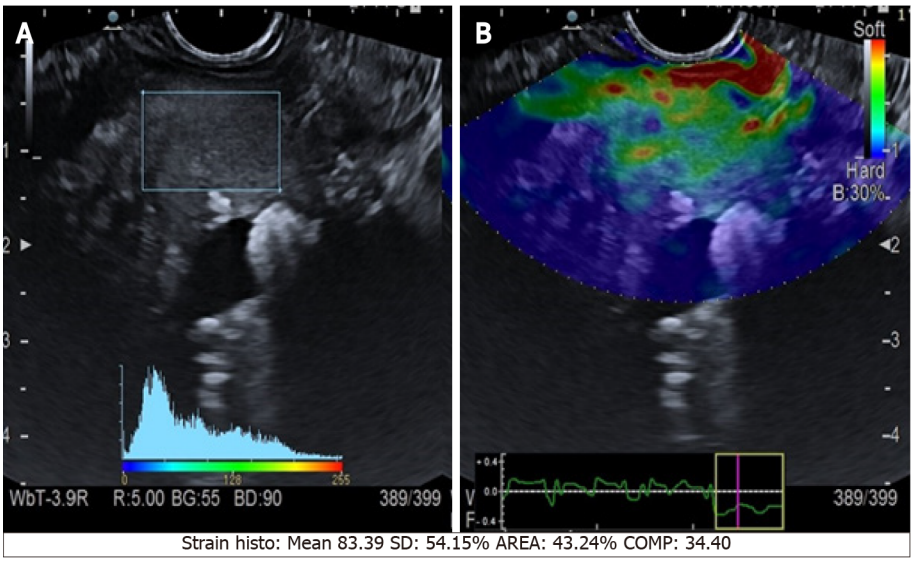

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Figure 1 Quantitative endoscopic ultrasound elastography of chronic pancreatitis. A: Endoscopic ultrasound B-mode with region of interest square; B: Elastographic image.

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

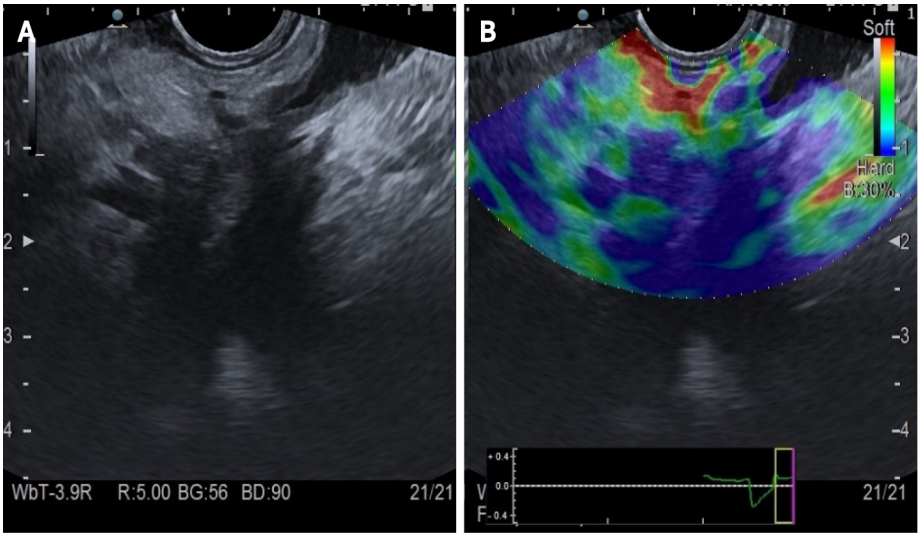

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Figure 2 Qualitative endoscopic ultrasound elastography of pancreatic adenocarcinoma of the head. A: Endoscopic ultrasound B-mode; B: Elastographic image.

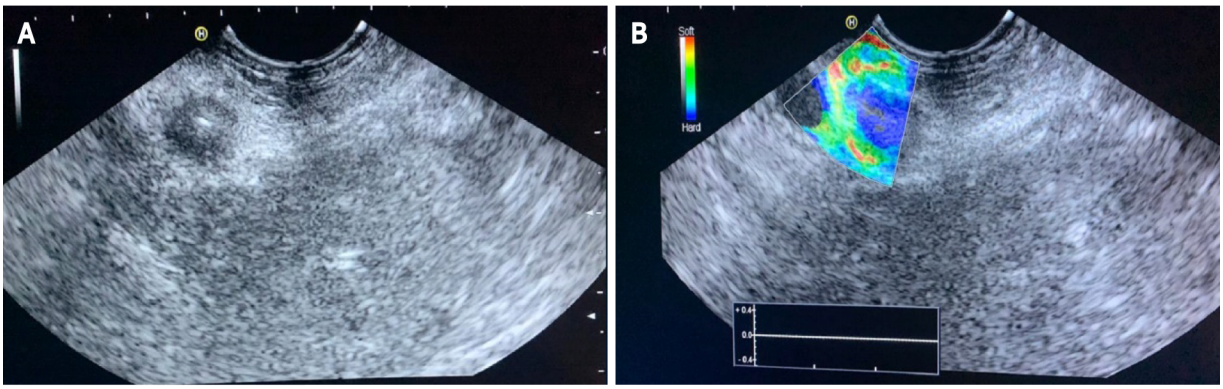

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Figure 3 Qualitative endoscopic ultrasound elastography of a neuroendocrine malignant tumor of the pancreatic tail. A: Endoscopic ultrasound B-mode; B: Elastographic image.

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.

