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REVIEW

Functional macrophages and gastrointestinal disorders

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Abstract

Macrophages (MΦ) differentiate from blood monocytes and participate in innate and adaptive immunity. Because of their abilities to recognize pathogens and activate bactericidal activities, MΦ are always discovered at the site of immune defense. MΦ in the intestine are unique, such that in the healthy intestine, they possess complex mechanisms to protect the gut from inflammation. In these complex mechanisms, they produce anti-inflammatory cytokines, such as interleukin-10 and transforming growth factor-β, and inhibit the inflammatory pathways mediated by Toll-like receptors. It has been demonstrated that resident MΦ play a crucial role in maintaining intestinal homeostasis, and they can be recognized by their unique markers. Nonetheless, in the inflamed intestine, the function of MΦ will change because of environmental variation, which may be one of the mechanisms of inflammatory bowel disease (IBD). We provide further explanation about these mechanisms in our review. In addition, we review recent discoveries that MΦ may be involved in the development of gastrointestinal tumors. We will highlight the possible therapeutic targets for the management of IBD and gastrointestinal tumors, and we also discuss why more details are needed to fully

understand all other effects of intestinal MΦ.

Key words: Macrophages; Homeostasis; Inflammatory bowel disease; Gastrointestinal tumors; Therapeutic targets

Core tip: The manuscript involves three components. First, after briefly describing the origin of macrophages (MΦ), it summarizes their general biologic features and common functions. The second component reveals the differences between resident MΦ in the intestine and those in other tissues. Notably, we depicted how resident MΦ participate in maintaining intestinal homeostasis and why they can maintain intestinal health by comparison between each of these distinct features. The third part discusses how the deficiency of this anti-inflammatory system leads to autoimmune diseases. However, we also discuss the many details of why intestinal MΦ and the underlying mechanism of inflammatory bowel disease and gut tumors remain obscure.

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INTRODUCTION

The intestine is organized into distinct specialized and functional tissues, such as the epithelium and lamina propria (LP). As the major site of bacterial colonization (10^2 cfu/mL in the duodenum, 10^2 cfu/mL in the jejunum, 10^3 cfu/mL in the proximal ileum, 10^7-10^8 cfu/mL in the distal ileum, and 10^{11}-10^{12} cfu/mL in the colon[1]), it is crucial to maintain intestinal homeostasis in which the intestinal immune system contributes to such maintenance under physiological conditions. Meanwhile, both commensal bacteria and their products play important roles[2].

The mammalian intestine is considered the largest immune organ in the body. It is estimated that 65%-80% of the immune cells, such as macrophages (MΦ), dendritic cells (DCs), T cells and B cells[3], exist in the intestine. There are many lymphocytes and natural killer (NK) cells in the region of the epithelial base[4,5]. Most of the intraepithelial lymphocytes are T cells, and they express CD3, CD8[6], TCRαβ[5] or TCRγδ[7] (mainly in mice). Goblet cells of the intestinal epithelium secrete net-like MUC2 mucins that compose the surface mucus layer, which can filter out microbes[8,9]. Both the intestinal epithelium and mucus layer constitute the double-protective barrier to maintain homeostasis at the entrance where pathogens invade. With the background described above, it seems that MΦ are insignificant in the intestinal immune system. In fact, they play a unique supporting role in maintaining the balance of intestinal immunity, and they are by no means as simple as we thought.

MΦ are one of the nonhematopoietic cells in all mammalian species that are distributed throughout the tissues of individuals. Their origin is relatively clear, and their biologic features have long been explored. In terms of immune defense, their name reveals their function: phagocytosis. They participate in innate immune responses and adaptive immune responses, especially in the intestine, which is the largest pool of MΦ and commensal bacteria. They can be considered as regulators instead of inflammation propellants (see below).

Emerging evidence suggests that intestinal resident MΦ contribute to maintaining intestinal homeostasis by several mechanisms (see below), and the production of immunosuppressive cytokines and their inhibitory biologic behavior suppress cascaded inflammatory responses. This is beneficial to the host because they protect the intestine from over-responding to commensal bacteria, resulting in severe tissue damage. Thus, they have attracted increasing attention in research on intestinal homeostasis and the correlative mechanisms of intestinal autoimmune diseases, represented by inflammatory bowel disease (IBD).

IBD includes two types of diseases: ulcerative colitis (UC) and Crohn's disease (CD). IBD has long been considered a typical autoimmune disease. Several reports have confirmed that multiple factors, for example, epithelial defects, disturbance of commensal or pathogenic bacteria and destruction of the mucus layer, lead to the development of IBD. In addition, intestinal MΦ highlight the defects of their protective function in IBD.

In addition, we propose some promising targets for the studies and treatments of IBD and gastrointestinal tumors. These comprehensive descriptions and findings of MΦ above have been summarized in figures of our manuscript to make the unique function of intestinal MΦ more understandable.

MACROPHAGES: DIFFERENTIATION AND BIOLOGY

Macrophages differentiate from blood monocytes

In 1884, Ilya Ilyich Mechnikov, an immunologist and pathologist in Russia, identified MΦ. Hereafter, the exploration of this cell type has never waned. Regarding the origin of MΦ, the mononuclear phagocyte system arises from hematopoietic stem cells in the bone marrow and from progenitors in the embryonic yolk sac[10], as well as from fetal liver during early development. As early as 1980, it was verified by using

the Chediak-Higashi marker that both interstitial and intraalveolar MΦ of the lung are derived from bone marrow precursor cells[11]. The family of mononuclear phagocytes consists of monocytes (Mo), MΦ, osteoclasts and DCs.

Granulocyte-macrophage colony stimulating factor (GM-CSF) is a major factor that can promote hematopoietic stem cell differentiation into granulocyte-monocyte cells, promonocytes and Mo[12,13]. Thereafter, Mo circulate in the blood stream in different types of tissues (the environment with different types of tissues controls the differentiation and maturation of resident MΦ by several molecular mechanisms[14-19]), a part of the blood MΦ undergo maturation, adapt to their local microenvironment and turn into various resident MΦ. Resident MΦ may remain as relatively long-life span cells, although they usually cease to proliferate[20]. The remaining blood Mo differentiate into free MΦ, migrating between diverse tissues like amoebae.

To be more rigorous, some researchers further showed that Mo in the bone marrow can be classified as $Ly6C^{hi}$ Mo and $Ly6C^{lo}$ Mo by their expression of Ly6C/Gr1, CCR2 and CX3CR1. $Ly6C^{hi}$ Mo express high levels of Ly6C/Gr-1, CCR2 and CD62L, but low levels of CX3CR1. CCR2 is a chemokine receptor, which is essential for $Ly6C^{+}Gr1^{+}CX3CL1^{-}$ Mo to enter the circulation. $Ly6C^{lo}$ Mo express low levels of Ly6C/Gr1, CCR2 and CD62L but high levels of CX3CR1[21]. $Ly6C^{lo}$ Mo are proposed to be the precursors of resident MΦ[4,22], but there are some conflicts about this hypothesis if the Mo entering the blood stream rely on expressing CCR2, and there is no abundant evidence to support this conclusion. Moreover, MΦ differentiate from blood Mo, a finding that has been challenged recently. Some researchers have suggested that blood Mo contribute little to MΦ in the steady state, and emerging evidence indicates that resident MΦ can undergo self-renewal[23]. However, other researchers demonstrated that blood $Ly6C^{hi}$ Mo are responsible for turning into resident MΦ because they convert into $Ly6C^{lo}$ Mo and can return to the bone marrow, differentiating into $Ly6C^{lo}$ Mo[21]. This explanation may be helpful to understand the origin of resident MΦ.

Biologic features and common functions of macrophages

The volume of MΦ is 5-10 times that of Mo, and they have more organelles (especially lysosomes), folds and pseudopodia. Resident MΦ are widely distributed throughout the body with distinctive phenotypes - for example, dust cells in lung, Langerhans cells in skin, histiocytes in connective tissue, Kupffer cells in the liver, mesangial cells in the kidney and microglial cells in the central nervous system.

A considerable amount of MΦ exists in the intestine, and specific markers expressed by MΦ can be used to study the heterogeneity. For instance, the F4/80[24] antigen and macrosialin in mice are proven to be useful markers in most of the tissues to define the distribution of MΦ, while several antigens such as sialoadhesin, a lectin-like receptor for sialylated glycoconjugates, are particularly strongly present in populations of MΦ in lymphoid organs that do not express F4/80 or CD68. In humans, the CD68 antigen (the human homolog of macrosialin) is widely found in MΦ expressing EMR2 (the human homolog of F4/80)[20].

Presently, many promising markers are awaiting identification, and some detected materials have already generated new hypotheses. For example, matrix metalloproteinase-9, produced by MΦ in the early phase of mouse peritonitis, may be used as an inflammatory marker[25]. In addition, the protein dehydrogenase/reductase-9 was identified as a specific and stable marker of human regulatory MΦ (Mregs)[26], which contributed greatly to the existing body of knowledge on immunosuppressive therapy.

MΦ can be classified as M_1 and M_2, functionally within the Mregs. M_1 MΦ produce high interleukin (IL)-12 and low IL-10, while M_2 MΦ show the opposite trend. Additionally, M_2 MΦ express IL-13α1, but M_1 MΦ do not[27]. A recent study has shown that a novel marker, MS4A4A (a member of the membrane-spanning 4A gene family), is only expressed in M_2 MΦ - that is, MS4A4A might be a surface marker of M_2 MΦ[28]. M_2 MΦ were largely mysterious in the past, while the importance of M_1 MΦ in mucosal biology has been appreciated for decades; the immune regulatory function of M_2 MΦ has only begun to be understood in the last few years. Additionally, their differentiation, as well as their differences from M_1 MΦ in cell biology, will become clearer in the future. Thus, regarding Mregs, it is also important that they are activated by different pathways and play diverse roles in the immune system, which will be described below.

MΦ, "big eaters", are named after their major function: phagocytosis, involving the uptake of particulate materials (> 5.0 μm) by opsonic (Fc receptors and C3b receptors) or non-opsonic receptors such as mannose receptors, scavenger receptors, formyl-methionine-leucyl-phenylalanine, and pattern recognition receptors (PRRs), especially the Toll-like receptors (TLRs). With the existence of these receptors, MΦ can participate in innate immunity and adaptive immunity (Figure 1).

MΦ dispose of approximately 2×10^{11} erythrocytes a day and clear damaged or dying cells[20]. Activated MΦ can recognize microorganisms that break into the epithelial or mucosal barriers with their special/nonspecial receptors and stretch the pseudopodia to swallow these microbes, followed by their digestion by oxygen-dependent/-independent pathways in phagolysosomes. Beyond that, MΦ can be activated by IL-8 and release chemotactic factors and mediators of inflammation (IL-1, IL-6, IL-12 and tumor necrosis factor (TNF)-α, which recruit neutrophils to the inflammatory site.

The neutrophils produce bactericidal compounds,

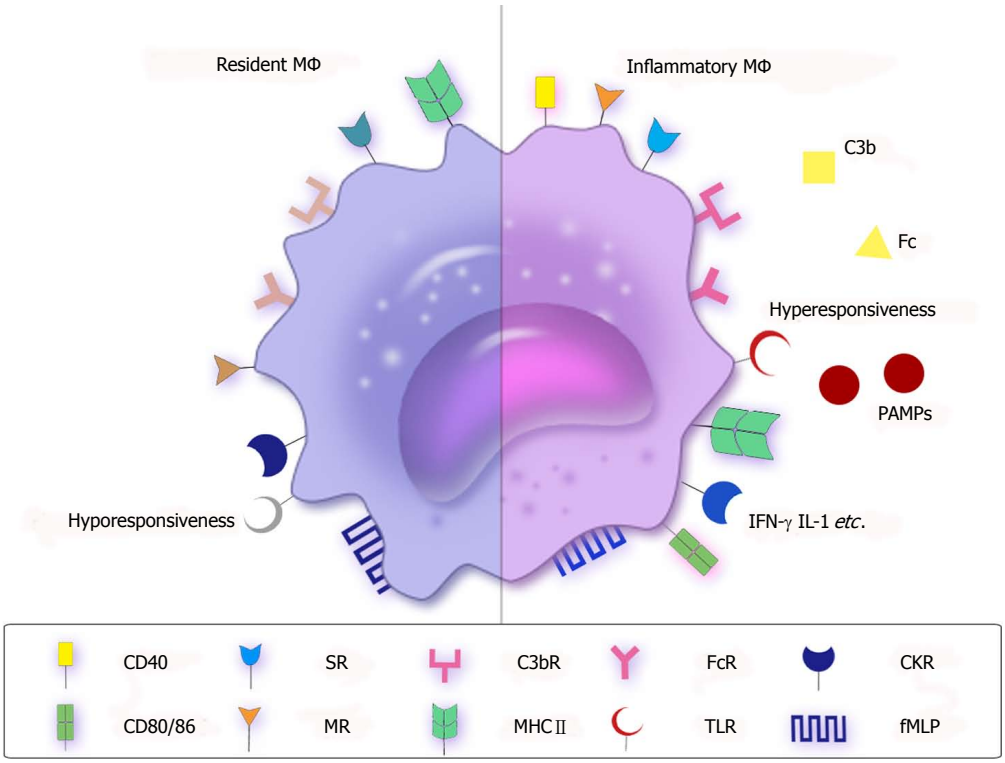

Figure 1 Receptors or molecules of resident and inflammatory macrophages. MΦ express opsonic (FcR and C3bR) or nonopsonic receptors, such as CKRs, MRs, SRs, fMLP and TLRs, as well as express high levels of MHC Ⅱ. However, there are some differences between resident MΦ and inflammatory MΦ. Resident MΦ (left side) do not express high levels of costimulatory molecules such as CD40, CD80 and CD86, and present hyporesponsiveness to TLRs to suppress inflammation. However, inflammatory MΦ (right side) show the opposite trend. The PAMPs lead to inflammation by connecting with hyperresponsive TLRs. CKR: Cytokine receptor; fMLP: Formyl-methionine-leucyl-phenylalanine; MR: Mannose receptor; MΦ: Macrophages; PAMP: Pathogen-associated molecular pattern; SR: Scavenger receptor; TLR: Toll-like receptor.

causing the liquefaction of tissue and formation of pus to eliminate the invading as well as missing pathogens. To complement MΦ, neutrophils secrete several preformed proteins stored in the granules, such as lactoferrin, lipocalin, lysozyme, IL-37, defensins and myeloperoxidase (converts H_2O_2 to hypochlorous acid)[20]. However, MΦ are not so bellicose. To maintain homeostasis of innate immunity, several self-regulative mechanisms restrain inflammation. NK cells inhibit the activation of MΦ by releasing IFN-γ or reducing the number of overactive MΦ by cytotoxicity. IL-1β, IL-10 and transforming growth factor (TGF)-β, produced by MΦ, are responsible for down-regulating the innate immune response. Moreover, the dead neutrophils are phagocytosed by mononuclear phagocytes, and lipoxins, protectins and resolvins contribute to the restoration of normal function[20].

In adaptive immunity, MΦ are an antigen-presenting cell type, like DCs. In the marginal sinus of a lymphoid organ, after digestion, MΦ present fragments at the cell surface on MHCII molecules. Indeed, MΦ are less effective than DCs in antigen presentation to naïve T cells because they only express appropriate costimulatory molecules (*e.g*., CD40, CD80 and CD86) following infection or contact with microbial productions. However, DCs express high levels of MHCII molecules as well as costimulatory molecules. In fact, several microbial productions promote the expression of MHCII molecules and costimulatory molecules in MΦ, which probably enhance the autoimmune response[29].

Gut-associated lymphoid tissues, including dispersed and aggregative tissues, are the primary part of the intestinal immune system[30-32]. The latter type is represented by Peyer's patches (PPs), settled in the LP of the appendix and small intestine, and the solitary lymphoid follicles, widely distributed in the intestinal LP[33,34]. The PPs look like an arch, and they are covered by follicle-associated epithelium, which involves special cells named microfold/membranous cells (M cells)[34,35]. T cells, B cells[36], DCs and MΦ exist in a pocket-like structure outside the base of M cells. M cells efficiently uptake antigens. However, instead of processing and presenting antigens, they are only responsible for transporting antigens and communicating with the resident B cells in the center of PPs.

Most PP cells are B cells, and only a few are T cells, which has been explored in mature mice. The B cells located in the germinal centers of PPs can produce IgA[37-40] (ingredient of sIgA) to participate in pathogen defense. In addition, M cells transport antigens to epithelial cells or antigen presenting cells (DCs and MΦ) to induce the adaptive immune response. It has been certified that the cell-bound antigen transportation can affect mucosal tolerance with the participation of

regional lymph nodes[41].

M_1 MΦ or classically activated MΦ develop in cell-mediated immune responses, which are mainly driven by interferon (IFN)-γ and TNF. IFN-γ can be produced in innate immunity and adaptive immunity. In the former, NK cells are important, but the production of IFN-γ in NK cells is too transient for the persistence of this population of MΦ. Consequently, it is necessary to depend on the adaptive immune response; T helper (Th)1 cells release sustainable IFN-γ and induce classical activated MΦ to kill the microbes indiscriminately[42].

Endogenously produced IFN-β is another factor that can replace IFN-γ to activate classically activated MΦ[43]. M_1 MΦ are the major component of host defense. They produce pro-inflammatory cytokines (*e.g.*, IL-1, IL-6 and IL-23) and associate with Th cells, but it has been reported that their connection with Th17 cells, which produce IL-17, results in serious tissue damage. Thus, their over-activation may be the cause of autoimmune diseases[42].

M_2 MΦ or alternatively-activated MΦ are produced during the innate or adaptive immune response. Basophils and mast cells produce innate IL-4, one of the first innate signals released during tissue injury, and IL-4 turns the resident MΦ into this population of cells to promote wound healing. IL-4 can also be released in adaptive immune responses that can be thought as particularly important pathways to develop and persist the alternatively-activated MΦ[42]. In addition, the Th2-type immune responses have been documented to work at the intestinal mucosal surface to respond to the disturbances by cytokines, such as IL-4 and IL-13[44]. However, compared with M_1 MΦ, there is no sufficient evidence to show that M_2 MΦ directly participate in the bactericidal activities, but they do have indirect regulatory effects[45], which may explain why it is hotly debated in the field of neoplasms[46-56], fibrosis[57-60], metabolic syndrome (might relate to insulin resistance)[61-65] and intestinal autoimmune diseases.

Mregs are a type of immunosuppressive cells, which have been illustrated comprehensively by Mosser *et al*[42]. Those authors summarized the mechanisms of producing Mregs in innate and adaptive immune responses and the stimuli of these processes. In addition, they mentioned that Mregs produce IL-10 and decrease the production of IL-12 to dampen inflammation. However, their helpful antiinflammatory function might be exploited by parasites to safely survive in the host's defense, which is an interesting point and powerful evidence to confirm the role of Mregs in the immune system.

To summarize, MΦ are extraordinarily complicated in their structure and functions. On the one hand, they are pioneers of pathogen defense *in vivo*, and one of the regulators that control the immune responses. On the other hand, they can be considered a bridge between innate immunity and adaptive immunity. It has been proven that they are very important in diseases such as asthma[66-70], atherosclerosis[71-76], retinopathy[77-80], neoplasm and autoimmune diseases.

MΦ PLAY A FUNCTIONAL ROLE IN INTESTINAL HOMEOSTASIS

General characteristics of intestinal MΦ

The differentiation of intestinal MΦ rely on intestinal epithelial cells, which have been proven by an extracorporeal three-dimensional coculture model[81]. MΦ are found in the intestinal tract of all mammals, both in the mucosa and deeper layers[82]. They are found mostly frequently in the LP and produce PGF2 to replenish deficient epithelial cells[23]. Several studies have summarized a rule about the quantity of intestinal MΦ, as follows: in different parts of the intestine, the numbers of MΦ correlate with the quantity of bacteria. An experiment provided the supporting evidence by recording the weight of each mouse organ or tissue and calculating their F4/80 antigen levels. The total F4/80 antigen levels in the small bowel were 1.3×10^7, and 1.4×10^7 in the large bowel. In the intestine of germ-free mice, the numbers of MΦ are decreased[24], likely indicating that the pathogen defense should also be the basic function of intestinal MΦ.

The general markers of MΦ have been mentioned above. Regarding intestinal MΦ, they can be recognized by their unique markers. Resident MΦ in the healthy mouse colon are F4/80^{hi}, class Ⅱ MHC^{hi} (also found in humans[83]), CX3CR1^{hi}, CD11c$^+$, CD103$^-$ and Siglec F$^-$[82]. Unlike resident MΦ in other tissues, the highly expressed CX3CR1 is unique. Furthermore, the intestinal MΦ express CD13[84], CD14 and CD70, and they can be subdivided according to their size[85]. Previously, it was difficult to distinguish between intestinal DCs and MΦ; however, a small population of mucosal MΦ has recently been found to express CD11c, which is a specific marker of DCs. The F4/80$^+$, CD11b$^+$, and CD68$^+$ cells are more likely to be MΦ rather than DCs. They do not present antigens to naïve T cells, and only the CD103$^+$CX3CR1$^-$ cells are classical DCs[82,86-90]. These findings resolved a few puzzles concerning intestinal DCs and MΦ-like cells with the emergence of a possible hypothesis about the relationship between intestinal MΦ and DCs.

Differences between macrophages in the intestine and other tissues are illustrated in Figure 1. Unlike MΦ in other tissues, resident MΦ[91] in the healthy intestine do not express high levels of costimulatory molecules, such as CD40, CD80 and CD86[83], and they do not up-regulate costimulatory molecules or induce a respiratory burst to exterminate microbes[92-94]. Additionally, their responses to TLR ligands are unexpected[83,95]. TLRs are membrane glycoproteins located at the cell surface or within endosomes. They have an extracellular region to bind ligand and an ectoplasmic domain to trigger the

intracellular signaling cascade. They can form hetero- or homodimers with each other, or complex with other receptors to recognize a wide range of microbes.

In general, with the TLRs, MΦ can be activated through many pathways mediated by MyD88, TRIF and NF-κB[20]. It is widely accepted that TLRs are the most characteristic PRRs. However, the intestinal resident MΦ do not respond to TLR ligands and produce proinflammatory cytokines or chemokines, such as IL-1, IL-6, IL-12, IL-23, TNF-α and CXCL10[82,91], which can be considered the inertia of mucosal MΦ. It has been conjectured that such is likely due to the absence of TLRs and other receptors (NOD-1/NOD-2) or malfunction of signaling pathways (*via* inhibitors or other mechanisms[96])[82,97,98]. However, this does not mean that the intestinal resident MΦ do not express TLRs or that TLRs are not necessary. In fact, they are essential to protect the intestinal epithelium under pathological circumstances[97,99,100].

These differences between intestinal mucosal MΦ and their homogeneity in other tissues reveal that they are more likely to control inflammation and maintain homeostasis in healthy individuals. However, what will occur if the balance has become broken?

Intestinal MΦ change dramatically under different situations

It is less rigorous to use the word "change[101]" in the subtitle because there is little detail to describe that the intestinal resident MΦ change into inflammatory MΦ (classical MΦ) under pathological circumstances with the changes in the environment, or that these two types of MΦ coexist in healthy intestine, working respectively. Nonetheless, there is another possibility. A credible concept has been explained[21] involving $CD14^{hi}CD16^{-}$ Mo, which can be considered to enter the intestinal LP only in a CCR2-dependent[102] manner and turn into the resident $CD14^{lo}$ $MHCII^{hi}CD163^{hi}CD64^{+}$ MΦ or inflammatory $CD14^{hi}MHCII^{hi}CD163^{lo}CD64^{+}$ MΦ in different circumstances. However, confusion concerning the relationship between $CD14^{hi}CD16^{-}$ Mo and $Ly6C^{hi}$/ $Ly6C^{lo}$ Mo has emerged and remains to be directly described.

It is clear that intestinal resident MΦ produce antiinflammatory cytokines, especially IL-10 and TGF-β[4,84,103-111], whereas inflammatory MΦ work at the inflammatory site and have strong bactericidal activity, as explained above. In healthy intestine, IL-10 is produced by mucosal MΦ themselves and is a component of T cells[112]. Vasoactive intestinal peptide (VIP) and pituitary adenylate cyclase-activating polypeptide increase the production of IL-10 by mucosal MΦ *in vitro* and *in vivo*[113]. IL-10 prevents the NF-κB pathway, and inhibiting the autocrine/paracrine production of IL-10 reverses TLR unresponsiveness in MΦ[82]. Maintaining Foxp3 expression of regulatory T cells (Tregs) has been reported as one of the important functions of IL-10 produced by MΦ[114]. $CD4^{+}Foxp3^{+}$ Tregs greatly contribute to the immune regulatory networks with the complement of other T cells and B cells, maintaining intestinal homeostasis[115]. Recently, research[107] on *Citrobacter rodentium*-infected mice with cell type-specific deletion of Il-10 demonstrated that IL-10 prevents excessive inflammation in acute bacterial infection by controlling IL-23[116,117] production to limit innate immunity. Another study indicated that the deficiency of IL-10 results in stable chromatin alterations in intestinal MΦ[118]. These results showed that IL-10 indeed plays a critical role in limiting inflammation.

Another factor for antiinflammation is TGF-β. Intestinal resident MΦ express high levels of TGF-β receptors and show constitutively-active TGF-β signaling[82]. TGF-β also connects with Foxp3, expressed by Tregs, and $CD4^{+}Foxp3^{+}$ Tregs decrease the ability of mucosal MΦ to activate and translocate NF-κB[115]. Intestinal resident MΦ do not respond to TLR ligands with the existence of TGF-β[82]. In contrast to IL-10, their production in murine MΦ is inhibited by VIP[111]. Moreover, the expression of Smad7 (a member of the Smad family that mediates a pathway for TGF-β and BMP-2 signal transduction) interrupts TGF-β signaling and activates inflammatory MΦ, a finding that was demonstrated in an experiment of necrotizing enterocolitis MΦ[110].

Currently, the study of CD200 for antiinflammation has received less attention. CD200L is a member of the protective system, with the ability to restrain the activity of MΦ. Inhibitory signaling of CD200L is triggered by the interaction with CD200 in nonhematopoietic cells as well as MΦ[20]. This process protects tissues from severe damage. A study reported that knock-out of CD200 or CD200R1 produces MΦ hyperactivity and autoimmune diseases[119]. Enlightened by this, it is possible to assume CD200 maintains intestinal homeostasis. There are some relevant studies in the respiratory system[120], but the existing evidence in the intestine remains insufficient.

The enteric nervous system (ENS) plays a crucial role in controlling gastrointestinal physiology and interacting with microbes and immune cells, functions that have been explored for decades. Accumulating evidence indicates they closely contact MΦ. The development of $CX3CR1^{hi}MHCII^{hi}$ $CD11b^{+}CD11c^{lo}CD103^{-}$ muscularis MΦ (MMs) requires CSF1, and enteric neurons selectively express bone morphogenetic protein (BMP; expressed by MMs) receptor 2, which produces CSF1. By contrast, the expression of BMP2 activates enteric neurons. The correlation of MMs and ENS contributes to gut motility[121]. Additionally, MMs have been found to express tissue-protective and wound-healing genes resembling M_2 MΦ, reacting in intestinal infection[122].

More importantly, neurotransmitters are essential for neuronal immune control. VIP is known to exhibit

antiinflammatory effects, depending on promoting the production of IL-10. Nitric oxide is well known for its antimicrobe ability in the respiratory burst. However, it suppresses excitability in neurons[121] and influences ENS during intestinal inflammation[91]. Interestingly, serotonin (5-HT), which was considered a trigger of inflammation, has been demonstrated to act, indirectly, on MMs by 5-HT_4 receptors in neurons and to stimulate an antiinflammatory cascade in MΦ. It has been indicated that 5-HT_2 and 5-HT_7 are related to the development of M_1 and M_2 MΦ[91]. In addition, γ-amino butyric acid has been suggested to have an immunosuppressive effect on resident MΦ of the central nervous system[91]. However, in the intestine, it remains unclear. It is worth investigating the functions of ENS and how they act on MΦ to understand the gut immune system and associated disease treatments in the future.

Current views about intestinal MΦ

First, Kennichi *et al*[123] provided an exhaustive experimental result concerning LP-resident $CD169^+$ MΦ that mainly persist in secondary lymphoid organs. They indicate that $CD169^+$ MΦ reside at the bottom-end of the LP microenvironment, far away from the epithelium-LP border. Most importantly, the $CD169^+$ MΦ recruit inflammatory monocytes by producing CCL8, selective depletion of $CD169^+$ MΦ and anti-CCL8 antibody promotion of dextran sulfate sodium-induced colitis in mice. The comparison of $CD109^-$ and $CD109^+$ MΦ led to an interesting hypothesis. Unlike $CD109^-$ MΦ, $CD109^+$ MΦ are located in a region distant from the perimeter where they can be interrupted by commensal bacteria and dead epithelial cells, and they can directly release CCL8 into the systemic circulation in the vascular-rich environment. $CD109^+$ MΦ probably respond to the collapse of the frontline defense - *i.e.* they can be considered as a "conservation corps" in the intestine (Figure 2).

Second, M_2 MΦ struggle for attention. As another regulative population, M_2 MΦ produce IL-10 and express CD163 and CD206 lectin receptors. They do not produce proinflammatory mediators with signals of stimulation. Certainly, they produce tissue-repairing factors, such as vascular endothelial growth factor (VEGF), actin and metalloproteinases, due to their function in wound healing. M_2 MΦ are $MHCII^+$, which may be helpful in exploring their potential in bactericidal activities[82,83,124,125]. Unlike M_2 MΦ, Mregs express high levels of costimulatory molecules, such as CD40, CD80 and CD86, to submit antigens to T cells more effectively[42], highlighting the hypothesis that the regulation of M_2 MΦ in the intestine might be different from that of Mregs. However, the antiinflammatory function of Mregs mentioned above has not been directly verified in the intestine. Therefore, we are unsure about the role of Mregs in intestinal homeostasis, and some questions remain concerning the meaning of the difference between M_2 MΦ and Mregs (Figure 2).

Finally, a novel finding[126] concerning GPBAR1 (a G protein-coupled receptor for secondary bile acids) suggests that GPBAR1 is essential to maintain intestinal immune homeostasis by regulating M_1/M_2 MΦ. BAR501 (a small-molecule stimulus of GPBAR1) contributes to this regulatory process, depending on the production control of IL-10. Absence of the GPBAR1 gene causes the recruitment of M_1 macrophages and severe inflammation in the colon. Exposure to BAR501 leads to the increased expression of IL-10 and TGF-β mRNA, and percentage of $CD4^+/Foxp3^+$ cells. Based on this study, GPBAR1 deserves attention for its potential to protect intestinal health (Figure 2).

MΦ AND GASTROINTESTINAL DISORDERS

MΦ and IBD

According to the mechanisms of intestinal MΦ in maintaining homeostasis, any defect of the antiinflammation system may bring the reduction of immune tolerance, resulting in IBD. In 1998, it was found that intestinal MΦ displayed low expression of class Ⅱ MHC molecules in mouse colitis[127]. A hypothesis arose from this study that there could be dysfunction of MΦ participating in adaptive immune responses when inflammation occurs.

From the origin of MΦ, emerging evidence suggests that GM-CSF plays a central role and has a protective effect in human CD and acute colitis by activating specific Mo[128,129]. Classical $CD14^{hi}CD16^-$ Mo differentiate into large numbers of inflammatory MΦ in the inflamed mucosa of patients with CD[21]. $CD14^+$ Mo in the mucosa from IBD patients increase the production of TNF-α[130,131], IL-1β and IL-6, and enhance respiratory burst activity[21]. Moreover, IL-10 knock-out mice develop spontaneous IBD[82]. An intrinsic resistance to TGF-β receptor signaling has been shown in the mucosa from patients with CD[132]. $CD4^+Foxp3^+$ T cells fail to protect the intestine from chronic inflammation without IL-10- and TGF-β-dependent mechanisms[115]. M_2 MΦ have been certified to be activated by the Wnt signaling pathway, which is associated with UC[133]. These studies showed that intestinal MΦ are of great value for IBD. Following this result, promising treatments for IBD, such as $CD109^+$ MΦ Tregs and GPBAR1, can be considered new therapeutic targets.

MΦ are clearly associated with IBD, but there remain a few puzzles regarding some details. The first study[134] observed that $RoRγ^+$ innate lymphoid cells (ILCs; the primary source of GM-CSF in the gut) promote MΦ to respond to the microbial signals and produce IL-1β, which enhances inflammation. By contrast, another study[135] discovered that with the regulation of $RoRγ^+$ ILCs, MΦ promote a negative feedback

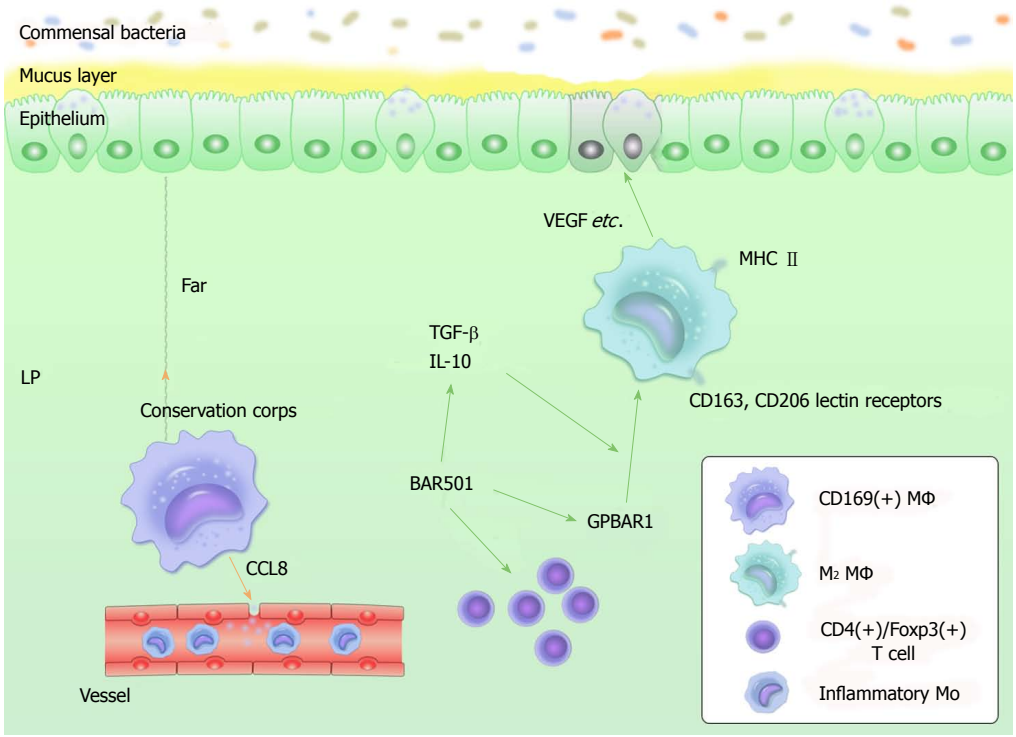

Figure 2 Current views about intestinal macrophages. 1. LP-resident CD169$^+$ MΦ reside at the bottom-end of the LP microenvironment, far away from the epithelium-LP border. CD169$^+$ MΦ recruit inflammatory monocytes by producing CCL8. CD109$^+$ MΦ can be considered as a "conservation corps" in the intestine because they likely respond to the collapse of frontline defense. 2. M_2 MΦ are MHC Ⅱ$^+$, producing IL-10 and expressing CD163, CD206 and lectin receptors. They do not produce proinflammatory mediators with signals of stimulation. In addition, they produce tissue-repairing factors, such as VEGF, actin and metalloproteinases. 3. GPBAR1 is essential to maintain intestinal immune homeostasis by regulating M_1/M_2 MΦ. BAR501 is a small-molecule stimulus for GPBAR1. It contributes to this regulative process, depending on the production control of IL-10. Exposure to BAR501 leads to increased expression of IL-10, TGF-β mRNA and the percentage of CD4$^+$/Foxp3$^+$ cells. IL: Interleukin; LP: Lamina propria; MΦ: Macrophages; TGF: Tumor growth factor; VEGF: Vascular endothelial growth factor.

pathway through the activation of IL-22 production, which might be protective. Indeed, the quantity of RoRγ$^+$ ILCs could increase in human CD. This finding inspires the question of whether the possibility exists that a portion of MΦ still tries to restore intestinal homeostasis when the intestine is trapped in a vicious cycle for inflammatory macrophages. The second item concerns CD200/CD200R1 mentioned above. Knock-out of CD200 results in MΦ hyperactivity *in vitro*, but CD200R1 knock-out mice have normal intestinal MΦ populations, and they neither develop spontaneous IBD nor become more susceptible to colitis induced by the dextran sulfate sodium model[82]. This indicates that CD200R1 may not be as important as we had previously considered, but the reasons remain unclear.

MΦ and gastrointestinal tumors

Since the end of the last century, many studies have certified the connection between MΦ and tumors in various systems. There are considerable numbers of investigations concerning tumor-associated macrophages (TAMs). They promote immunosuppression, tumor immune evasion[136], tumorigenesis, tumor metastasis and angiogenesis as well as invasion by releasing various cytokines and inflammatory mediators, such as IL-6, IL-10, TFG-β, CCL2, CCL17, VEGF and cathepsins[137]. However, different populations of TAM have different functions. M_1 MΦ have been confirmed to recognize and clear tumor cells, a function that is beneficial to health. By contrast, the development and movement of tumors benefit from M_2 MΦ. TAMs are one of the promising targets of tumor therapy, especially M_2 MΦ. Gut tumors are also included. We provide more details about TAMs and references in Box 4 to further illustrate the relationship between TAMs and tumors.

Similar to other MΦ, TAMs arise from hemato-poietic stem cells in the bone marrow and from progenitors in the embryonic yolk sac. With different environmental signals, Mo differentiate into distinctive macrophages[137,138]. Tumor signals contribute to the development of TAMs. Mantovani *et al*[139] summarized the signals associated with TAMs. For example, lactic acid, CCL2, CSF1, VEGF and TGF-1 from tumor cells, IL-1β from tumor-associated fibroblasts, and IL-10 from Tregs, all can drive TAMs into tumor-promoting MΦ. Moreover, they also list the products of TAMs which have different functions. For instance, IL-6, MFG-E8 and osteopontin from TAMs can active tumor stem cells; TAMs produce epidermal growth factor to promote tumor growth, invasion and metastasis. Nitric oxide and reactive oxygen species can be released to destroy tumor cells. However, they might

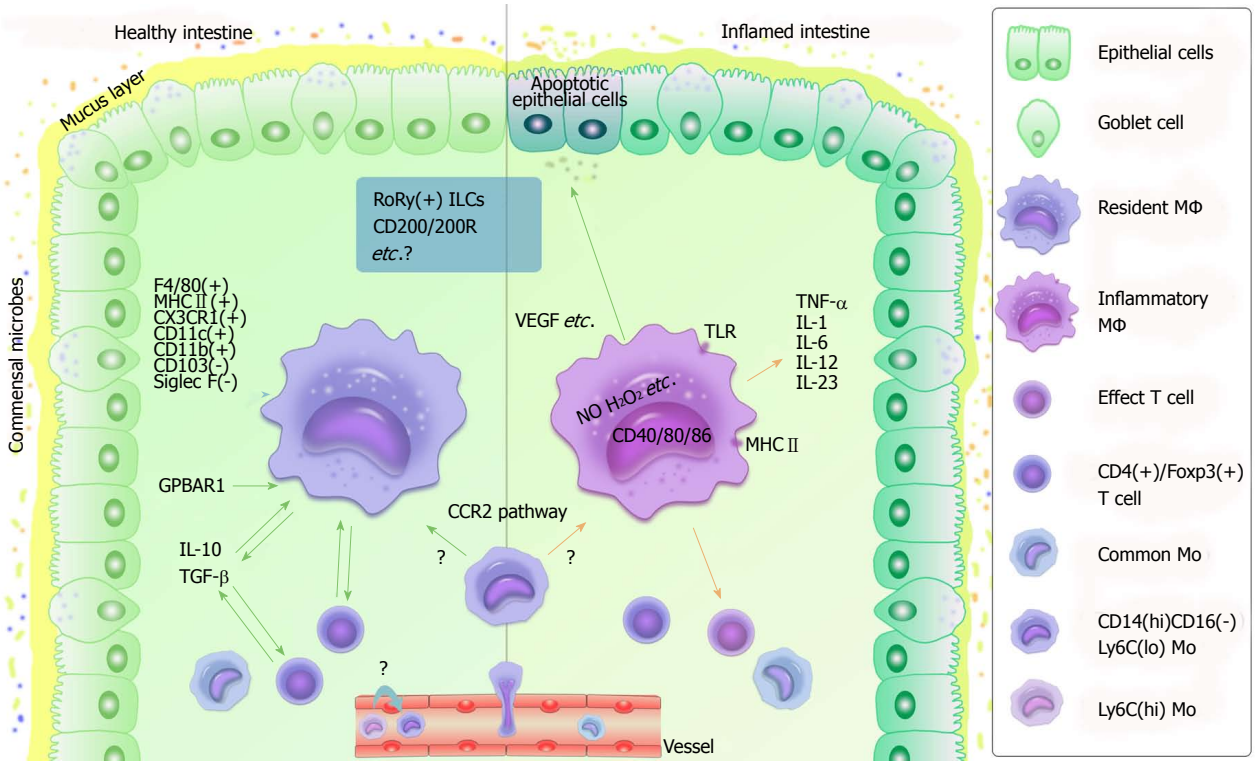

Figure 3 Functional role of macrophages in healthy or inflamed intestine. MΦ differentiate from blood Mo. Ly6c^{lo} Mo are proposed to be the precursors of resident MΦ. CD14^{hi}CD16^{-} Mo turn into resident or inflammatory MΦ according to different circumstances *via* the CCR2 pathway. In healthy intestine (left side), resident MΦ are F4/80^{hi}, class Ⅱ MHC^{hi} CX3CR1^{hi}, CD11c^{+}, CD103^{-} and Siglec F^{-}. They do not express high levels of costimulatory molecules such as CD40, CD80 and CD86. Their connections with CD4^{+}/Foxp3^{+} T cells, IL-10 and TGF-β are helpful to maintain intestinal homeostasis (green arrows). GPBAR1 is essential to maintain intestinal immune homeostasis by regulating M1/M2 MΦ. In inflamed intestine (right side), Mo change into inflammatory MΦ, which produce TNF-β, IL-1, IL-6, IL-12, and IL-23, and activate effective T cells with several specific receptors, such as TLR, as well as induce respiratory burst (*e.g.*, NO and H_2O_2 production), leading to inflammation (orange arrows). In addition, M2 MΦ produce tissue-repairing factors such as VEGF, which shows a positive effect in individuals during inflammation (green arrow). Regarding MΦ and intestinal immunity, many details remain unclear - for instance, the functions of RoRγ^{+} ILCs and CD200/200R (in blue rectangle) as well as that of Ly6C$^{hi/lo}$ Mo. IL: Interleukin; ILC: Innate lymphoid cell; Mo: Monocytes; MΦ: Macrophages; NO: Nitric oxide; TGF: Tumor growth factor; TLR: Toll-like receptor; TNF: Tumor necrosis factor; VEGF: Vascular endothelial growth factor.

result in genetic instability, causing tumor formation. Nevertheless, further studies have indicated that not all the macrophages that have emerged into the tumor microenvironment are tumor promoting.