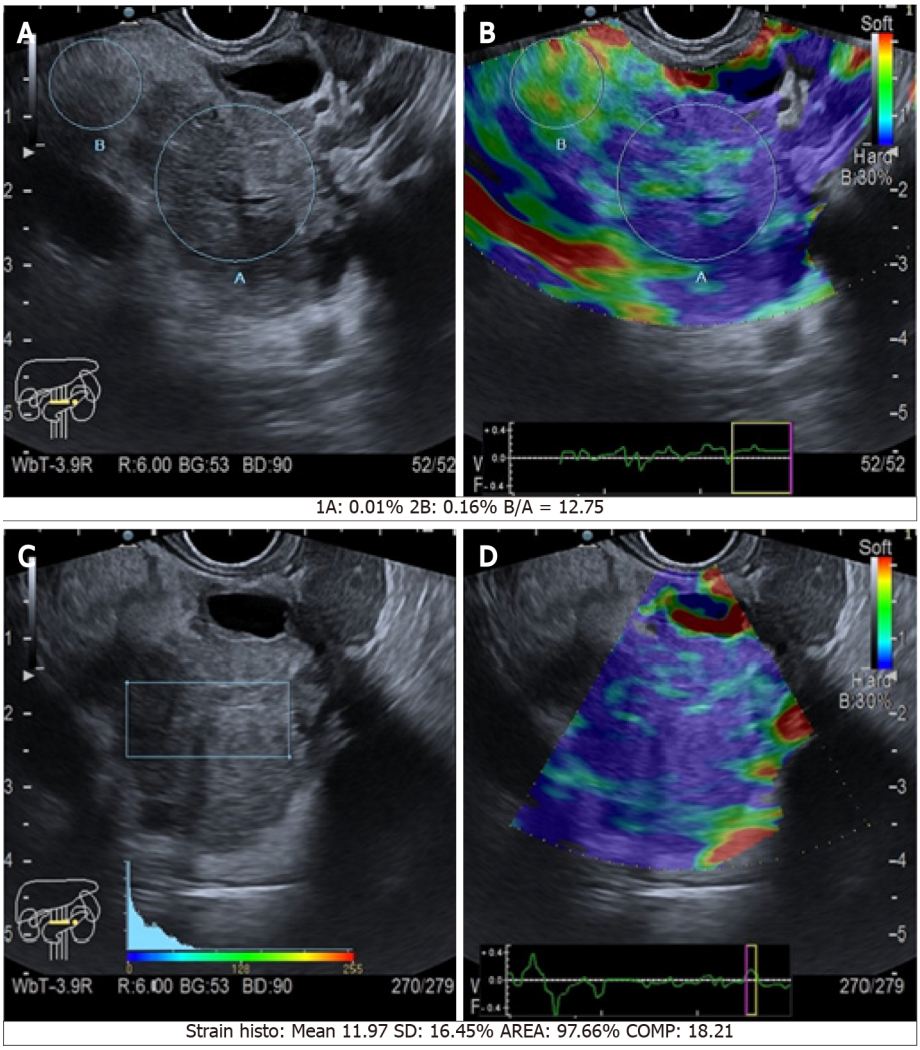

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Figure 4 Quantitative endoscopic ultrasound elastography of pancreatic cancers of the body. A and C: B-mode with region of interest circles and square; B and D: Elastographic images.

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.

Conflict-of-interest statement: Clara Benedetta Conti, Giacomo Mulinacci, Raffaele Salerno, Marco Emilio Dinelli, and Roberto Grassia have no conflict of interest.

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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

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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

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.

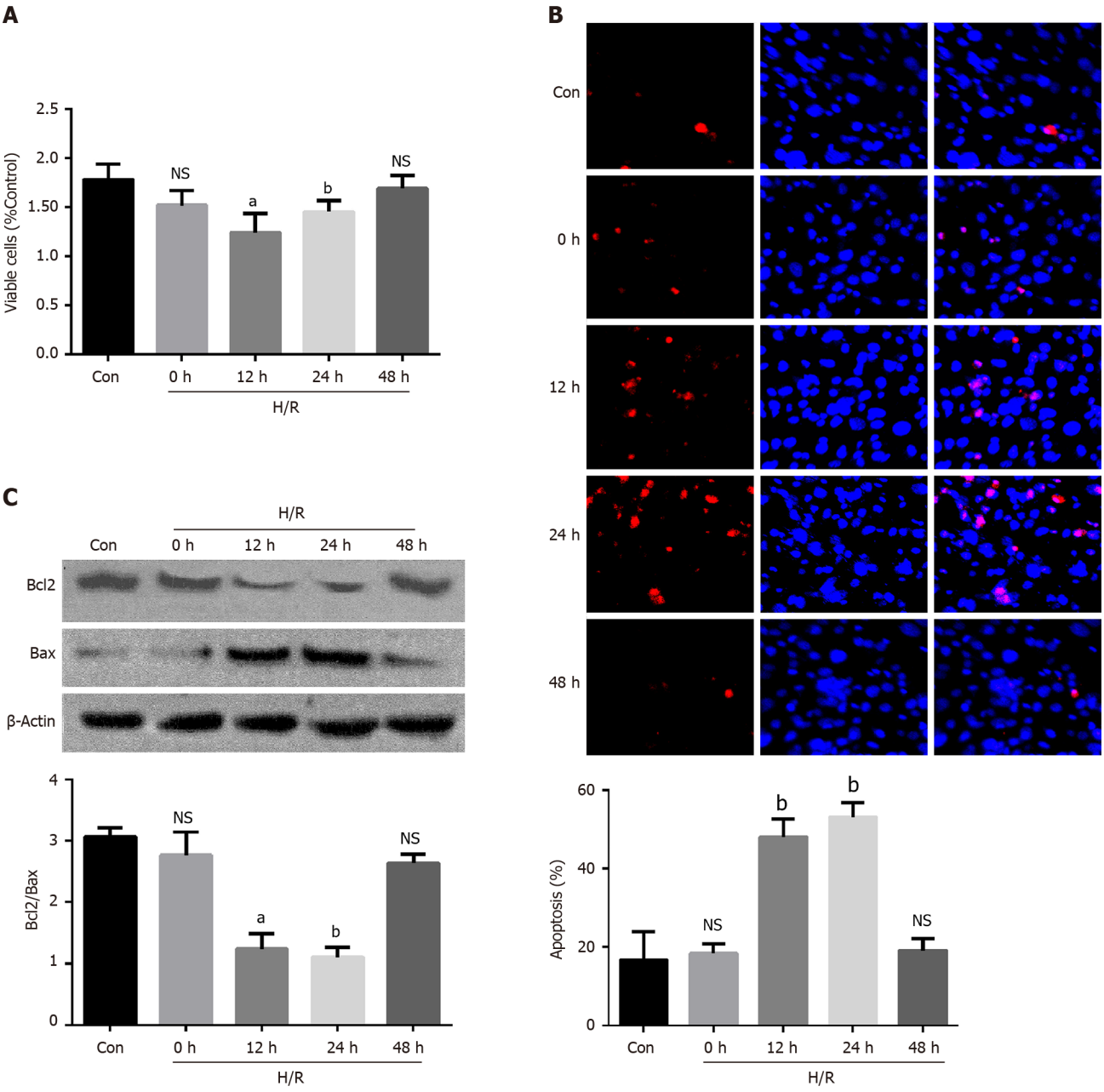


Figure 1 Apoptosis was induced in Caco2 cells after hypoxia/reoxygenation treatment. A: Cell viability was tested using the CCK8 kit on different times after the hypoxia/reoxygenation (H/R) treatment, $^{a}P < 0.01$ and $^{b}P < 0.001$ *vs* control ($n = 3$); B: The quantification data of apoptosis tested 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

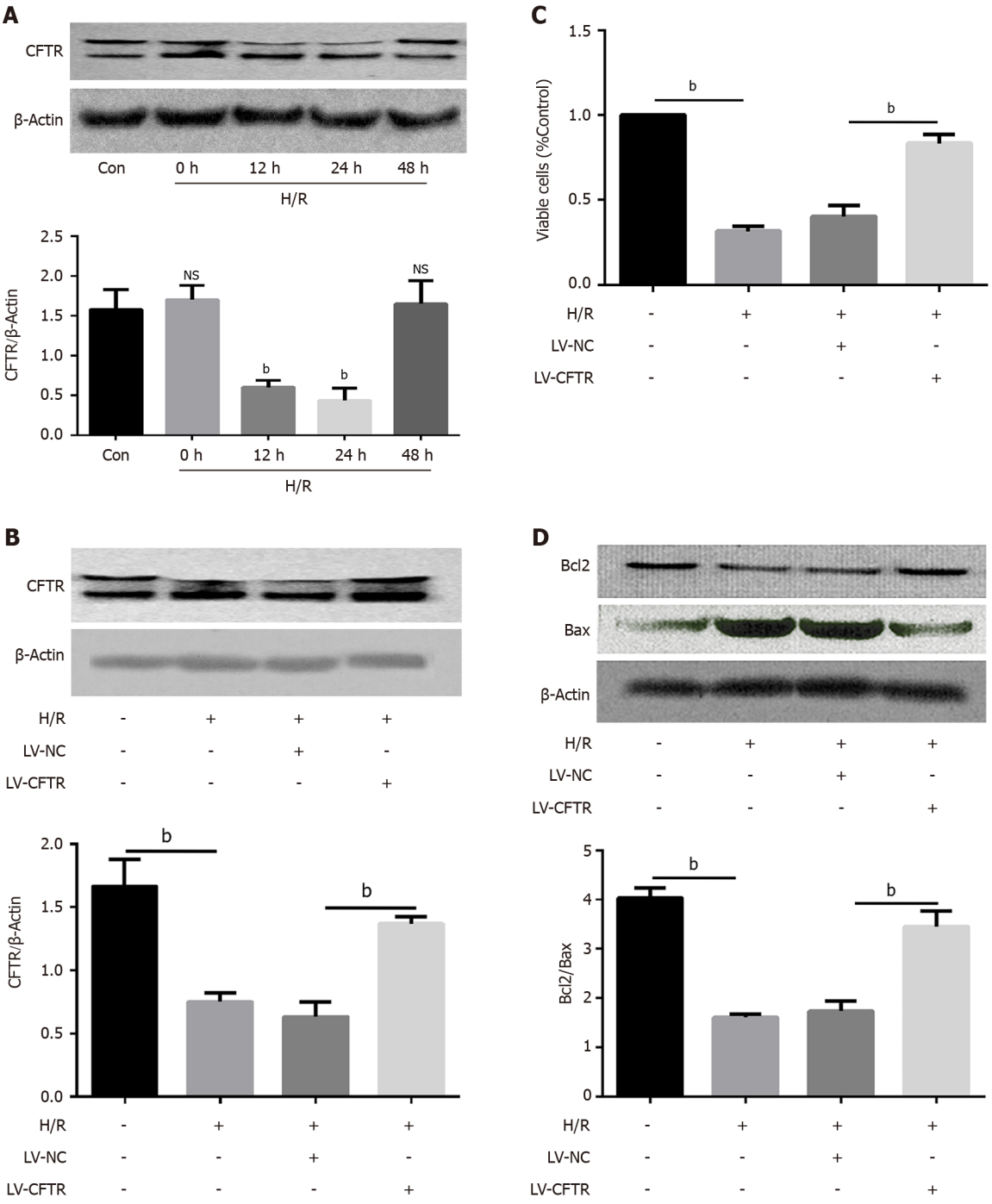
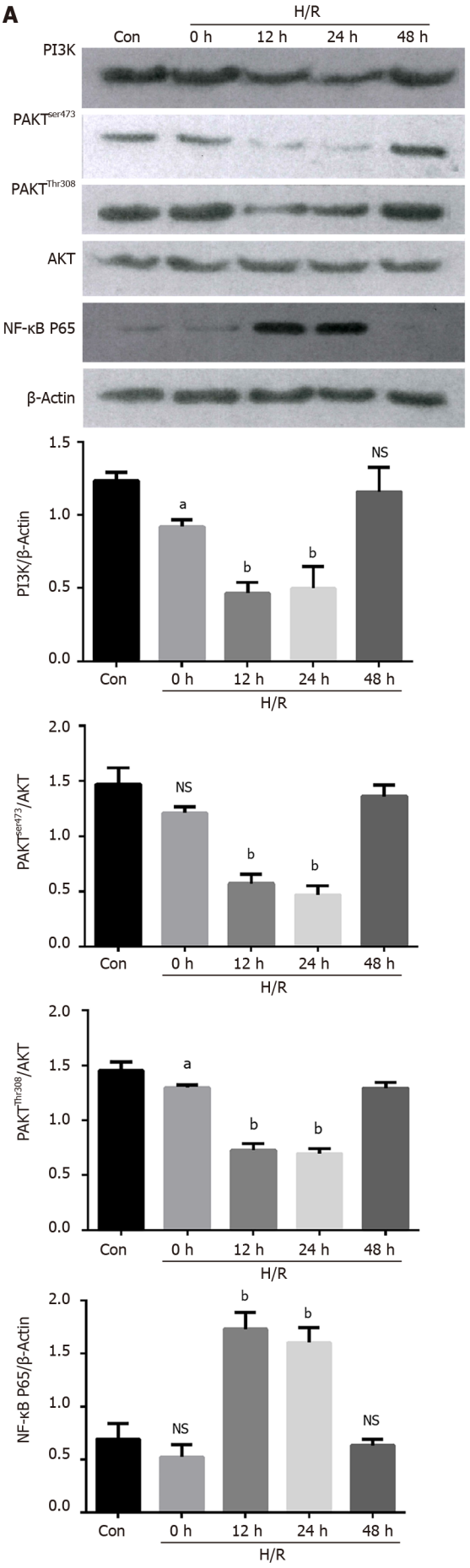