M1 MΦ (having antitumor function) can recognize tumors and kill tumor cells by the cytotoxic effect, representing a double-edged sword. They have been verified as an independent predictor of survival time in patients with non-small cell lung cancer[140]. M2 MΦ have a protumor function. They promote the metastasis of K7M2 wild-type osteosarcoma cells in mice. Additionally, all-trans retinoic acid dampens the profunction of M2 MΦ by suppressing the production of IL-13 or IL-14 (from M2 m MΦ) to inhibit the metastasis of osteosarcoma[141]. CHI3L1, a protein secreted by M2 MΦ, promotes the metastasis of gastric and breast cancer cells[55]. In addition, it was confirmed that patients with peritoneal dissemination in gastric cancer have more M2 MΦ and low expression of M1-related messengers[142]. MFG-E8, a powerful angiogenic factor, is induced by bone marrow-derived mesenchymal stromal cells in mice. Attenuated tumor growth and the decreasing function of M2 MΦ can be found in MFG-E8-deficient mice[143], which represent M2 MΦ that contribute to tumor angiogenesis; whether the correlation of M2 MΦ and MFG-E8 is parallel or antiparallel should be further clarified.

Above all, TAMs have advantages and disadvantages to both human physiology and tumors. They are members of our defensive line, but they are also tumor helpers. Compared with the favorable contributions of TAMs, such as M1 MΦ in tumor resistance, the promising therapeutic targets they provide might be more useful. In the 1990s, some scientists systematically revealed that TAMs were worth exploring for antitumor therapy[144], and more and more findings were uncovered during the last 50 years. On the one hand, TAMs are hopeful antitumor targets; on the other hand, as Mantovani and Allavena[139] illustrated, the mechanisms of TAMs in tumor development and antitumor processes are intricate, which limits researchers' ability to find the antitumor target precisely. This phenomenon is the yin-yang of antitumor therapy and the challenge[145] of future antitumor studies.

Several studies have presented recent research progress in gastrointestinal tumors. First, tumor angiogenesis and survival in intestinal-type gastric cancer is closely associated with the infiltration of thymidine

phosphorylase-positive MΦ[146]. Therefore, thymidine phosphorylase could be a useful marker for tumor angiogenesis, and the prognosis of intestinal-type gastric cancer. Second, there is a hotspot induced by M_2 MΦ. A portion of M_2 MΦ, cooperating with TNF, were shown to be recruited to tumors[56,147]. The macromolecular contrast agent PG-Gd-NIR813 shows a dual magneto-optical imaging probe of tumor-associated M_2 MΦ[50], and a few new factors have been evaluated as mediators of the development of gastrointestinal tumors, such as M_2 MΦ-secreted CHI3L1 protein[55] and monocyte chemoattractant protein-1[148]. All are likely to become novel approaches for antitumor therapy.

CONCLUSION

In summary (Figure 3), MΦ with their various receptors act as sentinels in innate immunity and adaptive immunity. In healthy intestinal mucosa, they are indispensable to suppress inflammation and play an essential role in maintaining homeostasis by producing many inhibitors, such as IL-10 and TGF-β. However, they show strong bactericidal activities. Intestinal resident MΦ create a harmonious environment for commensal bacteria and their host. Any defect in keeping this balance can reduce immune tolerance, causing acute tissue damage or chronic autoimmune diseases, explaining their close association with IBD. New findings concerning intestinal MΦ and IBD, as well as tumors, can be very helpful for studies and disease treatments. Meanwhile, there are many details awaiting clarification as well as many unresolved issues.

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ORIGINAL ARTICLE

Basic Study

NOD2- and disease-specific gene expression profiles of peripheral blood mononuclear cells from Crohn's disease patients

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Abstract

AIM

To investigate disease-specific gene expression profiles of peripheral blood mononuclear cells (PBMCs) from Crohn's disease (CD) patients in clinical remission.

METHODS
Patients with CD in clinical remission or with very low disease activity according to the Crohn's disease activity index were genotyped regarding nucleotide-binding oligomerization domain 2 (*NOD2*), and PBMCs from wild-type (WT)-*NOD2* patients, patients with homozygous or heterozygous *NOD2* mutations and healthy donors were isolated for further analysis. The cells were cultured with vitamin D, peptidoglycan (PGN) and lipopolysaccharide (LPS) for defined periods of time before RNA was isolated and subjected to microarray analysis using Clariom S assays and quantitative real-time PCR. *NOD2*- and disease-specific gene expression profiles were evaluated with repeated measure ANOVA by a general linear model.

RESULTS
Employing microarray assays, a total of 267 genes were identified that were significantly up- or downregulated in PBMCs of WT-*NOD2* patients, compared to healthy donors after challenge with vitamin D and/or a combination of LPS and PGN ($P < 0.05$; threshold: ≥ 2-fold change). For further analysis by real-time PCR, genes with known impact on inflammation and immunity were selected that fulfilled predefined expression criteria. In a larger cohort of patients and controls, a disease-associated expression pattern, with higher transcript levels in vitamin D-treated PBMCs from patients, was observed for three of these genes, *CLEC5A* ($P < 0.030$), *lysozyme* (*LYZ*; $P < 0.047$) and *TREM1* ($P < 0.023$). Six genes were found to be expressed in a *NOD2*-dependent manner (*CD101*, $P < 0.002$; *CLEC5A*, $P < 0.020$; *CXCL5*, $P < 0.009$; *IL-24*, $P < 0.044$; *ITGB2*, $P < 0.041$; *LYZ*, $P < 0.042$). Interestingly, the highest transcript levels were observed in patients with heterozygous *NOD2* mutations.

CONCLUSION
Our data identify *CLEC5A* and *LYZ* as CD- and *NOD2*-associated genes of PBMCs and encourage further studies on their pathomechanistic roles.

Key words: Peripheral blood mononuclear cells; Gene expression; *NOD2*; *Lysozyme*; Crohn's disease; *CLEC5A*

Core tip: Peripheral blood mononuclear cells (PBMCs) are a useful tool to study peculiarities of the immune response in the context of Crohn's disease (CD). Here, we investigated whether PBMCs from patients with CD, even at the stage of clinical remission, exhibit altered gene expression profiles after challenge with pathogen-associated molecular patterns and vitamin D. For *TREM1*, *lysozyme* and *CLEC5A*, disease-associated expression patterns, with higher transcript levels in patient-derived PBMCs, were observed. The two latter genes, along with four other transcripts, also showed *NOD2*-dependent expression profiles. *TREM1* and *CLEC5A* may act with *NOD2* in a regulatory network with a pathophysiological role in CD.

Schäffler H, Rohde M, Rohde S, Huth A, Gittel N, Hollborn H, Koczan D, Glass Ä, Lamprecht G, Jaster R. *NOD2*- and disease-specific gene expression profiles of peripheral blood mononuclear cells from Crohn's disease patients. *World J Gastroenterol* 2018; 24(11): 1196-1205 Available from: URL: http://www.wjgnet.com/1007-9327/full/v24/i11/1196.htm DOI: http://dx.doi.org/10.3748/wjg.v24.i11.1196

INTRODUCTION

Inflammatory bowel diseases (IBD) are chronic intestinal disorders and mainly consist of the two entities Crohn's disease (CD) and ulcerative colitis (UC)[1,2]. The clinical course of IBD is characterized by intermittent periods of relapses and remission, which are unpredictable in clinical practice. The pathogenesis of IBD is multifactorial, including genetic and environmental factors, and involves an inappropriate activation of the mucosal immune system, which is triggered by the intestinal microbiota in genetically predisposed individuals[1-5]. In Caucasian populations, nucleotide-binding oligomerization domain 2 (*NOD2*) has emerged as one of the main susceptibility genes for CD[6-8]. NOD2 is an intracellular pattern recognition receptor sensing muramyl dipeptide (MDP)[9,10], a fragment of peptidoglycan (PGN), but also PGN by itself[11,12] and, upon ligand binding, induces activation of the transcription factor NF-κB[13]. However, NOD2 activation via PGN is dependent on a TLR2 co-stimulatory signal[14].

In addition, the environment, *e.g.*, vitamin D deficiency, also affects the development and clinical course of IBD[15-17]. Vitamin D deficiency has a high prevalence in IBD patients[17,18]. We have recently shown that clinical factors, *e.g.*, the use of tumor necrosis factor (TNF)-α inhibitor, are associated with significant changes in vitamin D levels[19]. Vitamin D was originally mainly implicated in bone health, regulating calcium and phosphate metabolism[20,21], but recent evidence has shown that vitamin D also profoundly impacts the innate and adaptive immune system[22-24]. Underscoring its role in the pathogenesis of CD, vitamin D was shown to be an inductor of *NOD2* gene expression[25]. Using peripheral blood mononuclear cells (PBMCs) and dendritic cells, Dionne *et al*[26] showed that 1, 25-vitamin D acts as a modulator of the innate immune system. However, little is known about the effects of vitamin D and the presence of *NOD2* mutations on different gene expression levels in CD. The aim of our study was therefore to further characterize different gene expression profiles in CD patients and healthy controls correlating to *NOD2* mutation status and vitamin D pretreatment. We identified different genes associated with the presence of CD and mutations in the *NOD2*

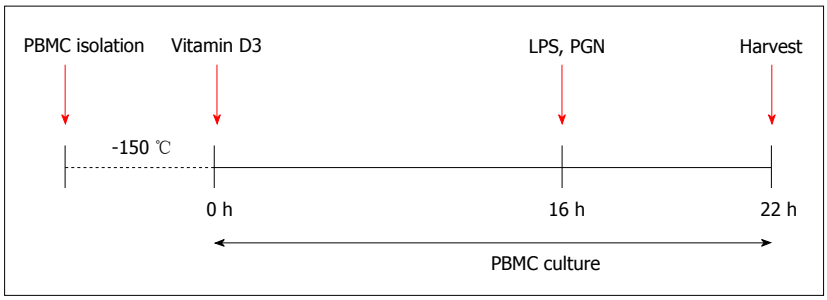

Figure 1 Protocol of peripheral blood mononuclear cells treatment.

gene. Follow-up studies on these genes may provide novel insights into the pathogenesis of CD and could contribute to the establishment of biomarkers to better predict the clinical course of the disease.

MATERIALS AND METHODS

Patients and controls

Sixteen patients with CD were recruited from the Rostock University Medical Center. The disease activity was determined via the Crohn's disease activity index (CDAI)[27]. Furthermore, all patients were classified according to the Montreal classification[28], and age, gender and disease-specific medication were recorded. Six healthy volunteers without immune-mediated gastrointestinal or other autoimmune disorders served as controls. EDTA blood samples were drawn from all participants for genotyping studies and isolation of PBMCs. Plasma levels of vitamin D and C-reactive protein (CRP) were determined using routine laboratory methods.

The study was approved by the ethics board of the University of Rostock (A-2015-0042). Written informed consent was obtained from each participant prior to enrollment.

Isolation, culture and treatment of PBMCs

PBMCs were isolated from EDTA venous blood using density-gradient centrifugation over Pancoll (PAN-Biotech, Aidenbach, Germany). Immediately after isolation, PBMCs were resuspended in cryopreservation medium [fetal calf serum (FCS) supplemented with 10% dimethyl sulfoxide (DMSO)] and stored at -150 ℃ until required. After thawing, the cells were cultured in RPMI-1640 medium supplemented with 10% FCS and 1% penicillin/streptomycin (all reagents from Biochrom/Merck, Berlin, Germany), and exposed to 1α,25-dihydroxyvitamin D3 (Santa Cruz Biotechnology, Dallas, TX, United States) at 40 nmol/L as indicated. After an incubation period of 20 h at 37 ℃ in a 5% CO_2 humidified atmosphere, lipopolysaccharide (LPS; 1 μg/mL; Sigma-Aldrich, Deisenhofen, Germany) and peptidoglycan (PGN; 10 μg/mL; Sigma-Aldrich) were added to the cells as indicated, and incubation continued for another 6 h (Figure 1). Subsequently, the cells were lysed in RTL Plus buffer, which was included in the RNeasy Plus Kit (Qiagen, Hilden, Germany), and subjected to RNA isolation (see below).

NOD2 genotyping

DNA was isolated from whole blood using a QIAamp DNA Blood Mini Kit (Qiagen) according to the manufacturer's protocol. All patients and controls were genotyped with respect to the three major mutations in the *NOD2* gene (SNP 8; R702W, NCBI reference SNP ID: rs2066844, SNP 12; G908R, NCBI reference SNP ID: rs2066845 and SNP 13; 1007fs, NCBI reference SNP ID: rs2066847). The corresponding regions of the *NOD2* gene were amplified by PCR using a Taq PCR Master Mix Kit (Qiagen) and primers as specified in Table 1. The following PCR conditions were used: 5 min, 94 ℃; 1 min, 94/60/72 ℃ (45 cycles); 7 min, 72 ℃; 4 ℃. After Sanger sequencing (Seqlab, Göttingen, Germany), the data were analyzed using the software Chromas, version 2.6. Individuals with no SNP mutations were considered wild-type (WT) for *NOD2*.

Microarray analysis of RNA expression profiles

RNA was extracted employing an RNeasy Plus Kit according to the manufacturer's protocol. Total RNA samples were quantified with a spectrophotometer (NanoDrop 1000, Thermo Fisher Scientific, Waltham, MA, United States), and their integrity was confirmed using the Agilent Bioanalyzer 2100 with an RNA Nano chip kit (both from Agilent Technologies, Waldbronn, Germany).

Expression profiling was performed using 200 ng RNA and the Affymetrix Human Clariom S Assay (Affymetrix/Thermo Fisher Scientific), which interrogates over 20000 well-annotated genes. Therefore, the so-called Whole Transcriptome protocol was employed. T7 promoter tags were introduced into all RNA molecules by using N6 3'-ends for DNA strand synthesis, before RNA strand replacement according to Eberwine[29] was conducted. Non-labeled aRNA was produced by *in vitro* transcription. All RNA molecules were amplified in a linear manner, avoiding a 3' bias. Using purified aRNA as a template, a new strand-identical single-strand DNA was produced by adding random primers and dNTPs (including dUTP, which replaced a limited

Table 1 Primer for *NOD2*-genotyping

SNP	Primer
8	Forward: 5′CCTCTTCAATTGTGGCAGGC-3′
	Reverse: 5′-CTCCTGCATCTCGTACAGGC-3′
12	Forward: 5′-ATGGAGGCAGGTCCACTTTG-3′
	Reverse: 5′-TTACCTGAGCCACCTCAAGC-3′
13	Forward: 5′-GATGGTACTGAGCCTTTGTTGA-3′
	Reverse: 5′-CAGACTTCCAGGATGGTGTCAT -3′

amount of dTTP). After digestion with RNase H, endpoint fragmentation was performed with uracil-DNA-glycosylase in combination with apurinic/apyrimidinic endonuclease 1, and biotinylated dNTPs were added to the 3′-ends of the single-stranded DNA fragments with deoxynucleotidyl transferase. Subsequently, hybridization of the microarrays was performed at 45 ℃ in a GeneChip® Hybridization Oven 645 (Affymetrix/Thermo Fisher Scientific). After overnight incubation, the microarrays were scanned using the GeneChip Scanner 3000 (Affymetrix/Thermo Fisher Scientific) at 0.7 μm resolution.

Primary data analysis was performed with the Affymetrix Transcriptome Analysis Console software version 3.1.0.5 including the Robust Multiarray Average module for normalization. Gene expression data were log-transformed. A change was considered significant when the ANOVA *P*-value met the criterion $P < 0.05$ at fold changes >|2|, *i.e.*, expression increments or declines larger than two. Along with the publication of the manuscript, our complete microarray data will be available in the Gene Expression Omnibus database (GEO accession number: GSE110186).

Quantitative reverse transcriptase-PCR using real-time TaqMan™ technology

Unless indicated otherwise, reagents from Thermo Fisher Scientific were used in all subsequent steps. Cellular RNA prepared as described above was treated with a DNA-free kit to remove traces of genomic DNA, and 250 ng of RNA per sample was reverse transcribed into cDNA using TaqMan™ Reverse Transcription Reagents and random priming. Using a ViiA 7 sequence detection system (Thermo Fisher Scientific), target cDNA levels were quantified by real-time PCR. Therefore, qPCR MasterMix (Eurogentec, Seraing, Liège, Belgium) and the following human-specific TaqMan™ gene expression assays with fluorescently labeled MGB probes were used: Hs00355476_m1 (*CCL20*), Hs00188627_m1 (*CD101*), Hs00370621_m1 (*CLEC12A*), Hs04398399_m1 (*CLEC5A*), Hs01902549_s1 (*CLEC7A*), Hs01099660_g1 (*CXCL5*), Hs01114274_m1 (*IL24*), Hs00167304_m1 (*ITGAM*), Hs00164957_m1 (*ITGB2*), Hs00426232_m1 (*LYZ*), Hs00234007_m1 (*MSR1*), Hs01065279_m1 (*PECAM1*), Hs00218624_m1 (*TREM1*), and Hs99999905_m1 (*GAPDH*). PCR conditions were as follows: 95 ℃ for 10 min, followed by 40 cycles of 15 s at 95 ℃/ 1 min at 60 ℃. Relative amounts of target mRNA in PBMCs were expressed as $2^{-(\Delta Ct)}$ values.

Statistical analysis

Real-time PCR data were analyzed with repeated-measures ANOVA. Mean group differences were compared for "disease" (patients with CD *vs* controls) and "*NOD2*-status" (WT, heterozygote, homozygote), as well as for (within-subject factors) "vitamin D application" (yes *vs* no) and "stimulation" (LPS, PGN, LPS + PGN, controls), employing a *general linear model* for repeated measurements. Age was considered a covariate in the disease model because disease groups were not balanced by age, and *NOD2* groups were tested post hoc by LSD. Normal distribution of measurements was assessed using the Kolmogorov-Smirnov test. $P < 0.05$ was considered statistically significant. All data were processed using IBM® SPSS® Advanced Statistics 22.0.

RESULTS

PBMCs provide an easily accessible tool to investigate disease-associated peculiarities of the antipathogenic immune response of patients with CD. To study transcripts in an unbiased manner, we initially chose a microarray approach. Therefore, PBMCs from healthy individuals and patients with CD in remission ($n = 3$ each; all of them *NOD2*-WT) were pretreated with vitamin D3 for 20 h before they were challenged simultaneously with LPS and PGN for 6 h. Subsequently, global gene expression was analyzed employing Clariom S assays, and data were compared with those of untreated controls. Table 2 gives an overview of the significant differences between patients with CD and controls under identical conditions of PBMC treatment.

Under basal conditions and any treatment regimen, genes upregulated in patients with CD exceeded downregulated genes both in number and maximum change. Complete lists of the 267 genes are presented as Supplementary Table 1.

Many of the differentially expressed genes are well-known modulators of immune cell functions in the context of innate and adaptive immunity, and unsurprisingly, some of them have previously been implicated in the pathogenesis of CD, including several immune cell receptors, cytokines/chemokines and their cognate receptors and the antimicrobial peptide lysozyme[30-36]. The latter transcript was found to be upregulated in PBMCs of patients with CD in response to LPS/PGN treatment, independent of the presence or absence of vitamin D3. Intriguingly, expression of various genes was synchronously up- or downregulated under different conditions, suggesting a robustness of the expression profile against external perturbations.

For in-depth analysis, we selected a panel of 11 genes from the list of candidates shown in Table 2 that fulfilled the following criteria: (1) differential expression in PBMCs from patients with CD and controls under basal conditions and/or under at least two

Table 2 Numbers and maximum changes of up- and downregulated genes in peripheral blood mononuclear cells from Crohn's disease patient *vs* identically treated controls

Treatment of PBMCs	Upregulated genes	Downregulated genes
Untreated	85 (59-fold)	39 (39-fold)
Vitamin D3	25 (21-fold)	12 (9-fold)
LPS/PGN	54 (6-fold)	15 (5-fold)
Vitamin D3 + LPS/PGN	29 (15-fold)	8 (5-fold)

$P < 0.05$; Threshold: ⩾ 2-fold change. PBMCs: Peripheral blood mononuclear cells; LPS: Lipopolysaccharide; PGN: Peptidoglycan.

Table 3 Genes selected for real-time PCR studies

Transcript	Fold changes: patients *vs* controls				Details on function/ reasons to study
	Basal	+D, -L/P	-D, +L/P	+D, +L/P	
MSR1	9.56	13.19		14.72	macrophage scavenger receptor[49]; differentially expressed in 3 of 4 groups
CD101	2.66				expressed on various immune cells; inhibits expansion of colitogenic T cells[30]
CLEC5A		2.82		3.11	C-type lectin member 5A, pattern recognition receptor; involved in antibacterial/ antiviral defense[43]
CLEC7A	4.53			3.94	C-type lectin member 7A, pattern recognition receptor; control of fungal infections[50]
CLEC12A	6.04	4.52			C-type lectin member 12A, pattern recognition receptor, inhibits cell death-induced inflammation[51]
ITGAM	2.18				CD11b; integrin αM; expressed by many immune cells; polymorphisms linked to autoimmunity[52]
LYZ			3.51	2.03	antimicrobial enzyme; essential role in innate immunity; increased production linked to CD[31]
PECAM1	3.15				CD31; implicated in transendothelial leukocyte migration in experimental colitis[32]
CCL20	-2.33		-5.08		chemokine expressed by neutrophils, enterocytes, B-cells and dendritic cell; IBD predilection gene[33]
CXCL5	-38.89		-5.32		regulates neutrophil homeostasis and chemotaxis; increased serum levels in IBD patients reported[34]
IL-24			-3.49	-4.82	Involved in host defence against bacteria and fungi; increased expression in patients with active IBD[35]
TREM1					amplifier of antimicrobial immune responses and inflammation in experimental colitis and IBD[36]

$P < 0.05$; Threshold: ⩾ 2-fold change. Positive values refer to genes upregulated and negative values to genes downregulated in CD patients. LPS: Lipopolysaccharide; PGN: Peptidoglycan; PBMCs: Peripheral blood mononuclear cells. CD: Crohn's disease; IBD: Inflammatory bowel diseases.

treatment regimens (vitamin D3, LPS+PGN and their combination, respectively) and (2) an established or potential role in inflammation and/or regulation of the immune response. Table 3 shows details regarding all selected genes as well as a twelfth gene, *TREM1*, that was included as a control as an established vitamin D-responsive gene with immunomodulatory function[37]. Interestingly, three pro-inflammatory mediators, *CCL20*, *CXCL5* and *IL-24*, displayed lower expression levels in patients with CD, which might be a consequence of their disease-specific medication (see below).

The expression profiles of the selected genes were subsequently studied by real-time PCR. In addition to WT-*NOD2* patients and healthy controls (n = 6 each, including the samples previously analyzed by microarray technology), we also included patients with heterozygous and homozygous mutations of *NOD2* (n = 5 each). Furthermore, we refined the protocol of PBMC treatment using LPS and PGN both in combination and as individual factors (Supplementary Table 2).

The clinical characteristics, laboratory findings and the medication of all 16 patients are shown in Table 4. Except for one person with a slightly increased CDAI of 166, all patients presented with a CDAI of < 150, indicating disease remission[27]. The CRP-values of 13 patients were in the normal range (below 5 mg/L). In the remaining three patients, modestly elevated CRP-values (all below 14 mg/L) were detected. Disease activity in all patients could still be considered low. All but two patients presented with vitamin D levels below 75 nmol/L, suggesting an insufficiency or even deficiency (levels below 50 nmol/L). This finding was not unexpected, as all of the samples were collected during the European winter season. As a consequence, a vitamin D substitution therapy was initiated, if appropriate. Three of the patients were on steroids (> 10 mg prednisolone/d) at the time of the study, 7 received azathioprine, and 12 were treated with anti-TFN-α antibodies. Healthy controls consisted of 3 males and 3 females with an age range from 25 to 53 years.

For statistical data analysis, a general linear model repeated measure was chosen to assess mean differ-

Table 4 Characteristics of the patients –nucleotide-binding oligomerization domain 2 status, classification and activity of the disease, C-reactive protein and vitamin D levels, and medication at the time of the study

No.	Sex	Age	NOD2	Montreal classification	CDAI	CRP (mg/L)	Vitamin D (nmol/L)	Prednisolon (> 10 mg/d)	Azathio-prine	Anti-TNF-α
1	M	24	WT[1]	A2 L3 L4 B1	121	2.58	104.0	Yes	No	Yes
2	M	28	WT[1]	A2 L3 B3p	46	< 1.0	62.9	No	No	Yes
3	M	64	WT[1]	A3 L3 B2p	111	4.58	27.0	Yes	No	Yes
4	F	60	WT	A2 L3 B2	100	2.27	72.5	No	No	Yes
5	F	62	WT	A3 L3 B3p	82	< 1.0	58.2	Yes	Yes	Yes
6	M	46	WT	A2 L3 L4 B2p	110	4.97	32.9	No	No	Yes
7	M	38	HO	A2 L3 B3p	92	13.30	40.2	No	Yes	No
8	M	64	HO	A3 L3 B1	34	< 1.0	53.0	No	No	Yes
9	F	48	HO[2]	A2 L3 B2	54	< 1.0	67.6	No	No	Yes
10	M	54	HO	A2 L3 B2p	103	1.63	51.9	No	Yes	No
11	M	26	HO	A1 L3 B2p	120	7.86	31.0	No	Yes	Yes
12	M	27	HT	A2 L3 B1	106	1.41	47.2	No	No	Yes
13	M	37	HT	A2 L3 B2p	166	4.56	37.9	No	Yes	No
14	F	48	HT	A2 L1 B3p	115	1.01	55.2	No	Yes	Yes
15	M	57	HT	A1 L3 B2p	132	12.30	52.3	No	No	Yes
16	M	47	HT	A2 L3 B3	60	2.62	92.0	No	Yes	No

[1]Patients who were also included into microarray analysis; [2]SNP8 mutation. All other HO/HT mutations refer to SNP13. NOD2: Nucleotide-binding oligomerization domain 2; CDAI: Crohn's disease activity index; CRP: C-reactive protein; TNF : Tumor necrosis factor; WT: Wild-type; HO: Homozygous mutation; HT: Heterozygous mutation.

Table 5 differentially expressed transcripts in vitamin D-pretreated peripheral blood mononuclear cells from Crohn's disease patients and controls (with and without additional stimulation with lipopolysaccharide and peptidoglycan, respectively, age adjusted)

Gene	*P* value	Upregulated in
CLEC5A	0.030	CD patients
LYZ	0.047	CD patients
TREM1	0.023	CD patients

CD: Crohn's disease.

ences between groups. Comparing controls to CD patients, no significant differences were detected when samples with and without vitamin D incubation were considered conjointly, due to the strong overlaying effect of vitamin D. However, focusing on the subgroup of vitamin D treated samples, disease-related differences were observed (no. 5-8 in Supplementary Table 2). Here, with age adjustment, three genes displayed a disease-dependent expression pattern (Table 5): consistent with the microarray data, significantly higher *CLEC5A* and *LYZ* transcript levels were observed in PBMCs of patients with CD. The same finding was observed for *TREM1*, which was included due to its vitamin D-dependent expression pattern but not because of the microarray results. Together, these findings suggest that CD-associated changes in gene expression could be attributed to treatment protocols that include vitamin D, as analyses in the remaining subgroup (w/o vitamin D) did not show any differences (all $P > 0.20$).

We also analyzed the influence of *NOD2* mutations on the expression of the gene panel described above. Considering all treated and untreated samples (Supplementary Table 2), a significant effect of the *NOD2* status was observed for 5 of these genes, including *CLEC5A* and *LYZ*, and one additional gene from Table 2, integrin subunit beta *2* (*ITGB2*) (Table 6). With a *P*-value of 0.053, *TREM1* just missed statistical significance.

Unexpectedly, heterozygous CD patients displayed the highest expression levels for all of the genes, whereas no statistically significant differences between persons with WT-*NOD2* (patients and controls) and homozygous *NOD2* mutations were detected. The phenomenon is apparently unrelated to medication, which was very similar in the groups of patients with heterozygous and homozygous *NOD2* mutations (Table 4). This conclusion is also supported by statistical evaluations, which did not show any significant association between treatment with prednisolone, azathioprine or anti-TNF-α and expression of *CLEC5A*, *LYZ* and *TREM1* in PBMCs of patients with CD.

DISCUSSION

Many studies have shown that numerous risk genes of CD code for molecules involved in host defense against pathogens, such as nucleotide-binding oligomerization domain 2 (*NOD2*), *ATG16L1*, and those implicated in the T helper type 17 (Th17) pathway[38-43]. Here, we tested the hypothesis that PBMCs of patients with CD, even at the stage of clinical remission, exhibit an

Table 6 Transcripts with a *NOD2*-dependent expression pattern

Gene	*P* value	Highest levels
CD101	0.002	Heterozygotes
CLEC5A	0.020	Heterozygotes
CXCL5	0.009	Heterozygotes
IL-24	0.044	Heterozygotes
ITGB2	0.041	Heterozygotes
LYZ	0.042	Heterozygotes

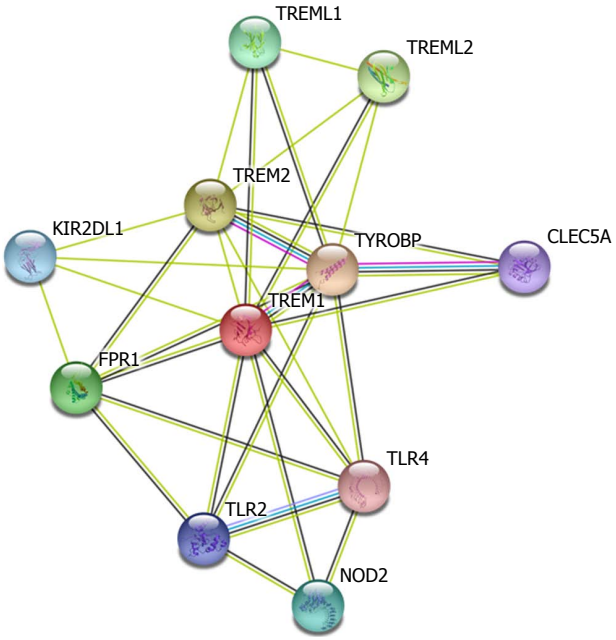

Figure 2 Network analysis using the STRING database[48]. The network was derived employing human TREM1 as the search term (https://string-db.org/cgi/network.pl?taskId=PmXpOD7RMwaM).

altered gene expression profile upon challenge with pathogen-associated molecular patterns (PAMPs) and/or the immunomodulatory hormone vitamin D, which has previously been shown to exert differential effects on the expression of NOD2- and TLR-induced cytokines in the context of CD[26].

Initial microarray experiments identified more than 200 genes with different expression patterns among patients with CD and controls. Based on predefined expression criteria, genes with roles in inflammation and immunity were selected for in-depth analysis by real-time PCR. A disease-associated expression pattern was identified for *CLEC5A*, *lysozyme* and *TREM1*. Six genes, including *CLEC5A* and *lysozyme*, displayed a *NOD2*-dependent expression pattern. With respect to lysozyme and TREM1, our findings are consistent with previous reports, which found that increased levels of both proteins in serum were implicated in the pathophysiology of IBD[44,45]. To the best of our knowledge, however, this is the first report of an association between *CLEC5A* expression and CD. CLEC5A has most recently been identified as an important receptor in innate immunity by neutrophil trap formation and secretion of different proinflammatory cytokines after stimulation with *Listeria monocytogenes*[43]. This finding is especially interesting, as defective bacterial clearance was shown to play a crucial role in the pathogenesis of CD[46,47]. Of note, both CLEC5A and TREM1 proteins can be linked to the product of the best-established CD risk gene, *NOD2*, by the STRING database[48] (Figure 2).

In conclusion, we found that PBMCs of patients with CD display alterations in their response to vitamin D and PAMPs. Disease-associated and *NOD2*-dependent gene expression profiles are preserved even at the stage of clinical remission. Our data identify *CLEC5A*, *LYZ* and *TREM1* as genes of particular interest for follow-up studies. We hypothesize that these genes may act in a common network relevant to CD pathogenesis. Establishment of biomarkers to better predict the clinical course of the disease remains a long-term goal of our studies.

ARTICLE HIGHLIGHTS

Research background

In Crohn's disease (CD), the interplay of genetic and environmental factors converges at the level of an altered antipathogenic immune response, which is incompletely understood. Peripheral blood mononuclear cells (PBMCs) provide a useful tool to study elements of the immunopathogenesis of the disease *in vitro*.

Research motivation

Currently, there is a lack of biomarkers to predict the clinical course of CD. Furthermore, the development of specific therapies would benefit from an improved mechanistic understanding of the pathogenesis of the disease.

Research objectives

The aim of this study was to identify disease-specific gene expression profiles of PBMCs from patients with CD in clinical remission. Specifically, we were interested in alterations of the gene expression profile after challenging PBMCs with pathogen-associated molecular patterns (PAMPs) and the immunomodulatory hormone vitamin D.

Research methods

PBMCs from patients with CD and healthy donors were cultured with vitamin D, peptidoglycan (PGN) and lipopolysaccharide (LPS), before RNA was isolated and subjected to microarray analysis and quantitative real-time PCR. Disease-specific gene expression profiles were evaluated by *general linear model repeated measure* analysis, paying particular attention to the well-established CD risk gene *NOD2*.

Research results

Microarray experiments yielded a total of 267 genes that were significantly up- or downregulated in PBMCs of patients with CD, compared to healthy donors, after challenge with vitamin D and/or a combination of LPS and PGN. For further analysis by real-time PCR, genes with roles in inflammation and immunity were selected. For three of these genes, *CLEC5A*, *lysozyme* and *TREM1*, a disease-associated expression pattern was validated. Six genes, including *CLEC5A* and *lysozyme*, were found to be expressed in a *NOD2*-dependent manner.

Research conclusions

PBMCs of patients with CD display alterations of their response to vitamin D and PAMPs that are preserved even at the stage of clinical remission. *CLEC5A*, *TREM1* and *NOD2* may act in a common network relevant to CD pathogenesis.

Research perspectives

Follow-up studies on alterations of the antipathogenic immune response may provide novel insights into the pathogenesis of CD and may also help to establish biomarkers to better predict the clinical course of the disease.

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ORIGINAL ARTICLE

Basic Study

Three-microRNA signature identified by bioinformatics analysis predicts prognosis of gastric cancer patients

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Abstract

AIM

To identify multiple microRNAs (miRNAs) for predicting the prognosis of gastric cancer (GC) patients by bioinformatics analysis.

METHODS

The original microarray dataset GSE93415, which included 20 GC and 20 tumor adjacent normal gastric mucosal tissues, was downloaded from the Gene Expression Omnibus database and used for screening differentially expressed miRNAs (DEMs). The cut-off criteria were $P < 0.05$ and fold change > 2.0. In addition, we acquired the miRNA expression profiles and clinical information of 361 GC patients from The Cancer Genome Atlas database to assess the prognostic role of the DEMs. The target genes of miRNAs were predicted using TargetScan, miRDB, miRWalk, and DIANA, and then the common target genes were selected for functional enrichment analysis.

RESULTS

A total of 110 DEMs including 19 up-regulated and 91 down-regulated miRNAs were identified between 20 pairs of GC and tumor adjacent normal tissues, and the Kaplan-Meier survival analysis found that a three-miRNA signature (miR-145-3p, miR-125b-5p, and miR-99a-5p) had an obvious correlation with the survival of GC patients. Furthermore, univariate and multivariate Cox regression analyses indicated that the three-

miRNA signature could be a significant prognostic marker in GC patients. The common target genes of the three miRNAs are added up to 108 and used for Gene Functional Enrichment analysis. Biological Process and Molecular Function analyses showed that the target genes are involved in cell recognition, gene silencing and nucleic acid binding, transcription factor activity, and transmembrane receptor activity. Cellular Component analysis revealed that the genes are portion of nucleus, chromatin silencing complex, and TORC1/2 complex. Biological Pathway analysis indicated that the genes participate in several cancer-related pathways, such as the focal adhesion, PI3K, and mTOR signaling pathways.

CONCLUSION

This study justified that a three-miRNA signature could play a role in predicting the survival of GC patients.

Key words: Gene functional enrichment; Prognosis; Bioinformatic analysis; Differentially expressed miRNAs; Gastric cancer

Core tip: We identified 110 differentially expressed miRNAs through mining the datasets of Gene Expression Omnibus database and acquired the miRNA expression profiles and clinical information of 361 gastric cancer (GC) patients from The Cancer Genome Atlas database. Multiple miRNAs together acting as biomarkers may have a stronger reliability in survival prediction. Our study found that a novel three-miRNA signature could be used for predicting the prognosis of GC patients.

Zhang C, Zhang CD, Ma MH, Dai DQ. Three-microRNA signature identified by bioinformatics analysis predicts prognosis of gastric cancer patients. *World J Gastroenterol* 2018; 24(11): 1206-1215 Available from: URL: http://www.wjgnet.com/1007-9327/full/v24/i11/1206.htm DOI: http://dx.doi.org/10.3748/wjg.v24.i11.1206

INTRODUCTION

Gastric cancer (GC) is the fourth most common cancer in incidence and the second in mortality among all cancers worldwide[1]. In 2008, a total of 989600 individuals were newly diagnosed with GC and 738,000 deaths occurred, therefore, this disease is a serious public health issue worldwide[2]. Research studies that explore the cellular and molecular mechanisms of GC development and the validation of novel biomarkers are urgently needed to achieve early diagnosis and treatment.

MicroRNAs (miRNAs), which are endogenous small noncoding RNAs (20-22 nt), have been identified as the key regulators of genes at the post-transcriptional level[3]. Increasing studies have found that miRNAs are associated with the development and progression of GC, and can act as important biomarkers in diagnosis[4,5], therapy[6], and prognosis[7,8]. Thus, the identification of differentially expressed miRNAs (DEMs) may contribute to the early diagnosis and the prediction of survival prognosis in GC.

Several studies have found that a number of miRNAs are differentially expressed in GC and are associated with survival prognosis. However, these studies lack a large sample size or an appropriate proportion of samples. A reliable survival prediction requires large-scale samples that include detailed clinical characteristics. The Gene Expression Omnibus (GEO) database is a public functional genomics data repository that includes array- and sequence-based data and allows users to query and download experiments or curated gene expression profiles[9]. The Cancer Genome Atlas (TCGA, http://cancergenome.nih.gov) project is one of the most useful cancer genomics programs and has generated, analyzed, and made available genomic sequence, expression, methylation, and copy number variation data on over 11,000 individuals who represent over 30 different types of cancer[10]. In the present study, we identified DEMs between GC and adjacent normal tissues by analyzing the miRNA data of GSE93415 from GEO. In addition, the associations between DEMs and survival prognosis were analyzed using the expression profiles and clinical features downloaded from TCGA.

MATERIALS AND METHODS

Microarray data processing and DEMs identification

The microarray data of GSE93415 were downloaded from the GEO database (https://www.ncbi.nlm.nih.gov/geo/) and the miRNA expression data were processed with the limma package in R. Statistically significant DEMs between GC and adjacent normal samples were identified with the cut-off criterion $P < 0.05$ and fold change > 2.0.

Association analysis between DEMs and GC patients' survival

TCGA (https://cancergenome.nih.gov/) stomach adenocarcinoma and adjacent normal tissue miRNA sequencing data and clinical information were downloaded for analysis. The inclusion criteria included: (1) samples with completed data for analysis; (2) patients had not received preoperative chemoradiation; and (3) overall survival time less than 80 mo. Consequently, 361 GC samples were included in the present study. The Kaplan-Meier method and log-rank test were conducted to test the prognostic value of DEMs. When $P < 0.05$, miRNAs were considered significantly associated with

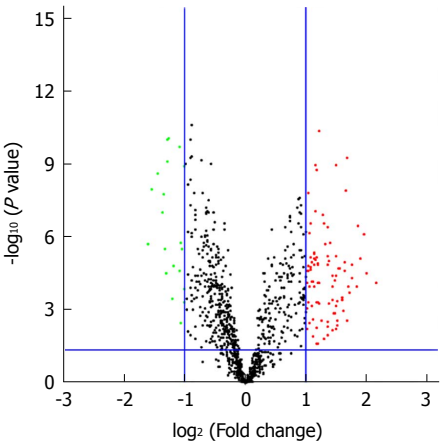

Figure 1 The volcano plot of the differentially expressed miRNAs. A total of 110 DEMs were identified between 20 pairs of GC patients and adjacent normal tissues (cut-off criteria are $P < 0.05$ and fold change > 2.0). The green and red spots represent downregulated and upregulated miRNAs, respectively. DEMs: Differentially expressed miRNAs; GC: Gastric cancer.

the prognosis of patients. Then, we ranked prognosis-related miRNAs according to the median expression level. Subsequently, we scored each GC patient in accordance with a high or low level of expression, and a risk grade was defined by the total scores. Finally, GC patients were sorted into high and low risk groups by the risk-score rank. The prognosis-related miRNA signature was used to analyze overall survival between high and low risk group patients using a Kaplan-Meier curve.

Target genes prediction of prognostic DEMs

We used four online tools to predict the potential target genes of the prognostic related DEMs, including TargetScan (http://www.targetscan.org/vert_71/), miRDB (http://www.mirdb.org/), miRWalk (http://zmf.umm.uni-heidelberg.de/apps/zmf/mirwalk2/index.html), and DIANA (http://www.microrna.gr/microT-CDS). In order to obtain the more reliable target genes, the Venn plot was performed to acquire the consensus genes of the four online tools.

Function analysis of target genes

FunRich [Functional Enrichment analysis tool (http://www.funrich.org/)] is a stand-alone software used for functional enrichment and interaction network analysis of genes and proteins[11]. Enrichment analysis was conducted on the consensus genes using the FunRich tool in the following categories: Biological Process, Cellular Component, Molecular Function, and Biological Pathways. $P < 0.05$ was considered statistically significant.

Statistical analysis

The data of miRNA expression in GC and adjacent normal samples were performed by unpaired t-test. The association between DEMs expression and clinical characteristics was analyzed by the chi-square and *t*-tests. Kaplan-Meier survival analysis and the univariate/multivariate Cox regression analysis were used to assess the expression levels of DEMs and prognostic features. All the statistical analyses were performed with IBM SPSS version 19.0 and $P < 0.05$ was considered statistically significant.

RESULTS

Identification of DEMs in GC

The microarray data of GSE93415, including 20 pairs of GC and adjacent normal tissue samples, were obtained from the NCBI-GEO database. After applying cut-off criteria of $P < 0.05$ and fold change > 2.0, a total of 110 DEMs were identified between GC and adjacent normal tissues (Table 1). The results of 19 downregulated miRNAs and 91 upregulated miRNAs are displayed in the volcano plot (Figure 1). A heat map of hierarchic cluster analysis showed that DEMs could be discriminated between GC and normal tissues (Figure 2).

Identification of DEMs related with overall survival in GC

To identify the DEMs which could be used to predict the overall survival of GC patients, we collected 361 samples from TCGA to assess the relationship between DEMs and the overall survival of GC patients. The patients' clinical characteristics including age at diagnosis, gender, race, TNM stage, and histologic grade are shown in Table 2. By using a log-rank test and Kaplan-Meier curve, we found that three DEMs (miR-145-3p, miR-125b-5p, and miR-99a-5p) were negatively associated with overall survival (Figure 3). The association analysis between the three DEMs and clinical characteristics indicated that miR-145-3p, miR-125b-5p, and miR-99a-5p were all significantly associated with histologic grade ($P < 0.05$). The detailed results are shown in Table 3.

Prognostic role of a three-DEM signature in GC patients

We ranked the three DEMs by the median of expression and then scored each GC patient in accordance with high or low-level expression. A risk grade was defined by the total scores. As a result, all the 361 GC patients were sorted into a high or low risk group. A survival analysis with the Kaplan-Meier method and log-rank test was conducted. The results indicated that the overall survival between the high risk and low risk groups was significantly different ($P = 0.045$). Interestingly, compared to patients in the high risk group, the low risk patients tended to have a better prognosis (Figure 4). Furthermore, we performed univariate and multivariate Cox regression analyses to verify the prognostic role of the three-DEM signature according to clinical features. The univariate analysis showed that pathologic stage (HR = 1.825, $P < 0.001$), T stage (HR = 1.864, $P = 0.006$), N stage (HR = 2.005, $P = 0.001$), and the three-DEM signature (HR = 1.422,

Table 1 The differentially expressed miRNAs identified between gastric cancer and adjacent normal tissues

Upregulated DEMs[1]	*P* value	Downregulated DEMs	*P* value
hsa-miR-199a-3p/hsa-miR-199b-3p	7.10E-05	hsa-miR-652-5p	0.000135
hsa-miR-125b-5p	2.99E-05	hsa-miR-1269b	1.13E-09
hsa-miR-199a-5p	7.65E-07	hsa-miR-665	2.86E-06
hsa-miR-223-3p	7.49E-06	hsa-miR-375	0.003304
hsa-miR-196a-5p	3.22E-07	hsa-miR-4501	1.64E-06
hsa-miR-27a-3p	0.000112	hsa-miR-4279	1.94E-10
hsa-miR-23b-3p	4.71E-05	hsa-miR-943	2.46E-05
hsa-miR-21-5p	1.36E-05	hsa-miR-148a-3p	1.53E-05
hsa-miR-100-5p	0.000197	hsa-miR-1275	0.000349
hsa-miR-20a-5p	0.000102	hsa-miR-4290	8.91E-11
hsa-miR-23a-3p	5.15E-10	hsa-miR-4268	9.16E-11
hsa-miR-1	0.002694	hsa-miR-891a	7.29E-10
hsa-miR-214-3p	1.23E-08	hsa-miR-4795-3p	3.07E-05
hsa-miR-10a-5p	2.33E-05	hsa-miR-1298	3.00E-06
hsa-miR-135b-5p	1.04E-05	hsa-miR-660-3p	1.75E-08
hsa-miR-99a-5p	0.001109	hsa-miR-4661-5p	9.22E-08
hsa-miR-20b-5p	0.000365	hsa-miR-4539	2.37E-09
hsa-miR-199b-5p	0.000291	hsa-let-7d-3p	1.10E-08
hsa-miR-10b-5p	1.83E-05	hsa-miR-4636	1.94E-06
hsa-miR-27b-3p	1.95E-05		
hsa-miR-126-3p	0.004079		
hsa-miR-130a-3p	0.000442		
hsa-miR-142-3p	0.002661		
hsa-miR-4291	1.12E-09		
hsa-miR-24-3p	6.14E-06		
hsa-let-7a-5p	0.0059		
hsa-miR-145-5p	0.00066		
hsa-miR-17-5p	3.84E-05		
hsa-miR-143-5p	6.07E-05		
hsa-let-7f-5p	0.001364		
hsa-miR-4328	0.001281		
hsa-miR-4324	3.91E-05		
hsa-miR-145-3p	0.000389		
hsa-miR-143-3p	0.006402		
hsa-miR-95	0.000106		

[1]Upregulated DEMs are listed according to the rank of fold changes. DEMs: Differentially expressed miRNAs.

P = 0.039) were significantly associated with the prognostic outcome of GC patients. The multivariate analysis revealed that T stage (HR = 1.623, *P* = 0.044) and the three-DEM signature (HR = 1.451, *P* = 0.032) were all independent factors in predicting the prognosis of GC patients (Table 4).

Target prediction of three DEMs and gene function analysis

The online target prediction tools TargetScan, miRDB, miRWalk, and DIANA were used to predict the targets genes of miR-145-3p, miR-125b-5p, and miR-99a-5p. We then obtained the consensus genes of each DEM from the four online predictions (Figure 5). As a result, we identified a 108 consensus target genes. Furthermore, we conducted gene enrichment analysis to identify the biological function of common target genes (Figure 6). The Biological Process analysis indicated that the genes were mostly enriched in cell recognition, regulation of nucleic acid, and gene silencing. Cellular Component analysis indicated that genes were enriched in the nucleus, RNA-induced silencing complex, chromatin silencing complex, and phosphoinositide 3-kinase complex. Molecular Function analysis showed that genes were enriched in transmembrane receptor, transcription regulator, and transcription factor activity. Biological Pathways were mainly enriched in the VEGFR, PI3K/Akt, and mTOR signaling pathways.

DISCUSSION

Due to the reduction in chronic *Helicobacter pylori* infection and improvement of sanitation, the incidence and mortality rates of GC have declined in recent years[12]. However, there are still almost 460,000 new GC cases and 350000 GC deaths each year in China[13]. The prognosis of GC patients is poor and the five-year survival rate is 5%-20% despite advances in GC therapy[14]. Thus, to improve the clinical treatment and management of GC patients, it is urgent to identify reliable prognostic biomarkers. In this study, we identified a total of 110 DEMs by analyzing the GSE93415 data and discovered that three miRNAs (miR-145-3p, miR-125b-5p, and miR-99a-5p) were

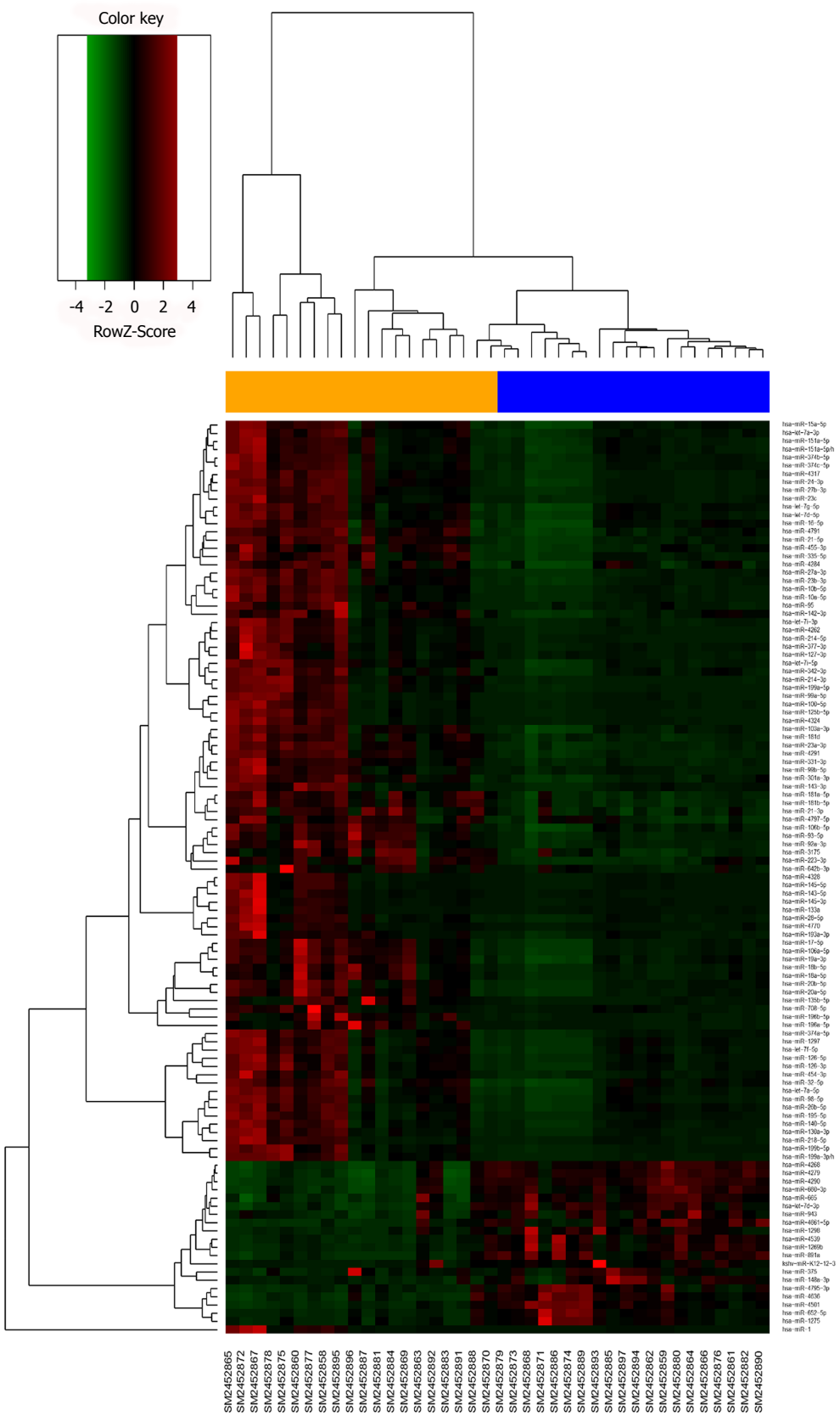

Figure 2 Hierarchical clustering analysis of the differentially expressed miRNAs between 20 pairs of gastric cancer and adjacent normal sample. Each row represents the expression of an miRNA and each column represents a sample: orange color for gastric cancer, blue for normal.

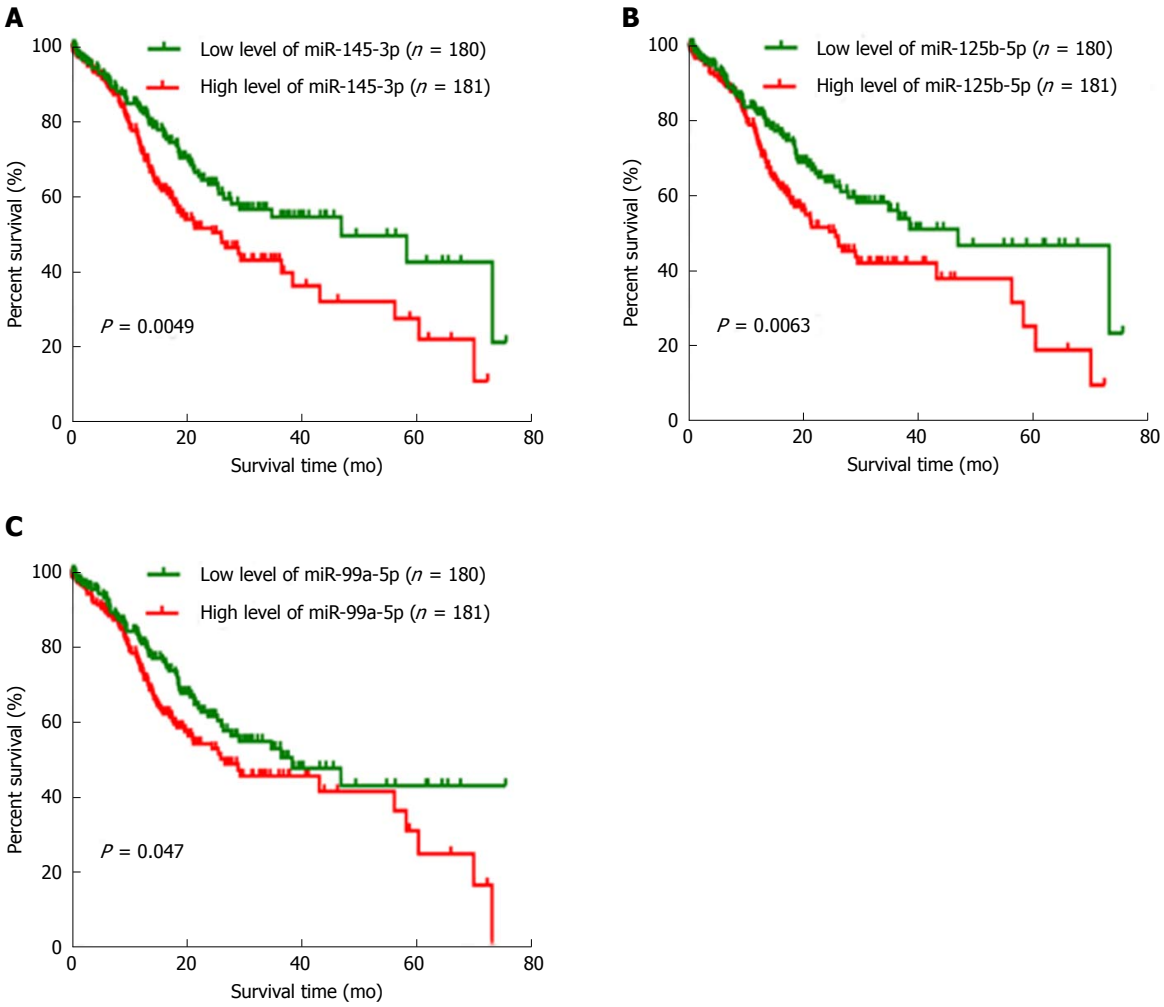

Figure 3 Three differentially expressed miRNAs are negatively associated with overall survival in gastric cancer. A: Patients with a high level of miR-145-3p (n = 180) had a poorer prognosis than those with a low level of miR-145-3p (n = 181) (P = 0.0049). B: Patients with a high level of miR-125b-5p (n = 180) had a poorer prognosis than those with a low level of miR-125b-5p (n = 181) (P = 0.0063). C: Patients with a high level of miR-99a-5p (n = 180) had a poorer prognosis than those with a low level of miR-99a-5p (n = 181) (P = 0.047).

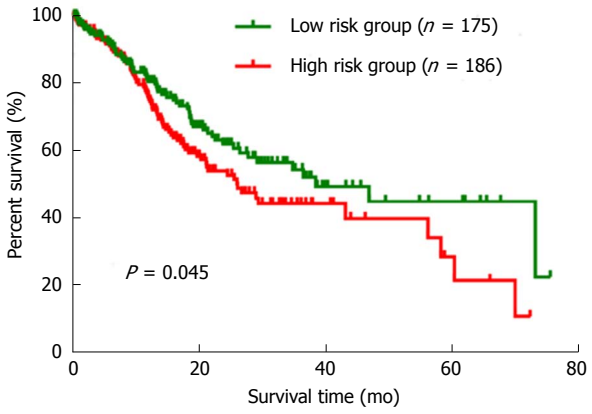

Figure 4 The Kaplan-Meier curve for the three-miRNA signature in gastric cancer. The three differentially expressed miRNAs were ranked by the median of expression and then scored for each gastric cancer patient in accordance with high or low-level expression. The low risk group (n = 175) and high risk group (n = 186) were defined by the total scores. Compared to the low risk group, patients in the high risk group had a poorer prognosis (P = 0.045).

negatively associated with overall survival. Additionally, we constructed a three-miRNA signature to predict the prognosis of GC patients.

For decades, a large number of studies have reported that miRNAs can play oncogenic or tumor-suppressing roles in regulating cell biological behavior of cells[15-18]. At present, several miRNAs are known to be useful in the early diagnosis of cancers, including miR-21[19], miR-486[20], miR-24[21], and miR-125a-5p[22]. In addition, miR-191[23], miR-1908[24], miR-200c[25], and miR-217[26] were found to be potential prognostic indictors in cancer. However, these studies only used a single indictor or a limited number of patients for survival analysis. In this study, we identified DEMs by analyzing the array data from the GEO database and found that three highly expressed miRNAs (miR-145-3p, miR-125b-5p, and miR-99a-5p) may be potential prognostic indictors in GC. Kaplan-Meier and Log-rank test survival analysis indicated that the three-miRNA

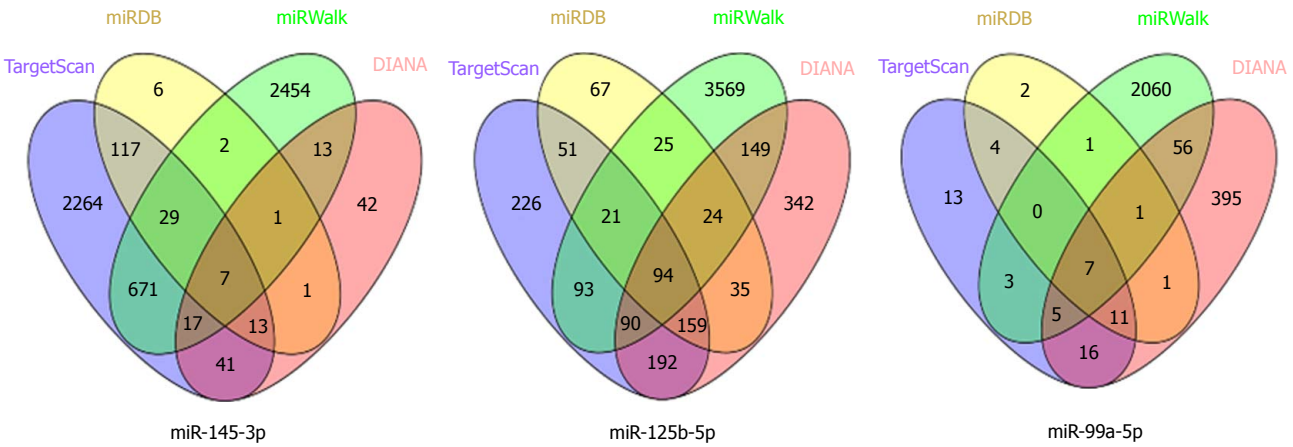

Figure 5 The consensus target genes of each differentially expressed miRNA. The target genes of the three miRNAs were predicted with four online tools (TargetScan, miRDB, miRWalk, and DIANA).

Table 2 Clinical features of gastric cancer patients

Variables	Case, *n* (%)
Age at diagnosis (yr)	
< 60	113 (31.3)
⩾ 60	248 (68.7)
Gender	
Male	241 (66.8)
Female	120 (33.2)
T stage	
T1 + T2	88 (24.4)
T3 + T4	273 (75.6)
Histologic grade	
G1 + G2	134 (37.1)
G3 + G4	227 (62.9)
Race	
White	234 (64.8)
Asian	83 (23.0)
Black or African American	12 (3.3)
NA	32 (8.9)
Pathologic stage	
Ⅰ	44 (12.2)
Ⅱ	117 (32.4)
Ⅲ	162 (44.9)
Ⅳ	30 (8.3)
NA	8 (2.2)
Node status	
N0	111 (30.7)
N1-3	249 (69.0)
NA	1 (0.3)
Metastasis	
M0	325 (90.0)
M1	22 (6.1)
Mx	14 (3.9)

NA: Not available.

signature can be used to predict the prognosis of GC patients.

We then searched present publications online to compare and test our findings. Chang *et al*[27] showed that miR-125b-5p was overexpressed in GC patients and promoted invasion and metastasis of GC by targeting *STARD13* and *NEU1*. It was also indicated that miR-125b-5p could be a potential biomarker for predicting prognoses and clinical outcomes in patients with HER2-positive GC that receive trastuzumab treatment[28]. Wu *et al*[29] found that miR-125b-5p promotes cell migration and invasion by targeting PPP1CA-Rb signal pathways and acts as an independent prognostic factor in GC. Furthermore, Zhang *et al*[30] demonstrated that miR-99a-5p might function as a novel molecule to regulate cisplatin resistance by directly targeting the calpain small subunit 1 (CAPNS1)-associated pathway in GC. Interestingly, there are no studies describing relations between miR-145-3p and GC. However, in non-small cell lung cancer, miR-145-3p was found to inhibit cancer cell migration and invasion by targeting *PDK1 via* the mTOR signaling pathway[31]. Moreover, miR-145-3p was also identified to be down-regulated in metastatic castration-resistant prostate cancer and target four molecules which can significantly predict survival in prostate cancer[32]. These results may suggest that miR-145-3p has a complicated effect in different cancers and, in future research, we will investigate the role of miR-145-3p in GC.

Dysregulated genes may participate in tumorigenesis and progression by aberrant signaling pathways. In this study, we predicted the target genes of the three miRNAs and performed gene functional enrichment analysis. The results showed that these target genes were associated with the process of gene silencing and cell recognition, as well as focal adhesion, EGFR, PI3K/Akt, and mTOR signaling pathways. Xu *et al*[33] suggested that the EGFR-Akt signaling pathway regulates drug resistance in GC patients. The PI3K/Akt pathway was demonstrated to be associated with poor prognosis, tumor progression, and resistance to systematic therapy in many cancers including GC[34,35]. In addition, the PI3K/Akt/mTOR pathway is a key signaling pathway that is reported to be involved in GC[36]. Thus, the results of our functional enrichment analysis are in accordance with present studies.

Above all, we identified a three-miRNA signature for predicting the prognosis of patients with GC and

Table 3 Association between the three differentially expressed miRNAs and clinical features

Variables	miR-145-3p expression		*P* value	miR-125b-5p expression		*P* value	miR-99a-5p expression		*P* value
	Low	High		Low	High		Low	High	
Age at diagnosis (yr)									
< 60	51	62	0.225	42	71	0.001[a]	49	64	0.096
⩾ 60	129	119		138	110		131	117	
T stage									
T1 + T2	40	48	0.342	51	37	0.081	46	42	0.603
T3 + T4	140	133		129	144		134	139	
N stage									
N0	58	53	0.567	57	54	0.731	57	54	0.731
N1-3	119	124		120	123		120	123	
M stage									
M0	163	162	0.670	165	160	0.191	164	161	0.386
M1	10	12		8	14		9	13	
Histologic grade									
G1 + G2	77	57	0.026[a]	87	47	< 0.001[a]	80	54	0.004[a]
G3 + G4	103	124		93	134		100	127	
Pathologic stage									
Ⅰ + Ⅱ	81	80	0.876	86	75	0.221	80	81	0.954
Ⅲ + Ⅳ	95	97		90	102		96	96	

[a]$P < 0.05$, statistically significant.

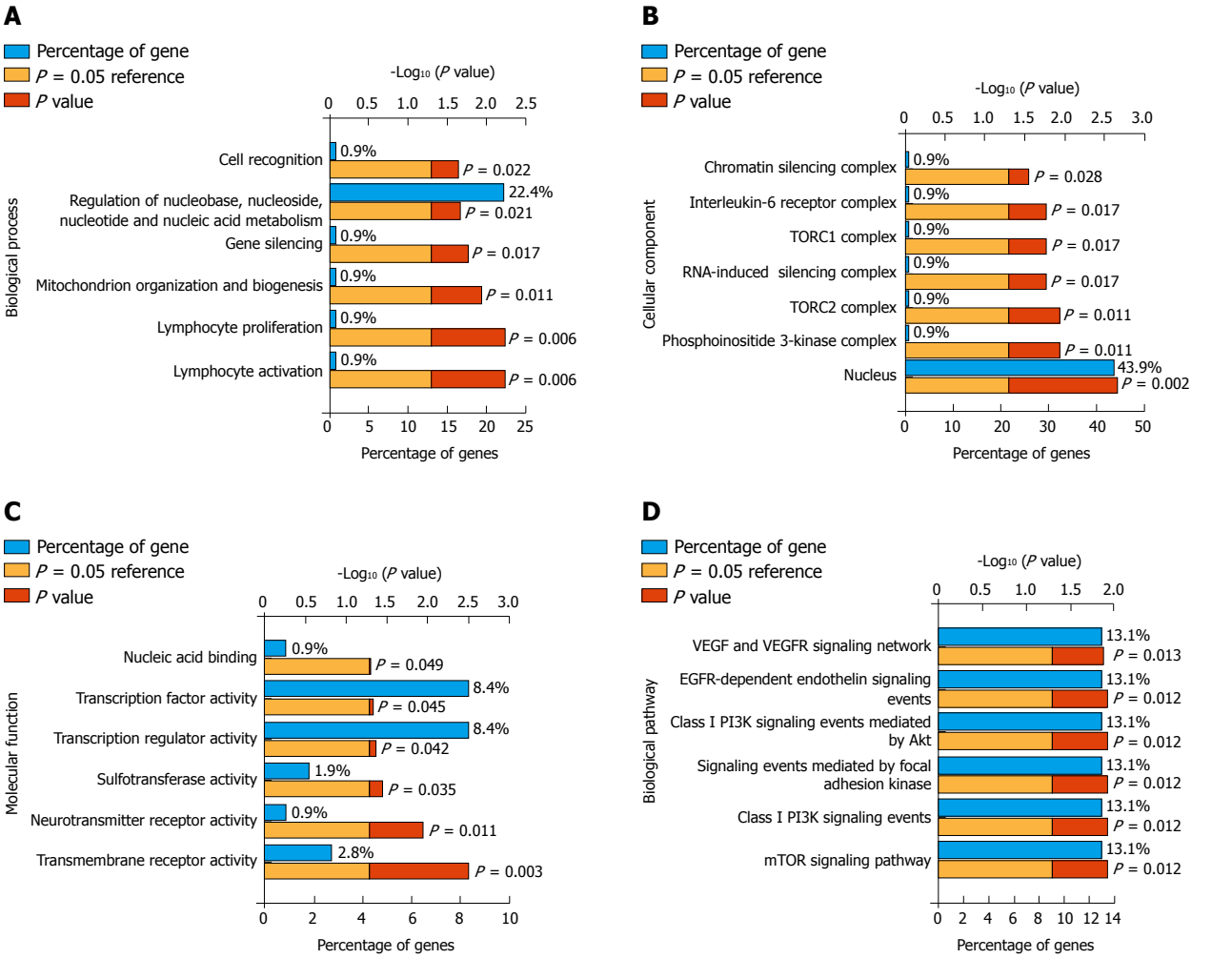

Figure 6 Genetic functional enrichment analysis. A total of 108 consensus genes were used for functional enrichment analysis with the tool of FunRich. A: Biological process analysis showed that the genes are involved in cell recognition and gene silencing; B: Cellular component analysis indicated that the genes are portion of nucleus, chromatin silencing complex, and TORC1/2 complex; C: Molecular function results showed that the genes are involved in nucleic acid binding, transcription factor activity, and transmembrane receptor activity; D: Biological pathway analysis indicated that the genes participate in focal adhesion, PI3K, and mTOR cancer-related signaling pathways. TORC1/2: Target of rapamycin 1/2; PI3K: Phosphatidylinositide 3-kinase; mTOR: Mammalian target of rapamycin.

Table 4 Univariate and multivariate Cox regression analyses of the association between the three differentially expressed miRNAs and clinical features

Variables	Univariate analysis		Multivariate analysis	
	HR (95%CI)	*P* value	HR (95%CI)	*P* value
Age at diagnosis (≥ 60 *vs* < 60)	1.373 (0.948-1.988)	0.094	1.642 (1.122-2.401)	0.011[a]
Pathologic stage (Ⅲ + Ⅳ *vs* Ⅰ + Ⅱ)	1.825 (1.332-2.499)	< 0.001[a]	1.252 (0.812-1.929)	0.309
T stage (T3 + T4 *vs* T1 + T2)	1.864 (1.197-2.902)	0.006[a]	1.623 (1.012-2.603)	0.044[a]
N stage (N1-2 *vs* N0)	2.005 (1.328-3.026)	0.001[a]	1.602 (0.935-2.744)	0.086
M stage (M1 *vs* M0)	1.368 (0.964-1.941)	0.080	1.313 (0.919-1.875)	0.134
Three-DEM signature (high *vs* low risk)	1.442 (1.018-1.988)	0.039[a]	1.451 (1.033-2.040)	0.032[a]

[a]$P < 0.05$, statistically significant. DEMs: Differentially expressed miRNAs.

analyzed potential signaling pathways in the development and progression of GC. However, to determine the genesis and development mechanism of GC, more large-scale and systematic investigations are required.

ARTICLE HIGHLIGHTS

Research background

Increasing studies have reported that microRNAs (miRNAs) play an important role in the development and progression of cancers, including gastric cancer (GC). Furthermore, miRNAs can also act as accurate biomarkers in diagnosis and prognosis prediction. In this study, we found that a three-miRNA signature could be used for predicting the prognosis of GC patients and multiple miRNAs together acting as biomarkers may have a stronger reliability in survival prediction.

Research motivation

The worldwide incidence and mortality rates of GC are fairly high. Most of GC patients have been in the advanced stage when diagnosed and endure a poor prognosis. Identifying accurate biomarkers in predicting prognosis of patients is an urgent issue to be solved, so that patients could have an individualized treatment and an improvement in prognosis.

Research objectives

We aimed to identify multiple miRNAs for predicting the prognosis of patients with gastric cancer. In the present study, we found that a three-miRNA (miR-145-3p, miR-125b-5p, and miR-99a-5p) signature could be used for predicting the prognosis of patients with gastric cancer. This objective could be applied to clinical practice and have a guidance role in improving the prognosis of patients with gastric cancer.

Research methods

We obtained the differentially expressed miRNAs by analyzing a microarray dataset from the Gene Expression Omnibus database with the limma package in R. The Kaplan-Meier method and log-rank test were used for describing the survival curve. The target genes of the three miRNAs (miR-145-3p, miR-125b-5p, and miR-99a-5p) were predicted with the online tools of TargetScan, miRDB, miRWalk, and DIANA. Venn plot was performed to obtain the common target genes from these four online tools. Enrichment analysis was conducted on the consensus genes using the FunRich tool.

Research results

In the present study, we found that a three-miRNA (miR-145-3p, miR-125b-5p, and miR-99a-5p) signature could be used for predicting the prognosis of patients with gastric cancer. Multiple miRNAs together acting as prognosis-related biomarkers may have a stronger reliability and this finding could be useful in clinical treatment according to gastric cancer patients with different prognoses. However, the role of miR-145-3p in the tumorigenesis and progression of gastric cancer remains unclear. Thus, this problem remains to be solved in our future study.

Research conclusions

Our study identified that the three miRNAs (miR-145-3p, miR-125b-5p, and miR-99a-5p) were up-regulated in gastric cancer patients by analyzing a microarray dataset. Besides, the novel three-miRNA signature could be used for predicting the prognosis of patients with gastric cancer. Multiple miRNAs together acting as prognosis-related biomarkers may have a stronger reliability, so that our finding could be useful in clinical treatment according to gastric cancer patients with different prognoses.

Research perspectives

This study provides us with a new insight that multiple miRNAs can be together used for predicting the prognosis of patients with gastric cancer. In order to further confirm the prognostic value of the three-miRNA signature, our future research may focus on exploring the relation between miR-145-3p and gastric cancer.

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ORIGINAL ARTICLE

Retrospective Cohort Study

Differing profiles of people diagnosed with acute and chronic hepatitis B virus infection in British Columbia, Canada

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Abstract

AIM

To describe the characteristics of people diagnosed with acute and chronic hepatitis B virus (HBV) infection in British Columbia (BC).

METHODS

We used data from the BC Hepatitis Testers Cohort (BC-HTC), which includes all individuals tested for hepatitis C virus (HCV) or human immunodeficiency virus (HIV) or those diagnosed with HBV or active tuberculosis in BC since 1990. These data were integrated with prescription drug, medical visit, hospitalization and mortality data. HBV cases were classified as acute or chronic according to provincial guidelines. We compared characteristics of individuals by HBV infection group (acute, chronic and negative). Factors associated with acute or chronic HBV infection were assessed with multinomial logistic regression models in comparison to the HBV negative group.

RESULTS

46498 of the 1058056 eligible BC-HTC participants were diagnosed with HBV infection. 4.3% of HBV positive individuals were diagnosed with acute HBV infections while 95.7% had chronic infections. Problematic alcohol use, injection drug use, and HIV or HCV co-infection were more common among individuals diagnosed with acute HBV compared to those with chronic infections and HBV negative individuals. In multivariable multinomial logistic regression models, we observed significant associations between acute or chronic HBV diagnosis and being male, age at HBV diagnosis or birth cohort, South and East Asian ethnicity, HCV or HIV infection, and injection drug use. The odds of acute HBV decreased with increasing age among people who inject drugs, while the opposite was true for chronic HBV. Persons with acute HBV were predominantly White (78%) while those with chronic HBV were mostly East Asian (60%). Relative to Whites, East Asians had 12 times greater odds of being diagnosed with chronic HBV infection. These odds increased with increasing socioeconomic deprivation.

CONCLUSION

Differences in the profiles of people diagnosed with acute and chronic HBV infection necessitate differentiated screening, prevention, care and treatment programs.

Key words: Hepatitis B virus; Ethnicity; Drug use; Acute hepatitis B; BC Hepatitis Testers Cohort; North America

Core tip: Substance use, major mental illness and hepatitis C virus or human immunodeficiency virus co-infection were more common among individuals with acute HBV compared with those diagnosed with chronic hepatitis B virus (HBV). Acute HBV was mainly diagnosed in the White population, while chronic HBV was mostly diagnosed among people with East Asian ethnicity. The risk of acute HBV was highest among the younger population who injected drugs, while the risk of chronic HBV infection was highest among East Asian people with lower socioeconomic status. Differences in the profiles of people diagnosed with acute and chronic HBV suggest the need for different interventions for both population groups.

Binka M, Butt ZA, Wong S, Chong M, Buxton JA, Chapinal N, Yu A, Alvarez M, Darvishian M, Wong J, McGowan G, Torban M, Gilbert M, Tyndall M, Krajden M, Janjua NZ. Differing profiles of people diagnosed with acute and chronic hepatitis B virus infection in British Columbia, Canada. *World J Gastroenterol* 2018; 24(11): 1216-1227 Available from: URL: http://www.wjgnet.com/1007-9327/full/v24/i11/1216.htm DOI: http://dx.doi.org/10.3748/wjg.v24.i11.1216

INTRODUCTION

Hepatitis B virus (HBV) affects 257 million people worldwide, including approximately 200000 Canadians[1-4]. Chronic HBV infection is associated with 66% of the 1.34 million viral hepatitis-related deaths reported worldwide and is responsible for a substantial disease burden from liver cancer and end-stage liver disease[2].

HBV vaccination is highly effective in preventing infection[1,3,5]. Mother to child transmission during childbirth is the most common mode of infection in HBV-endemic countries, while infection typically occurs through sexual transmission and injection drug use in Canada and other developed countries[1,3,5]. Over 90% of children and 50% of adults display no symptoms with acute infection[1,3,5]. The asymptomatic nature of this disease makes diagnosis difficult, leading to chronic illness in 5% of infected adults, 30% to 50% of children, and 90% of infected neonates[1,3,5]. Childhood vaccination programs have led to a dramatic reduction in the occurrence of acute infections in developed countries, including Canada and the United States[1,3,6]. The record low number of reported acute HBV infections in British Columbia (BC) in 2015 have been attributed to successful vaccination programs[7]. However, certain high risk groups, including men who have sex with men (MSM) and people who inject drugs (PWID),

continue to acquire and transmit HBV infection[8,9]. As HBV infection is mostly asymptomatic, identifying the factors associated with acute and chronic infection could determine avenues for closing gaps in screening, vaccination and other prevention programs.

In many developed countries, a relatively larger number of people are diagnosed with chronic HBV, compared with acute HBV, each year[5,10-13]. Data from Canada, the United States and other developed countries indicate that most chronic HBV infections are diagnosed among immigrants from HBV-endemic Asia-Pacific countries[10,12-14]. Our previous work suggests that 49% of persons with chronic HBV and decompensated cirrhosis and 46% of those with HCC in BC were diagnosed late in the course of their infections[15]. The rate of late diagnosis has declined but is still substantially higher for HBV compared to hepatitis C virus (HCV)[15]. Therefore, establishing the characteristics of individuals who are more likely to be infected with HBV could enhance the planning of screening programs to further reduce late diagnoses within the province.

Individuals diagnosed with acute and chronic HBV infections may differ with regards to demographics and risk behavior[1,5]. These distinctions may have implications for interventions targeted at either population. Analyses based on these differences could also identify areas for the optimal integration of such HBV programs with currently available health services. We are unaware of any study comparing large population level data for both acute and chronic HBV. Previous studies have mainly focused on chronic HBV epidemiology, with limited data on acute HBV, or acute HBV only[10,11,16-18]. In this study, we describe the characteristics of individuals with acute and chronic HBV infections and identify the factors associated with HBV infection within the BC Hepatitis Testers Cohort (BC-HTC).