Figure 2 Cystic fibrosis transmembrane conductance regulator can protect the Caco2 cells from apoptosis induced by the hypoxia/reoxygenation. A: Representative Western blotting and quantification data for cystic fibrosis transmembrane conductance regulator (CFTR) after the hypoxia/reoxygenation (H/R) 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
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PAKTThr308/AKT
NF-κB P65/β-Actin
NS
a
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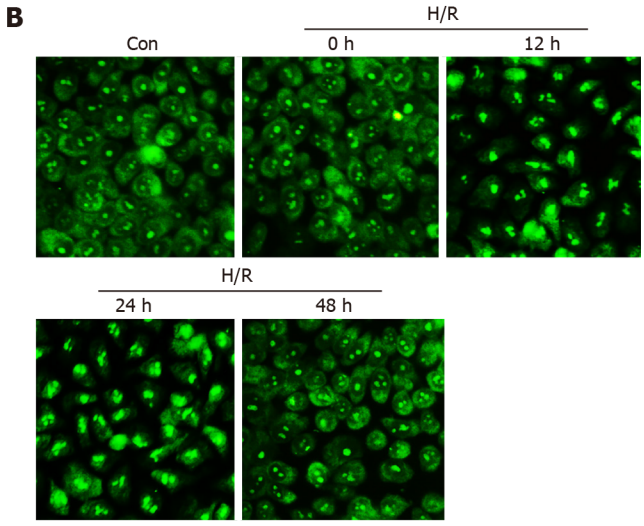
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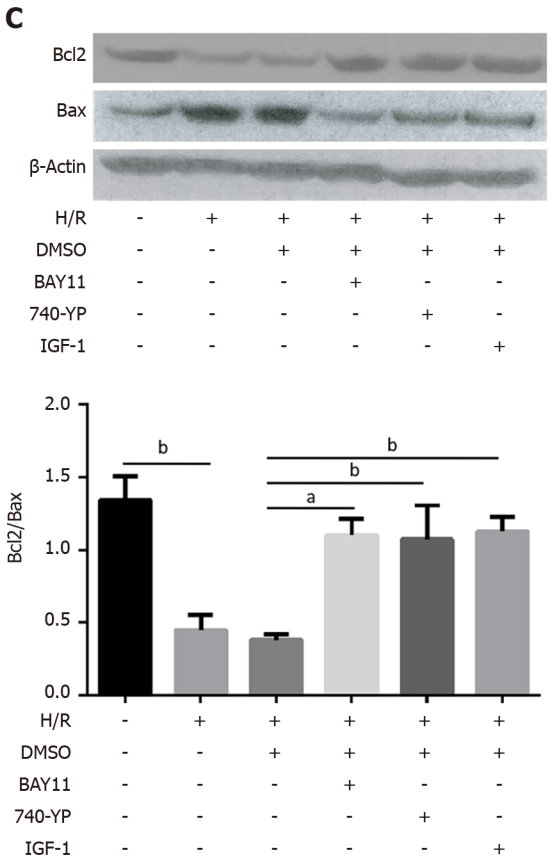
C
Bcl2
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β-Actin
H/R
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740-YP
IGF-1
Bcl2/Bax
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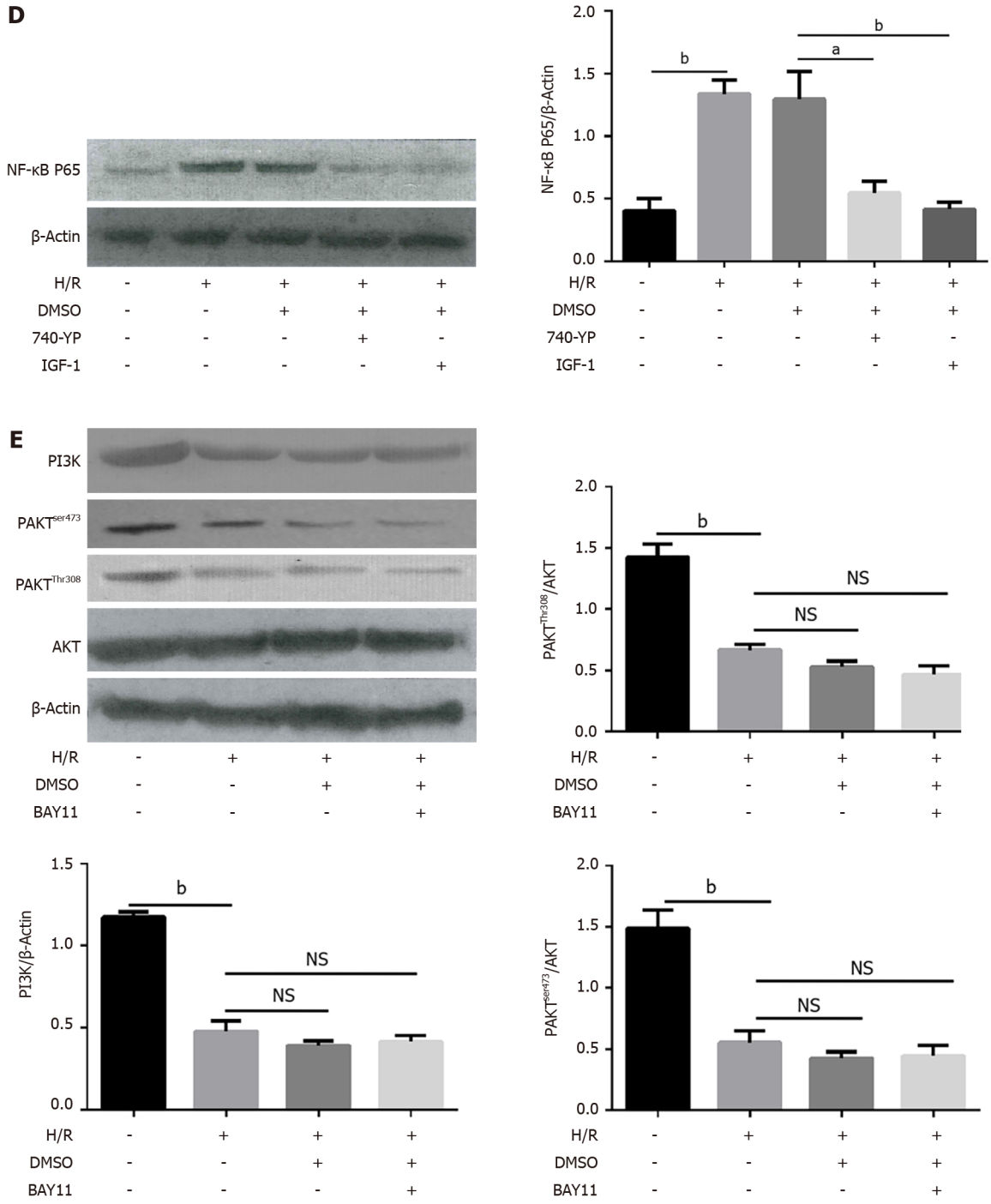


Figure 3 PI3K/AKT/NF-κB pathway was involved in hypoxia/reoxygenation-induced apoptosis. A: Representative western blotting and quantification data for PI3K, p-AKT^{ser473}, p-AKT^{Thr308}, NF-κB P65, and β-actin at different time points after hypoxia/reoxygenation (H/R) treatment. ns, no significant 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