MATERIALS AND METHODS

The cohort

The BC-HTC includes over 1.7 million individuals tested for hepatitis C virus (HCV) or human immunodeficiency virus (HIV) at the BC Centre for Disease Control Public Health Laboratory, or reported to BC public health as a confirmed case of HCV, HBV, HIV/AIDS or active tuberculosis (TB) since 1990[19]. Cohort data are integrated with data on medical visits, hospitalizations, prescription drugs, cancers and deaths[19]. Details of cohort creation and epidemiological characteristics have been reported previously and are summarized in Supplementary Table 1[19]. This analysis is based on data collected between April 1, 1990 and December 31, 2015.

Study population

BC-HTC participants who were included in the provincial registry of reported HBV cases, those who tested positive for HBV DNA or HBeAg, and those who were recorded as having received treatment for HBV were considered as cases of HBV. HBV cases recorded in the BC provincial registry with an acute diagnosis were classified as acute HBV infections, while the remainder were designated as having chronic HBV (Supplementary Table 2). Individuals who were not diagnosed with HBV but were tested for HBsAg or anti HBV core total were denoted as being HBV negative.

Definitions and covariates

We assessed potential risk factors at HBV diagnosis or at the last negative test, including age, birth cohort, sex, infection with HIV, HCV or TB, problematic alcohol use, illicit drug use, major mental illness, and material and social deprivation. HIV, HCV or TB diagnoses were based on laboratory confirmation or being reported as a confirmed case in the public health reportable disease database. Assessment of problematic alcohol and illicit drug use, and major mental illness was based on associated diagnostic codes in administrative health datasets evaluated across the entire dataset prior to the first positive or the last negative test (Supplementary Table 2).

Ethnicity classification was based on the validated name recognition software Onomap[20,21]. Onomap sensitivity and specificity relative to self-identified ethnicity, determined on a subset of the BC-HTC (n = 5962), was 93% and 98.6% for South Asians, and 67% and 99.5% for East Asians. Race/ethnicity was grouped as White, South Asian (Pakistani, Indians, Bangladeshis, Nepalese and Sri Lankans), East Asian (Chinese, Filipinos, Japanese, Korean and South-East Asians), and Others (Black, Central Asian, Latin American, Pacific Islander and West Asian individuals). Socioeconomic status was assessed using the Quebec Material and Social deprivation Index[22].

Statistical analysis

We compared characteristics of individuals by HBV infection group (acute, chronic and negative) with Pearson's chi-square tests for categorical variables and Kruskal Wallis tests for median age. Factors associated with acute or chronic HBV infection were assessed with multivariable multinomial logistic regression models in comparison with the HBV negative group.

RESULTS

1058056 individuals were eligible for inclusion in this analysis. Of these, 46498 individuals were diagnosed with HBV infection while 1011558 were HBV negative. 4.3% of HBV positive individuals were diagnosed with acute HBV infections while 95.7% had chronic infections (Table 1).

Characteristics of acute, chronic and hepatitis B negative individuals

About two-thirds of acute infections (70.7%) and half

Table 1 Characteristics of hepatitis B testers in the British Columbia Hepatitis Testers Cohort by hepatitis B diagnosis, 1990-2015 *n* (%)

	HBV positive			HBV negative	
	Acute	Chronic	All positive		*P* value
	n = 2015	*n* = 44483	*n* = 46498	*n* = 1011558	
Sex					
Female	590 (29.3)	20094 (45.2)	20684 (44.5)	563440 (55.7)	0.000
Male	1425 (70.7)	24387 (54.8)	25812 (55.5)	448072 (44.3)	
Unknown	0 (0.0)	2 (0.0)	2 (0.0)	44 (0.0)	
Birth Cohort					
< 1945	204 (10.1)	5385 (12.1)	5589 (12.0)	103498 (10.2)	0.000
1945-1964	1001 (49.7)	20316 (45.7)	21317 (45.8)	297768 (29.4)	
1965-1974	577 (28.6)	9852 (22.1)	10429 (22.4)	199121 (19.7)	
> 1974	233 (11.6)	8930 (20.1)	9163 (19.7)	411171 (40.6)	
Age group at HBV diagnosis (yr)					
< 25	306 (15.2)	5163 (11.6)	5469 (11.8)	137379 (13.6)	0.000
25-34	653 (32.4)	9963 (22.4)	10616 (22.8)	264809 (26.2)	
35-44	552 (27.4)	11835 (26.6)	12387 (26.6)	220745 (21.8)	
45-54	317 (15.7)	9202 (20.7)	9519 (20.5)	165884 (16.4)	
55-64	115 (5.7)	4954 (11.1)	5069 (10.9)	117550 (11.6)	
> 64	72 (3.6)	3366 (7.6)	3438 (7.4)	105191 (10.4)	
Median [IQR]	35 [28-45]	41 [31-51]	40 [31-50]	39 [29-53]	0.000
Year of HBV diagnosis					
1990-1999	1388 (68.9)	14068 (31.6)	15456 (33.2)	47343 (4.7)	0.000
2000-2004	362 (18.0)	11246 (25.3)	11608 (25.0)	126715 (12.5)	
2005-2009	196 (9.7)	8968 (20.2)	9164 (19.7)	219195 (21.7)	
2010-2015	69 (3.4)	10201 (22.9)	10270 (22.1)	618305 (61.1)	
Ethnicity					
East Asian	278 (13.8)	26578 (59.7)	26856 (57.8)	139306 (13.8)	0.000
Other	35 (1.7)	1651 (3.7)	1686 (3.6)	41452 (4.1)	
South Asian	125 (6.2)	1420 (3.2)	1545 (3.3)	83200 (8.2)	
White	1577 (78.3)	14834 (33.3)	16411 (35.3)	747600 (73.9)	
HCV					
Negative	1296 (64.3)	39575 (89.0)	40871 (87.9)	964780 (95.4)	0.000
Positive	719 (35.7)	4908 (11.0)	5627 (12.1)	46778 (4.6)	
HIV					
Negative	1888 (93.7)	43370 (97.5)	45258 (97.3)	1006511 (99.5)	0.000
Positive	127 (6.3)	1113 (2.5)	1240 (2.7)	5047 (0.5)	
HCV and HIV					
Positive	82 (4.1)	712 (1.6)	794 (1.7)	1768 (0.2)	0.000
Active tuberculosis					
Negative	2004 (99.5)	44101 (99.1)	46105 (99.2)	1008555 (99.7)	0.000
Positive	11 (0.5)	382 (0.9)	393 (0.8)	3003 (0.3)	
Problematic alcohol use					
No	1807 (89.7)	42544 (95.6)	44531 (95.8)	959326 (94.8)	0.000
Yes	208 (10.3)	1939 (4.4)	2147 (4.6)	52232 (5.2)	
Illicit drug use					
No	1617 (80.2)	41558 (93.4)	43175 (92.9)	944910 (93.4)	0.000
Yes	398 (19.8)	2925 (6.6)	3323 (7.1)	66648 (6.6)	
Injection drug use					
No	1718 (85.3)	42040 (94.5)	43758 (94.1)	960849 (95.0)	0.000
Yes	297 (14.7)	2443 (5.5)	2740 (5.9)	50709 (5.0)	
Major mental illness					
No	1788 (88.7)	41410 (93.1)	43198 (92.9)	874387 (86.4)	0.000
Yes	227 (11.3)	3073 (6.9)	3300 (7.1)	137171 (13.6)	
Material deprivation quintile					
Q1 (most privileged)	320 (15.9)	7076 (15.9)	7396 (15.9)	228694 (22.6)	0.000
Q2	303 (15.0)	6753 (15.2)	7056 (15.2)	189225 (18.7)	
Q3	320 (15.9)	7797 (17.5)	8117 (17.5)	189823 (18.8)	
Q4	425 (21.1)	9564 (21.5)	9989 (21.5)	198263 (19.6)	
Q5 (most deprived)	585 (29.0)	12226 (27.5)	12811 (27.6)	185193 (18.3)	
Unknown	62 (3.1)	1067 (2.4)	1129 (2.4)	20360 (2.0)	
Social deprivation quintile					
Q1 (most privileged)	247 (12.3)	9952 (22.4)	10199 (21.9)	184233 (18.2)	0.000
Q2	267 (13.3)	9032 (20.3)	9299 (20.0)	180059 (17.8)	
Q3	314 (15.6)	7574 (17.0)	7888 (17.0)	173461 (17.1)	
Q4	383 (19.0)	7336 (16.5)	7719 (16.6)	199229 (19.7)	
Q5 (most deprived)	742 (36.8)	9522 (21.4)	10264 (22.1)	254216 (25.1)	
Unknown	62 (3.1)	1067 (2.4)	1129 (2.4)	20360 (2.0)	

Data collected at baseline (date of diagnosis for HBV positive or last negative test for HBV negative) unless otherwise indicated. HIV: Human immunodeficiency virus; HCV: Hepatitis C virus; HBV: Hepatitis B virus; IDU: Injection drug use; OST: Opioid substitution therapy; Q: Quintile.

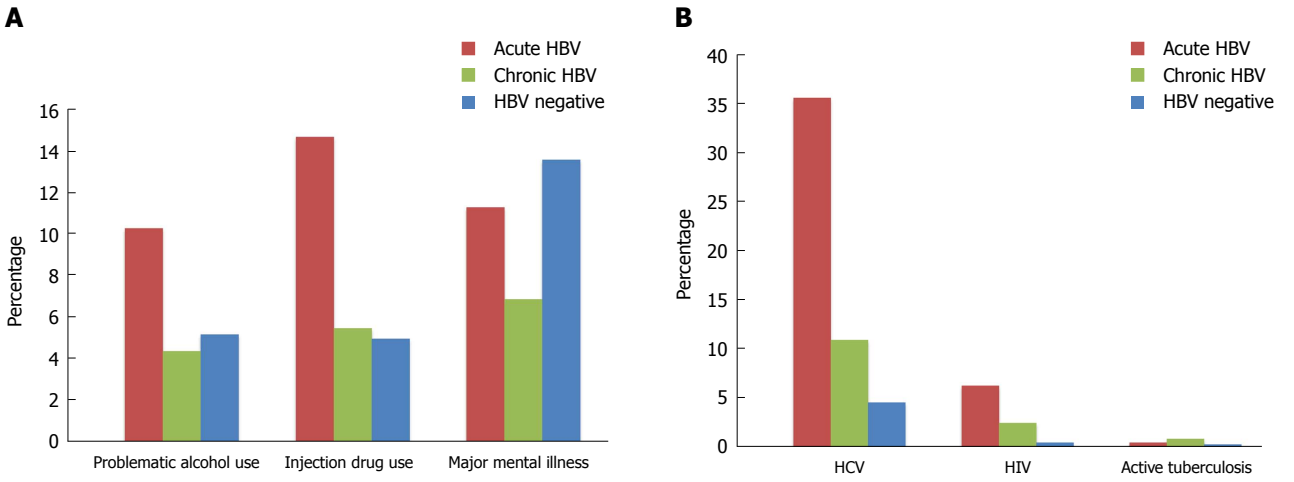

Figure 1 Comorbidities and co-infections in the British Columbia Hepatitis Testers Cohort, 1990-2015. A: Distribution of problematic alcohol use, injection drug use, and major mental illness by hepatitis B diagnosis; B: Distribution of co-infections by HBV diagnosis. HBV: Hepatitis B virus; HCV: Hepatitis C virus; HIV: Human immunodeficiency virus.

of chronic HBV infections (54.8%) were diagnosed among males. In contrast, HBV negative individuals were predominantly female (55.7%). The 1945-1964 birth cohort formed the majority of persons with either acute or chronic HBV infections (acute: 49.7%; chronic: 45.7%) but represented a smaller proportion of the HBV negative group (29.4%). Age at HBV testing or diagnosis was significantly lower among persons with acute HBV compared to those with chronic infections and HBV negative individuals (median age: 35 *vs* 41 and 39 years; $P < 0.001$). Additionally, females were more likely to be tested or diagnosed at a younger age compared to males (median age: 37 *vs* 41 years, $P < 0.001$).

The majority of HBV negative individuals (73.9%) and those with acute HBV (78.3%) were White. Chronic HBV infections, however, were more frequently diagnosed among East Asians (59.7%). Material deprivation was more common among HBV positive individuals than among HBV negative persons [Q5 (most deprived): acute HBV: 29.0%; chronic HBV: 27.5%; HBV negative: 18.3%]. In contrast, social deprivation was predominant within each HBV group, though highest among individuals with acute HBV [Q5 (most deprived): acute HBV: 36.8%; chronic HBV: 21.4%; HBV negative: 25.1%] (Table 1).

A larger proportion of persons with acute HBV experienced problematic alcohol use, illicit drug use, and injection drug use relative to those with chronic infections and HBV negative individuals (problematic alcohol use: 10.3%, 4.4%, 5.2%, $P < 0.001$; illicit drug use: 19.8%, 6.6%, 6.6%, $P < 0.001$; injection drug use: 14.7%, 5.5%, 5.0%, $P < 0.001$) (Figure 1A, Table 1). Conversely, major mental illness was most prevalent among HBV negative individuals (HBV negative: 13.6%; acute HBV: 11.3%, chronic HBV: 6.9%; $P < 0.001$).

HCV and HIV co-infections were more common among people diagnosed with acute HBV than among persons with chronic HBV and HBV negative individuals (HCV: 35.7%, 11.0%, 4.6%, $P < 0.001$; HIV: 6.3%, 2.5%, 0.5%, $P < 0.001$) (Figure 1B, Table 1). Active TB was less prevalent in the cohort, but more common among those with chronic HBV relative to HBV negative individuals and those with acute HBV (0.9%, 0.3%, 0.5%; $P < 0.001$). The cohort also consisted of 749 persons with HBV/HCV/HIV triple infection, who mostly had acute HBV infections (acute HBV: 4.1%, chronic HBV: 1.6%; HBV negative: 0.2%, $P < 0.001$) (Table 1).

Factors associated with acute and chronic HBV infection

In multivariable multinomial logistic regression models, we observed significant associations between acute or chronic HBV diagnosis and being male, age at HBV diagnosis or birth cohort, South and East Asian ethnicity, HCV or HIV infection, and injection drug use (Table 2 and Supplementary Table 3). However, the magnitude and direction of association for various variables differed for acute and chronic HBV.

South and East Asians had higher odds of acute or chronic HBV compared to Whites, with East Asians having 12 times greater odds of chronic HBV (ORacute: East Asian: 1.76, 95%CI: 1.53-2.02; South Asian: 1.66, 95%CI: 1.37-2.02; ORchronic: East Asian: 12.45, 95%CI: 12.15-12.77; South Asian: 1.26, 95%CI: 1.19-1.33) (Table 2).

Individuals with HCV or HIV infection had 5 times the odds acute HBV infection compared to their HBV negative counterparts (OR: HIV: 5.29, 95%CI: 4.30-6.51; HCV: 5.23, 95%CI: 4.65-5.87). For chronic infections, however, the odds of infection were slightly elevated among people with HIV co-infection (OR: 5.73, 95%CI: 5.29-6.20), but much lower among those infected with HCV (OR: 2.89, 95%CI: 2.77-3.01) (Table 2).

Injection drug use was associated with increased odds of both acute and chronic HBV (ORacute: 1.84, 95%CI: 1.57-2.17; ORchronic: 1.67, 95%CI: 1.58-1.77). In contrast, individuals with major mental

Table 2 Multivariable multinomial regression model for factors associated with acute or chronic hepatitis B virus in the British Columbia Hepatitis Testers Cohort, 1990-2015

	Adjusted OR (95%CI)	
	Acute HBV	Chronic HBV
Sex		
Male	2.28 (2.06-2.52)	1.43 (1.40-1.46)
Female	1.00	1.00
Age at HBV diagnosis (yr)		
< 25	2.38 (1.97-2.87)	0.89 (0.85-0.92)
25-34	2.62 (2.21-3.09)	1.09 (1.05-1.13)
35-44	1.82 (1.53-2.16)	1.19 (1.15-1.23)
45-54	1.49 (1.23-1.79)	1.22 (1.18-1.27)
55 +	1.00	1.00
Ethnicity		
East Asian	1.76 (1.53-2.02)	12.45 (12.15-12.77)
Other	0.88 (0.63-1.24)	3.06 (2.90-3.23)
South Asian	1.66 (1.37-2.02)	1.26 (1.19-1.33)
White	1.00	1.00
HCV		
Positive	5.23 (4.65-5.87)	2.89 (2.77-3.01)
Negative	1.00	1.00
HIV		
Positive	5.29 (4.30-6.51)	5.73 (5.29-6.20)
Negative	1.00	1.00
Active tuberculosis		
Yes	0.59 (0.32-1.09)	0.97 (0.85-1.10)
No	1.00	1.00
Problematic alcohol use		
Yes	0.87 (0.74-1.04)	1.02 (0.96-1.08)
No	1.00	1.00
Injection drug use		
Yes	1.84 (1.57-2.17)	1.67 (1.58-1.77)
No	1.00	1.00
Major mental illness		
Yes	0.73 (0.62-0.85)	0.74 (0.71-0.78)
No	1.00	1.00
Material deprivation quintile		
Q1 (most privileged)	1.00	1.00
Q2-4	1.06 (0.94 -1.21)	1.10 (1.06-1.13)
Q5 (most deprived)	1.35 (1.17 -1.55)	1.35 (1.30-1.39)
Social deprivation quintile		
Q1 (most privileged)	1.00	1.00
Q2-4	1.20 (1.04-1.39)	0.97 (0.95-1.00)
Q5 (most deprived)	1.56 (1.34-1.82)	1.00 (0.97-1.03)
Year of HBV diagnosis		
1990-1999	1.00	1.00
2000-2004	0.10 (0.09-0.12)	0.27 (0.26-0.28)
2005-2009	0.03 (0.03-0.04)	0.14 (0.13-0.14)
2010-2015	0.00 (0.00-0.01)	0.05 (0.05-0.06)

HIV: Human immunodeficiency virus; HCV: Hepatitis C virus; HBV: Hepatitis B virus; Q: Quintile.

illness had lower odds of either infection (ORacute: 0.73, 95%CI: 0.62-0.85; ORchronic: 0.74, 95%CI: 0.71-0.78). Material deprivation was also associated with increased odds of acute and chronic HBV infection (Q5: ORacute: 1.35, 95%CI: 1.17-1.55; ORchronic: 1.35, 95%CI: 1.30-1.39) (Table 2). Although social deprivation was associated with higher odds of acute HBV infection (ORacute: 1.56, 95%CI: 1.34-1.82), it was not significantly associated with a chronic infection (ORchronic: 1.00, 95%CI: 0.97-1.03).

In an additional model, composite variables (age at HBV diagnosis and injection drug use, and race/ethnicity and material deprivation) were used to determine joint effects on HBV infection (Figure 2, Table 3). In this model, there was a graded decrease in the odds of acute HBV with increasing age at diagnosis among PWID (OR: < 25 years: 7.55, 95%CI: 5.13-11.10; 25-34 years: 5.32, 95%CI: 4.08-6.94; 35-44 years: 2.98, 95%CI: 2.26-3.9; 45-54 years: 1.77, 95%CI: 1.19-2.62; 55 + years: 1.56, 95%CI: 0.78-3.10) (Figure 2A, Table 3). The opposite pattern was observed for chronic HBV infections, as the odds of chronic HBV increased with age at diagnosis (OR: < 25 years: 1.43, 95%CI: 1.18-1.73; 25-34 years: 1.72, 95%CI:

Table 3 Multivariable multinomial regression model (with interaction terms) for factors associated with acute or chronic hepatitis B virus in the British Columbia Hepatitis Testers Cohort, 1990-2015

	Adjusted OR (95% CI)	
	Acute HBV	Chronic HBV
Sex		
Male	2.30 (2.08-2.54)	1.43 (1.4-1.46)
Female	1.00	1.00
HCV		
Positive	5.22 (4.65-5.86)	2.88 (2.77-3.00)
Negative	1.00	1.00
HIV		
Positive	5.25 (4.26-6.47)	5.75 (5.31-6.22)
Negative	1.00	1.00
Active tuberculosis		
Yes	0.58 (0.31-1.08)	0.97 (0.86-1.10)
No	1.00	1.00
Problematic alcohol use		
Yes	0.90 (0.75-1.07)	1.01 (0.95-1.07)
No	1.00	1.00
Major mental illness		
Yes	0.72 (0.61-0.84)	0.75 (0.71-0.78)
No	1.00	1.00
Social deprivation quintile		
Q2-Q4	1.21 (1.05-1.40)	0.97 (0.94-1.00)
Q5 (most deprived)	1.58 (1.36-1.84)	0.99 (0.96-1.03)
Q1 (most privileged)	1.00	1.00
IDU*Age at HBV diagnosis (yr)		
IDU* < 25	7.55 (5.13-11.10)	1.43 (1.18-1.73)
IDU*25-34	5.32 (4.08-6.94)	1.72 (1.55-1.91)
IDU*35-44	2.98 (2.26-3.93)	1.87 (1.71-2.05)
IDU*45-54	1.77 (1.19-2.62)	2.06 (1.87-2.28)
IDU*55+	1.56 (0.78-3.10)	2.34 (2.05-2.65)
No IDU* < 25	2.23 (1.84-2.71)	0.90 (0.86-0.93)
No IDU*25-34	2.53 (2.13-3.01)	1.11 (1.07-1.15)
No IDU*35-44	1.84 (1.54-2.19)	1.21 (1.17-1.25)
No IDU*45-54	1.57 (1.30-1.90)	1.24 (1.20-1.28)
No IDU*55+	1.00	1.00
Ethnicity*Material Deprivation quintile		
East Asian*Material deprivation Q2-Q4	1.71 (1.37-2.12)	13.39 (12.82-13.98)
East Asian*Material deprivation Q5	2.61 (2.06-3.32)	15.98 (15.23-16.77)
East Asian*Material deprivation Q1	2.05 (1.49-2.81)	11.69 (11.07-12.33)
Other*Material deprivation Q2-Q4	0.97 (0.60-1.57)	3.32 (3.07-3.60)
Other*Material deprivation Q5	1.15 (0.59-2.26)	4.23 (3.78-4.74)
Other*Material deprivation Q1	0.89 (0.44-1.81)	2.62 (2.32-2.95)
South Asian*Material deprivation Q2-Q4	1.81 (1.35-2.42)	1.42 (1.30-1.54)
South Asian*Material deprivation Q5	2.44 (1.81-3.30)	1.41 (1.28-1.56)
South Asian*Material deprivation Q1	1.02 (0.48-2.19)	1.55 (1.32-1.82)
White*Material deprivation Q2-Q4	1.08 (0.94-1.25)	1.03 (0.98-1.08)
White*Material deprivation Q5	1.33 (1.13-1.56)	1.36 (1.29-1.43)
White*Material deprivation Q1	1.00	1.00
Year of HBV diagnosis		
1990-1999	1.00	1.00
2000-2004	0.10 (0.09-0.12)	0.27 (0.26-0.28)
2005-2009	0.03 (0.03-0.04)	0.14 (0.13-0.14)
2010-2015	0.00 (0.00-0.01)	0.05 (0.05-0.06)

IDU: Injection drug use; HIV: Human immunodeficiency virus; HCV: Hepatitis C virus; HBV: Hepatitis B virus; Q: Quintile.

1.55-1.91; 35-44 years: 1.87, 95%CI: 1.71-2.05; 45-54 years: 2.06, 95%CI: 1.87-2.28; 55 + years: 2.34, 95%CI: 2.05-2.65) (Figure 2B, Table 3). This model also demonstrated that the relatively high odds of chronic HBV among East Asians increased further with worsening material deprivation [OR: Material deprivation, Q1 (most privileged): 11.69, 95%CI: 11.07-12.33; Q2-Q4: 13.39, 95%CI: 12.82-13.98; Q5 (most deprived): 15.98, 95%CI: 15.23-16.77) (Figure 2B, Table 3).

DISCUSSION

In this large population based cohort study, we assessed over 1 million individuals for risk factors associated with acute and chronic HBV infection in British Columbia. This

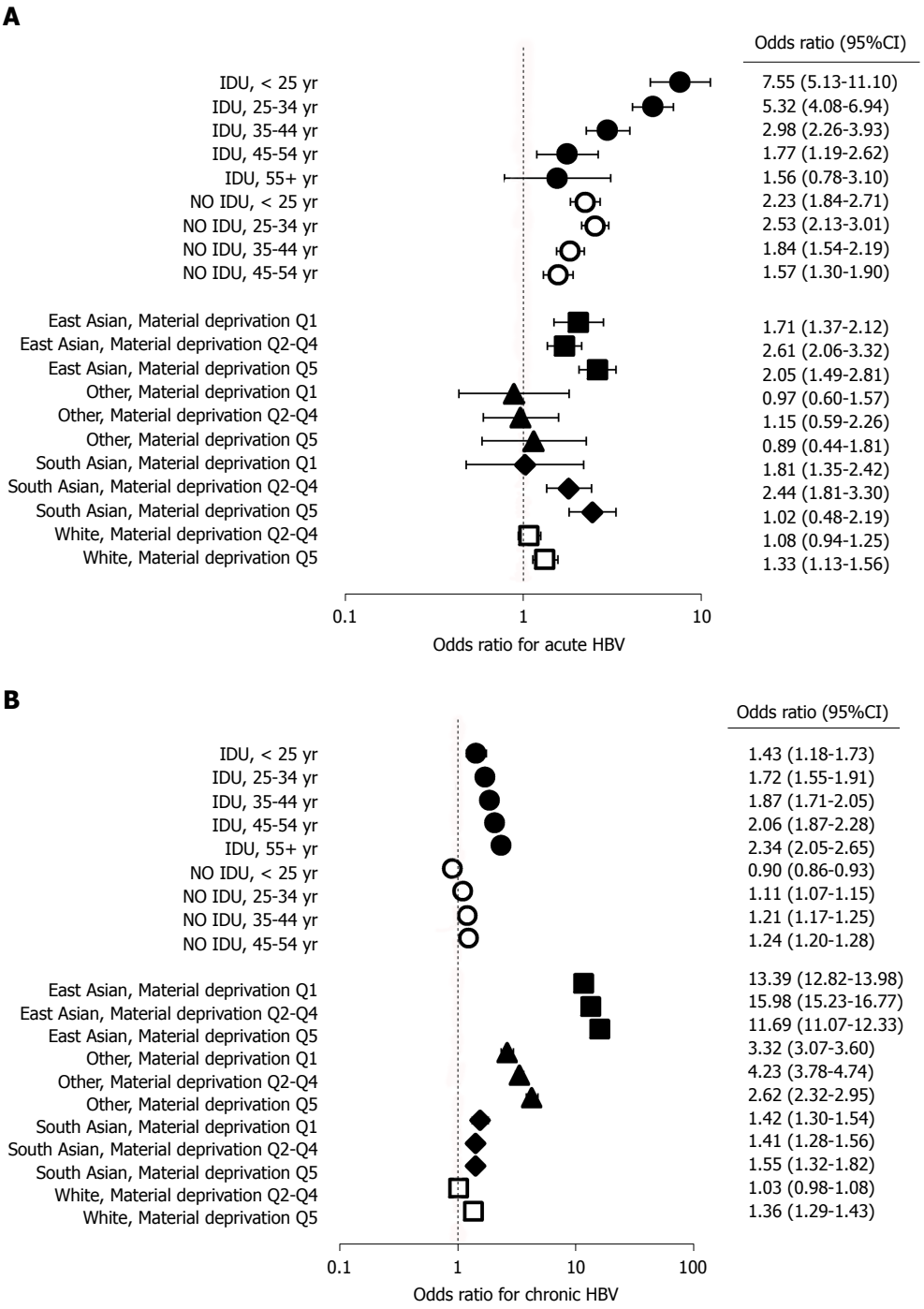

Figure 2 Odds ratios for interactions of factors associated with hepatitis B infection in the British Columbia Hepatitis Testers Cohort, 1990-2015. A: Acute hepatitis B infection; B: Chronic hepatitis B infection.

study shows two distinct patterns of risk factors among people diagnosed with acute and chronic infections. Acute HBV infections, indicative of new transmission events, occurred predominantly among males, persons aged between 25 and 34 years, White individuals, and socioeconomically disadvantaged persons. Problematic alcohol use, injection drug use, HIV and HCV co-infection were also common within this group. Individuals diagnosed with chronic HBV infection had similar characteristics, but were predominantly older, that is, aged between 35 to 44 years, and East Asian. Additionally, substance use and HIV or HCV co-infection were relatively low within this group. These findings highlight distinct risk patterns for individuals with acute and chronic HBV infections within the province and underscore the need for different strategies to prevent, diagnose and treat HBV within these groups.

Persons with acute and chronic infections had distinct co-infections and concurrent social condition profiles. Problematic alcohol use, illicit drug use and major mental illness were more common among individuals diagnosed with acute HBV than among

those with chronic HBV. Similarly, HBV/HCV, HBV/HIV and HBV/HCV/HIV co-infection occurred 3 times more frequently among individuals diagnosed with acute infections. These findings are consistent with those of studies in the United States and with observations made among seroconverters and chronically infected HCV positive individuals in the BC-HTC[17,18,23]. The HBV vaccination rate in BC is high and the incidence of acute HBV infections gradually declined to just 6 reported cases in 2015[7,24]. However, the current opioid epidemic may lead to localized HBV transmission among unvaccinated PWID, as was seen in suburban United States[18,25]. In our study, the odds of acute HBV were highest among younger persons and these age-dependent odds were greatly elevated by injection drug use among younger age groups. Younger PWID had 8-fold greater odds of being diagnosed with acute HBV than older non-PWID and up to 3 times the odds of their non-PWID of identical age. These findings demonstrate the interconnected nature of HBV, HCV and HIV infection, mental illness and alcohol/drug addiction and, therefore, highlight the need for a syndemic approach to significantly reducing new HBV infections. As the incidence of vaccine preventable HBV infection typically occurs among populations that are already affected by many social conditions and infections, such an approach should involve the integration of HBV prevention, screening and treatment programs with those of HIV, HCV, mental health and addiction programs.

Ethnicity was the most distinguishing factor between individuals diagnosed with acute and chronic HBV in BC. While over 75% of persons with acute HBV in the BC-HTC were White, 60% of chronically infected persons were East Asian. East Asian individuals had 12-fold greater odds of being diagnosed with chronic HBV than White persons, which worsened with declining socioeconomic status. Recent studies from the US on acute HBV infection also indicated that 57%-89% of acute infections were among the White population, while the majority of prevalent HBV infections were among people from the Asia-Pacific region[12,13,17,18]. Similarly, most individuals with chronic HBV in Australia in 2011 (38%) were born in the Asia-Pacific region[10]. These results mirror those observed in Canada (2007-2011), where rates of chronic HBV among foreign-born and non-White Canadians were estimated to be 4 and 5 times the national rates, respectively[4]. Low socioeconomic status within the East Asian community may act as a double-edged sword; increasing their risk of chronic HBV, while acting as a barrier to accessing health care. East Asian immigrants may also face additional barriers to health care, including language and cultural barriers, and insufficient information to make full use of the health care system[26-28]. Lack of awareness about HBV and its effects on health, a large proportion of unvaccinated individuals and low awareness of vaccination status may also affect HBV screening and treatment within this population[28,29]. In general, the asymptomatic nature of HBV infection leads to late diagnosis after complications have developed[1]. Studies have shown that immigrants in Canada are disproportionately affected by HCC and decompensated cirrhosis and have up to 5 times greater risk of death from these causes than their Canadian-born counterparts[14,30,31]. Therefore, screening for HBV within the East Asian population is vital for early diagnosis. As the East Asian population in BC faces the additional challenge of a high burden of TB, the integration of HBV screening with TB screening and treatment programs should create additional avenues for the identification of undiagnosed HBV carriers within the East Asian population in BC[32]. The ethnicity-related differences in acute and chronic HBV and related comorbidities and social conditions emphasize the need for differentiated programing for prevention, diagnosis and treatment by ethnicity.

The differences in HBV risk factor patterns among individuals diagnosed with acute and chronic HBV infection in BC are also mirrored by the distinct patterns of circulating HBV genotypes in both populations[33]. Between 2006 and 2012, genotype C viruses were predominantly isolated from individuals with chronic HBV, while genotype D viruses were prevalent among persons diagnosed with acute HBV in Canada[33]. As a high rate of chronic HBV diagnoses in Canada occur among immigrants form HBV endemic countries, these distinctions in circulating HBV genotypes may be related to the primary circulating strains in their home countries as well as to differing modes of acquisition[4,33]. In contrast to developed countries, major risk factors for HBV acquisition in HBV endemic developing countries include unsafe medical practices, for example, during circumcision and dental procedures[34,35]. These variations in circulating genotypes may have serious implications for disease progression and treatment outcomes among individuals diagnosed with either acute or chronic HBV in Canada[33,36]. Indeed, the elevated risk of HCC among African, Asian and Alaskan populations may be linked to circulating HBV genotypes[36]. This reinforces the need for ethnicity-based screening programs that go beyond the ongoing prenatal screening, and neonatal/preadolescent vaccination programs for immigrants originating from HBV endemic countries in certain parts of Canada[37].

The findings of this study should be interpreted in the light of following methodological considerations. As inclusion in the BC-HTC is dependent on either testing for HCV or HIV at the public health laboratory or being reported as confirmed cases of HIV, HCV, HBV or TB, individuals who test negative for HBV and do not meet any of the aforementioned criteria were excluded from our dataset. However, given the large number of HBV negative individuals in the study, we do not expect any major impact on our findings. Data on vaccination status, household contacts, sexual transmission and orientation were not available, which could have further enhanced our understanding of HBV epidemiology within the province. Additionally, ethnicity classifications

in our analysis may be affected by the variable sensitivity of Onomap in assigning Asian ethnicities[20]. Onomap was validated on a subset of the BC-HTC and showed low sensitivity for Filipinos. This may have resulted in the misclassification of East Asians from the Philippines as Whites and in the underestimation of the HBV in East Asian population. Previous studies have shown that foreign-born and Indigenous Canadians have a higher prevalence of chronic HBV relative to Canadian-born and non-Indigenous Canadians, respectively[5,30,38]. Similar findings have been reported in Australia and the United States[10,39]. Therefore, future analyses incorporating Indigenous and immigration status would provide additional insights for more tailored prevention and screening programs.

In summary, our findings show distinct characteristics of people diagnosed with acute and chronic infection in BC. Persons diagnosed with acute infection had a high level of substance use, co-infection with HIV or HCV, and were predominantly young White males. As acute HBV infection co-occurs with other infections and social conditions, a syndemic approach, where HBV prevention, screening, and treatment programs are integrated with those of other sexually transmitted and blood-borne infections as well as with mental health and addiction care would be an optimal approach for further reducing the incidence of acute HBV in the province and providing care to those diagnosed with acute HBV.

In contrast, the majority of chronic HBV infections in BC were diagnosed among East and South Asian individuals, who had very low levels of illicit drug and problem alcohol use, major mental illness and co-infection with HIV or HCV. Since traditional risk behavior and viral co-infection are less common among persons with chronic HBV infections, risk-based screening and prevention programs may impact only a fraction of such individuals. Given the asymptomatic nature of the disease and the grave health-related consequences of untreated HBV infection, organized screening programs are urgently needed to facilitate early diagnosis within the immigrant population in BC. Therefore, HBV screening and treatment programs focusing specifically on foreign-born East and South Asian population within BC would be necessary to have a significant impact on HBV-related disease burden within the province.

ARTICLE HIGHLIGHTS

Research background

Hepatitis B virus (HBV) affects approximately 200000 Canadians and 257 million people worldwide. In many developed countries, a relatively larger number of people are diagnosed with chronic HBV compared to acute HBV, each year. Chronic HBV infection is associated with 66% of the 1.34 million viral hepatitis-related deaths reported worldwide. It is responsible for a substantial disease burden from liver cancer and end-stage liver disease.

Research motivation

Data from Canada, the United States and other developed countries indicate that most chronic HBV infections are diagnosed among immigrants from HBV-endemic Asia-Pacific countries, while acute infections are predominant among White individuals. Persons diagnosed with acute and chronic HBV infections may differ with respect to demographics and risk behavior. These distinctions may have implications for interventions targeted at either population. Additionally, 49% of persons with chronic HBV and decompensated cirrhosis and 46% of those with HCC in British Columbia (BC) were diagnosed late in the course of their infections. Therefore, establishing the characteristics of individuals who are more likely to be infected with HBV could enhance the planning of prevention and screening programs to further reduce late diagnoses within the province.

Research objectives

In this study, we describe the characteristics of individuals diagnosed with acute and chronic HBV infections and identify the factors associated with HBV infection within the BC Hepatitis Testers Cohort (BC-HTC). We are unaware of any study comparing large population level data for both acute and chronic HBV. Study findings should inform prevention and screening programs within BC.

Research methods

We used data from the BC Hepatitis Testers Cohort (BC-HTC), which includes all individuals tested for HCV or HIV and those diagnosed with HBV or TB in BC since 1990. These data were integrated with prescription drug, medical visit, hospitalization and mortality data. HBV cases were classified as acute or chronic in accordance with provincial guidelines. We compared characteristics of individuals by HBV infection group (acute, chronic and negative). Factors associated with acute or chronic HBV infection were assessed with multivariable multinomial logistic regression models in comparison with the HBV negative group.

Research results

46498 of the 1058056 eligible BC-HTC participants were diagnosed with HBV infection; 95.7% with chronic infections at HBV diagnosis. Acute HBV infections, indicative of new transmission events, were diagnosed predominantly among males, persons aged between 25 and 34 years, White individuals, and socioeconomically disadvantaged persons. Problematic alcohol use, injection drug use, HIV and HCV co-infection were also common within this group. Individuals diagnosed with chronic HBV infection were predominantly older and East Asian. Additionally, substance use and HIV or HCV co-infection were relatively low within this group. Relative to Whites, East Asians had 12 times greater odds of being diagnosed with chronic HBV infection. These odds increased with increasing socioeconomic deprivation.

Research conclusions

These findings highlight distinct risk patterns for individuals with acute and chronic HBV infections and underscore the need for different strategies to prevent, diagnose and treat HBV within these groups. Optimal care for acute HBV would require the integration of HBV prevention, screening, and treatment programs with programs for mental health, addiction and other blood-borne infections. Managing chronic HBV, on the other hand, may require screening programs focusing on at-risk ethnic groups, including foreign-born East and South Asians with low prevalence of traditional risk factors, for early diagnosis and treatment initiation.

Research perspectives

We found clear differences in the characteristics of individuals diagnosed with acute and chronic HBV in BC. Consequently, we propose two distinct interventions for the management of acute and chronic HBV in the province: the integration of HBV-related public health programs with those of blood borne infection programs and mental health services to provide optimal care for populations at risk for acquiring acute HBV, and the implementation of targeted screening programs for early diagnosis among ethnic groups at risk for chronic HBV.

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All inferences, opinions, and conclusions drawn in this publication are those of the authors, and do not necessarily reflect the opinions or policies of the (British Columbia) Ministry of Health.

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ORIGINAL ARTICLE

Retrospective Cohort Study

Role of relevant immune-modulators and cytokines in hepatocellular carcinoma and premalignant hepatic lesions

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Abstract

AIM

To assess the levels of different immune modulators in patients with hepatocellular carcinoma (HCC), in relation to other hepatic diseases.

METHODS

Eighty-eight patients were included in the current study and represented patients with HCC (20), liver cirrhosis (28) and chronic hepatitis (CH; 25), and normal controls (NC; 15). Peripheral blood was isolated for immunophenotyping of active myeloid dendritic cells (mDCs; CD1c and CD40), mature inactive myeloid cells (CD1c and HLA), active plasmacytoid cells (pDCs; CD303 and CD40), mature inactive pDCs (CD30 and HLA), active natural killer (NK) cells (CD56 and CD161), active NK cells (CD56 and CD314) and inactive NK cells (CD56 and CD158) was done by flow cytometry. Serum levels of interleukin (IL)-2, IL-10, IL-12, IL-1β, interferon (IFN)-α, IFN-γ and tumor necrosis factor (TNF)-αR2 were assessed by ELISA.

RESULTS

Active mDCs (CD1C+/CD40+) and inactive mDCs (CD1c+/HLA+) were significantly decreased in HCC patients in relation to NC ($P < 0.001$). CD40+ expression on active pDCs was decreased in HCC patients ($P < 0.001$), and its level was not significantly changed among other groups. Inactive pDCs (CD303+/HLA+), inactive NKs (CD56+/CD158+) and active NKs (CD56+/CD161+) were not statistically changed among the four groups studied; however, the latter was increased in CH ($P < 0.05$). NKG2D was statistically decreased in HCC, CH and cirrhosis ($P < 0.001$), and it was not expressed in 63% (12/20) of HCC patients. There was significant decrease of IL-2, IFN-α and IFN-γ ($P < 0.001$), and a significant increase in IL-10, IL-1β, and TNF-αR2 ($P < 0.01$, $P < 0.001$ and $P < 0.001$; respectively) in HCC patients. There was inverted correlation between IL-12 and IL-1β in HCC ($r = -0.565$, $P < 0.01$), with a strong correlation between pDCs (CD303+/CD40+) and NKs (CD56+/CD161+; $r = 0.512$, $P < 0.05$) as well as inactive mDCs (CD1c+/HLA+) and inactive NK cells (CD56+/CD158+; $r = 0.945$, $P < 0.001$).