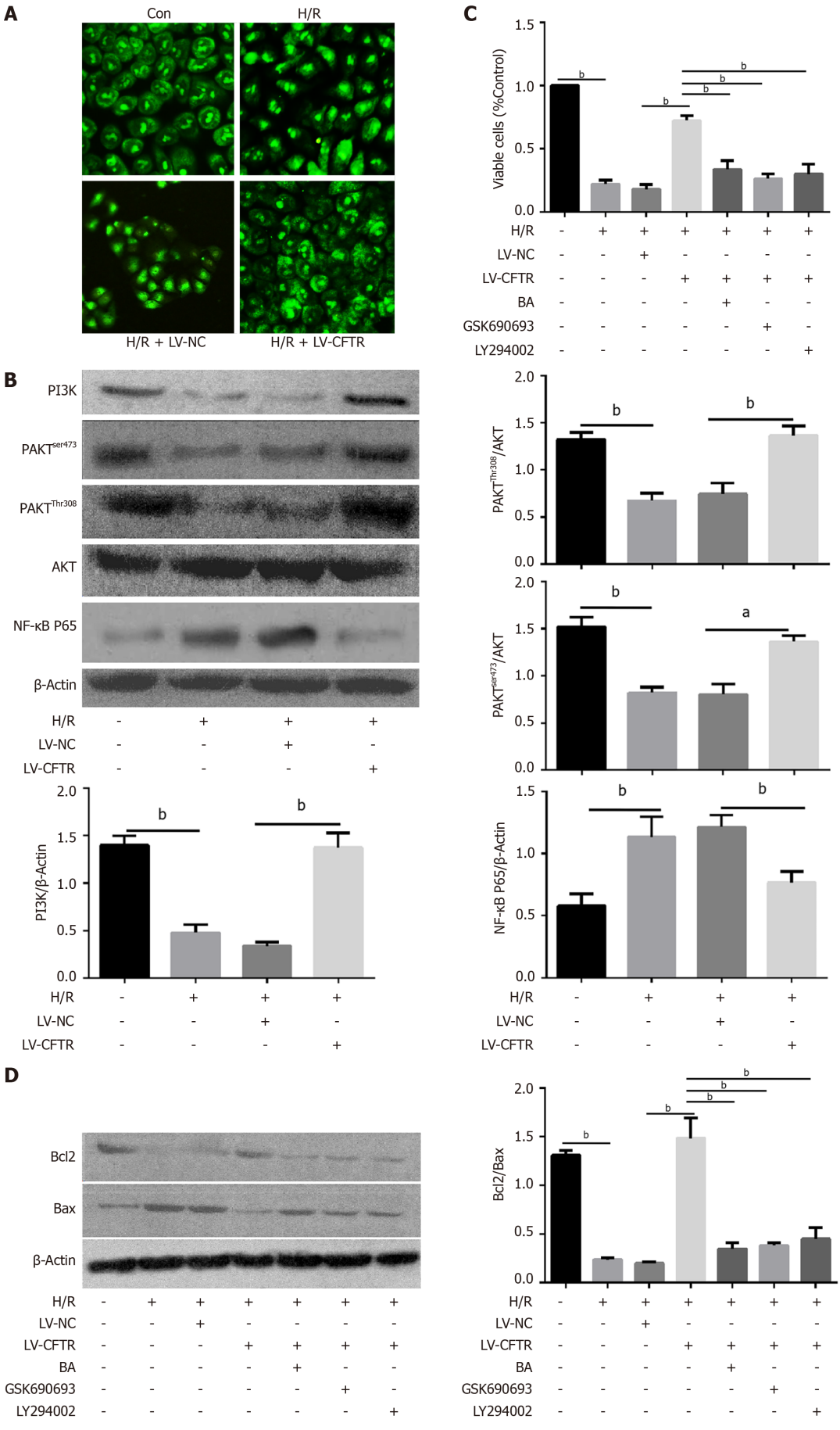


Figure 4 Cystic fibrosis transmembrane conductance regulator protected the Caco2 cells from hypoxia/reoxygenation-induced apoptosis

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

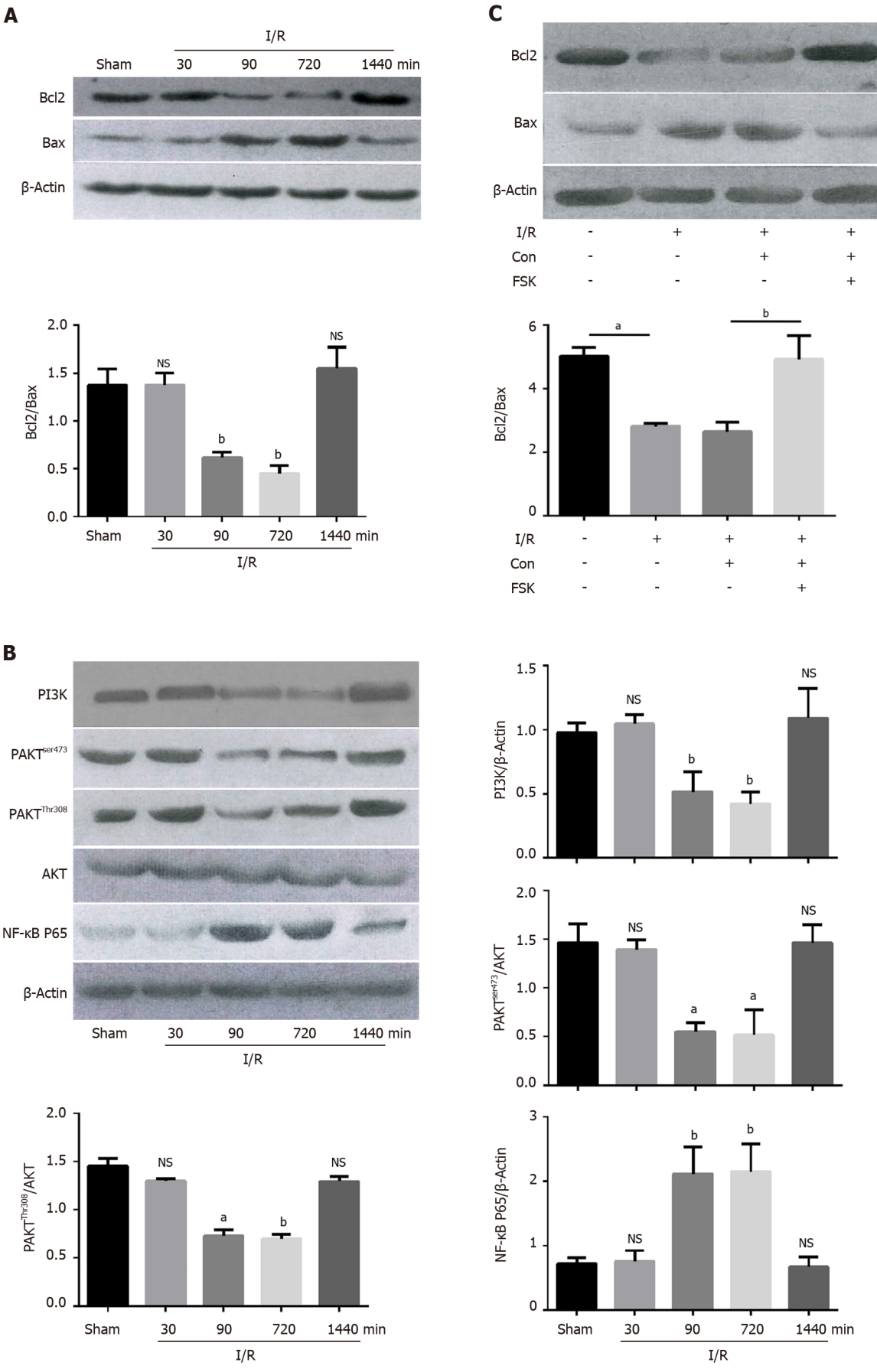
To assess whether the findings of CFTR in Caco2 cell lines were relevant to animal models *in vivo*, we performed the I/R-induced intestinal injury in animal models. Results from the western blotting demonstrated that the ratio of Bcl-2/Bax was significantly decreased at 90 min and 720 min, indicating 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
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Bax
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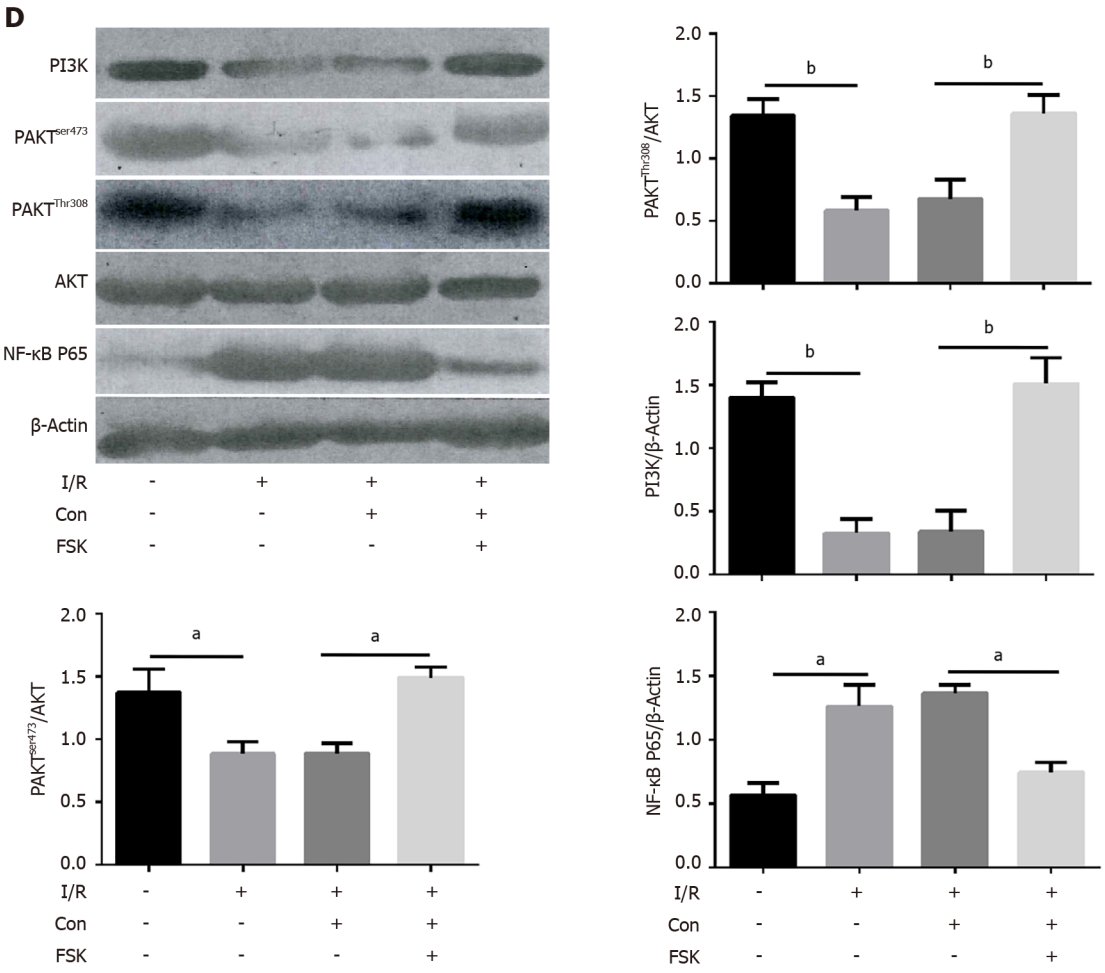