CONCLUSION

NKG2D, CD40, IL-2 and IL-10 are important modulators in the development and progression of HCC.

Key words: Hepatocellular carcinoma; Hepatitis C virus; NKG2D; CD40; Interleukin-2; Interleukin-10; Myeloid dendritic cells; Plasmacytoid cells; Natural killer cell; Cytokines

Core tip: We assessed the levels of different immune modulatory cytokines and innate immune cells as natural killer (NK) cells and dendritic cells (DCs) in patients with disease progression of hepatocarcinogenesis. Our results showed significant down-regulation in active mDCs and pDCs expressing CD40 as well as NK cells expressing NKG2D. The expression of NKG2D on NKs was not expressed in 63% of hepatocellular carcinoma (HCC) patients. Also, there was significant decrease of interleukin (IL)-2, interferon-α and interferon-γ, and a significant increase in IL-10, IL-1β, and TNF-αR2 in HCC patients. These factors could be implicated in the pathogenesis of HCC, and represent attractive targets for therapy in chronic hepatitis C virus hepatitis and HCC.

Zekri AN, El Deeb S, Bahnassy AA, Badr AM, Abdellateif MS, Esmat G, Salama H, Mohanad M, El-dien AE, Rabah S, Abd Elkader A. Role of relevant immune-modulators and cytokines in hepatocellular carcinoma and premalignant hepatic lesions. *World J Gastroenterol* 2018; 24(11): 1228-1238 Available from: URL: http://www.wjgnet.com/1007-9327/full/v24/i11/1228.htm DOI: http://dx.doi.org/10.3748/wjg.v24.i11.1228

INTRODUCTION

Hepatocellular carcinoma (HCC) is a major health problem worldwide. It is the sixth most common cancer and the second leading cause of cancer-related death in the world[1]. In Egypt, HCC ranks the first among cancers in males (33.6%), and the 2nd in females after breast cancer (13.5%)[2]. This high incidence is attributed to the high prevalence of hepatitis C virus (HCV) infection, especially of genotype IV, in Egypt[3]. Despite advances in treatment modalities, sorafenib is still the only treatment approved by the Food and Drug Administration (FDA) for HCC; however, it extends the overall survival by 3 mo only. Hence, it is crucial to understand the underlying biological and immunological changes in HCC and to develop new treatment modalities based on these data[4,5].

The body's immune defense mechanism(s) plays an important role in the inhibition or progression of cancer. However, tumors can escape immune surveillance by producing a local and/or systemic immune-suppressive environment[6]. Although the underlying molecular mechanisms are not yet fully clear, recent studies show that the immune response in cancer patients is usually down-regulated by immunosuppressive cells, mainly T regulatory cells (Tregs) and myeloid-derived suppressor cells (MDSCs), which suppress the immune system and promote immunologic tolerance[7]. These inhibitory cells accumulate during advanced cancer stages. They are involved in chronic inflammation and tumor progression and they can inhibit many immune cells

(*e.g.*, CD4+, CD8+, natural killer (NK) cells)[8]. They also secrete many immune-suppressive cytokines, such as interleukin (IL)-6, IL-10, and transforming growth factor (TGF)-β, creating a tolerogenic and suppressive environment[9].

The NK cells represent 25%-30% of the human liver lymphocytes, compared to 10%-20% of the total peripheral blood lymphocytes[10]. They are the main effector cells of innate immunity, which play an important role in tumor surveillance. The NK cells achieve their functions through the release of cytolytic mediators, such as perforin and granzymes, or the induction of apoptosis *via* the expression of tumor necrosis factor (TNF) ligands and interferon (IFN)-γ[11]. Activation of NK cells is strictly regulated by a balance between activating and inhibitory signals, whereas the inhibitory signals are mainly induced by receptors for MHC class I molecules (KIRs, CD94/NKG2A). The activating signals are mainly achieved through NKG2D, a C-type lectin-like receptor which is expressed on NK cells, γδ T cells and CD8+ T cells[12,13]. NKG2D is responsible for detection and elimination of transformed cells *via* binding to MICA (MHC class I–related chain), MICB and the UL16-binding proteins[14]. Activation of NKG2D provides unique costimulation to antigen-specific CD8 T cells that is non-redundant to CD28 costimulation[15].

Dendritic cells (DCs) are the most professional antigen-presenting cells which respond rapidly to microenvironment signals. Upon maturation by tumor antigen, cross-priming to T and B lymphocytes occurs to produce antitumor adaptive immune responses[16]. There are two subsets of DCs: the myeloid dendritic cells (mDCs), which are characterized by their ability to produce IL-12; and, the plasmacytoid cells (pDCs), which are responsible for the production of over 95% of type-I IFNs in response to viral infection[17]. On activation, the CD8+ cytotoxic T cells (CTLs) eliminate tumor cells by the production of IFN-γ, whereas CD4+ T helper cells stimulate B cells to support the cytotoxic and humoral immune response[18].

IL-1β is a regulatory cytokine produced by tumor and immune cells, such as MDSCs. It also induces COX-2 expression, which prevents maturation and activation of antigen presenting cells at the tumor site[19].

In the current study, we assessed the role(s) of different immune regulatory cells and cytokines in the development and progression of chronic liver disease (chronic active hepatitis, cirrhosis and HCC) compared to the normal healthy volunteers. We believe that this could allow for better understanding of different pathways implicated in the development and progression of hepatocarcinogenesis, and could also help in designing new immune-therapeutic drugs.

MATERIALS AND METHODS

Patients

The current study included 88 patients who attended the medical oncology clinics of the National Cancer Institute (NCI), Cairo University during the period from 2014 to 2016. Patients were divided into four groups: HCC (20 patients - G1), liver cirrhosis (28 patients - G2), chronic hepatitis (CH; 25 patients - G3) and normal healthy volunteers as a control group (NC; 15 persons, matched for age and sex - G4). The Ethical Committee of the NCI, Egypt approved the study protocol and an informed consent was obtained from all participants before enrollment in the study.

Characterization of immune cells by flow cytometry

Peripheral blood samples were isolated from patients and healthy subjects for immunophenotyping of active mDCs (CD1c and CD40), mature inactive myeloid cells (CD1c and HLA), active pDCs (CD303 and CD40), mature inactive pDCs (CD303 and HLA), active NK cells (CD56 and CD161), active NK cells (CD56F CD314) and inactive NK cells (CD56 and CD158) using the specific monoclonal antibodies according to manufacturer's protocols (all monoclonal antibodies were purchased from Invitrogen, eBioscience, San Diego, CA, United States). Samples were then analyzed by flow cytometry (FACSCaliber; Becton and Dickenson, Franklin Lakes, NJ, United States).

Detection of cytokine levels by ELISA

Serum levels of IL-2, IL-10, IL-12, IL-1β, IFN-α, IFN-γ and TNF-αR2 were measured by the ELISA technique, according to manufacturer's instructions (Invitrogen, eBioscience), using ELISA reader (Sunrise; Tecan, Mannedorf, Switzerland).

Statistical analysis

Data were expressed as mean ± SE of mean for continuous variables. Comparison between cytokine levels and immune cells were performed using one-way analysis of variance followed by Tukeys' post hoc test. Pearson's correlation was used to assess the strength of correlation between different variables and a two-tailed *P*-value was determined. The *P*-value was considered significant at < 0.05. All statistical analyses were performed using SPSS, version 22 (IBM SPSS, Armonk, NY, United States).

RESULTS

Patients' characteristics

The mean age of the patients included in the current study was 57.1 ± 4.76 for HCC, patients, 51.29 ± 9.03 for liver cirrhosis patients, 49.64 ± 7.2 for CH patients and 42.9 ± 10.2 for NC (Table 1).

Flow cytometry data

There was a significant decrease in active mDCs (CD1C+/CD40+) and inactive mDCs (CD1c+/HLA+) in HCC patients compared to the NC group ($P < 0.001$), as well as in active mDCs (CD1C+/CD40+) in cirrhotic patients compared to CH patients ($P = 0.003$; Table

Table 1 Patient characteristics

	Normal	Chronic hepatitis	Cirrhosis	HCC
Sex				
Male	11 (73.3)	9 (36.0)	19 (67.9)	19 (95.0)
Female	4 (26.7)	16 (64.0)	9 (32.1)	1 (5.0)
Age, mean ± SD	42.9 ± 10.2	49.64 ± 7.2	51.29 ± 9.03	57.1 ± 4.76
HCV	+ve	+ve	+ve	+ve
Total	15	25	28	20

Data are presented as *n* (%), unless otherwise specified. HCC: Hepatocellular carcinoma; HCV: Hepatitis C virus.

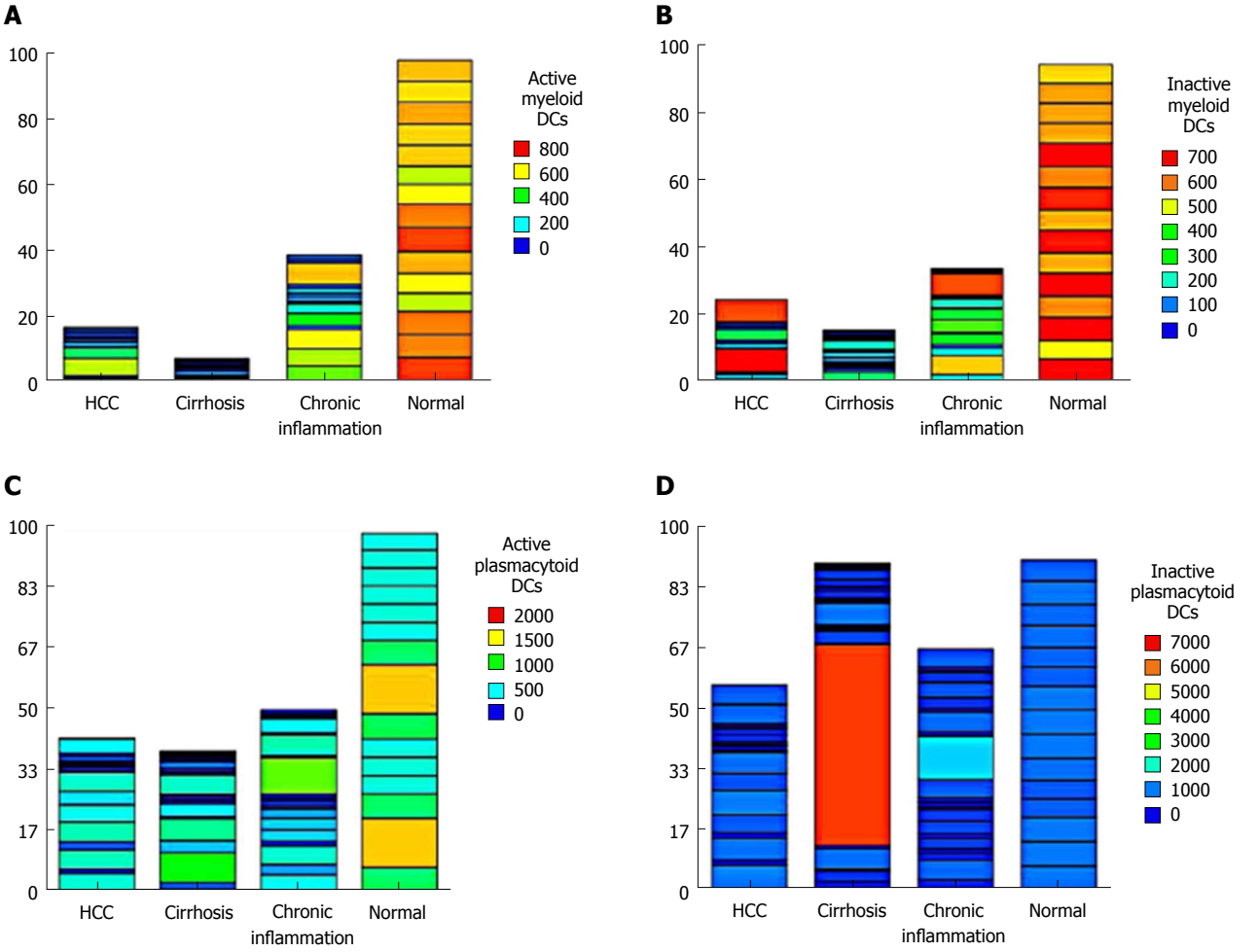

Figure 1 Heatmap of the differential levels of (A) active myeloid dendritic cells, (B) inactive myeloid dendritic cells, (C) active plasmacytoid cells, and (D) inactive plasmacytoid cells in the four groups studied. HCC: Hepatocellular carcinoma.

2). Also, the expression level of CD40+ on active pDCs (CD303+) was significantly decreased in HCC compared to the NC group ($P < 0.001$). However, there was no significant difference from the other groups (cirrhosis and CH). Meanwhile, the level of inactive pDCs (CD303+/HLA+) did not differ significantly between the four groups studied (Table 2, Figure 1).

The level of the nonactive NK cells (CD56+/CD158+) did not differ significantly among groups. However, the active NK cells (CD56+/CD161+) showed a significant increase in the CH group ($P < 0.05$), whereas the level of active NK cells (CD56+/CD314+) was statistically decreased in HCC, CH and cirrhotic patients compared to the NC group ($P < 0.001$), indicating an important role of these cells in the pathogenesis of HCC (Table 2). The expression of all cell types in each patient showed that active NK cells (CD56+/CD314+) were not expressed in 63% (12/20) of HCC patients. However, 2 patients showed unexplainable high variability (Figure 2).

We also found a significant decrease in the active NK cells (CD56+/CD314+; $P < 0.05$) and active pDCs (CD303+, CD40+; $P < 0.05$) compared to the inactive NK cells (CD56+/CD158+) and inactive pDCs (CD303+/HLA+) respectively in HCC. To the contrary,

Table 2 Differential counts of nature killer cells and dendritic cells among the studied groups

	Normal	Chronic hepatitis	Cirrhosis	HCC	*P* value
Active mDCs (CD1C+/CD40+)	652.4 ± 15.9^{A1}	154.1 ± 40.9^{B}	25.5 ± 4.3^{C}	82.8 ± 28.7^{BC}	< 0.001
Inactive mature mDCs (CD1c+/HLA+)	627.1 ± 14.8^{A}	134.4 ± 37.1^{B}	54.7 ± 12.3^{B}	121.8 ± 44.8^{B}	< 0.001
Active pDCs (CD303+/CD40+)	782.2 ± 89.6^{A}	237.8 ± 56.2^{B}	164.1 ± 45.9^{B}	251 ± 55.9^{B}	< 0.001
Inactive mature pDCs (CD303+/HLA+)	727.5 ± 18.7^{A}	318.5 ± 61.4^{A}	385.7 ± 233.3^{A}	339.2 ± 67.5^{A}	0.339
Active NK cells (CD56+/CD161+)	535 ± 83.4^{AB}	1166.3 ± 426.3^{B}	399.8 ± 139.8^{AB}	145.5 ± 30.2^{A}	< 0.05
Inactive NK cells (CD56+/CD158+)	710.3 ± 127.9^{A}	656.3 ± 235.7^{A}	462.6 ± 105.3^{A}	456.7 ± 218.1^{A}	0.708
Active NK cells (CD56+/CD314+)	585.5 ± 35.9^{A}	188.5 ± 69.4^{B}	123.5 ± 47.6^{B}	196 ± 110.8^{B}	< 0.001

Data are presented as mean ± SEM. [1]Groups having the same letters in the same variable are statistically insignificant. HCC: Hepatocellular carcinoma; mDCs: Myeloid dendritic cells; NK: Natural killer; pDCs: Plasmacytoid cells.

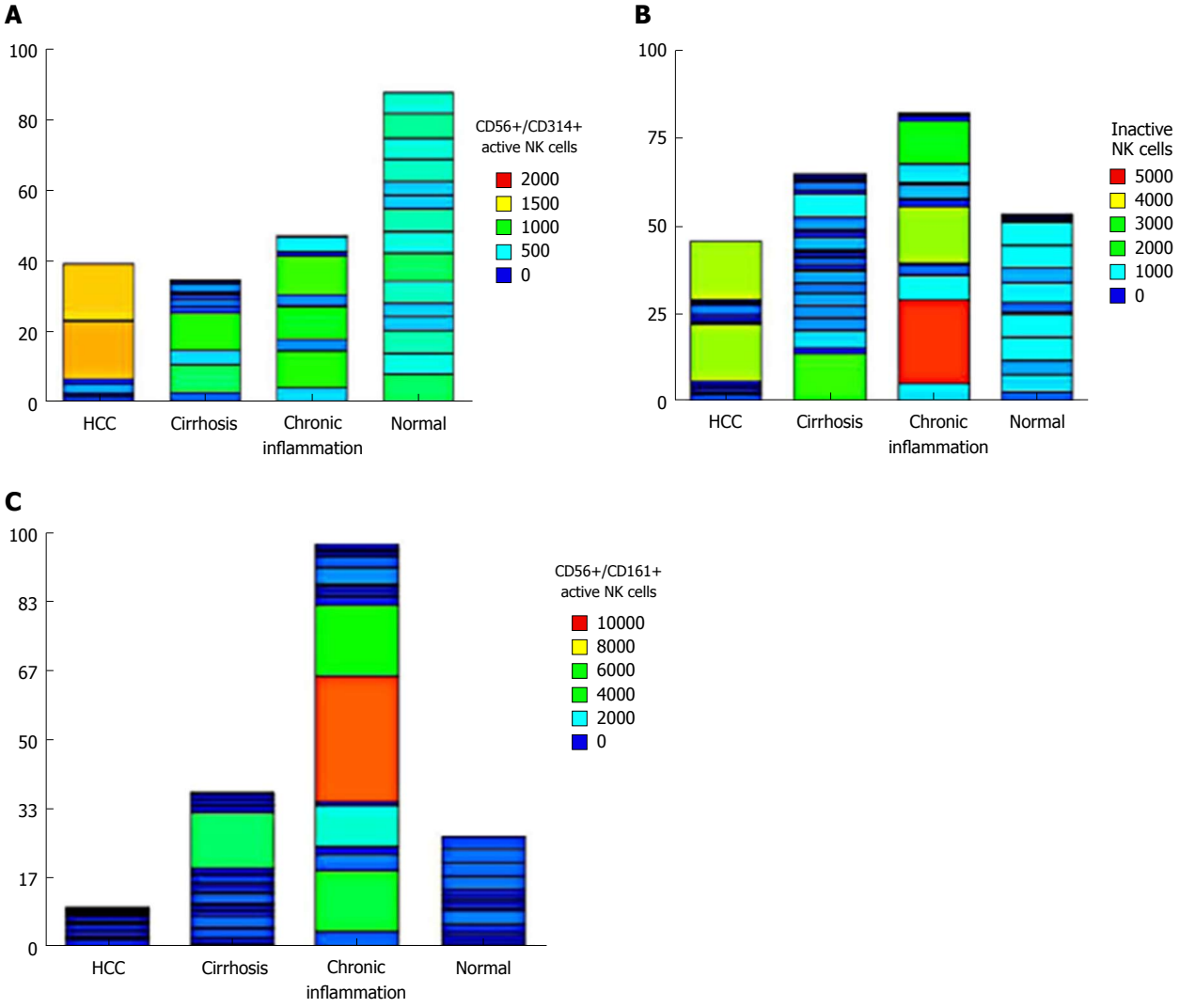

Figure 2 Heatmap of the differential levels of (A) active natural killer cells (CD56+/CD314+), (B) inactive natural killer cells (CD56+/CD158+), and (C) natural killer cells (CD56+/CD161+) in the four groups studied. HCC: Hepatocellular carcinoma.

the NK cells (CD56+/CD161+) and the mDCs did not differ significantly between groups (Figure 3).

Serum levels of cytokines

There was a significant decrease in serum levels of IL-2, IFN-α and IFN-γ in HCC patients compared to the NC group ($P < 0.001$), and a significant increase in serum levels of IL-10, IL-1β and TNF-αR2 in HCC compared to the NC group ($P < 0.01$, $P < 0.001$ and $P < 0.001$ respectively). However, there was no statistically significant change in the serum level of IL-12 ($P = 0.393$) among the four groups studied (Table 3, Figure 4).

Significant correlation was also reported in HCC patients between (1) serum level of IL-12 and IL-1β ($r = -0.565$, $P < 0.01$), (2) expression of CD40 on

Table 3 Levels of the studied cytokines among different studied groups

	Normal	Chronic hepatitis	Cirrhosis	HCC	*P* value
IL-2	22.13 ± 2.21^{A1}	16.28 ± 2.12^{B}	11.36 ± 0.95^{C}	10.24 ± 0.64^{C}	< 0.001
IL-10	74.8 1 ± 1.34^{A}	98.46 ± 4.01^{AB}	104.11 ± 9.35^{B}	132.1 ± 14.26^{C}	< 0.01
IL-12	0.06 ± 0.01^{A}	0.09 ± 0.01^{A}	0.15 ± 0.06^{A}	0.08 ± 0.01^{A}	0.393
IL-1β	3.46 ± 0.15^{A}	6.48 ± 0.58^{A}	6.71 ± 2.39^{A}	21.38 ± 4.88^{B}	< 0.001
IFN-α	31.20 ± 0.67^{A}	32.28 ± 1.44^{A}	21.31 ± 1.01^{B}	18.49 ± 1.37^{B}	< 0.001
IFN-γ	20.47 ± 0.49^{A}	29.75 ± 0.68^{B}	17.51 ± 1.18^{C}	10.46 ± 0.56^{D}	< 0.001
TNF-αR2	6.03 ± 0.07^{A}	13.25 ± 1.73^{A}	10.09 ± 0.97^{A}	47.42 ± 6.74^{B}	< 0.001

Data are presented as mean ± SEM. [1]Groups having the same letters in the same variable are statistically insignificant. HCC: Hepatocellular carcinoma; IFN: Interferon; IL: Interleukin; TNF: Tumor necrosis factor.

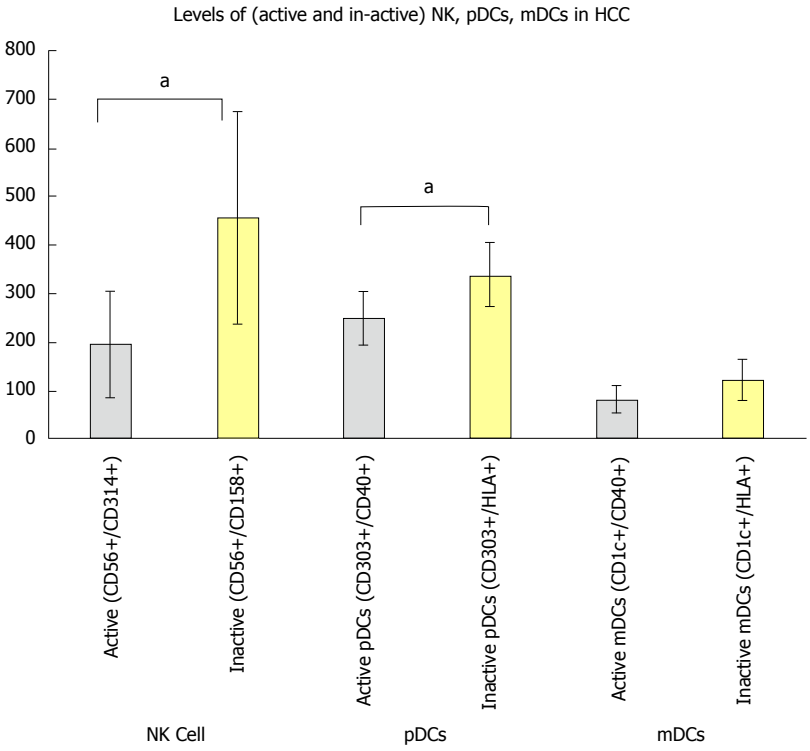

Figure 3 Balance between active and inactive natural killer cells, plasmacytoid cells, and myeloid dendritic cells in hepatocellular carcinoma patients in relation to the normal group. [a]*P* < 0.05. HCC: Hepatocellular carcinoma; mDCs: Myeloid dendritic cells; NK: Natural killer; pDCs: Plasmacytoid cells.

pDCs (CD303+, CD40+) and active NK cells (CD56+/CD161+; $r = 0.512$, $P < 0.05$), and (3) inactive mDCs (CD1c+/HLA+) and inactive NK cells (CD56+/CD158+; $r = 0.945$, $P < 0.001$) (Figure 5A-C).

Patients with chronic active hepatitis showed strong significant correlation between active NK cells (CD56+/CD161+) and both IL-2 and IL-12 ($r = 0.549$, $P = 0.004$ and $r = 0.660$, $P < 0.001$ respectively). Strong correlations were also reported between the expression of CD314 on NK cells and IL-2 ($r = 0.548$, $P < 0.01$) and active mDCs CD1c+/CD40+ ($r = 0.577$, $P = 0.003$). The level of IL-10 was strongly correlated with (1) increased inactive NK cells (CD56+/CD158+; $r = 0.604$, $P = 0.001$) and (2) inactive mDCs CD1c+/HLA+ ($r = 0.588$, $P = 0.002$; Table 4).

DISCUSSION

Identification of inflammatory mediators which promote or prevent the progression of cancer depends on the tumor microenvironment. Thus, this study was designed to assess the levels of different immune modulators and cytokines in patients with HCC, nonmalignant hepatic diseases (*e.g.*, CH and liver cirrhosis) compared to a normal group. This would enable us to study different pathways that help in identifying immune cells or biomarkers that may be implicated in hepatocellular carcinogenesis. This may also explain why liver disease progresses more rapidly in some patients than in others and provides additional therapeutic modalities for viral infections and HCC (immunotherapy).

One of the most powerful antigen presenting cells is the DC, which plays an important role in antitumor immune response. Our data revealed that active and inactive mDCs were significantly decreased in HCC patients compared to the NC group ($P < 0.001$); however, the level of active mDCs (CD1C+/CD40+) was significantly decreased in cirrhosis compared to CH

Table 4 Correlation between dendritic cell and nature killer cell count and the levels of studied cytokines in chronic active hepatitis

	IL-2	IL-12	IL-10	Active mDCs (CD1c+/CD40+)
Active NK cells (CD56+/CD161+)	$r = 0.549^{b}$ $P = 0.004$	$r = 0.660^{b}$ $P < 0.001$		
Active NK cells (CD56+/CD314+)	$r = 0.548^{a}$ $P = 0.005$			$r = 0.577^{b}$ $P = 0.003$
Inactive NK cells (CD56+/CD158+)			$r = 0.604^{b}$ $P = 0.001$	
Inactive mDCs (CD1c+/HLA+)			$r = 0.588^{b}$ $P = 0.002$	

$^{a}P < 0.05$ (2-tailed); $^{b}P < 0.01$ (2-tailed). IL: Interleukin; mDCs: Myeloid dendritic cells; NK: Natural killer.

patients ($P = 0.003$). These data are consistent with Obermajer *et al*[20], who reported that the inhibition of DC maturation in HCC may prove to be a critical feature of tumor escape, as well as with another two recent studies which reported numerical and functional defects in the peripheral DCs in hepatitis B- and C-associated HCC patients[21,22].

The pDC (CD303+, CD40+) represents the major cell responsible for antiviral immunity, and the main source of IFN-α in the body. Our data revealed that the expression level of CD40+ on active pDCs was significantly decreased in HCC patients compared to NC ($P < 0.001$). Meanwhile, inactive pDCs (CD303+/HLA+) did not change significantly among the four groups studied. This was also confirmed by the significant decrease of serum IFN-α, and the increase of IL-10 in HCC patients compared to the NC group ($P < 0.001$ and $P < 0.01$ respectively). This is in concordance with previous reports showing that IL-10 inhibits IFN-α production and promotes apoptosis of human pDCs[23,24]. Moreover, Gonzalez-Carmona *et al*[25] demonstrated that cancer patients had low CD40 expression on DCs or CD40L on T cells, which is associated with an impaired immune response, and Shurin *et al*[26] reported that the tumor-derived IL-10 inhibits CD40 expression on DCs and DC precursors and suppresses their maturation and function.

NK cells play an important role in controlling viral hepatitis, liver fibrosis and carcinogenesis. Their functions are modulated by different classes of receptors present on its surface[27]. Thus, it is essential to identify the roles of these cells in different stages of HCC development. Among these, we investigated the role of the killer cell immunoglobulin-like receptors (KIR; CD158), NKR-P1A (CD161) and NKG2D (CD314) in HCV CH patients, and in liver cirrhosis and its progression to HCC.

Our results revealed that the expression of the inhibitory KIR (CD158+) receptors and the activating NKR-P1A (CD161) on NK cells was not statistically changed between HCC and NC ($P = 0.827$ and $P = 0.788$ respectively). Thus, these two pathways may not be involved in the pathogenesis of HCC. However, the latter were statistically increased in CH ($P < 0.05$). This result was supported by our finding of a strong statistical correlation between the expression of CD161 on NK cells and IL-12 ($r = 0.77$, $P < 0.001$) in patients with HCV chronic active hepatitis. This illustrates the role of NK cells (CD56+/CD161+) in viral hepatitis.

On the other hand, the level of active NK cells expressing NKG2D were statistically decreased in HCC, CH and cirrhosis in relation to the NC group ($P < 0.001$), which could indicate the implication of these cells in the progression of HCC. This was clarified by representing its expression in each patient, it was not expressed in 63% (12/20) of HCC patients, emphasizing its important role in HCC development. These data were consistent with Lanier *et al*[28]. Yoon *et al*[29] reported that direct interaction between human NK cells and HCV-infected hepatoma cells down-regulates NK cell NKG2D expression and effector function with diminished IFN-γ production. Another study carried out by Kamiya *et al*[30] demonstrated that NKG2D was an important mediator of antiHCC activity. On the contrary, Oliviero *et al*[31] found a decreased percentage of NK cells expressing the inhibitory receptor KIR3DL1 and a concomitant increase in the proportion of $NKG2D^{+}$ NK cells in Italian patients with chronic HCV. This could be explained by racial and environmental differences with different viral genotypes, and also according to the clinical stage.

Our results regarding DCs and NK cells were supported by assessment of the cytokine serum levels in the four groups studied. We found that there was a significant increase in IL-1β ($P < 0.001$) in HCC patients compared to the NC group. However, there was no statistically significant change in the serum level of IL-12 ($P = 0.393$) among the four groups studied. Furthermore, we had a significant increase in the serum level of IL-10 in the cirrhosis and HCC groups compared to the NC group. This is consistent with Accapezzato *et al*[32]. IL-10 is important for allowing liver NK cells to maintain immune-tolerant states[33]. Its significant increase in HCC patients is responsible for the abnormal tolerant NK cells that cannot eliminate infected or transformed cells, which leads to immune evasion.

In the current study, there was a significant decrease of the serum levels of IFN-γ in chronic HCV hepatitis, cirrhosis and HCC patients in comparison to NC ($P < 0.001$). This is in agreement with Li *et al*[34],

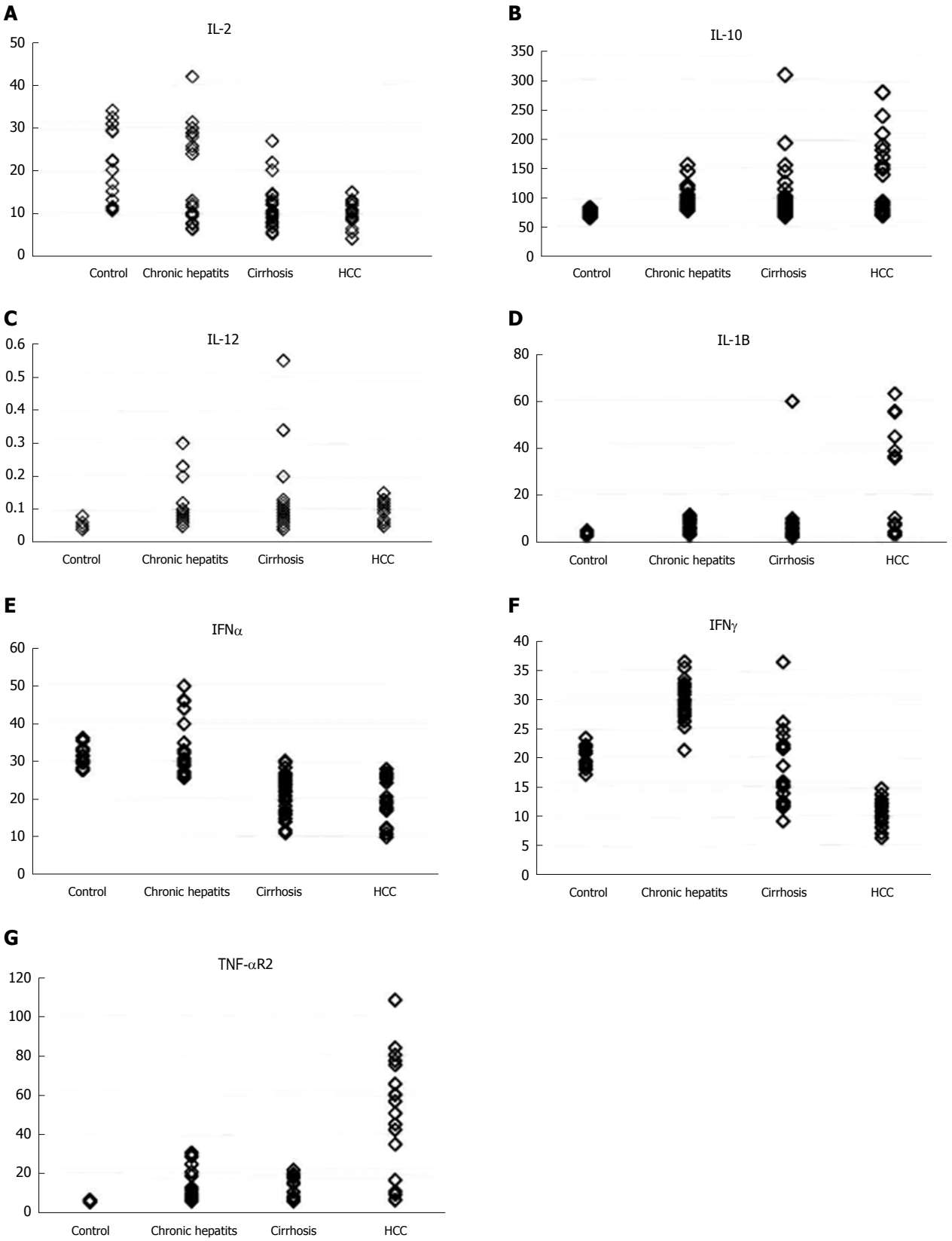

Figure 4 Different levels of serum cytokines in the four groups studied. A: IL-2; B: IL-10; C: IL-12; D: IL-1B; E: IFN-α; F: IFN-γ; G: TNF-αR2. IFN: Interferon; IL: Interleukin; TNF: Tumor necrosis factor.

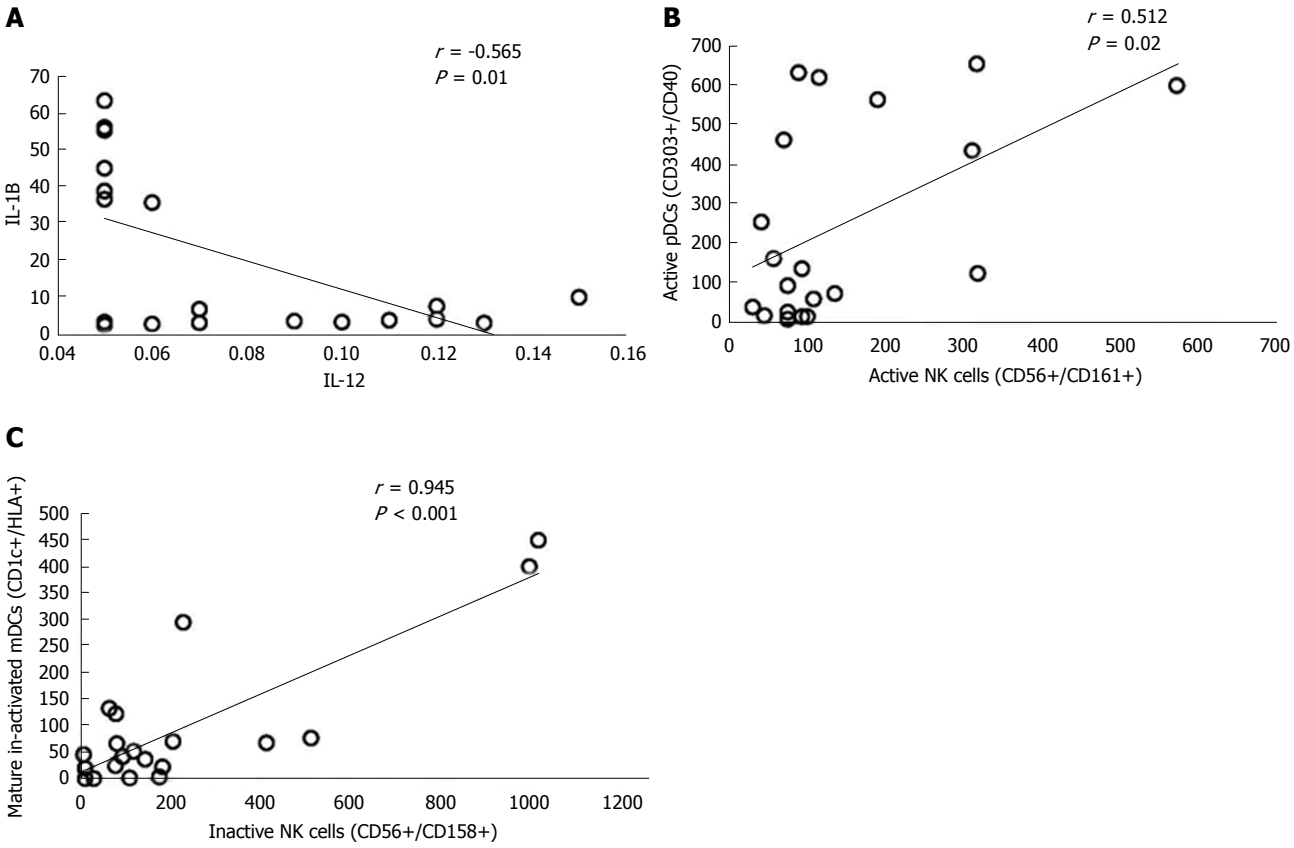

Figure 5 Correlation between immune cells and cytokine levels in hepatocellular carcinoma patients. IL: Interleukin; NK: Natural killer.

who found that NK cells cultured with cancer-associated fibroblasts from HCC (H-CAFs) down-regulate NKG2D and NKp46 and decrease expression of granzyme B, perforin and INF-γ. Later, another study showed that TGF-β and IL-10 in hepatic carcinoma patients induce the expression of microRNA (miR)-146a, which causes reduced IFN-γ production and cytotoxicity, resulting in a poorer prognosis[35]. Meanwhile, Zhang *et al*[36] found that MDSCs selectively suppressed the IFN-γ production deriving from NKT cells through membrane-bound TGF-β.

IL-2 plays a critical role in activation of the immune system, which could be used to eradicate cancer. It has been demonstrated as a monotherapy, and was approved for metastatic renal cell carcinoma and metastatic melanoma by the FDA[37]. Our results showed a significant increase in TNF-αR2 ($P < 0.001$) and significant decrease in IL-2 ($P < 0.001$) in HCC patients in relation to other groups, consistent with our previous results[38,39] that concluded the possible use of serum TNFR-II, IL-2Ra and IL-8 as combined biomarkers in HCV-infected patients at high risk of developing HCC.

Another important finding in the current study was that HCC patients had a significant decrease of active NK cells (CD56+/CD314+; $P < 0.05$) and active pDCs (CD303+, CD40+; $P < 0.05$) in comparison to inactive NK cells (CD56+/CD158+) and inactive pDCs (CD303+/HLA+) respectively. NK cells expressing CD56+/CD161+ and mDCs were not statistically affected. This revealed the importance of these two subsets of cells [active NK cells (CD56+/CD314+), and active pDCs (CD303+, CD40+)] in the pathogenesis of HCC, and allows for further research around the state of this imbalance that emerges in HCC patients.

We conclude that there are immunological changes occurring in HCC patients which could be possible candidate(s) for future immunotherapy for these patients. Among these are the NK cells expressing NKG2D and pDCs expressing CD40, IL-2 and IL-10. These factors could be implicated in the pathogenesis of HCC, and provide an attractive target for therapeutics in chronic HCV hepatitis and liver cancer.

ARTICLE HIGHLIGHTS

Research background

Hepatocellular carcinoma (HCC) is a major health problem worldwide and mainly in Egypt due to the high prevalence of hepatitis C virus (HCV) infection, especially of genotype IV. It ranks the first among cancers in males (33.6%), and the 2nd in females after breast cancer. Sorafenib is still the only treatment approved by the Food and Drug Administration for HCC; however, it extends the overall survival by 3 mo only. Hence, it is crucial to understand the underlying biological and immunological changes in HCC Egyptian patients, and to develop new treatment modalities based on these data.

Research motivation

The immune system plays an important role in suppression of cancer. However, tumors can escape immune surveillance by producing an immune-suppressive

environment, for which the underlying mechanisms are not fully clear. Recent studies show that the immune response in HCC patients is usually down-regulated by immunosuppressive cells (myeloid-derived suppressor cells and T regulatory cells) that are involved in chronic inflammation and tumor progression. Also, these inhibitory cells secrete many immune-suppressive cytokines, such as interleukin (IL)-6, IL-10 and transforming growth factor-β, creating a tolerogenic and suppressive environment[9].

Research objectives

The objective of this study was to assess the levels of different immune modulators and cytokines that may play a role in the pathogenesis of HCC and other hepatic diseases (*e.g.*, chronic hepatitis and liver cirrhosis) compared to a normal group. This would help in identifying additional therapeutic modalities for viral infections and HCC in the context of immunotherapy.

Research methods

This retrospective cohort study included 88 patients who attended the medical oncology clinics of the National Cancer Institute, Cairo University during the period from 2014 to 2016. Patients were divided into the HCC group (20 patients-G1), liver cirrhosis group (28 patients-G2), chronic hepatitis group (CH; 25 patients-G3) and normal healthy volunteers as a control group (NC; 15 persons). The immune system of the patients was assessed through immunophenotyping of CD1c and CD40, CD1c and HLA, CD303 and CD40, CD303 and HLA, CD56 and CD161, CD56 and CD314, and CD56 and CD158 by flow cytometry. On the other hand, serum levels of IL-2, IL-10, IL-12, IL-1β, interferon (IFN)-α, IFN-γ and tumor necrosis factor (TNF)-αR2 were measured by the ELISA technique, according to the manufacturer's instructions. Data were expressed as mean ± SE of mean, and statistical comparison between cytokine levels and immune cells were performed.

Research results

There was a significant decrease in active and inactive mDCs in HCC patients compared to the NC group, as well as active mDCs (CD1C+/CD40+) in cirrhotic patients compared to CH patients. The expression level of CD40+ on active pDCs (CD303+) was significantly decreased in HCC compared to the NC. However, there was no significant difference with the other groups (cirrhosis and CH). Meanwhile, the level of inactive pDCs (CD303+/HLA+) and inactive NK cells (CD56+/CD158+) did not differ significantly between the four groups studied.

The active NK cells (CD56+/CD161+) showed a significant increase in the CH group, whereas the level of active NK cells (CD56+/CD314+) was statistically decreased in HCC, CH and cirrhotic patients compared to the NC group, indicating an important role of these cells in the pathogenesis of HCC. The individual expression of each cell type in patients showed that active NK cells (CD56+/CD314+) were not expressed in 63% (12/20) of HCC patients.

There was a significant decrease in serum levels of IL-2, IFN-α and IFN-γ in HCC patients compared to the NC group, and a significant increase in serum levels of IL-10, IL-1β and TNF-αR2 in HCC compared to the NC group. However, there was no statistically significant change in the serum level of IL-12 among the four groups studied.

Research conclusions

We conclude that there are immunological changes occurring in HCC patients in relation to other liver diseases. The related immunological factors are NKG2D expressed on NK cells, and pDCs expressing CD40, IL-2 and IL-10. These factors could be implicated in the pathogenesis of HCC, and represent attractive targets for therapeutics in chronic HCV hepatitis and liver cancer.

Research perspectives

NKG2D, CD40, IL-2 and IL-10 could be a possible candidate for future immunotherapy for HCC patients. However, further studies are recommended regarding correlation of these factors and clincopathological features as well as the overall survival of the patients.

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ORIGINAL ARTICLE

Retrospective Study

Serum autotaxin levels are correlated with hepatic fibrosis and ballooning in patients with non-alcoholic fatty liver disease

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Abstract

AIM

To examine the relationship between serum autotaxin (ATX) concentrations and clinicopathological findings in non-alcoholic fatty liver disease (NAFLD) patients.

METHODS
One hundred eighty-six NAFLD patients who had undergone liver biopsy between 2008 and 2017 were retrospectively enrolled. Serum samples were collected at the time of biopsy and ATX was measured by enzyme immunoassays. Sera obtained from 160 healthy, non-obese individuals were used as controls. Histological findings were graded according to an NAFLD scoring system and correlations with serum ATX were calculated by Spearman's test. Diagnostic accuracy was evaluated using the area under the receiver operating characteristic curve (AUC). Cut-off values were identified by the Youden index, and the nearest clinically applicable value to the cutoff was considered the optimal threshold for clinical convenience.

RESULTS
Serum ATX levels were significantly higher in NAFLD patients than in controls (0.86 mg/L *vs* 0.76 mg/L, $P < 0.001$) and correlated significantly with ballooning score and fibrosis stage ($r = 0.36$, $P < 0.001$ and $r = 0.45$, $P < 0.001$, respectively). Such tendencies were stronger in female patients. There were no remarkable relationships between ATX and serum alanine aminotransferase, lipid profiles, or steatosis scores. The AUC values of ATX for predicting the presence of fibrosis (≥ F1), significant fibrosis (≥ F2), severe fibrosis (≥ F3), and cirrhosis (F4), were all more than 0.70 in respective analyses.

CONCLUSION
Serum ATX levels may at least partially reflect histological severity in NAFLD.

Key words: Autotaxin; Non-alcoholic fatty liver disease; Fibrosis; Ballooning

Core tip: Patients with non-alcoholic fatty liver disease (NAFLD) exhibited significantly higher serum levels of autotaxin (ATX) than did healthy subjects. Serum ATX levels correlated significantly with ballooning score and fibrosis stage in NAFLD patients and may therefore reflect histological severity in NAFLD.

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INTRODUCTION

The prevalence of non-alcoholic fatty liver disease (NAFLD) is increasing worldwide[1,2]. NAFLD exhibits a wide spectrum, ranging from non-alcoholic fatty liver to non-alcoholic steatohepatitis (NASH) and ensuing cirrhosis and hepatocellular carcinoma[1-3]. Since the concept of NASH was developed using pathological characteristics, *i.e.*, the presence of hepatocyte ballooning and lobular inflammation in addition to macrovesicular steatosis, liver biopsy is currently considered the gold standard for evaluating NAFLD/NASH activity. However, general limitations of liver biopsy are the costs and invasiveness, but also sampling error and inter- and intra-observer variability[4]. So, simple, accurate, non-invasive, quantitative alternatives are needed. Several studies have attempted to estimate histological severity in NAFLD using various serum biomarkers[5-8], but the accuracy of these techniques remains unsatisfactory.

Autotaxin (ATX) was originally discovered in conditioned medium from human melanoma cell cultures[9]. The protein is encoded by ectonucleotide pyrophosphatase/phosphodiesterase family member 2 gene (*ENPP2*) and catalyzes the hydrolysis of lysophosphatidylcholine (LPC) to lysophosphatidic acid (LPA), which functions as a phospholipase[10,11]. Signaling *via* a family of six G-protein-coupled receptors (LPA_{1-6}) regulates the diverse cellular processes of ATX, including proliferation, migration, neurogenesis, angiogenesis, fibrogenesis, glucose homeostasis, insulin action, and cancer progression[12-18]. Disrupted LPC metabolism has been reported in murine NASH models[19,20].

ATX is synthesized by a variety of normal cells and tissues, secreted into the circulation as a glycoprotein, and later degraded by liver sinusoidal endothelial cells[21]. Serum ATX levels are reportedly increased during the progression of pregnancy[22] and in patients with idiopathic pulmonary fibrosis or some kinds of cancers[23-25]. Recently, elevated serum ATX has also been implicated in fibrosis progression in chronic hepatitis C[26,27], for which the retarded degradation of circulating ATX due to liver sinusoidal endothelial cell dysfunction from liver fibrosis was considered a main mechanism[28]. Perisinusoidal fibrosis is more frequently detected in alcoholic and non-alcoholic steatohepatitis than in viral hepatitis, with sinusoidal endothelial dysfunction also being reported in NAFLD[29].

Based on the above reports, we have hypothesized that serum ATX is increased in advanced stage NASH patients, but evidence is scarce on the relationship between circulating ATX concentration and histological severity in NAFLD. Accordingly, we measured serum ATX levels in 186 NAFLD patients who had undergone liver biopsy and examined for associations with clinicopathological findings.

MATERIALS AND METHODS

Patients and clinical examinations

This retrospective, cross-sectional study was approved by the Committee for Medical Ethics of Shinshu University School of Medicine (ID number: 3244) and performed

in accordance with the Helsinki declaration of 1975, 1983 revision. Informed consent was obtained from all patients. We enrolled 186 biopsy-proven Japanese NAFLD patients who were admitted to Shinshu University Hospital (Matsumoto, Japan) between November 2008 and May 2017. NAFLD was suspected based on the following criteria: (1) the presence of hepatorenal contrast and increased hepatic echogenicity on abdominal ultrasonography; (2) An average daily consumption of < 20 g/d of ethanol; And (3) the absence of other causes of liver dysfunction, such as viral hepatitis, drug-induced liver injury, autoimmune liver disease, primary sclerosing cholangitis, Wilson's disease, hereditary hemochromatosis, and citrin deficiency[30,31]. The diagnosis of NAFLD/NASH was confirmed with the histological findings of biopsied specimens. Body weight and height were measured before liver biopsy in a fasting state. All laboratory data were obtained in a fasting state on the day of liver biopsy. Homeostasis model assessment for insulin resistance (HOMA-IR), fibrosis-4 index (FIB-4), and aspartate aminotransferase (AST) to platelet ratio index (APRI) were calculated according to the following formulae: HOMA-IR = [fasting blood glucose (mg/dL) × fasting insulin (μU/mL)]/405[32,33], FIB-4 = [age (years) × AST (IU/L)]/[platelet count (10^9/L) × alanine aminotransferase (ALT) (IU/L)$^{1/2}$][34], and APRI = [AST/upper limit of normal; 28 (IU/L)] × [100/platelet count (10^9/L)][35]. One hundred sixty subjects (80 male and 80 female) whose liver function tests and body mass index (BMI) were within normal levels and having no past medical history of NAFLD were selected as healthy controls, with equal age distribution among the male and female individuals (twenties: 20 subjects, thirties: 20 subjects, forties: 20 subjects, fifties: 20 subjects). These healthy controls were same as our previous report[26]. Sera were obtained after overnight fasting on the day of the liver biopsy and stored at -80 ℃ until testing.

Measurement of ATX

Serum ATX concentrations were determined with a specific two-site enzyme immunoassay using the automated immunoassay analyzer AIA-2000 system (Tosoh Co., Tokyo, Japan), as described previously[36]. To prepare the 2-site immunoassay, R10.23 was digested with pepsin and the purified F(ab)$_2$ form using phenyl-5PW (Tosoh Co.) hydrophobic column chromatography in order to avoid the nonspecific binding of human antibodies against various animal IgG in human specimens, like human anti-mouse antibodies. Magnetic beads were coated with R10.23 F(ab)$_2$ and placed in the reaction cup, and 35 ng of alkaline phosphatase-labeled R10.21 in assay buffer (5% BSA, 5% sucrose, 10 mmol/L Tris-HCl, 10 mmol/L $MgCl_2$, pH 7.4) was added to the reaction cup. ATX assay reagent was prepared by immediate freeze-dry procedure of the reaction cup. The ATX assay reagent thus prepared can be used with AIA-system.

Histological findings

Liver specimens of at least 1.5 cm in length were obtained from segment 5 or 8 using 14-gauge needles, as described previously, and immediately fixed in 10% neutral formalin. Sections of 4 μm in thickness were cut and stained by means of the hematoxylin and eosin and Azan-Mallory methods. The histological activity of NAFLD was assessed by an independent expert pathologist (KS) in a blinded manner according to the NAFLD scoring system proposed by Kleiner *et al*[37]. Steatosis was graded as 0 to 3 based on the rate of steatotic hepatocytes (< 5%, 5%-33%, > 33-66%, and > 66%, respectively). Lobular inflammation was graded as 0 to 3 based on the overall assessment of all inflammatory foci (no foci, < 2 foci/200 × field, 2-4 foci/200× field, and > 4 foci/200× field, respectively). Ballooning grade was scored as 0-2 by the frequency of ballooned hepatocytes (none, few, and many, respectively). NAFLD activity score (NAS) was calculated as the sum of steatosis, lobular inflammation, and ballooning scores, and NASH was defined as the presence of macrovesicular steatosis (> 5% of hepatocytes affected) and hepatocyte ballooning with or without lobular inflammation and fibrosis. Fibrosis stage was scored as follows: F0, none; F1, perisinusoidal or periportal; F2, perisinusoidal and portal/periportal; F3, bridging fibrosis; and F4, cirrhosis.

Statistical analysis

Clinical data are expressed as the number (percentage) or median (interquartile range). Statistical analyses were performed using StatFlex Ver. 6.0 (Artech Co., Ltd., Osaka, Japan) and SPSS 24.0 (IBM, Chicago, IL, United States) software. The Mann-Whitney *U* test was used for comparisons between two groups. Bonferroni's correction test was performed for multiple comparisons. Correlation analysis was conducted by Spearman's test. Diagnostic accuracy was evaluated using the area under the receiver operating characteristic (ROC) curve (AUC). Cut-off values were identified by the Youden index, with the nearest clinically applicable value to the cutoff being considered as the optimal threshold for clinical convenience. All statistical tests were two-sided and evaluated at the 0.05 level of significance.

RESULTS

Serum ATX levels were higher in NAFLD patients

The clinicopathological features of the 186 NAFLD patients enrolled in this study are summarized in Table 1. Eighty (43%) were male, and median age was 56 years. The number of patients according to fibrosis stage F0, F1, F2, F3, and F4 was 35, 89, 19, 34, and 9, respectively. Comparisons between genders revealed

Table 1 Clinicopathological features of 186 patients with non-alcoholic fatty liver disease

	All (n = 186)	Male (n = 80)	Female (n = 106)	
	Median (IQR)/n	Median (IQR)/n	Median (IQR)/n	P value[1]
Age (yr)	56 (46-65)	50 (38-59)	61 (54-66)	< 0.001
BMI (kg/m^2)	26.2 (23.8-29.6)	26.1 (24.3-29.4)	26.5 (23.6-29.7)	NS
Laboratory data				
Albumin (g/dL)	4.5 (4.3-4.7)	4.6 (4.4-4.8)	4.4 (4.2-4.7)	< 0.001
T-bil (mg/dL)	0.87 (0.69-1.17)	0.94 (0.74-1.26)	0.81 (0.67-1.07)	< 0.05
AST (IU/L)	41 (30-65)	39 (30-62)	42 (30-69)	NS
ALT (IU/L)	63 (38-97)	68 (43-103)	53 (33-89)	NS
γ-GT (IU/L)	54 (35-92)	64 (43-99)	50 (32-81)	< 0.05
TG (mg/dL)	122 (92-159)	122 (91-159)	121 (95-159)	NS
LDL-C (mg/dL)	130 (107-151)	132 (105-154)	130 (109-149)	NS
HDL-C (mg/dL)	51 (44-60)	48 (44-56)	55 (47-63)	
Plt (× 10^4/μL)	23.1 (18.5-26.8)	23.0 (19.6-26.7)	23.3 (17.6-26.9)	NS
HbA1c (%)	5.9 (5.7-6.6)	5.9 (5.6-6.5)	5.9 (5.7-6.6)	NS
FBG (mg/dL)	108 (98-121)	108 (98-121)	108 (97-121)	NS
IRI (mU/L)	11.2 (7.2-16.7)	10.5 (6.8-16.3)	11.5 (7.4-17.2)	NS
HOMA-IR	3.0 (1.9-4.6)	2.9 (1.8-4.5)	3.2 (2.0-4.7)	NS
Fe (μg/dL)	111 (90-137)	120 (92-146)	104 (88-129)	< 0.05
Ferritin (ng/mL)	146 (79-274)	172 (126-293)	113 (58-236)	< 0.001
AFP (ng/mL)	3.2 (2.2-4.8)	2.8 (2.1-4.0)	3.4 (2.6-5.2)	< 0.01
Fibrosis markers				
HA (ng/mL)	51 (28-91)	41 (25-62)	63 (34-118)	< 0.001
4C7S (ng/mL)	4.6 (3.8-5.7)	4.5 (3.8-5.5)	4.7 (3.8-6.6)	NS
FIB-4	1.35 (0.94-2.18)	1.12 (0.77-1.88)	1.53 (1.13-2.51)	< 0.001
APRI	0.69 (0.46-1.13)	0.66 (0.44-1.03)	0.71 (0.46-1.25)	NS
Histological findings				
Steatosis (1/2/3)	57/90/39	24/41/15	33/49/24	NS
Lobular inflammation (0/1/2/3)	9/101/69/7	6/48/23/3	3/53/46/4	< 0.05
Ballooning (0/1/2)	43/98/45	22/44/14	21/54/31	NS
Fibrosis (0/1/2/3/4)	35/89/19/34/9	16/43/8/13/0	19/46/11/21/9	NS

[1]Comparison between male and female subjects. IQR: Interquartile range; BMI: Body mass index; T-bil: Total bilirubin; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; γ-GT: Gamma-glutamyltransferase; TG: Triglyceride; LDL-C: Low density lipoprotein cholesterol; HDL-C: High density lipoprotein cholesterol; Plt: Platelet; FBG: Fasting blood glucose; IRI: Immunoreactive insulin; HOMA-IR: Homeostasis model assessment of insulin resistance; AFP: Alpha-fetoprotein; HA: Hyaluronic acid; 4C7S: Type 4 collagen•7S; FIB-4: Fibrosis-4 index; APRI: AST to platelet ratio; NS: Not significant.

significant differences in fibrosis-related parameters, such as age, albumin, hyaluronic acid (HA), and FIB-4, but fibrosis stage distribution was comparable.

Median serum ATX levels were significantly higher in NAFLD patients than in healthy controls (0.86 *vs* 0.76 mg/L, $P < 0.001$) (Figure 1A). In agreement with a previous report demonstrating a gender difference in serum ATX levels[26], serum ATX levels were higher in female patients and controls than in their male counterparts (Figure 1B). The degree of a serum ATX concentration increase was significant in female NAFLD patients (Figure 1B).

Relationship between serum ATX levels and clinicopathological features in NAFLD patients

We observed significant but weak correlations between ATX and glucose metabolism, BMI, and iron status, but none with lipid profiles. ATX was significantly and positively correlated to the factors of age, AST, HA, type 4 collagen 7S (4C7S), FIB-4, and APRI and was significantly and negatively correlated to platelet count (Table 2), which supported an association with fibrosis stage in NAFLD[38]. Indeed, ATX was significantly and positively correlated with ballooning grade (r = 0.36, $P < 0.001$) and fibrosis stage (r = 0.45, $P < 0.001$) overall, with no significant relationships for steatosis grades (Table 2, Figure 2). These correlations were stronger for women than for men, as were the correlation coefficients for ballooning score and fibrosis stage (Table 2, Figure 3).

Performance of ATX for diagnosing fibrosis status

To assess the significance of ATX as a predictor of fibrosis stage, ROC analysis was performed. Cut off values, sensitivities, specificities, positive predictive values, negative predictive values, and accuracies for predicting the presence of fibrosis (≥ F1), significant fibrosis (≥ F2), severe fibrosis (≥ F3), and cirrhosis (F4) in overall, male, and female NAFLD patients are shown in Table 3, and these ROC curves are shown in Figure 4. The AUC values of ATX for predicting the presence of fibrosis (≥ F1), significant fibrosis (≥ F2), severe fibrosis (≥ F3), and cirrhosis (F4), were all more than 0.70 in respective analyses.

For comparison, ROC analysis of serum ATX and conventional fibrosis indicators (HA, 4C7S, APRI, and FIB-4) for determination of severe fibrosis (≥ F3) were performed (Table 4). Although sensitivity of ATX

Table 2 Correlation between autotaxin and clinicopathological findings

	All (n = 186)		Male (n = 80)		Female (n = 106)	
	r	P value	r	P value	r	P value
Age (yr)	0.48	< 0.001	0.45	< 0.001	0.28	< 0.01
BMI (kg/m^2)	0.18	< 0.05	0.06	NS	0.31	< 0.01
Platelet (× 10^4/μL)	-0.32	< 0.001	-0.28	< 0.05	-0.43	< 0.001
Albumin (g/dL)	-0.32	< 0.001	-0.10	NS	-0.31	< 0.01
AST (IU/L)	0.31	< 0.001	0.34	< 0.01	0.40	< 0.001
ALT (IU/L)	0.06	NS	0.14	NS	0.24	< 0.05
TG (mg/dL)	-0.09	NS	-0.14	NS	-0.08	NS
LDL-C (mg/dL)	-0.04	NS	-0.01	NS	-0.06	NS
HDL-C (mg/dL)	0.13	NS	-0.04	NS	-0.04	< 0.001
FBG (mg/dL)	0.22	< 0.01	0.36	0.001	0.21	< 0.05
IRI (mU/L)	0.20	< 0.01	0.15	NS	0.31	0.002
HOMA-IR	0.22	< 0.01	0.22	< 0.05	0.31	0.001
Fe (μg/dL)	0.09	NS	0.12	NS	0.35	< 0.001
Ferritin (ng/mL)	0.04	NS	0.22	NS	0.31	0.002
HA (ng/mL)	0.49	< 0.001	0.47	< 0.001	0.46	< 0.001
4C7S (ng/mL)	0.40	< 0.001	0.30	< 0.01	0.50	< 0.001
FIB-4	0.58	< 0.001	0.51	< 0.001	0.60	< 0.001
APRI	0.43	< 0.001	0.45	< 0.001	0.55	< 0.001
Histological findings						
Steatosis score	0.02	NS	0.12	NS	-0.03	NS
Lobular inflammation score	0.22	< 0.01	0.06	NS	0.25	< 0.01
Ballooning score	0.36	< 0.001	0.34	< 0.01	0.38	< 0.001
NAS	0.27	< 0.001	0.27	< 0.05	0.26	< 0.01
Fibrosis stage	0.45	< 0.001	0.44	< 0.001	0.53	< 0.001

Correlations were calculated using Spearman's test. ATX: Autotaxin; BMI: Body mass index; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; TG: Triglyceride; LDL-C: Low density lipoprotein cholesterol; HDL-C: High density lipoprotein cholesterol; FBG: Fasting blood glucose; IRI: Immunoreactive insulin; HOMA-IR: Homeostasis model assessment of insulin resistance; HA: Hyaluronic acid; 4C7S: Type 4 collagen•7S; FIB-4: Fibrosis-4 index; APRI: AST to platelet ratio; NAS: NAFLD activity score; NS: Not significant.

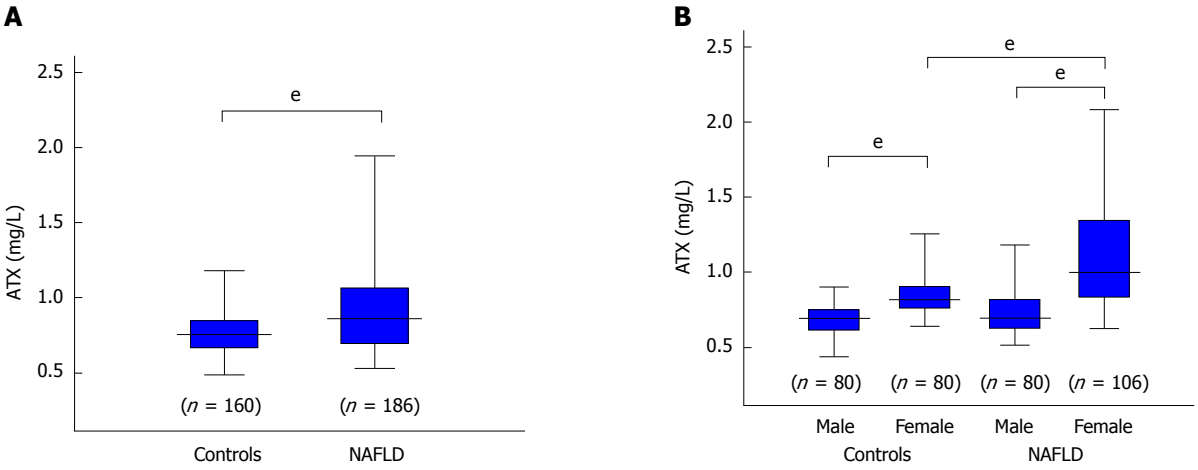

Figure 1 Comparison of autotaxin levels between controls and all patients with non-alcoholic fatty liver disease (A) and according to gender (B). The box plot shows the interquartile range, 95% confidence interval, and median. The difference between each group was tested with the Mann Whitney U test. ^eP < 0.001. ATX: Autotaxin; NAFLD: Non-alcoholic fatty liver disease.

is lower than those of HA, 4C7S, APRI, and FIB-4, specificity of ATX was highest (91%) compared to others.

DISCUSSION

Rachakonda *et al*[39] recently reported increased serum ATX levels in NAFLD patients. In severely obese and non-diabetic women, serum ATX was higher in those with NAFLD compared with those without NAFLD and positively correlated with insulin resistance. However, they did not assess liver pathology in their cohort of female subjects only. In this study, we compared serum ATX levels with clinicopathological background factors in biopsy-proven NAFLD patients and found that serum ATX levels were significantly related to hepatic fibrosis stage and ballooning score, implicating at least a partial reflection of histological severity in NAFLD.

Table 3 Diagnostic performance of autotaxin for predicting liver fibrosis stage in patients with non-alcoholic fatty liver disease

	Cut off	AUC	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Accuracy (%)
All patients							
≥ F1	0.73	0.71	77	57	89	36	73
≥ F2	1.19	0.75	45	94	80	77	78
≥ F3	1.19	0.75	51	91	63	86	82
F4	1.20	0.87	78	85	21	99	84
Male							
≥ F1	0.70	0.73	58	94	97	36	65
≥ F2	0.71	0.75	81	68	47	91	71
F3	0.82	0.74	62	82	40	92	79
Female							
≥ F1	1.03	0.76	53	95	98	31	60
≥ F2	1.19	0.80	66	91	82	81	81
≥ F3	1.19	0.78	73	86	67	89	82
F4	1.20	0.78	78	74	22	97	75

ATX: Autotaxin; AUC: Area under the receiver operating characteristic curve; PPV: Positive predictive value; NPV: Negative predictive value.

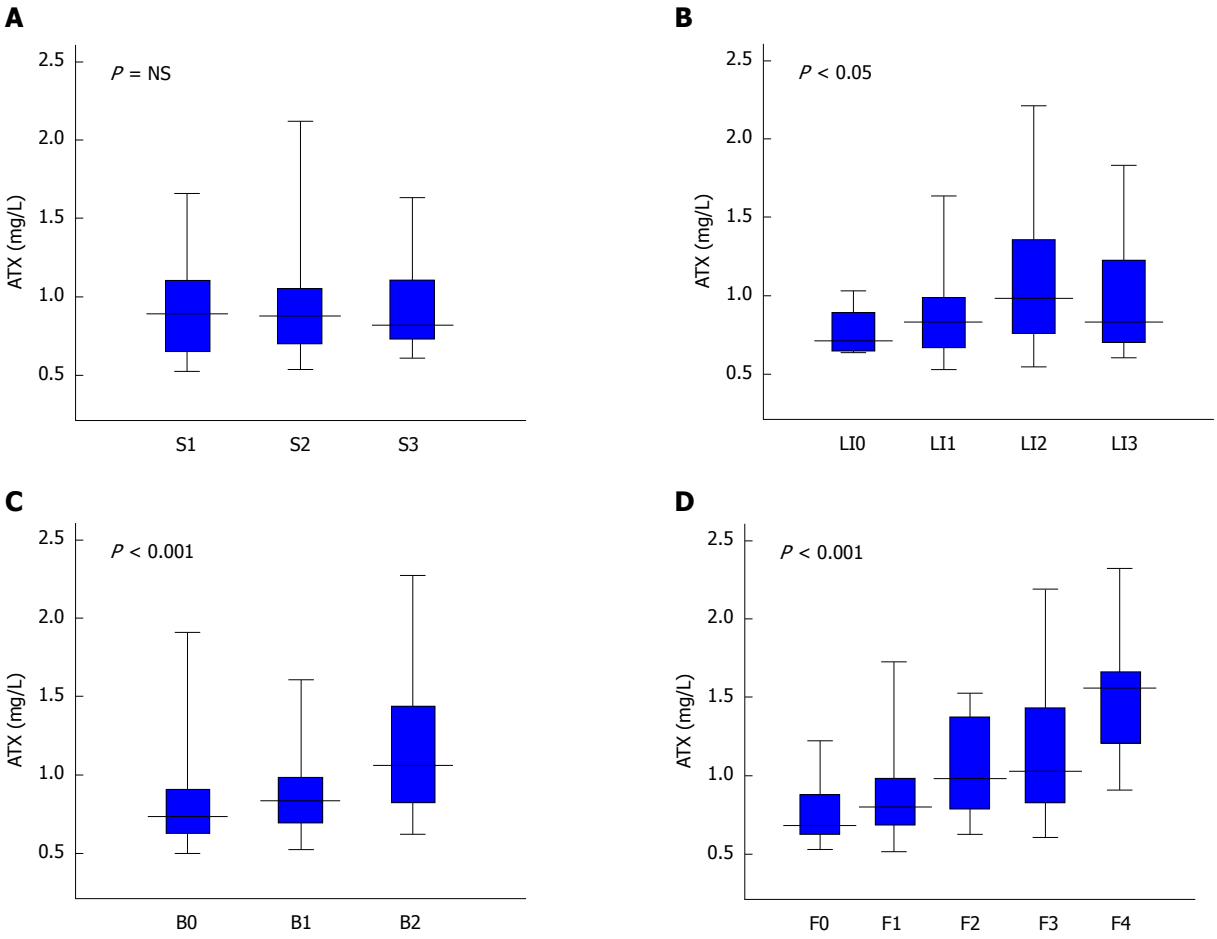

Figure 2 Relationship between autotaxin and histological grade in non-alcoholic fatty liver disease patients for steatosis (A), lobular inflammation (B), ballooning (C), and fibrosis (D). Table 1 presents the number of subjects for each histological stage. The Kruskal-Wallis test was used for multi-group simultaneous comparisons. *P* values are displayed in the upper left of each graph. ATX: Autotaxin; NAFLD: Non-alcoholic fatty liver disease; NS: Not significant.

The correlation between serum ATX levels and the severity of hepatic fibrosis has been explained by a mechanism of impaired circulating ATX degradation in damaged or impaired sinusoidal endothelial cells[28]. However, a recent study documented that ATX expression in hepatocytes activated hepatic stellate cells and amplified the fibrotic process, suggesting direct fibrosis-promoting properties of ATX[40]. Since ATX is a novel biomarker for hepatic fibrosis in chronic hepatitis C patients[26,27], we presumed similar results in NAFLD patients, but the correlation between ATX and fibrosis stage was comparatively weaker.

Table 4 Diagnostic performance of autotaxin and conventional fibrosis indicators for predicting severe fibrosis (≥ F3) in patients with non-alcoholic fatty liver disease

	AUC	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Accuracy (%)
All patients						
ATX	0.75	51	91	63	86	82
HA	0.82	93	63	44	96	70
4C7S	0.87	75	88	64	92	85
APRI	0.82	60	89	62	88	82
FIB-4	0.85	79	74	48	92	75
Male						
ATX	0.74	62	82	40	92	79
HA	0.76	85	72	41	95	75
4C7S	0.81	69	89	56	94	86
APRI	0.74	77	64	29	93	66
FIB-4	0.81	92	75	41	98	78
Female						
ATX	0.78	73	86	67	89	82
HA	0.86	78	86	68	91	83
4C7S	0.89	78	90	75	92	87
APRI	0.86	63	95	83	87	86
FIB-4	0.85	80	75	56	90	76

AUC: Area under the receiver operating characteristic curve; PPV: Positive predictive value; NPV: Negative predictive value; ATX: Autotaxin; HA: Hyaluronic acid; 4C7S: Type 4 collagen•7S; APRI: AST to platelet ratio; FIB-4: Fibrosis-4 index.

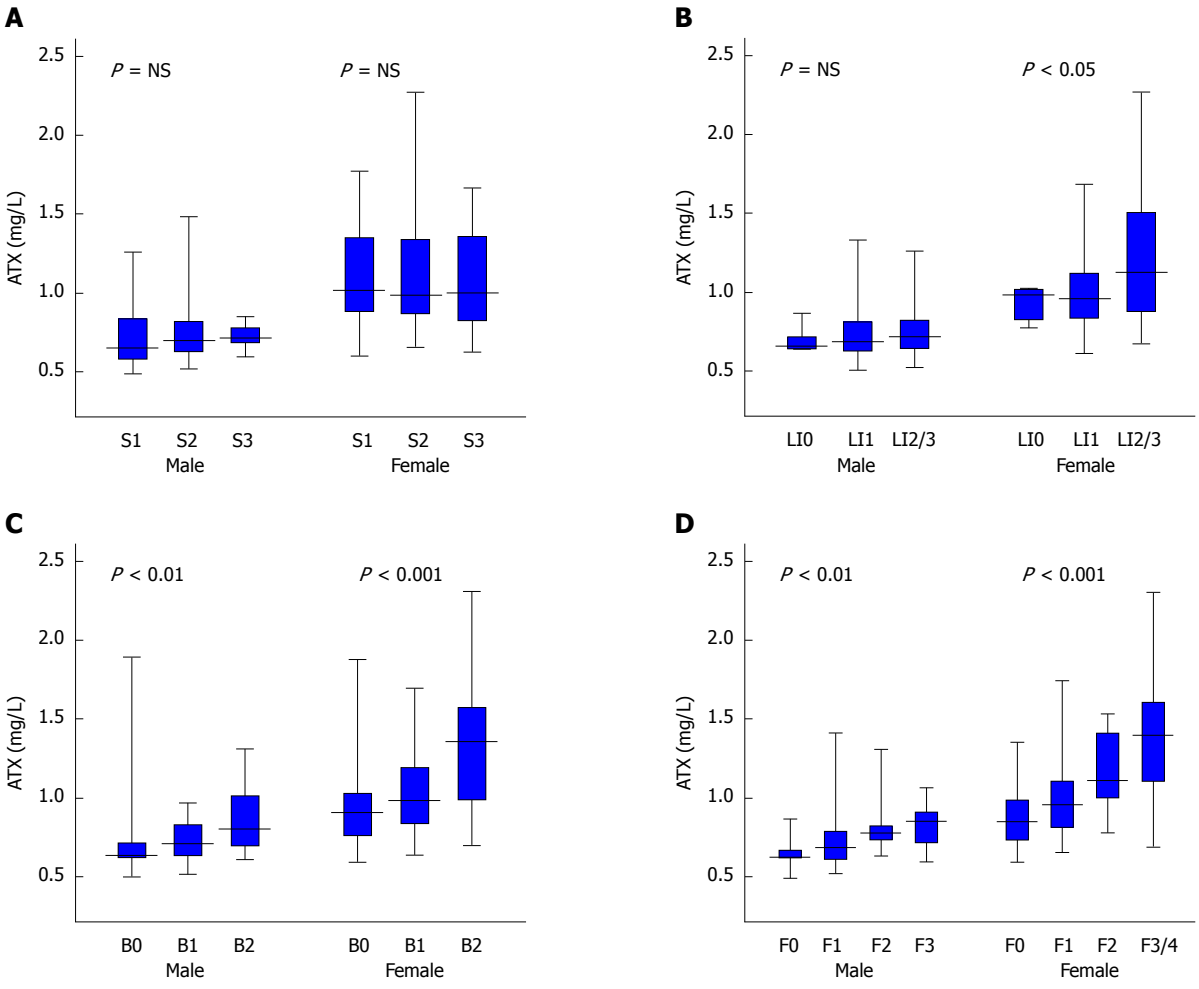

Figure 3 Relationship between autotaxin and histological grade in non-alcoholic fatty liver disease patients by gender for steatosis (A), lobular inflammation (B), ballooning (C), and fibrosis (D). Table 1 presents the number of subjects for each histological stage. The Kruskal-Wallis test was used for multi-group simultaneous comparisons. *P* values are displayed in the upper left of each graph. ATX: Autotaxin; NAFLD: Non-alcoholic fatty liver disease; NS: Not significant.

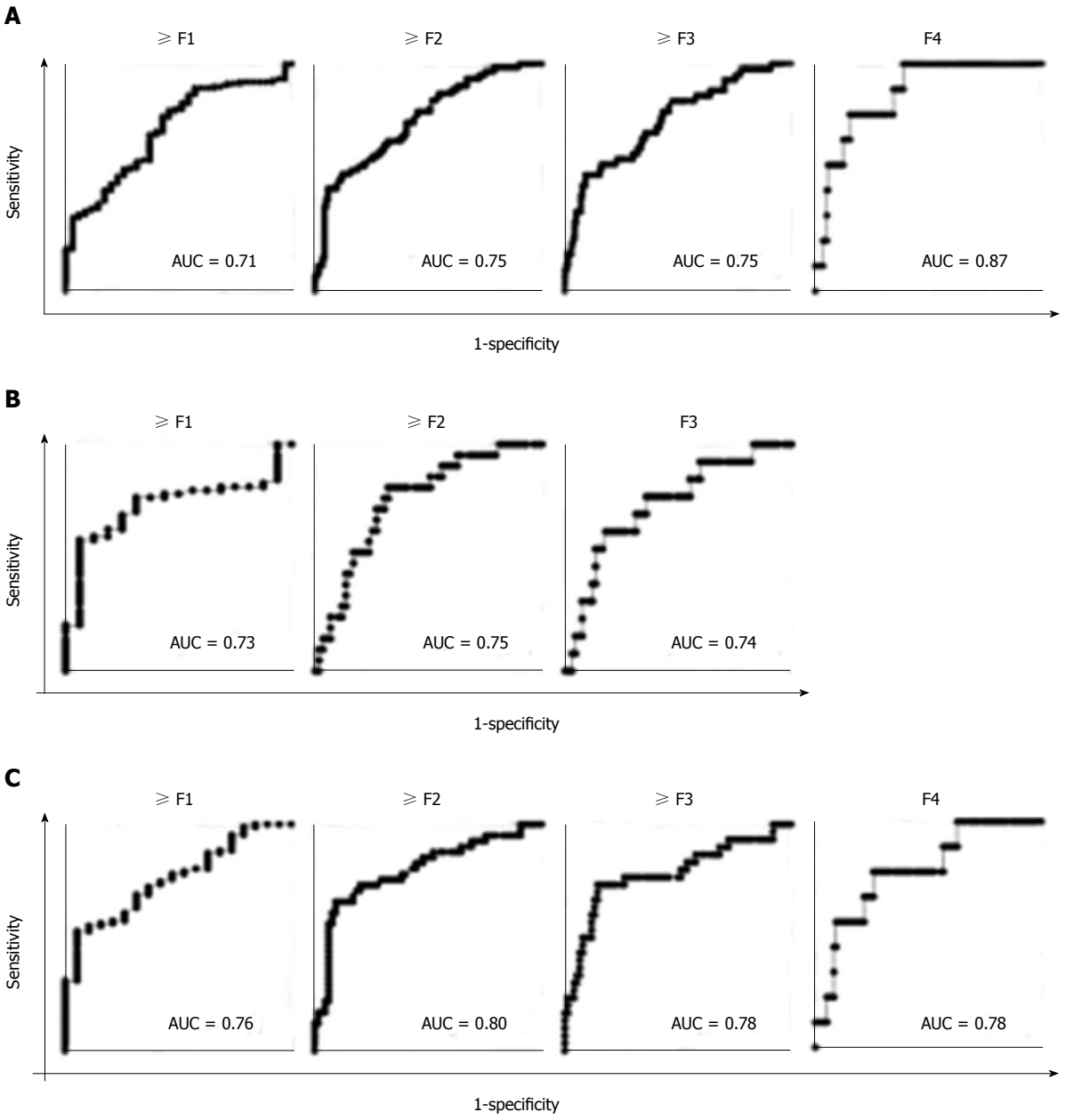

Figure 4 Receiver operating characteristic analysis of autotaxin for the estimation of the presence of fibrosis (≥ F1), significant fibrosis (≥ F2), severe fibrosis (≥ F3), and cirrhosis (F4) in all (A), male (B), and female (C) patients. The areas under the receiver operating characteristic curve are displayed in the lower right of each graph. AUC: Receiver operating characteristic curve; F: Fibrosis.