Figure 5 Cystic fibrosis transmembrane conductance regulator protects the intestinal cell from apoptosis induced by ischemia/reperfusion by inhibiting PI3K/AKT/NF-κB pathway *in vivo*. A: Representative Western blotting and quantification data for cell apoptotic proteins Bcl-2 and Bax at different time 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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ORIGINAL ARTICLE

Retrospective Cohort Study

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

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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

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*.

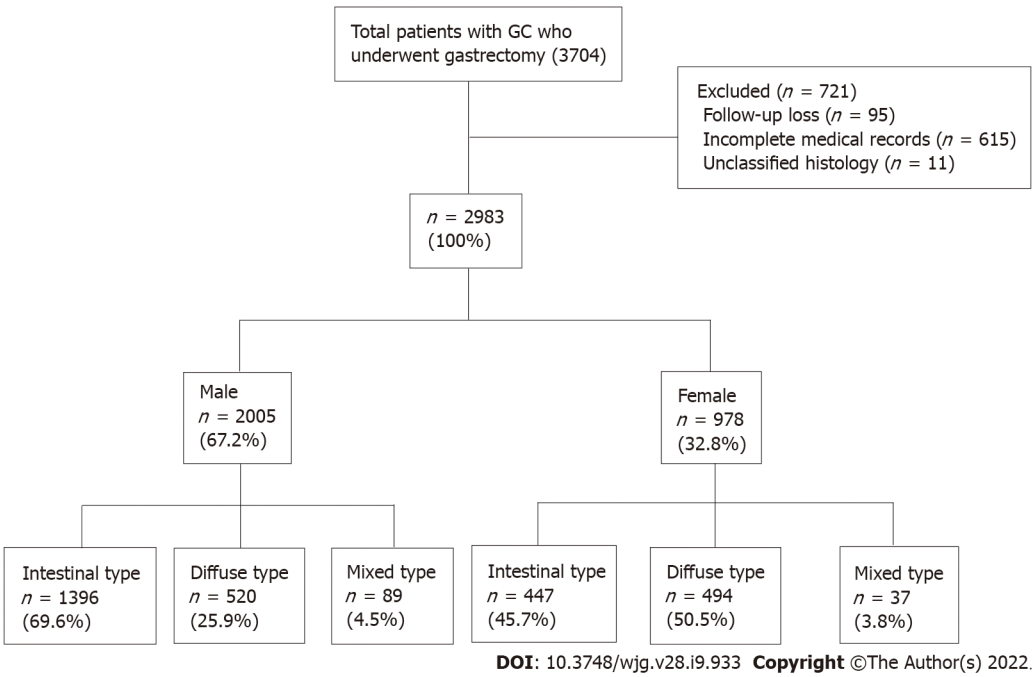

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Figure 1 Study flow chart of patient enrollment and exclusion process. GC: Gastric cancer.

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,

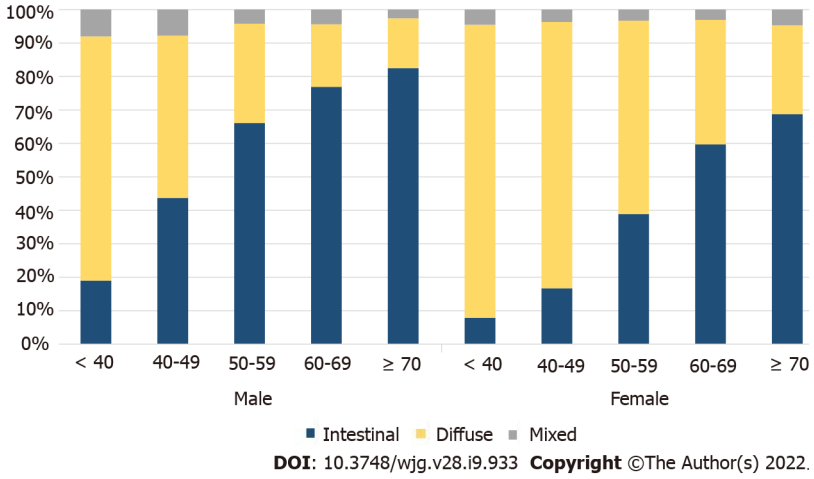

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Figure 2 Proportion of histological types of gastric cancer according to sex and age. The trend of an increasing proportion of intestinal-type cancers with increasing age was observed in both males and females. In males, the proportion of intestinal-type 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.

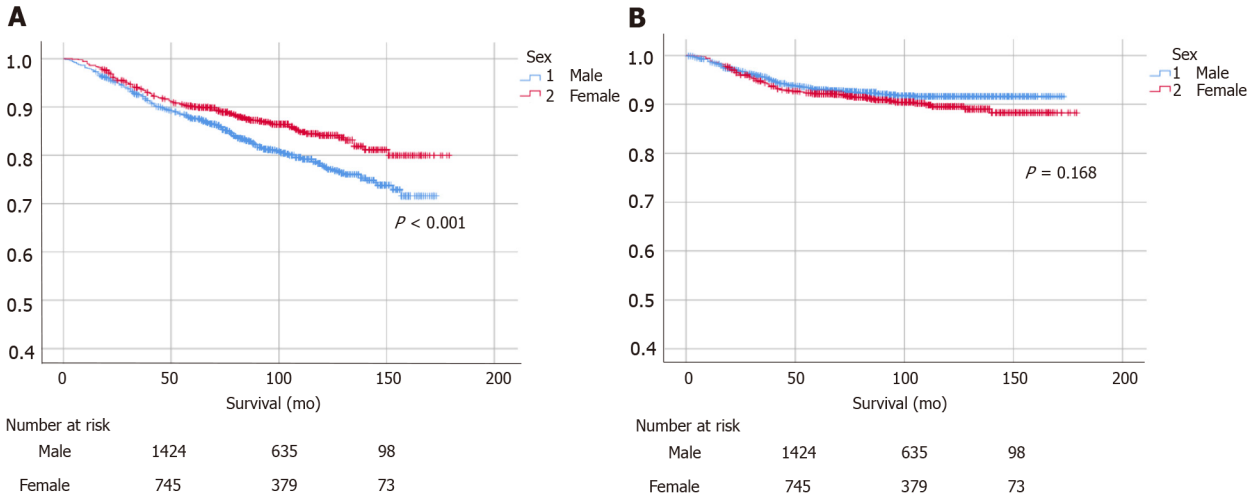

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Figure 3 Survival according to sex and initial cancer stage. (A) Overall and (B) gastric cancer–specific survival. A statistically significant female predominance in overall survival was identified ($P < 0.001$), while a non-significant male predominance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