Thus, other mechanisms determining circulating ATX concentrations may exist as ATX is present in various tissues, such as white adipose tissue and the nervous system[41-43]. The importance of visceral fat has also been discussed[44], but in this study, we have not been able to examine waist circumference or waist-to-hip ratio, so this point is the limitation of this study.

In this study, we also conducted AUC analysis of ATX for determination of severe fibrosis (≥ F3) compared to conventional fibrosis indicators (HA, 4C7S, APRI, and FIB-4). AUC values and sensitivity of ATX was inferior to those other indicators[41], but specificity of ATX was highest among those other indicators. So ATX might be useful as a biomarker to exclude severe hepatic fibrosis.

Serum ATX levels were significantly associated with hepatocyte ballooning in our cohort, and a correlation was detected between fibrosis stage and ballooning grade (r = 0.56, P < 0.001). Ballooning degeneration is caused by an impaired intracellular cytoskeleton and resultant protein transport and appears after exposure to oxidative and endoplasmic reticulum stresses and during lipoapoptotic processes[45]. ATX expression was up-regulated by oxidative stress in microglia[46] and by LPC (18:1), an inducer of lipoapoptosis[47], in isolated hepatocytes[42]. Additionally, intravenous injection of LPC (18:1) into mice increased hepatic *Enpp2* mRNA expression and hepatocyte apoptosis[40]. These findings may explain how circulating ATX concentrations are

positively correlated with the prevalence of hepatocytes with ballooning degeneration.

In this study, we examined the relationship between NAFLD activity score as the severity of NAFLD/NASH and ATX, the correlation coefficient was significant but not high (r = 0.27, P < 0.001, Table 2). It seems difficult to predict the histological severity of NAFLD with ATX alone.

In conclusion, serum ATX levels were significantly higher in NAFLD patients over controls and correlated with ballooning score and fibrosis stage, especially in female patients. Further prospective research in larger cohorts is necessary for understanding the metabolism of circulating ATX in NAFLD.

ARTICLE HIGHLIGHTS

Research background

The prevalence of non-alcoholic fatty liver disease (NAFLD) is increasing worldwide. NAFLD exhibits a wide spectrum, ranging from non-alcoholic fatty liver to non-alcoholic steatohepatitis (NASH) and ensuing cirrhosis and hepatocellular carcinoma. Although the evaluation of NAFLD/NASH depends on the histological findings, there is a limitation and an alternative method is required.

Research motivation

Several studies have attempted to estimate histological severity in NAFLD using various serum biomarkers, but the accuracy of these techniques remains unsatisfactory.

Research objectives

Recently, elevated serum autotaxin (ATX) has been implicated in fibrosis progression in chronic liver disease, especially hepatitis C. So, we examine the relationship between serum ATX concentrations and clinicopathological findings in NAFLD patients.

Research methods

One hundred eighty-six NAFLD patients who had undergone liver biopsy between 2008 and 2017 were retrospectively enrolled. Serum samples were collected at the time of biopsy and ATX was measured by enzyme immunoassays. Sera obtained from 160 healthy, non-obese individuals were used as controls. Histological findings were graded according to an NAFLD scoring system and correlations with serum ATX were calculated by Spearman' s test. Diagnostic accuracy was evaluated using the area under the receiver operating characteristic curve (AUC). Cut-off values were identified by the Youden index, and the nearest clinically applicable value to the cutoff was considered the optimal threshold for clinical convenience.

Research results

Serum ATX levels were significantly higher in NAFLD patients than in controls (0.86 *vs* 0.76 mg/L, P < 0.001) and correlated significantly with ballooning score and fibrosis stage (r = 0.36, P < 0.001 and r = 0.45, P < 0.001, respectively). Such tendencies were stronger in female patients. There were no remarkable relationships between ATX and serum alanine aminotransferase, lipid profiles, or steatosis scores. The AUC values of ATX for predicting the presence of fibrosis (≥ F1), significant fibrosis (≥ F2), severe fibrosis (≥ F3), and cirrhosis (F4), were all more than 0.70 in respective analyses.

Research conclusions

Serum ATX levels may at least partially reflect histological severity in NAFLD.

Research perspectives

In order to evaluate the severity of NAFLD, it is considered that a method that can simultaneously evaluate activity and fibrosis is necessary.

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ORIGINAL ARTICLE

Retrospective Study

Epidemiological features of chronic hepatitis C infection caused by remunerated blood donors: A nearly 27-year period survey

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Author contributions: Tan YW, Tao Y and Liu LG contributed equally to this work; Tan YW designed the research; Tao Y, Ye Y, Zhou XB, Chen L, He C and Liu LG collected and analyzed the data and drafted the manuscript; Tao Y and Ye Y performed the research; Ye Y and He C interpreted the data and revised the statistical analysis; Tan YW and Tao Y wrote and revised the article; all authors have read and approved the final version to be published.

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Abstract

AIM

To understand the prevalence of hepatitis C virus (HCV) infection in blood donors over a nearly 27-year interval and to explore the factors that affect the outcome of HCV infection.

METHODS

A retrospective and cross-sectional study was conducted. The participants, mostly plasma donors, were selected from three administrative villages in the Jiangsu province in Eastern China. A questionnaire was administered among the villagers who had a history of blood donation from the late 1980s to the early 1990s. All participants underwent physical examination, liver B-ultrasonography, and liver stiffness measurement. In addition, 10 mL of blood was collected from each participant to measure simple liver function parameters (albumin, alanine aminotransferase, aspirate aminotransferase), blood factors (platelet), and for hepatitis B surface antigen, antiHCV, and antihuman immunodeficiency virus detection. HCV RNA detection, HCV genotyping, and other tests were carried out in antiHCV-positive patients.

RESULTS

After a median of 27 years (25-31 years) from the last blood donation to the time of survey, a total of 1694 participants were investigated, and the antiHCV-positive individuals were categorized into three groups: blood donors (*n* = 12, 3.3%), plasma donors (*n* = 534, 68.5%), and mixed donors (*n* = 324, 58.8%). A total of 592 (68.05%) patients had detectable HCV RNA, and 91.9% had genotype 1b. A total of 161 (27.2%, 161/592) patients with chronic HCV were considered to have cirrhosis with a liver stiffness measurement level higher than 12 kPa. Multiple logistic (binary) regression analysis results showed that platelet and IgG levels were associated with cirrhosis.

CONCLUSION

The nearly 27-year interval investigation revealed that chronic hepatitis C infection is a very serious public health problem in Eastern China. Plasma donation and subsequent return of blood cells to the donor are the main causes of hepatitis C infection. The main HCV genotype is 1b. Nearly 28% of cases progressed to cirrhosis. Age, especially over 60 years, and regular drinking habits were risk factors associated with cirrhosis.

Key words: Blood donor; Hepatitis C; Cross-sectional study; Epidemiologic; China

Core tip: A retrospective and cross-sectional study was conducted. A total of 1694 participants were investigated and categorized into three groups: 2 (3.3%), 534 (68.5%), and 324 (58.8%) patients positive for anti-hepatitis C virus (HCV) in blood donor, single plasma donor, and mixed donor groups, respectively. A total of 592 (68.05%) cases had detectable HCV RNA, and genotype 1b accounted for 91.9%. A total of 161 (27.2%, 161/592) patients with chronic HCV were considered to have cirrhosis with a liver stiffness measurement level of more than 12 kPa. Multiple logistic (binary) regression analysis results showed that platelet and IgG levels were associated with cirrhosis.

Tan YW, Tao Y, Liu LG, Ye Y, Zhou XB, Chen L, He C. Epidemiological features of chronic hepatitis C infection caused by remunerated blood donors: A nearly 27-year period survey. *World J Gastroenterol* 2018; 24(11): 1250-1258 Available from: URL: http://www.wjgnet.com/1007-9327/full/v24/i11/1250.htm DOI: http://dx.doi.org/10.3748/wjg.v24.i11.1250

INTRODUCTION

Hepatitis C infection is a major global public health problem. The World Health Organization estimated that the global hepatitis C virus (HCV) infection rate is about 2.8% and that about 170 million people are infected with chronic HCV. Approximately 350000 people die each year from hepatitis C-related liver diseases[1,2]. However, because of the occult nature of HCV, most people who are infected have no knowledge of their HCV infection; thus, the global incidence of chronic hepatitis C (CHC) is not clear. A Serum Hepatitis C Epidemiology Survey carried out in 2006 in China showed that the general population aged 1-59 years has an antiHCV-carrying rate of 0.43% and in the global range, HCV infection has low prevalence in some areas[3].

HCV is mainly transmitted through contact with the blood of an infected person; thereby, blood donors, especially plasma donors, are high-risk groups for HCV infection[4]. A study in remunerated blood donors reported an increased HCV infection rate of 15.53%[5] due to the use of nonsterile medical devices and other reasons.

The phenomenon of remunerated blood donation has been reported to occur in underdeveloped rural areas with low economic status, from the late 1980s to the early 1990s. Moreover, most of these hepatitis C-infected individuals had no history of seeking any medical assistance and had no knowledge about their HCV status; although, a considerable proportion of infections among those who have progressed to cirrhosis or even to hepatocellular carcinoma (HCC) were found.

The natural history of HCV has not been as fully delineated as that of hepatitis B virus[6]. Some epidemiological studies suggest that an estimated nearly 55%-85% of the individuals infected with acute hepatitis C will develop CHC, and nearly 5%-15% of patients with CHC will progress to cirrhosis after 20 years[7]. However, the conclusions of these epidemiological studies differ widely and lack longer epidemiological surveys. The main reason is the lack of a relatively fixed CHC epidemiological population.

A CHC population infected through plasma apheresis donation has a relatively consistent infection time and place. Most of these patients with hepatitis C infection did not seek medical assistance. These characteristics have created a unique advantage for the study of the natural history of hepatitis C.

We, therefore, chose to study the natural administrative villages in Jiangsu, a province in Eastern China where most villagers are plasma donors, in order to further understand the prevalence and the prognosis of HCV infection over nearly 30 years and to explore the factors that affect the outcome of this infection.

MATERIALS AND METHODS

Ethics statement

The study was approved by the Medical Ethics Committee of the Third Hospital of Zhenjiang Affiliated Jiangsu University, and written informed consent was obtained from each patient prior to participation. The study was conducted in compliance with the Declaration of Helsinki.

Participation and methods

A retrospective and cross-sectional study was conducted. The research team was composed of a staff of more than 20 trained individuals, including specialist doctors, technicians, community doctors, nurses, epidemiological researchers, medical graduate students, *etc*. Before the survey, a formal survey plan was drafted in advance and a standard questionnaire formulated. Two weeks prior to the survey, a research representative informed participants about the questionnaire and their physical and ultrasound examinations, and provided information about any matter requiring attention. Signed informed consent was obtained before the study started in the community hospital at the appointed time.

Research participation: The participants were selected from three administrative villages in the Jiangsu province in Eastern China, where most people are plasma donors, and the questionnaires were carried out among the villagers who had a history of plasma extraction. The participants had signed written informed consent. The inclusion criteria were the following: (1) a history of remunerated blood donation from the late 1980s to the early 1990s; (2) age above 40 years; (3) voluntary provision of contact information; and (4) no HCV treatment performed. Qualified subjects participated in the health examination and questionnaire from March to May 2017.

Investigation methods: The researchers conducted a unified training. The questionnaire submitted to the patients included: social demographic characteristics; history of common diseases, viral hepatitis, family diseases, and remunerated blood donations; and blood transfusion methods. All participants underwent physical examination, liver B-ultrasound and liver stiffness measurement (LSM). In addition, 10 mL of blood were collected for simple liver function parameter analysis [albumin (ALB), alanine aminotransferase (ALT); aspartate aminotransferase (AST)], blood routine [platelet (PLT)], and hepatitis B surface antigen (HBsAg), antiHCV, and antihuman immunodeficiency virus (HIV) detection.

Detection for HCV RNA, HCV genotyping, and other tests were carried out in antiHCV-positive patients. HCV RNA from subjects' sera was quantified in fresh or well-preserved stored samples by commercial quantitative assays, such as real-time PCR (COBAS AmpliPrep/COBAS *Taq*Man HCV Test; Roche, DaAn Gene Co., Nanjing, China). The HCV genotype was assessed in all patients with detectable HCV RNA. We used a PCR assay based on reverse transcription of the HCV core region with genotype-specific primers, in accordance with the international classification (*i.e.* Ⅰa, Ⅰb, Ⅱa, Ⅱb, Ⅲ, Ⅳ, Ⅴ and Ⅵ) (DaAn Gene Co,). Antinuclear antibody (ANA) and smooth muscle actin (SMA) determination was carried out using indirect immunofluorescence.

LSM

LSM using transient elastography (TE) (FibroScan502®; Echosens, Paris, France) was performed with the 3.5 MHz standard probe operated by a skillful operator (experience: > 10000 measurements) in a blinded manner. As previously described, the examination was carried out with the patient lying down in a supine position with the right arm placed behind the head. The tip of the probe transducer was placed on the skin between the ribs at the level of the right lobe of the liver, exerting an adequate pressure on it. The results were expressed in kPa, and each LSM value corresponds to the median of 10 validated measurements[8]. An examination was considered successful and reliable if the interquartile range (IQR)/median for LSM was ⩽ 30% or the LSM was < 7.1 kPa when the IQR/median for LSM was > 30%[9]. For the diagnosis of liver cirrhosis, a cut-off value of 12 kPa was used.

Statistical analysis

Continuous variables are given as median (range) or mean ± SD and categorical variables as frequencies or percentages (%) of patients. All data of demographic and clinical features were analyzed using the Statistical Package for the Social Sciences (SPSS) Version 21.0 (IBM Corp., Armonk, NY, United States). Chi-squared and Fisher's exact tests were performed for categorical variables, while Student's *t*-test or one-way analysis of variance was used for group comparisons of parametric quantitative data. Multinomial (binary) logistic regression was performed to evaluate factors predicting CHC and cirrhosis. All *P* values were two-

Table 1 Demographic and clinical characteristics of remunerated blood donors

	Blood donors, *n* = 363	Single plasma donors, *n* = 780	Blood and plasma donors, *n* = 551	*P* value
Age in yr	56.8 ± 13.2	57.2 ± 11.3	57.1 ± 9.4	0.882[1]
⩾ 40, < 50	56 (15.4)	83 (10.6)	68 (12.3)	0.07
⩾ 50, < 60	203 (55.9)	501 (64.2)	336 (61.0)	
⩾ 60	104 (28.7)	196 (25.1)	147 (26.7)	
Sex				
Male	123 (33.92)	315 (36.5)	211 (38.3)	0.109[2]
Female	240 (66.1)	465 (63.5)	340 (61.5)	
BMI	25.52 ± 4.32	25.36 ± 4.11	25.45 ± 3.22	0.353[1]
< 25	178 (49)	395 (50.6)	294 (53.4)	0.167[2]
⩾ 25, < 28	130 (35.8)	284 (36.4)	169 (30.7)	
⩾ 28	55 (15.2)	101 (12.9)	88 (16)	
PLT as × 10^9/L	207.3 ± 64.8	161.8 ± 55.4	176.3 ± 63.1	< 0.001[1]
ALB in g/L	42.3 ± 3.5	41.4 ± 4.7	43.3 ± 4.5	0.513[1]
ALT in U/L	27.4 ± 6.5	63.2 ± 18.7	52.6 ± 15.4	< 0.001[1]
AST in U/L	23.5 ± 7.4	55.4 ± 12.9	44.5 ± 22.6	< 0.001[1]
Anti-HCV				
Positive	12 (3.3)	534 (68.5)	324 (58.8)	< 0.001[2]
Negative	351 (96.7)	246 (31.5)	227 (41.29)	
HBsAg				
Positive	2 (0.6)	3 (0.4)	1 (0.2)	0.643[3]
Negative	361 (99.4)	777 (99.6)	550 (99.8)	
LSM in kPa	5.56 ± 2.64	7.37 ± 3.62	6.54 ± 3.54	< 0.001[1]
⩾ 9	2 (0.6)	224 (28.7)	122 (22.1)	< 0.001[2]
< 9, ⩾ 6	20 (5.5)	313 (40.1)	287 (52.1)	
< 6	341 (93.9)	243 (31.2)	142 (25.8)	
Blood donated frequency times				
⩾ 10	212 (58.4)	245 (31.4)	187 (33.9)	< 0.001[2]
< 10, ⩾ 5	120 (33.1)	352 (45.1)	202 (36.7)	
< 5	31 (8.5)	183 (23.5)	162 (29.4)	
Interval time from last donated blood to survey in yr	27.56 ± 2.11	27.65 ± 3.02	27.84 ± 2.54	0.453[1]
Refused donated by elevated ALT				
Yes	37 (10.2)	317 (40.6)	195 (35.4)	< 0.001[2]
No	326 (89.8)	463 (59.4)	356 (64.6)	

Data are presented as *n* (%). The normal range of ALT and AST are 5-40 U/L, PLT is 100-300 × 10^9/L, ALB is 35-55 g/L. [1]One-way analysis; [2]Pearson Chi-Squared; [3]Fisher's exact test. ALB: Albumin; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; BMI: Body mass index; LSM: Liver stiffness measurement; PLT: Platelet.

sided.

RESULTS

Demographic and clinical characteristics of remunerated blood donors

In this survey, we investigated a total of 1694 participants after a median of 27 years (25-31 years) from the last blood donation to the moment of survey, including 363 blood donors, 780 plasma donors and 551 mixed blood donors. We detected 870 antiHCV-positive cases, 6 HBsAg-positive cases and no cases of HIV infection. As shown in Table 1, we analyzed age, sex, body mass index (BMI; < 25; ⩾ 25, < 28; ⩾ 28), PLT, ALB, ALT, AST, antiHCV (positive, negative), HBsAg (positive, negative), LSM (< 6; ⩾ 6, < 9; ⩾ 9), frequency of blood donation (< 5 ; ⩾ 5 < 10; ⩾ 10), and rejection of blood donation owing to elevated ALT (yes, no). The differences in PLT, ALT, AST, LSM, frequency of blood donation, and rejection of blood donation owing to elevated ALT were statistically significant ($P < 0.05$) among different blood donation mode groups. In particular, we observed 12 (3.3%), 534 (68.5%) and 324 (58.8%) antiHCV-positive patients in the blood donor, plasma donor and mixed donor groups, respectively.

Demographic and clinical characteristics of CHC

A total of 870 participants were antiHCV-positive; among them, 592 (68.05%) had detectable HCV RNA, were diagnosed with CHC and categorized to the CHC group, whereas 278 (31.95%) had undetectable HCV RNA and were categorized to the no CHC group. Table 2 shows an analysis of age, sex, BMI, (< 25; ⩾ 25, < 28; ⩾ 28), PLT, ALB, ALT, AST, SMA (positive, negative), ANA (positive, negative), immunoglobulin (IgG; normal, elevated), LSM (< 6; ⩾ 6, < 9; ⩾ 9), frequency of blood donation (< 5; ⩾ 5 < 10; ⩾ 10), and rejection of blood donation due to elevated ALT (yes, no). Differences in age, BMI, homeostatic model assessment of insulin resistance (HOMA-IR), ALT, AST, PLT and LSM were statistically significant ($P < 0.05$)

Table 2 Demographic and clinical characteristics of hepatitis C virus in remunerated blood donors and multiple logistic regression analysis of factors associated with hepatitis C virus

	CHC, *n* = 592	No CHC, *n* = 278	*P* value	Multivariate[4]			
				OR	95%CI	Wald	*P* value
Age in yr	55.4 ± 13.2	58.5 ± 9.4	< 0.001[1]	1.642	0.426-11.164	3.012	0.013
⩾ 40, < 50	121 (20.4)	35 (12.6)	0.003[2]		1		
⩾ 50, < 60	356 (60.1)	168 (60.4)		3.542	0.521-13.254	1.534	0.435
⩾ 60	115 (19.4)	75 (27.0)		11.226	0.065-137.53	5.322	0.004
Sex							
Male	277 (46.8)	111 (39.9)	0.058[2]		1		
Female	315 (53.2)	167 (60.1)		0.233	0.054-6.634	1.004	0.364
Alcohol consumption				0.532	0.147-1.647	0.853	0.547
Never	441 (74.5)	175 (62.9)	0.002[2]	0.436	0.124-1.006	1.075	0.443
Occasional	95 (16.0)	68 (24.5)		0.876	0.857-1.354	1.446	0.374
Often	56 (9.5)	35 (12.6)		1.231	0.843-1.556	0.667	0.432
BMI	24.12 ± 2.32	25.45 ± 3.22	< 0.001[1]	0.889	0.674-1.327	0.896	0.547
< 25	278 (47.0)	194 (69.8)	< 0.001[2]	1.216	0.536-1.625	0.034	0.646
⩾ 25, < 28	230 (38.9)	69 (24.8)		7.233	0.054-66.63	1.534	0.343
⩾ 28	84 (14.2)	15 (5.4)		4.365	0.643-22.534	1.543	0.113
HOMA-IR	1.53 ± 0.48	1.31 ± 0.52	< 0.001[1]	1.002	0.864-1.007	0.984	0.657
PLT as × 10^9/L	164.3 ± 64.8	196.3 ± 73.1	< 0.001[1]	3.112	1.475-121.153	16.886	< 0.001
ALB in g/L	42.3 ± 3.5	43.3 ± 4.5	0.513[1]	0.576	0.645-1.2147	0.543	0.674
ALT in U/L	67.4 ± 26.5	22.6 ± 15.4	< 0.001[1]	3.216	1.036-121.625	25.034	< 0.001
AST in U/L	53.5 ± 17.4	24.5 ± 10.6	< 0.001[1]	2.578	0.937-76.354	26.332	< 0.001
SMA							
Negative	517 (87.3)	262 (94.2)	0.002[2]		1		
Positive	75 (12.7)	16 (5.8)		1.146	0.545-1.654	0.543	0.653
ANA							
Negative	477 (80.6)	244 (87.8)	0.009[2]		1		
Positive	115 (19.4)	34 (12.2)		1.423	0.587-1.001	0.123	0.886
IgG							
Normal	271 (45.8)	261 (93.9)	< 0.001[2]		1		
Elevated	321(54.2)	17 (6.1)		6.001	0.957-12.353	6.075	< 0.001
LSM in kPa	7.67 ± 4.43	4.12 ± 2.25	< 0.001[1]	0.233	0.054-6.634	1.004	0.364
< 6	155 (26.2)	241 (86.7)	< 0.001[2]		1		
< 9, ⩾ 6	211 (35.6)	31 (11.2)		0.532	0.147-1.647	0.853	0.547
⩾ 9	226 (38.2)	6 (2.2)		2.436	0.124-11.776	7.075	< 0.001
Blood donated frequency times	8.67 ± 6.43	8.42 ± 6.25	0.107[1]	1.233	0.874-1.134	1.032	0.832
< 5	139 (23.5)	62 (22.3)	0.101[2]		1		
< 10, ⩾ 5	252 (42.6)	102 (36.7)		0.932	0.927-1.433	1.032	0.883
⩾ 10	201 (34.0)	114 (41.0)		0.247	0.257-1.754	1.054	0.664
Refused donated by elevated ALT							
No	377 (63.7)	148 (53.2)	0.003[3]		1		
Yes	215 (36.3)	130 (46.8)		1.668	1.061-3.143	4.804	0.027

Data are presented as *n* (%). Alcohol consumption: Often, the ethanol intake per week was more than 140 g in men (70 g in women) in the past 12 mo; Occasional, the ethanol intake per week was less than 140 g in men (70 g in women) in the past 12 mo. [1]One-way analysis; [2]Pearson's chi-square; [3]Fisher's exact test; [4]Binary logistic regression. ALB: Albumin; ALT: Alanine aminotransferase; ANA: Antinuclear antibody; AST: Aspartate aminotransferase; BMI: Body mass index; CI: Confidence interval; LSM: Liver stiffness measurement; OR: Odds ratio; SMA: Smooth muscle actin; PLT: Platelet.

between the HCV and no HCV groups. However, ALB, frequency of blood donation and refusal of donation by elevated ALT were not significantly different.

Demographic and clinical characteristics of cirrhosis caused by HCV infection and multiple logistic regression analysis associated with cirrhosis

A total of 161 (27.2%, 161/592) patients with CHC were diagnosed with cirrhosis, having an LSM value higher than 12 kPa. Among them, 431 patients were diagnosed with CHC. Table 3 shows an analysis of the age, sex, alcohol consumption (never, occasional, often), BMI (< 25; ⩾ 25, < 28; ⩾ 28), PLT, ALB, ALT, AST, HCV RNA (LgIU/mL, ⩾ 3,< 5; ⩾ 5), genotype (Ⅰ, Ⅱ, Ⅲ), frequency of blood donation (< 5 ; ⩾ 5 < 10; ⩾ 10), and rejection of blood donation due to elevated ALT (yes, no). Differences in age, alcohol consumption, PLT and IgG were statistically significant ($P < 0.05$) between the cirrhosis and CHC groups. However, sex, BMI, ALB, ALT, AST, SMA, ANA, HCV RNA, genotype, frequency of blood donation and rejection of blood donation due to elevated ALT were not significantly different. When the LSM level higher than 12 kPa was considered a binary dependent variable, multiple logistic (binary) regression analysis was used to assess factors associated with cirrhosis and CHC (Table 3).

Table 3 Demographic and clinical characteristics of cirrhosis by hepatitis C virus infection in remunerated blood donors and multiple logistic regression analysis of factors associated with cirrhosis

	Cirrhosis by HCV, *n* = 161	CHC, *n* = 431	*P* value	Multivariate[4]			
				OR	95%CI	Wald	*P* value
Age in yr	58.4 ± 13.2	56.5 ± 9.4	< 0.001[1]	2.143	0.553-6.453	4.543	0.002
⩾ 40, < 50	41 (25.43)	80 (18.6)	0.034[2]		1		
⩾ 50, < 60	82 (50.9)	270 (62.6)		2.443	0.242-7.345	1.423	0.065
⩾ 60	38 (23.6)	81 (18.8)		3.223	0.124-14.344	3.153	0.021
Sex							
Male	75 (46.6)	202 (46.9)	0.951[2]		1		
Female	86 (53.4)	229 (53.1)		1.223	0.112-6.765	0.653	0.445
Alcohol consumption							
Never	97 (60.2)	344 (79.8)	< 0.001[2]		1		
Occasional	40 (24.8)	55 (12.8)		0.879	0.647-2.654	2.753	0.152
Often	24 (14.9)	32 (7.4)		1.004	0.875-1.744	3.057	0.005
BMI	24.12 ± 2.32	25.45 ± 3.22	0.353[1]	0.647	0.465-1.632	4.135	0.432
< 25	78 (48.4)	200 (46.4)	0.108[2]		1		
⩾ 25, < 28	68 (42.2)	162 (37.6)		1.242	0.574-1.735	0.536	0.438
⩾ 28	15 (9.3)	69 (16)		0.665	0.426-1.645	0.476	0.537
HOMA-IR	1.53 ± 0.48	1.51 ± 0.52	0.556	0.023	0.772-1.423	0.365	0.221
PLT as × 10^9/L	147.3 ± 55.7	176.3 ± 84.2	< 0.001[1]	1.314	0.022-1.463	3.647	0.013
ALB in g/L	42.3 ± 3.5	43.3 ± 4.5	0.513[1]	0.864	0.707-1.364	1.557	0.675
ALT in U/L	67.4 ± 26.5	62.6 ± 25.4	0.113[1]	1.643	0.463-1.755	0.634	0.247
AST in U/L	53.5 ± 27.4	54.5 ± 22.6	0.201[1]	1.425	0.428-1.254	0.546	0.664
SMA							
Negative	144 (89.4)	373 (86.5)	0.346[2]		1		
Positive	17 (10.6)	58 (13.5)		0.526	0.537-1.843	1.034	0.536
ANA							
Negative	130 (80.7)	347 (80.5)	0.945[1]		1		
Positive	31 (19.3)	84 (19.5)		2.123	0.132-5.563	0.843	0.246
IgG							
Normal	60 (37.3)	205 (47.6)	0.025[2]		1		
Elevated	101 (62.7)	226 (52.4)		1.352	0.663-12.267	3.537	0.012
HCV RNA in LgIU/mL	7.12 ± 2.43	6.73 ± 2.533	0.067[1]	0.657	0.536-1.523	0.863	0.536
⩾ 3, < 5	22 (13.7)	51 (11.8)	0.546[2]		1		
⩾ 5	139 (86.3)	380 (88.2)		1.325	0.972-1.445	0.143	0.782
Genotype							
Ⅰ	152 (94.4)	390 (90.5)	0.310[2]		1		
Ⅱ	8 (5.0)	37 (8.6)		0.753	1.003-1.664	0.623	0.242
Ⅲ	1 (0.6)	4 (0.9)		1.862	1.182-1.635	0.845	0.118
Blood donated frequency times	8.67 ± 5.43	8.42 ± 6.25	0.107[1]	1.536	0.874-2.154	0.923	0.101
< 5	36 (22.4)	103 (23.9)	0.698[2]		1		
< 10, ⩾ 5	66 (41.0)	186 (43.2)		0.354	0.274-1.203	0.991	0.783
⩾ 10	59 (36.6)	142 (32.9)		1.024	0.154-2.163	0.332	0.224
Refused donated by elevated ALT							
No	94 (58.4)	243 (56.4)	0.661[2]		1		
Yes	67 (41.6)	188 (43.6)		0.012	0.037-1.002	0.682	0.563

Data are presented as *n* (%). Alcohol consumption: Often, the ethanol intake per week was more than 140 g in men (70 g in women) in the past 12 mo; Occasional, the ethanol intake per week was less than 140 g in men (70 g in women) in the past 12 mo. [1]One-way analysis; [2]Pearson's chi-square; [3]Fisher's exact test; [4]Binary logistic regression. ALB: Albumin; ALT: Alanine aminotransferase; ANA: Antinuclear antibody; AST: Aspirate aminotransferase; BMI: Body mass index; LSM: Liver stiffness measurement; SMA: Smooth muscle actin.

Using the "enter" method, the results suggested that age, alcohol consumption and PLT levels were associated with cirrhosis.

DISCUSSION

Hepatitis C is a blood-borne disease mainly transmitted by percutaneous exposure to contaminated blood and by unprotected sexual intercourse[10,11]. In the last century, from the late 1980s to the early 1990s, a large number of paid blood donors emerged in underdeveloped rural areas with a low economic status in Eastern China. Many blood donors were infected with HCV because of the use of contaminated medical devices. A total of 1694 participants were investigated, and 870 cases were positive for anti-HCV. In particular, we found 12 (3.3%), 534 (68.5%) and 324 (58.8%) patients positive against antiHCV in the blood donor, plasma donor and mixed donor groups, respectively.

The results showed that the blood donation method is the main cause of transmission of hepatitis C, and plasma donation in particular is the main causes of

hepatitis C infection. The rate of HCV infection in blood donors is 3.3%, quite similar to the average antiHCV-positivity rate of 3.2% in the general Chinese population according to the national epidemiological survey of HCV conducted from 1992 to 1995[12,13]. Some studies reported the transmission of hepatitis C in blood donors in the last decade in China[14-18]. However, this survey revealed that the blood donation method, in particular plasma apheresis, is the main cause of transmission of hepatitis C.

We also found that the frequency of blood donation in the plasma donor group was lower compared to the blood donor group, due to the more frequent rejection of blood donation in the plasma group because of elevated ALT. In other words, more plasma donors are likely to have been infected with HCV. The response of serum markers (ALT, AST and PLT) to liver damage in the plasma and mixed donor groups is higher than in the whole blood donor group. The HBsAg-positivity rate decreased because of the beginning of hepatitis B screening for blood donation.

HCV RNA was first detected in peripheral blood 1-3 wk after exposure to HCV[19]. Hepatitis C viremia not yet cleared 6 mo after exposure will progress to chronic infection. The hepatitis C chronicity rate is approximately 55%-85%[20-22]. Our survey interval of nearly 30 years shows that there are still 68% cases of detectable HCV RNA. Some studies have suggested that chronic predictive factors of HCV infection include male sex, age > 25 years, lack of symptoms after infection, race (African American), HIV infection, and immunosuppression[21]. The genetic background of the host may affect chronicity. IL-28B gene, human leukocyte antigen class 1 molecule HLA B57, and class Ⅱ molecules HLA DRB1 and DQBI allele polymorphism can affect HCV clearance[23-25]. For example, CC genotype at the rs12979860 site of the IL-28B gene leads to virus clearance, whereas TT is associated with a very low virus clearance[26,27].

In our study, age was a factor in the spontaneous clearance of the virus, but no sex-related differences in terms of HCV clearance were found. The increased levels of indicators of liver damage such as PLT, ALT, AST and LSM are considered the result of a chronic hepatitis C. Interestingly, blood donation due to elevated ALT reflects the activity of hepatitis C and indicates whether its current activity is beneficial to its spontaneous clearance.

HCV infection progresses slowly, up to 20 years after infection. The incidence of cirrhosis in children and young women is 2%-4%[28], in middle aged people infected due to blood transfusion 18%-30%[29], in plasma donors 1.4%-10.0%[7,30], and in the general population 5%-15%[26]. The factors that can promote disease progression include infection with HCV at age over 40 years, male sex, alcohol use (50 g/d or more in men, 70 g in women), HCV with HIV infection which leads to immune dysfunction[31,32], obesity, insulin resistance, hepatitis B virus infection, nonalcoholic fatty liver, high iron load in the liver, accompaniment of schistosomiasis infection, hepatotoxic drugs, and environmental pollution caused by toxic substances. Genetic factors can also promote disease progression[33,34].

Baseline liver tissue inflammation, necrosis and fibrosis stage are the best predictors of progression to cirrhosis. The incidence rate of cirrhosis of patients with CHC after a nearly 30-year interval is 27.2%, which was higher than in related studies[7,30]. Studying the incidence rate involved a long observation period, age, especially higher than 60 years, and regular drinking were risk factors for cirrhosis. Significantly increased levels of PLT and immunoglobulin are seen in cirrhosis.

HCV Ib and 2a genotypes were the most common in China, with genotype 1b (56.8%) being the highest, followed by genotypes 2 (24.1%) and 3 (9.1%). Genotypes 4 and 5 were not found, whereas genotype 6 (6.3%)[3] was found to be low. However, our study found that genotype 1b accounted for 91.9%, which shows heterogeneity in the distribution of hepatitis C genotypes in China.

In conclusion, this research over 27 years revealed that CHC infection remains a serious public health problem in Eastern China. Plasma donation is the main causes of hepatitis C infection. The main HCV genotype is 1b. After nearly 30 years of CHC, nearly 28% of cases progressed to cirrhosis. Age, especially greater than 60 years, and regular drinking habits were risk factors associated with cirrhosis.

ARTICLE HIGHLIGHTS

Research background

The natural history of hepatitis C virus (HCV) is still unclear. One of the main reasons why natural history is not clear is that the time of establishment of the infection is unclear. In this report, the authors followed many patients with HCV who can estimate the time of infection.

Research motivation

In the last century, from the late 1980s to the early 1990s, a large number of paid blood donors emerged in underdeveloped rural areas with a low economic status in Eastern China. Many blood donors were infected with HCV because of the use of contaminated medical devices.

Research objectives

The study aimed to understand the prevalence of HCV infection in blood donors over a nearly 27-year interval and to explore the factors that affect the outcome of HCV infection.

Research methods

A retrospective and cross-sectional study was conducted. The participants, mostly plasma donors, were selected from three administrative villages in the Jiangsu province in Eastern China. A questionnaire was administered among the villagers who had a history of blood donation from the late 1980s to the early 1990s. All participants underwent physical examination, liver B-ultrasonography, and liver stiffness measurement (LSM). In addition, 10 mL of blood was collected from each participant to measure simple liver function parameters [albumin (ALB), alanine aminotransferase (ALT), aspartate aminotransferase (AST)], blood factors [platelet (PLT)], and for hepatitis B surface antigen (HBsAg), antiHCV, and antihuman immunodeficiency virus

detection. HCV RNA detection, HCV genotyping, and other tests were carried out in antiHCV-positive patients.

Research results

After a median of 27 years (25-31 years) from the last blood donation to the time of survey, a total of 1694 participants were investigated, and the antiHCV-positive individuals were categorized into three groups: blood donors (*n* = 12, 3.3%), plasma donors (*n* = 534, 68.5%), and mixed donors (*n* = 324, 58.8%). A total of 592 (68.05%) patients had detectable HCV RNA, and 91.9% had genotype 1b. A total of 161 (27.2%, 161/592) patients with chronic hepatitis C (CHC) were considered to have cirrhosis, with an LSM level higher than 12 kPa. Multiple logistic (binary) regression analysis results showed that PLT and IgG levels were associated with cirrhosis.

Research conclusions

The nearly 27-year interval investigation revealed that CHC infection is a very serious public health problem in Eastern China. Plasma donation and subsequent return of blood cells to the donor are the main causes of hepatitis C infection. The main HCV genotype is 1b. Nearly 28% of cases progressed to cirrhosis. Age, especially over 60 years, and regular drinking habits were risk factors associated with cirrhosis.

Research perspectives

This research over 27 years revealed that CHC infection remains a serious public health problem in Eastern China. The epidemiological data in the present investigation may play an important role in focusing on the significance of public health in chronic HCV infection.

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ORIGINAL ARTICLE

Clinical Trials Study

Low-FODMAP *vs* regular rye bread in irritable bowel syndrome: Randomized SmartPill® study

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Author contributions: Pirkola L, Freese R, Laatikainen R, Hongisto SM, Loponen J, Linderborg KM, and Hillilä M contributed to the design and practical implementation of the study; the research was performed by Pirkola L; the University of Turku team (Linderborg KM, Nuora A, and Yang B) provided the SmartPill equipment and related technical assistance; data analyses were performed by Pirkola L and Freese R. The manuscript was written by Laatikainen R, Pirkola L, and Freese R; all authors critically revised the manuscript; the authors had complete access to the data that supports the publication; all authors have approved the final version of the manuscript.

Clinical trial registration statement: ISRCTN registry (ISRCTN11005234).

Informed consent statement: All participants signed the informed consent form.

Conflict-of-interest statement: Laatikainen R has written a Finnish book on irritable bowel syndrome and diet; He is also founder and owner of Booston Ltd, which provides IBS-related dietetic services to IBS patients, healthcare professionals, and various organizations; Pirkola L, Hongisto SM, and Loponen J are employees of Fazer Bakeries; At the time of the research, Pirkola L was working at the University of Helsinki; Others have no personal interests to declare; Fazer Bakeries funded the study and provided the breads.

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Abstract

AIM

To compare the effects of regular *vs* low-FODMAP rye bread on irritable bowel syndrome (IBS) symptoms and to study gastrointestinal conditions with SmartPill®.

METHODS

Our aim was to evaluate if rye bread low in FODMAPs would cause reduced hydrogen excretion, lower intraluminal pressure, higher colonic pH, different transit times, and fewer IBS symptoms than regular rye bread. The study was a randomized, double-blind, controlled cross-over meal study. Female IBS patients (n = 7) ate study breads at three consecutive meals during one day. The diet was similar for both study periods except for the FODMAP content of the bread consumed during the study day. Intraluminal pH, transit time, and pressure were measured by SmartPill, an indigestible motility capsule.

RESULTS

Hydrogen excretion (a marker of colonic fermentation) expressed as area under the curve (AUC)$_{(0\text{-}630\ min)}$ was [median (range)] 6300 (1785-10800) ppm·min for low-FODMAP rye bread and 10 635 (4215-13080) ppm·min for regular bread (P = 0.028). Mean scores of gastrointestinal symptoms showed no statistically significant differences but suggested less flatulence after low-FODMAP bread consumption (P = 0.063). Intraluminal pressure correlated significantly with total symptom score after regular rye bread (ρ = 0.786, P = 0.036) and nearly significantly after low-FODMAP bread consumption (ρ = 0.75, P = 0.052). We found no differences in pH, pressure, or transit times between the breads. Gastric residence of SmartPill was slower than expected. SmartPill left the stomach in less than 5 h only during one measurement (out of 14 measurements in total) and therefore did not follow on par with the rye bread bolus.

CONCLUSION

Low-FODMAP rye bread reduced colonic fermentation *vs* regular rye bread. No difference was found in median values of intraluminal conditions of the gastrointestinal tract.

Key words: Colonic pressure; FODMAP; Irritable bowel syndrome; Rye; Wireless motility capsule; Symptom severity

Core tip: Our study confirmed that low-FODMAP rye bread reduces colonic fermentation in irritable bowel syndrome (IBS) patients compared with regular rye bread. The observed correlation between increased intracolonic pressure and symptom severity underlines the central role of visceral sensitivity in IBS and suggests that some IBS symptoms might be exacerbated by any pathophysiological reason that leads to increased colonic pressure. The study also suggests that SmartPill might not be an optimal device to evaluate gastrointestinal circumstances during meal studies lasting less than 24 h, due to device's inability to measure effects of a singular food bolus in a timely manner.