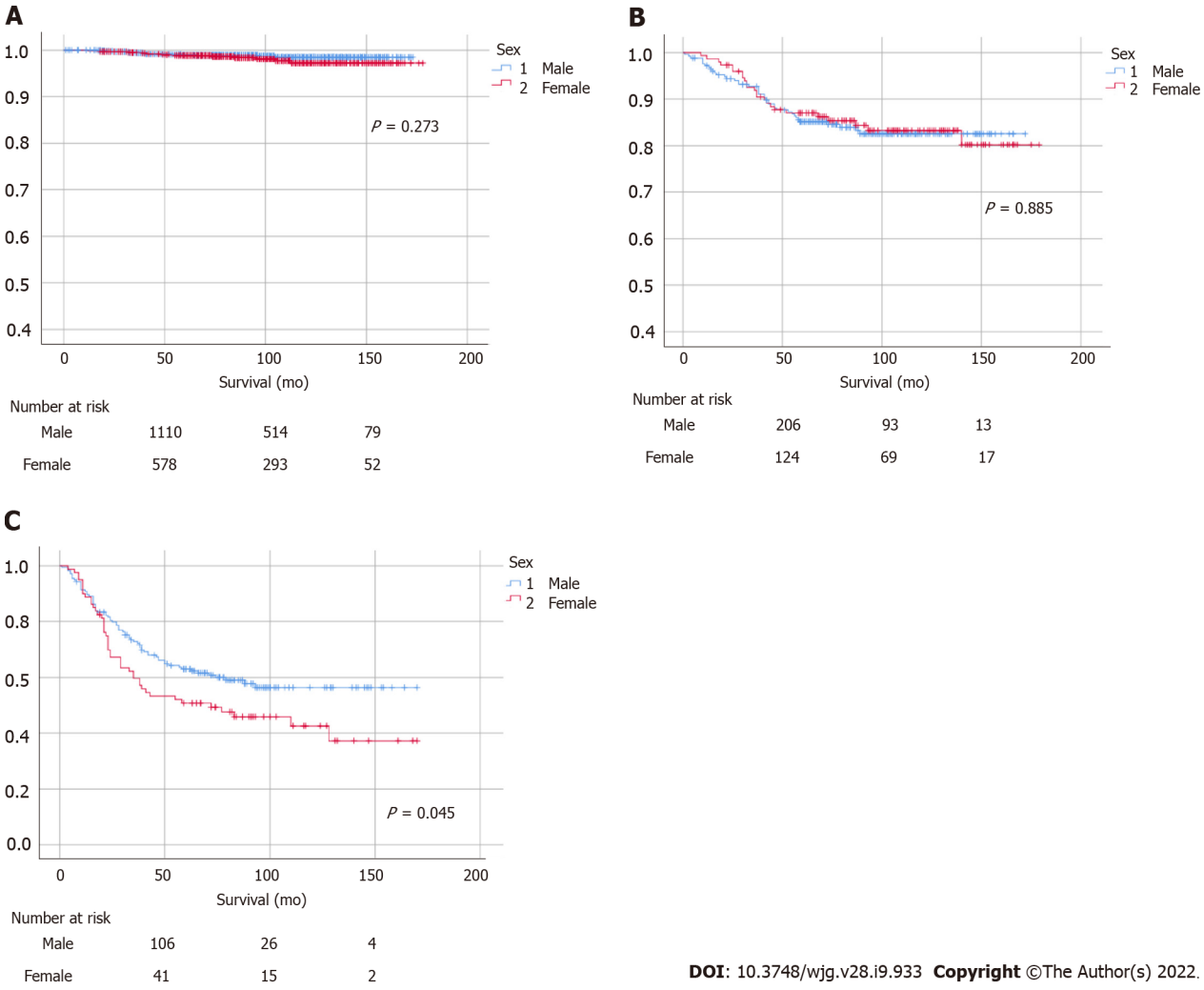

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Figure 4 Gastric cancer-specific survival in (A) stage I, (B) stage II, and (C) stage above III. There were no significant differences between males and females in stages I and II, but a statistically significant male predominance was observed in advanced-stage 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.

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ORIGINAL ARTICLE

Retrospective Study

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

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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

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

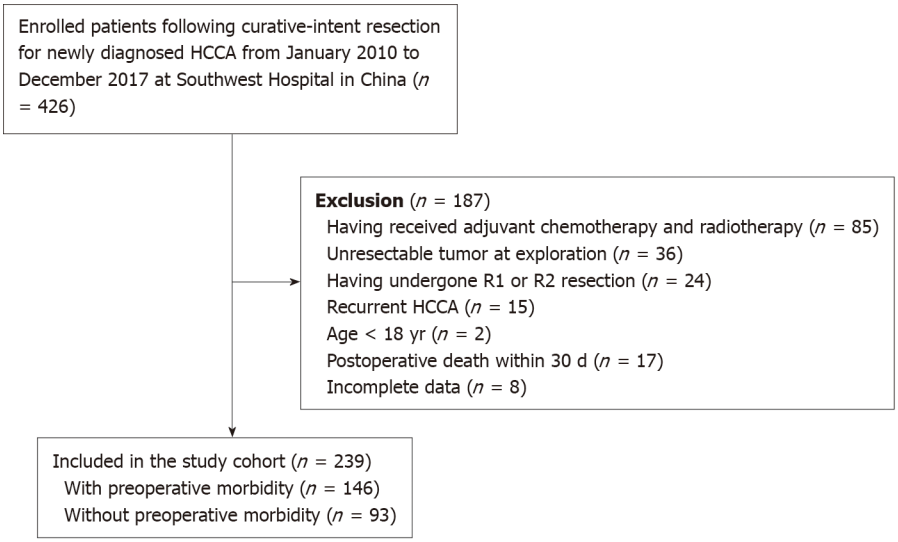

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Figure 1 Selection of the study population. HCCA: Hilar cholangiocarcinoma.

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.

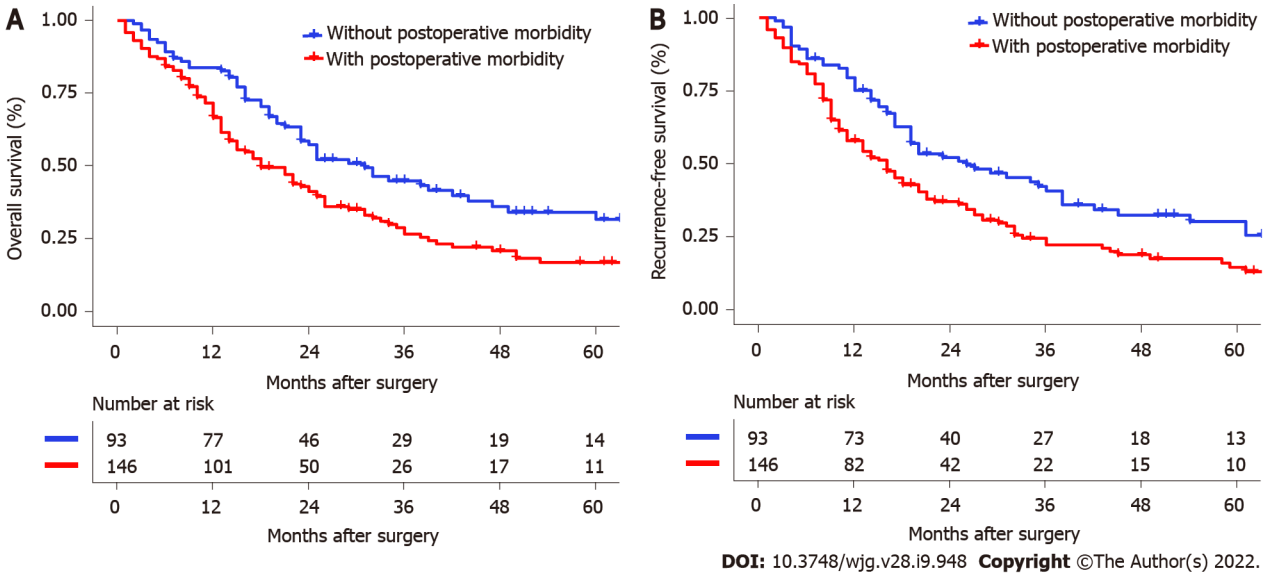
Table 5 Univariable and multivariable Cox regression analyses of risk factors associated with overall survival following curative resection for hilar cholangiocarcinoma

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.

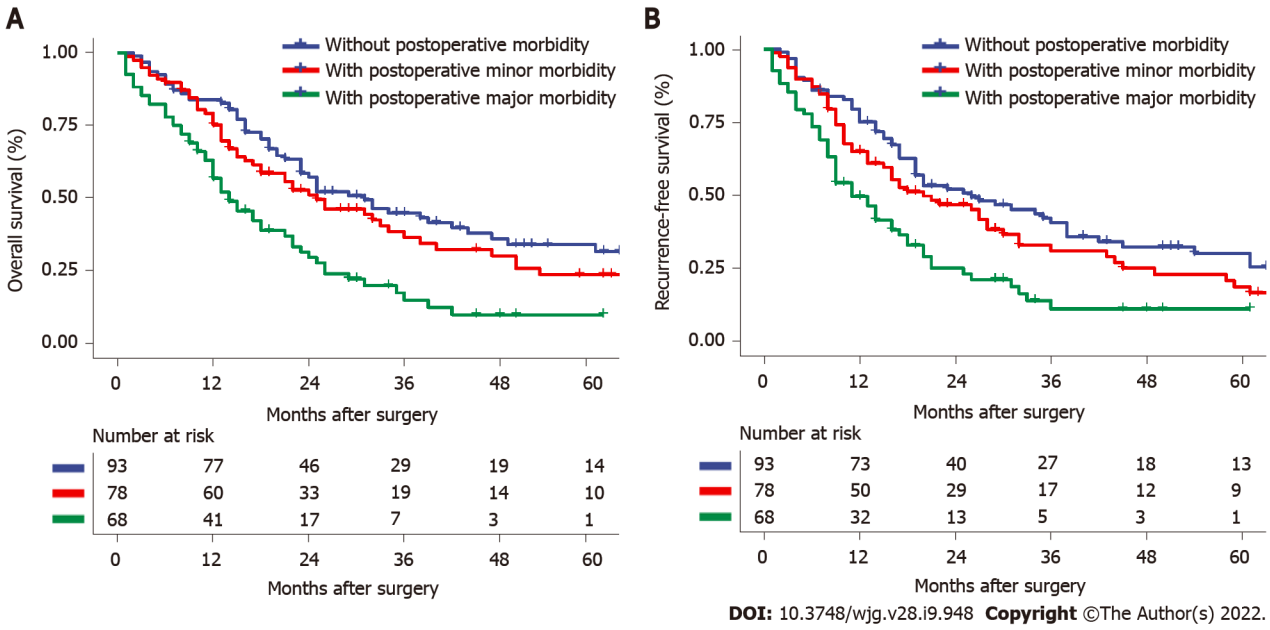
In other cancers, postoperative morbidity may be an independent predictor of poor prognostic outcome, including colorectal liver metastasis[37], HCC[8,38,39], pancreatic cancer[40], and esophageal cancer[41]. Although the precise association between postoperative morbidity and unfavorable prognostic 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.



DOI: 10.3748/wjg.v28.i9.948

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, $P = 0.002$.



DOI: 10.3748/wjg.v28.i9.948

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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Conflict-of-interest statement: Nothing to disclose.

Data sharing statement: Technical appendix, statistical code, and dataset available from the corresponding author at daihaisu@163.com. Participants gave informed consent for data sharing.

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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

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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

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.

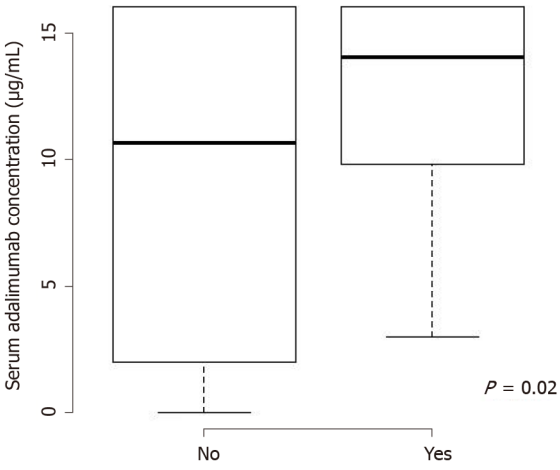

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Figure 1 Serum adalimumab concentrations according to clinical remission status.

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).

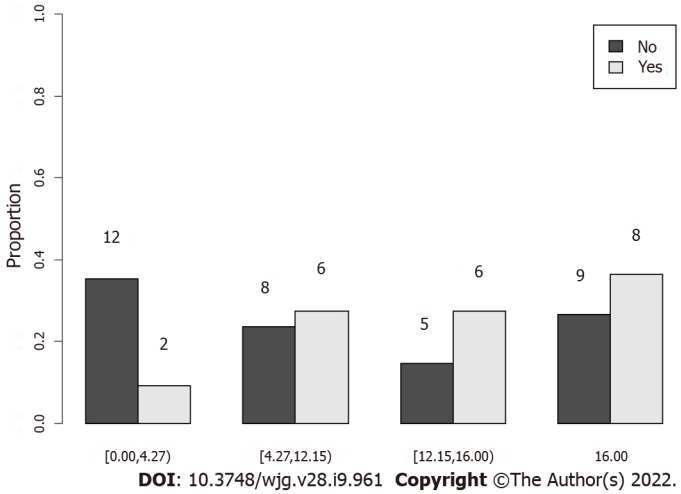

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Figure 2 Clinical remission according to quartiles of serum adalimumab concentrations.

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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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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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

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.

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