Pirkola L, Laatikainen R, Loponen J, Hongisto SM, Hillilä M, Nuora A, Yang B, Linderborg KM, Freese R. Low-FODMAP *vs* regular rye bread in irritable bowel syndrome: Randomized SmartPill® study. *World J Gastroenterol* 2018; 24(11): 1259-1268 Available from: URL: http://www.wjgnet.com/1007-9327/full/v24/i11/1259.htm DOI: http://dx.doi.org/10.3748/wjg.v24.i11.1259

INTRODUCTION

Irritable bowel syndrome (IBS) is a common functional gastrointestinal disorder[1]. Symptoms include bloating, abdominal pain, flatulence, constipation, and diarrhea. Visceral sensitivity[2], low-grade inflammation[3], and impaired gas handling[4] contribute to the etiology of IBS.

Many patients consider food as a trigger of their symptoms[5,6] and half of IBS patients report postprandial exacerbation of symptoms[7]. FODMAPs (Fermentable Oligo-, Di-, Monosaccharides and Polyols) are poorly absorbable carbohydrates that are rapidly fermented in the proximal colon[8]. A low-FODMAP diet decreases colonic fermentation, which in turn parallels a reduction of IBS symptoms[9]. One of the major food groups excluded during a low-FODMAP diet is fiber-rich gluten-containing grain products[8]. Elimination of whole-grain products from the diet may, however, lead to decreased fiber intake and increase the risk of chronic diseases in the long term[10].

Rye, a widely-consumed grain in the Nordic countries, is very high in fiber that is mainly composed of arabinoxylan, lignin, cellulose, β-glucan, and fructans[11]. Fructans are classified as FODMAPs[8]. Typical rye bread contains more than 10% fiber and is the most important source of fiber (28%-35% of total intake) of Finnish adults[12]. Rye bread induces gastrointestinal symptoms in some individuals, possibly due to the high fructan content[13]. Therefore, IBS patients may avoid rye products.

Efforts are being made to develop grain products that are low in FODMAPs but high in other fibers[14]. In a recent study, an innovative low-FODMAP high-fiber (fiber > 10% of weight) rye bread caused less colonic fermentation and led to fewer IBS symptoms than regular rye bread[15]. The low-FODMAP rye bread was lower both in fructans and mannitol. Mannitol is a FODMAP compound that is formed during the rye sourdough breadmaking process.

A major challenge in the research of functional gastrointestinal disorders is the lack of objective markers of disease activity. Excretion of hydrogen and methane and colonic fermentation markers are among the rare easily available and objective markers of gastrointestinal circumstances during the consumption of FODMAPs or other poorly absorbable carbohydrates[16]. However, a recent study utilized a wireless motility capsule (SmartPill®) and demonstrated that pH in the colon of IBS patients is lower than in healthy subjects[17]. This may indicate more intensive fermentation in the colon of IBS patients. SmartPill measures intraluminal pH, temperature, motility, and pressure and might thus offer the means to gather objective data on gastrointestinal conditions in IBS patients. A combination of SmartPill data and measurements of perceived symptoms may improve our understanding on the etiology of IBS symptoms.

The aim of this pilot study was to compare a low-FODMAP rye bread and a regular rye bread with regards to postprandial abdominal symptoms, breath hydrogen concentration, and gastrointestinal transit times, pH, and pressure as measured by SmartPill. Our hypothesis was that a low-FODMAP rye bread would induce less hydrogen excretion, lower pressure, and increase pH in colon compared with regular rye bread, which would subsequently parallel with fewer IBS symptoms. The secondary objective was to evaluate the feasibility of the SmartPill capsule in associating IBS symptoms with physiologic responses in the gastrointestinal tract in a meal study.

MATERIALS AND METHODS

Study subjects

Patients with IBS were recruited from the Helsinki metropolitan area *via* the Internet. The eligibility inclusion criteria were the following: (1) female, (2) aged 18 to 65 years, (3) BMI 18.5-30 kg/m^2, and (4) IBS defined by the Rome III criteria[18]. The exclusion criteria were celiac disease, Crohn's disease, diverticulitis, severe dyspepsia, stomach bezoar, bowel obstruction, severe constipation, medication used in the management of intestinal motility, major abdominal surgery, dysphagia, pregnancy or breastfeeding, regular smoking, implanted medical device, and hormonal, renal, hepatic, or hematologic disease or participation in another clinical trial during the past two months.

The study candidates were pre-screened with questionnaires on health and diet and IBS diagnostic criteria. Candidates meeting the preliminary inclusion criteria received laboratory tests (blood count, sedimentation rate, thyroid function tests, transglutaminase antibodies and immunoglobulins for celiac disease, calprotectin, and gene test for lactose intolerance) and a clinical evaluation by a gastroenterologist. Weight and height were also measured. All participants signed an informed consent form. The study protocol was approved by the Ethics Committee of the Helsinki and Uusimaa Hospital District, Finland. The study was registered at ISRCTN registry (ISRCTN11005234).

Study design

This study was a randomized, double-blind, postprandial cross-over meal study. All participants attended on two occasions with a washout period of $\geqslant$ 2 wk between the study periods. Each study period consisted of a run-in period of 12 h (standardized dinner and overnight fast), a test day with study meals (breakfast, lunch, and dinner with low-FODMAP or regular rye bread) and a follow-up of 1 to 3 d depending on the transit time of the SmartPill capsule. The order of the interventions (low-FODMAP or regular rye bread) was randomized for each patient with a random number table. Both investigators and participants were blinded to the identity of the bread. The study events during the study period are detailed in Supplemental Table 1.

Study diets

The diet was standardized from the evening (-12 h) before the test breakfast (0 h) until the following morning (+24 h). The standardized diet consisted of regular grocery products and had a low FODMAP content (Supplemental Table 1). The diet was similar for both study periods except for the FODMAP content of the bread eaten during the study day. Participants kept a food diary from the day before the test day until the end of the study period.

On the morning of the test day, the volunteers ingested the SmartPill capsule with water and ate four slices (approximately 120 g) of bread with spread, cheese, vegetables, and coffee or tea. Lunch with two slices of the test bread was consumed six hours later and dinner with an additional two slices of bread 10-12 h after the breakfast. Thus, participants consumed a total of eight slices (approximately 240 g) of bread during each study period. Breads were developed and supplied by Fazer Bakeries (Vantaa, Finland). The low-FODMAP rye bread was prepared using a specific sourdough that contains unique lactobacilli that efficiently consume fructans and also results in low mannitol content. The control rye bread was prepared using a traditional rye sourdough. The breads were similar in appearance and taste.

The nutrient composition of the breads (Table 1) was analyzed by Eurofins scientific Finland, Raisio (Food and Agro), Finland. The dietary fiber content of the breads was determined by using the AOAC method 2011.25 that discriminates soluble and insoluble, low, and high molecular weight dietary fibers. The mannitol content was analyzed by the HPLC method used by Eurofins Food and Agro, Lidköping, Sweden. Fructan content was measured by using the AOAC 999.03 method (Megazyme assay kit K-FRUC, Megazyme

Table 1 Nutritional composition of the study breads

	Low-FODMAP rye bread		Regular rye bread	
	Per 100 g	Per slice (30 g)	Per 100 g	Per slice (30 g)
Energy (kJ/kcal)	1031/245	309/74	1037/246	311/74
Fat (g)	2.6	0.8	1.1	0.3
Protein (g)	7.5	2.3	7.5	2.3
Carbohydrates (g)	42.4	12.7	45.1	13.5
Dietary fiber (g)	10.8	3.2	12.8	3.8
Soluble fiber (g)	2.6	0.8	2.9	0.9
Insoluble fiber (g)	6.7	2.0	7.7	2.3
Fructans (g)	0.4	0.12	1.2	0.36
Mannitol (g)	0.09	0.03	0.26	0.08

international Ireland Ltd, Bray, Ireland).

SmartPill

SmartPill® (Given Imaging ltd, Yoqneam, Israel) is an indigestible wireless capsule that contains sensors for temperature, pH, and pressure. The capsule is 26.8 mm long and its diameter is 11.7 mm. The capsule sends the measured data to a receiver worn by the subject. Measurement data is uploaded to a computer and analyzed by MotiliGI® program. The program calculates mean pressure, median pH, contractions/min, and transit times based on changes in pH and temperature for the different parts of the gastrointestinal tract. In the present study, the capsule was swallowed with the test breakfast and the subject was prohibited from eating for six hours after the meal so the capsule would proceed to small intestine. Each study period ended when the capsule was defecated.

Breath hydrogen and IBS symptoms

Breath hydrogen was analyzed with Gastrolyzer® (Bedfont Scientific Ltd, Kent, England) before the test breakfast (0 min) and every 30 min for 11 h (660 min) on the test day and every three hours on the following days. Breath hydrogen was analyzed as a marker for colonic fermentation[19].

IBS symptoms during the study periods were collected with a visual analogue scale (VAS) questionnaire. We did not formally validate the questionnaire, but it follows the concept as described by Francis *et al*[20]. A similar scoring system has been used previously in other diet studies in IBS[21,22]. Symptoms were recorded once before the test breakfast (0 min) and every 30 min for 11 h on the test day and every 3 h on the following days. The questionnaire consisted of nine individual 100 mm VAS lines of the following different symptom classes: abdominal pain, abdominal cramps, bloating, flatulence, belly rumbling, nausea, heartburn, unpleasant sensation in the upper abdomen, and continuous urge to defecate. Additionally, participants kept a diary of defecation times and form of stool.

Missing values in gastrointestinal symptoms and hydrogen measurements were imputed with the mean value of the previous and following measurements. Two subjects failed to report the 660 min measurements for all symptoms and hydrogen concentration, and thus values were included only until 630 min for all subjects when calculating the outcome variables. The baseline (0 min) of symptoms was not included in outcome variables as this was measured before eating the test breakfast.

The outcome variables calculated from the symptom questionnaire scores were the following: mean of the scores for each symptom during the follow-up period (30-630 min), sum of total symptom scores at each time point, and the area under the curve (AUC) of the total symptom score during the follow up (30-630 min). AUC of breath hydrogen was calculated using the absolute breath hydrogen values (0-630 min). AUC values were calculated following trapezoidal rule[23] without respect to increase because symptom severity and hydrogen concentration may have a baseline value of zero. Symptom and H_2 measurements that were conducted after the test day were used to calculate the mean of total symptom score and mean of breath hydrogen content for different parts of the gastrointestinal tract.

Statistical analysis

Breath hydrogen was the primary outcome variable used in the study power calculations. Suitable previously published data was not available, and thus the study power was calculated based on a preliminary test performed in Fazer Bakeries in which healthy participants ate regular rye bread or low-FODMAP rye bread followed by analysis of breath hydrogen content during the six-hour postprandial period. The difference in breath hydrogen content (ppm) between fasting and at six hours was used to evaluate the number of subjects in the current study. Based on the power calculation, a sample size of eight would have 80% power to detect a 25-ppm difference in breath hydrogen using a paired *t*-test with a two-sided significance level of 0.05.

The patient characteristics and outcome variables are expressed as a median (range) and as number of cases for categorical variables. The difference in outcome variables between study periods was analyzed using the Wilcoxon signed-rank test for related samples. Correlations between mean symptom severity and

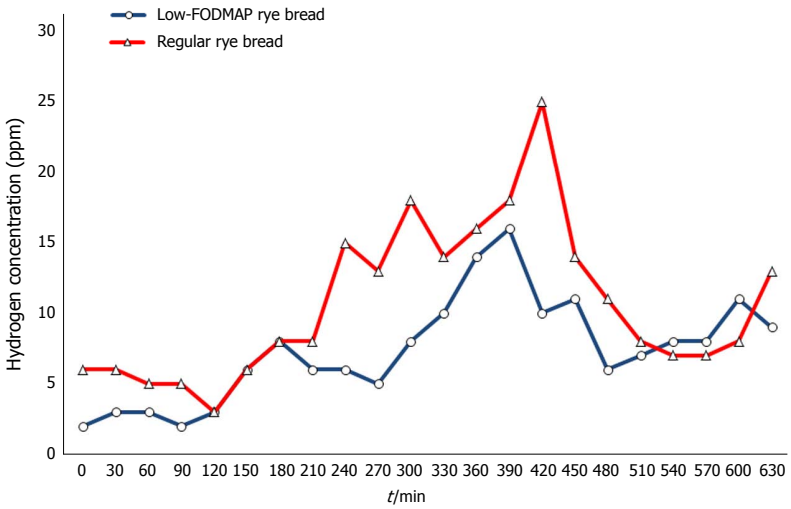

Figure 1 Medians of expired hydrogen concentration (ppm·min) during the test days.

mean breath hydrogen and between mean symptoms and SmartPill indices during the colonic phase were analyzed using the Spearman's rho. Statistical analysis was performed with IBM SPSS Statistics (Version 23, IBM Co., New York, United States) and Microsoft Office Excel 2013 (Microsoft Co., Washington, United States).

RESULTS

A total of nine female subjects (median age 39 years, range 29-51 years) with IBS were recruited into the study, of whom two withdrew during the study for personal reasons. The BMI of the subjects was [median (range)], 26.4 (19.5-30.4) kg/m^2. Three participants suffered from diarrhea-predominant IBS, two from constipation-predominant IBS and two from mixed type IBS. A flowchart of the recruitment process and the study is shown in Supplemental Figure 1.

Postprandial excretion of hydrogen expressed as $AUC_{(0\text{-}630\ min)}$ was [median (range)] 6300 (1785-10800) ppm·min for low-FODMAP rye bread and 10635 (4215-13080) ppm·min for regular bread. The two bread tests differed significantly (P = 0.028), indicating more intensive colonic fermentation after consumption of the regular rye bread. Median expired hydrogen concentrations are shown in Figure 1.

The means of the VAS measurements of individual gastrointestinal symptoms during the follow-up (30-630 min) did not reveal any statistically significant differences between the breads (Table 2). However, the flatulence severity was nearly significantly lower after low-FODMAP rye bread consumption (P = 0.06). Furthermore, there was a significant (P = 0.034) difference between the low-FODMAP bread (15 mm; range 5-34 mm) and the regular rye bread (34 mm; range 8-56 mm) in the maximum severity of flatulence (data for other maximum values not shown). Figure 2 shows the development of the total symptoms during the course of the test day. The difference in AUCs of total symptom score between low-FODMAP (23520 mm·min; range 6885-113610 mm·min) and regular rye breads (41130 mm·min; range 10785-83220 mm·min) was not statistically significant (P = 0.866).

All patients could swallow the SmartPill device without major challenges. In eight out of a total of 14 measurements the device stayed in the stomach for an unexpectedly long period (*i.e.*, > 10 h; in 6 measurements out of a total of 14 the device resided in the stomach > 15 h, data not shown). SmartPill left the stomach in less than five hours only during one measurement. Therefore, the devices did not follow on par with the rye bread bolus. All devices were defecated within three days of swallowing.

Transit times, median pH values, mean pressures, and contractions are shown in Table 3. SmartPill-derived transit times, pH values, mean pressure, or contractions/min in any part of the gastrointestinal tract did not differ between the bread tests. The association between colonic pressure and overall symptom severity during the time when the device was transiting the colon is shown in Figure 3. The correlation was significant after the participants had consumed regular rye bread (ρ = 0.786, P = 0.036) and was nearly significant after low-FODMAP bread consumption (ρ = 0.750, P = 0.052). The correlation coefficients between symptom severity and colonic contraction frequency were 0.775 (P = 0.041) and 0.786 (P = 0.036) after regular and low-FODMAP bread consumption, respectively. Colon pH and H_2 excretion were associated with symptom severity after regular bread (ρ = 0.821, P = 0.023 and ρ = 0.857, P = 0.014, respectively) but not after low-FODMAP bread (ρ = 0.342, P = 0.452 and ρ = 0.536, P = 0.215, respectively) consumption.

DISCUSSION

In this meal study, we demonstrated that consumption of low-FODMAP rye bread leads to reduced hydrogen

Table 2 Means (mm) of gastrointestinal symptoms from 30-630 min after the test meals median (range)

	Low-FODMAP rye	Regular rye bread	*P* value[1]
Abdominal pain	2.4 (0.0-28.2)	4.8 (0.1-21.9)	0.735
Cramps	1.2 (0.1-29.2)	3.0 (0.1-10.0)	0.917
Bloating	12.3 (1.0-48.4)	23.1 (1.1-37.8)	0.866
Flatulence	3.3 (0.5-7.6)	4.2 (0.5-28.2)	0.063
Belly rumbling	3.0 (0.1-6.6)	3.8 (1.4-11.4)	0.398
Nausea	1.0 (0.0-22.4)	2.1 (0.0-15.5)	1.000
Heartburn	1.4 (1.0-20.8)	1.8 (0.1-20.3)	1.000
Unpleasant sensation in the uppemen	7.4 (1.0-26.1)	11.8 (1.2-23.2)	0.310
Urge to defecate	1.5 (0.0-29.5)	2.1 (0.3-22.4)	0.735

[1]Wilcoxon signed-rank test.

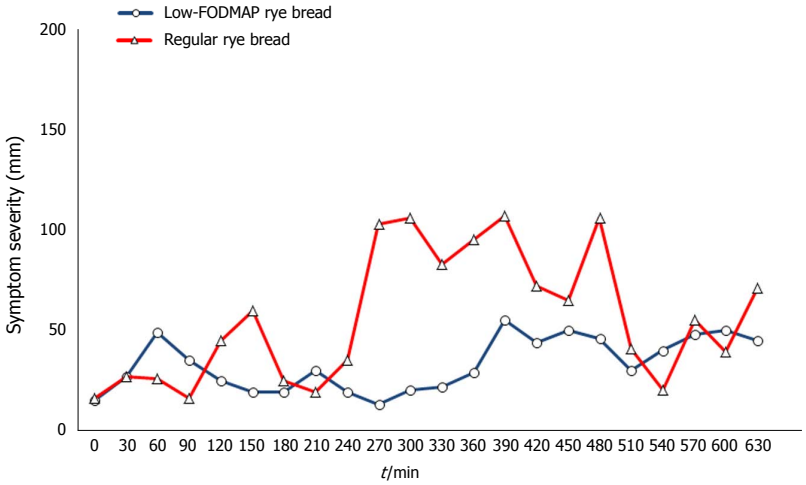

Figure 2 Medians of total symptom scores (mm) for both breads during the test days. The theoretical maximum of the symptom score sum is 900 mm.

expiration (a marker of colonic fermentation) when compared with regular rye bread consumption. Furthermore, the maximum flatulence severity was lower for the low-FODMAP rye bread compared with regular rye bread. No difference was found in other symptoms, pH, contractions, or total gastrointestinal tract pressure. Interestingly, intracolonic pressure and contraction frequency, rather than total gastrointestinal pressure, were associated with symptom severity during the colonic transition period of SmartPill. This finding suggests that the colon is most affected and is the origin of IBS symptoms. The exceedingly long gastric transit time measured by SmartPill was an unexpected finding.

Our finding of lower hydrogen excretion during the low-FODMAP rye bread test is consistent with previous studies; grain products that are high in fructans increase hydrogen excretion (*i.e.*, colonic fermentation) when compared with grain products low in fructans[15,24]. Additionally, the difference in the maximum flatulence value suggests a higher level of gas formation after regular rye bread consumption. There were no differences between the treatments in the perceived severity of other gastrointestinal symptoms, although the median values tended to favor the low-FODMAP rye bread. The perceived severity of symptoms was overall low. A larger sample size most likely would have been needed to reach statistical significance in symptom severity. The power calculation for the study was based on hydrogen excretion rather than change in symptoms.

A previous scintigraphy study has shown that after consumption of a medium-sized solid meal, 98%-99% of ingested food should leave the stomach within four hours and 84% within two hours[25]. The transition time of the SmartPill from stomach to small intestine varied from 4.5-22 h in the present study. These results are in contrast with previous studies[26,27], which have shown that gastric emptying of SmartPill takes place within five hours in most study subjects. On the other hand, sporadic prolonged gastric residence times of SmartPill in healthy volunteers have been previously reported[28,29]. SmartPill is a non-digestible capsule that presumably leaves the stomach during phase III of the migrating motor complex (MMC)[26]. The long gastric emptying times in the present study indicate that the six-hour lag time between the study breakfast and lunch was not long enough for the SmartPill to leave the stomach. The subsequent meal probably terminated the MMC cycle before the capsule was transported to the duodenum.

It is possible that gastric emptying times, at least

Table 3 SmartPill-derived transit times, pH values, mean pressure, and contractions/min median (range)

	Low-FODMAP rye bread	Regular rye bread	*P* value[1]
Transit time (h)			
Stomach	18.1 (5.3-22.3)	5.6 (4.5-18.0)	0.091
Small intestine	4.0 (2.1-5.6)	4.6 (3.2-6.6)	0.866
Colon	25.2 (12.2-50.0)	32.1 (14.7-47.6)	0.176
Whole GI tract	46.5 (22.6-73.5)	45.8 (24.3-70.4)	0.612
Median pH			
Stomach	1.5 (0.8-4.1)	1.5 (1.0-2.4)	0.671
Small intestine	7.5 (5.0-8.0)	7.6 (7.0-7.8)	0.915
Colon	7.2 (5.8-7.5)	6.5 (5.9-8.5)	0.612
Mean pressure (mmHg)			
Stomach	2.2 (1.9-2.7)	2.5 (2.0-3.0)	0.610
Small intestine	3.1 (1.6-8.6)	4.5 (2.4-7.0)	0.398
Colon	4.8 (3.2-6.3)	4.0 (2.0-6.7)	0.310
Contractions/min			
Stomach	1.2 (0.6-1.7)	1.0 (0.8-1.8)	0.553
Small intestine	3.2 (0.5-6.1)	4.9 (1.9-6.5)	0.176
Colon	1.7 (1.3-2.9)	1.7 (0.6-3.3)	0.495

[1]Wilcoxon signed-rank test.

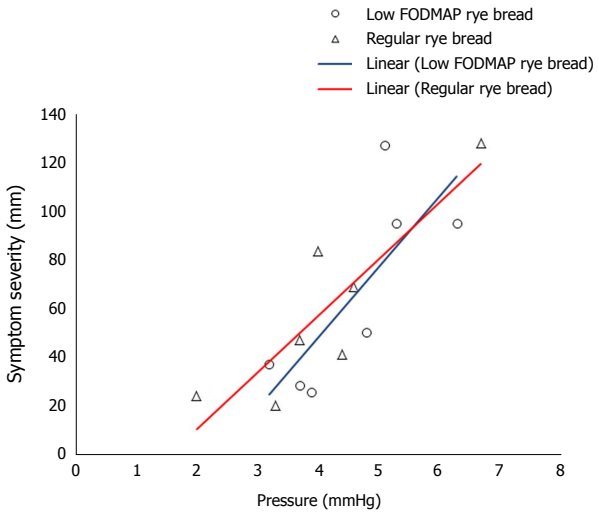

Figure 3 Association between intracolonic pressure and simultaneous total symptom score after consumption of test breads. The theoretical maximum symptom score sum is 900 mm.

those measured with SmartPill, are increased in IBS. Indeed, another study by Dupont *et al*[30] also reported gastric emptying times longer than 5 h in IBS patients. Furthermore, in study from Ringel-Kulka *et al*[17], patients with constipation-predominant IBS had prolonged gastric emptying times (mean 8 h). Although there were three bread meals during the study day, in many cases SmartPill followed the rye bolus with a delay of several hours. This is important information for future studies. SmartPill might fail to measure gastrointestinal circumstances in a timely manner, at least in patients with IBS. Based on our study, it is possible that SmartPill is more applicable for feeding studies lasting at least two days rather than one-day meal studies.

Due to the unexpectedly long residence of SmartPill in the stomach, the device was usually in the small intestine during the night. For this reason, we could not compare perceived symptoms and measured conditions in the small intestine. However, the residence time was longer in the large intestine, and thus we could link symptom ratings and conditions in the colon. Intracolonic pressure correlated with IBS symptom severity in our study. One of the key underlining reasons why IBS patients experience pain and discomfort is their lower threshold to sense pain and distension, a phenomenon called visceral hypersensitivity[2,31]. Therefore, any dietary or physio-anatomic factors that increase pressure in gut might worsen symptoms. Theoretically, intracolonic pressure might increase due to many factors, such as consumption of gas-forming FODMAPs, impaired handling of colonic gas, intestinal microbiota disturbances, fecal retention, difficulties in expelling gas or anatomical abnormalities that obstruct gas. However, with our methods we cannot explain the underlining cause of the increased intracolonic pressure. Nonetheless, hydrogen excretion did not correlate with intracolonic pressure in the present study, suggesting that other reasons such as intestinal abnormalities or microbiota disturbances might play a larger role. Intestinal abnormalities previously associated with IBS symptoms include small intestinal constriction, dilated transverse colon, and redundant colon[32,33]. Further research is warranted in the area of intracolonic pressure and its role in IBS symptoms. Interestingly, Rogers *et al*[34] have shown that intraluminal pressure was higher among subjects with IBS when compared with healthy subjects. These results, together with our findings, suggest that colonic gas amplitude may have a causal role in IBS symptom generation.

We also found a correlation between colonic contraction frequency and symptom severity during both bread periods. Previously, Hasler *et al*[35] demonstrated

in their SmartPill study that colonic motor activity (*i.e.*, colonic contraction frequency and duration of contractions) is increased in constipation-predominant IBS patients when compared with non-IBS subjects. Increased motoric activity might be one mechanism for how intracolonic pressure is increased in IBS. We also observed that pH and hydrogen excretion during the colonic phase were associated with symptom severity after consumption of regular rye bread; these findings might simply reflect the degree of colonic fermentation and act as surrogates of colonic pressure. Taken together, our findings with previous SmartPill-gathered data[17,35] provide further evidence for the role of intraluminal physiological conditions in triggering IBS symptoms. This observation is of relevance to clinicians as IBS is sometimes considered primarily as a psychosocial condition[36]. Interestingly, spasmolytics, such as peppermint oil capsules, relax smooth muscles of the intestinal wall and thus reduce colonic motility, which may explain the reductions in pain, discomfort, and feeling of bloating reported in several clinical trials[37]. Attempts to reduce intraluminal pressure and contractions might continue to be important therapeutic targets in IBS.

Our study has limitations. As stated previously, the number of subjects might have been too small to detect all true differences between the breads. The long residence of SmartPill in the stomach is a potential bias in our study. The observation period was rather short (630 min). On the basis of our experience in this study, we recommend longer studies when using SmartPill in diet-related research. The strengths of our study include the double-blind randomized setting and standardization of the evening snack before the trial and all food consumed during the first 24 h of the test period.

In conclusion, our meal study demonstrated that consumption of low-FODMAP rye bread led to reduced colonic fermentation and maximum flatulence values in IBS patients when compared with regular rye bread. No differences could be found in other symptoms, pH, colonic pressure, or gut contractions. Due to its inability to measure effects of a singular food bolus in a timely manner, this study also showed that SmartPill might not be an optimal device in evaluating gastrointestinal circumstances during meal studies lasting less than 24 h. The observed correlation between increased intracolonic pressure and symptom severity underlines the central role of visceral sensitivity in IBS. Further studies are needed to understand the role of intracolonic pressure formation in IBS.

ARTICLE HIGHLIGHTS

Research background

FODMAPs are rapidly fermentable carbohydrates shown to aggravate gastrointestinal symptoms in irritable bowel syndrome (IBS). A major challenge in the research of IBS is the lack of objective markers of disease activity. Excretion of hydrogen and methane and colonic fermentation markers are among the rare easily available and objective markers of gastrointestinal circumstances during the consumption of FODMAPs or other poorly absorbable carbohydrates. Grains are often considered as triggers of irritable bowel syndrome symptoms but less is known about the effects of grain products with differing content of FODMAPs on gastrointestinal transit times, pH and intraluminal pressure in patients with IBS.

Research motivation

SmartPill, a motility monitoring capsule, which measures intraluminal pH, transit time and pressure, and might thus offer the means to gather objective data on gastrointestinal conditions in IBS patients. A combination of SmartPill data and measurements of perceived symptoms may improve our understanding on the etiology of IBS symptoms when consuming grains high in FODMAPs.

Research objectives

Our aim was to evaluate if rye bread low in FODMAPs would cause less hydrogen excretion, lower intraluminal pressure, higher colonic pH and less IBS symptoms than regular rye bread.

Research methods

The study was conducted as a randomized double blind controlled cross-over meal study. Female IBS patients (n = 7) ate study breads on 3 consecutive meals. Intraluminal conditions were measured by SmartPill®, an indigestible motility capsule.

Research results

Postprandial hydrogen excretion, a marker of colonic fermentation, expressed as $AUC_{(0\text{-}630\ min)}$ was [median (range)] 6300 (1785-10800) for low-FODMAP rye bread and 10635 (4215-13080) ppm·min for regular bread (P = 0.028). The means of the visual analogue scale measurements of individual gastrointestinal symptoms did not show any statistically significant differences between the breads. Intraluminal pressure correlated significantly with total symptom score after regular rye bread (ρ = 0.786, P = 0.036) and nearly significantly after low-FODMAP bread consumption (ρ = 0.75, P = 0.052). We found no differences in pH, contractions or transit times between the breads. Gastric emptying of SmartPill was slower than expected on the basis of majority of research literature.

Research conclusions

Our meal study demonstrated that low-FODMAP rye bread reduces colonic fermentation but no difference was found in median values of symptoms, pH, colonic pressure of gastrointestinal tract when compared to regular rye bread. Our observation on the correlation between increased intra-colonic pressure and symptom severity warrants further studies in IBS.

Research perspectives

Our finding on the correlation of intracolonic pressure and symptom severity suggests that IBS symptoms might be worsened by any reason that leads to increased colonic pressure in IBS. Consequently, any therapeutic attempts to reduce intraluminal pressure and contractions might continue to be important therapeutic targets in IBS. The study also implied that SmartPill might not be an optimal device to evaluate the gastrointestinal circumstances during meal studies among IBS patients lasting less than 24 h, due to device's inability to measure effects of a singular food bolus in a timely manner. Observation and feeding periods longer than 38 h are recommended for future research utilizing SmartPill, especially among people with IBS.

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ORIGINAL ARTICLE

Prospective Study

Fatty liver in hepatitis C patients post-sustained virological response with direct-acting antivirals

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Abstract

AIM

To determine steatosis and fibrosis prevalence in hepatitis C patients after a sustained virological response achieved with direct-acting antivirals.

METHODS

Transient elastography with controlled attenuation parameter (CAP) was used to assess hepatic steatosis post-sustained virological response (SVR); the CAP technology was not available in the United States at study initiation. Liver stiffness/fibrosis was measured before and 47 wk after treatment completion. Patients with genotype 3 and patients with cirrhosis were excluded.

RESULTS

One hundred and one patients were included in the study. Post-SVR there were decreases from baseline in alanine aminotransferase (ALT) (63.1 to 17.8 U/L), aspartate aminotransferase (51.8 to 21.5 U/L) and fibrosis score (7.4 to 6.1 kPa) ($P < 0.05$). Post-SVR, 48 patients (47.5%) had steatosis on CAP; of these, 6.25% had advanced fibrosis. Patients with steatosis had higher body mass index (29.0 *vs* 26.1 kg/m^2), glucose (107.8 *vs* 96.6 mg/dL), ALT (20.4 *vs* 15.3 mg/dL), CAP score (296.3 *vs* 212.4 dB/m) and fibrosis score (7.0 *vs* 5.3 kPa); $P < 0.05$. Interestingly, compared to baseline, both patients with and without steatosis had change in fibrosis score post-SVR (7.7 kPa *vs* 7.0 kPa and 7.0 kPa *vs* 5.3 kPa); alternatively, ($P < 0.05$) and therefore patients with steatosis continued to have clinically significant stiffness ($\geqslant$ 7 kPa).

CONCLUSION

Fatty liver is very common in hepatitis C virus (HCV) patients post-SVR. These patients continue to have elevated mean fibrosis score ($\geqslant$ 7 kPa) compared to those without fatty liver; some have advanced fibrosis. Long term follow up is needed to assess steatosis and fibrosis in HCV patients post-SVR.

Key words: Nonalcoholic fatty liver disease; Hepatitis C; Fibrosis; Steatosis; Sustained virological response; Direct-acting antivirals

Core tip: This is the first prospective study to assess the prevalence of fatty liver in hepatitis C patients who have achieved a sustained virological response with direct-acting antivirals. The study's findings that fatty liver is present in 47.5% of these patients and that some steatotic patients have clinically significant fibrosis despite normal liver enzymes should raise awareness of the post-sustained virological response (SVR) prevalence of fatty liver and the importance of post-SVR assessment of steatosis and fibrosis and long-term follow up with these patients.

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INTRODUCTION

With the growing epidemic of obesity and type 2 diabetes mellitus, nonalcoholic fatty liver disease (NAFLD) currently has a worldwide prevalence of 25.24% (approximately 1.8 billion people)[1], making it the most common cause of chronic liver disease (CLD), followed by chronic hepatitis B (CHB, 257 million people), and chronic hepatitis C (CHC, 71 million people)[2]. In the United States, NAFLD and CHC are the two most common CLD causes[3], and nonalcoholic steatohepatitis (NASH)-associated cirrhosis is the second leading indication for liver transplant (LT) after hepatitis C virus (HCV)-associated end-stage liver disease[4]. With the recent study that showed that between 2004 and 2013 the number of adult patients with NASH awaiting LTs almost tripled[4], combined with the rapidly expanding population of CHC patients achieving sustained virological responses (SVRs) with direct-acting antivirals (DAAs), it is thought that NASH may soon become the leading indication for LT. NAFLD prevalence is now estimated to be approximately 30% in the United States[5].

NAFLD is usually diagnosed by detecting steatosis after excluding other causes of liver disease. However, hepatic steatosis may occur in patients with other liver diseases, often in those with obesity and other metabolic factors typical of NAFLD, potentially creating an additive or synergistic combination of steatosis, oxidative damage, cellular impairment and other factors that may worsen liver injury[6]. Steatosis is known to escalate liver necroinflammatory activity and accelerate fibrosis in CHC patients[7]. The hepatic steatosis prevalence in

CHC patients has been reported to be approximately 50% (range 30%-70%)[8]. The mechanisms leading to steatosis in CHC have not been fully elucidated but may include host factors leading to insulin resistance and interactions between lipid metabolism pathways and the HCV core protein[9,10]. It has been proposed that HCV's effects on hepatic lipid metabolism may inhibit the export proteins needed for the assembly and secretion of very low density lipoproteins (VLDL), resulting in triglyceride accumulation in the liver[8]. Therefore, hepatic steatosis in HCV patients may result from some combination of viral and metabolic factors, other than in genotype 3 (GNT3) patients in which the steatosis may be due to direct effects of genotype 3 viral proteins[11].

Historically, an SVR with interferon was not associated with steatosis resolution except in GNT3 patients which has a different steatosis etiology[10]. In patients with an SVR achieved with DAAs steatosis prevalence is unknown. In this prospective, cross-sectional study, we assessed steatosis prevalence and degree of fibrosis in CHC patients who achieved an SVR through treatment with DAAs.

MATERIALS AND METHODS

Study design

This is a prospective, cross-sectional study of patients with CHC who achieved an SVR after treatment with DAAs. The patients in this cohort had been treated with a variety of direct-acting antiviral regimens: ledipasvir/sofosbuvir (Harvoni), 75 patients; elbasvir/grazoprevir (Zepatier), 1 patient; dasabuvir/ombitasvir/paritaprevir/ritonavir (Viekira), 7 patients; dasabuvir/ombitasvir/paritaprevir/ritonavir with ribavirin, 2 patients; sofosbuvir (Sovaldi) with ribavirin, 9 patients; sofosbuvir with daclatasvir (Daklinza), 1 patient; sofosbuvir with simeprevir (Olysio), 2 patients; sofosbuvir/velpatasvir (Epclusa), 4 patients. Between January 2016 and March 2017, 101 adult patients were enrolled, excluding patients with other liver diseases, secondary causes of steatosis (*e.g.*, medications, excessive alcohol), and GNT3 which has a different steatosis etiology. After achieving an SVR, patients were invited to undergo standardized history and anthropometric examination, laboratory testing, and transient elastography (TE) at the California Liver Research Institute in Los Angeles. This study received approval and was done under IRB protocol CLRI-01. Ethical guidelines for human research were followed. All patients signed informed consent.

Transient elastography

TE was performed using the FibroScan 502 Touch model (M Probe, XL Probe; Echosens, Paris, France) by an experienced TE-certified technician blinded to clinical data. Patients were asked to fast for at least 4 h prior to the examination. The procedure was performed in the supine position with the right arm adducted while holding the breath for 10 s. All patients were first scanned with the M probe (3.5 MHz) over the right liver lobe. If indicated by the machine, patients were re-evaluated using the XL probe (2.5 MHz). Ten measurements were made and the interquartile range was less than 30%. We defined test failure when no stiffness measurement was obtained or there were unreliable measurements (success rate < 60% or interquartile range/median > 30%)[12-14].

Liver stiffness/fibrosis scores were measured before and within one year after completion of HCV treatment with DAAs; the median time interval between treatment completion and post-SVR TE was 47 wk, with no significant difference between patients with and without steatosis. Simultaneous liver steatosis measurements were obtained using controlled attenuation parameter (CAP) values in dB/m only after SVR achievement as the technology was not available in the United States at the study's initiation. Based on the recent large patient data meta-analysis of studies containing histology-verified CAP data for grading of steatosis that determined optimal cut-offs for CAP[15], steatosis was defined as $\geqslant$ 248 dB/M. Clinically significant stiffness was defined as $\geqslant$ 7 kilopascal (kPa)[16,17].

Patients' specifications

We included patients if they were 18 years or older, were treated for CHC using DAAs and were able to provide informed consent. We excluded patients if they (1) had a history of significant alcohol intake within 2 years of recruitment (14 drinks/wk for men or 7 drinks/wk for women) as assessed by the hepatologist as well as the Alcohol Use Disorders Identification Test-Consumption (AUDIT-C) questionnaire; (2) had secondary causes of fatty liver such as medications (for example, methotrexate) or other infectious causes (for example, human immunodeficiency virus); (3) had evidence of liver diseases other than hepatitis C; (4) were HCV GNT3 as it is thought to have a different underlying etiology of steatosis related to the virus (viral steatosis) and we sought to investigate this genotype separately; or (5) had cirrhosis based on imaging or FibroScan. All the following information was collected: medical history, age, sex, height, weight, body mass index (BMI), ethnic background, and vital signs.

Laboratory measurements

The biochemical tests that were measured included aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase, total bilirubin, direct bilirubin, albumin, fasting glucose, hemoglobin A1c, triglycerides, total cholesterol, high-density lipoprotein, and low-density lipoprotein. Other measurements included platelets, prothrombin time, and international normalized ratio.

Statistical analysis

The chi-square test was used to compare between categorical variables, and a paired *t* test to compare

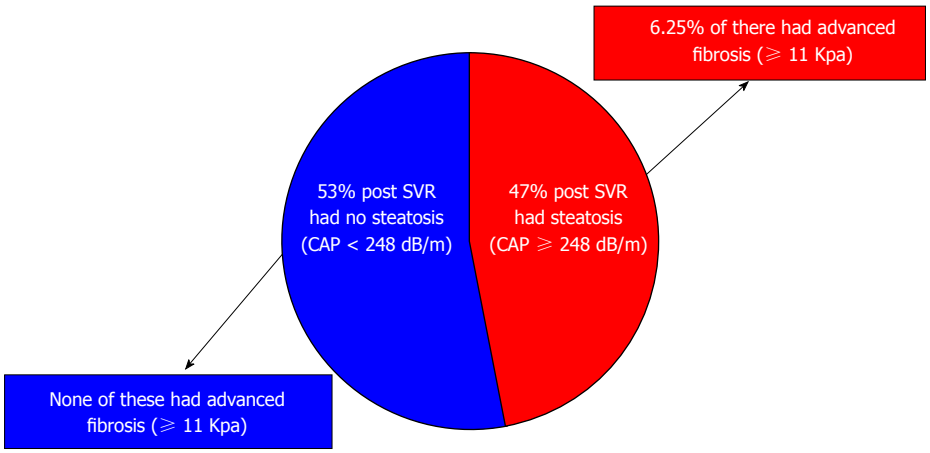

Figure 1 Post-sustained virological response steatosis prevalence in hepatitis C virus patients and advanced fibrosis prevalence in those with and without steatosis. SVR: Sustained virological response; CAP: Controlled attenuation parameter.

mean differences between continuous variables. Primary and secondary comparisons within groups were calculated with paired *t* tests, two-tailed, independent-sample *t* tests, or nonparametric tests including Wilcoxon signed-rank test as applicable. A two-tailed $P < 0.05$ was considered statistically significant. Statistical analyses were performed with SPSS 21.

RESULTS

Patient characteristics

Between January 2016 and March 2017, 101 adult CHC patients who achieved SVR were enrolled. At baseline the average age for the entire cohort was 60.3 ± 10.7 years and BMI was 27.6 ± 6.9 kg/m^2; 37% were Caucasian and 26% were Hispanic. The average fibrosis score was 7.4 ± 1.9 kPa. HCV genotypes were: GNT1 (85%), GNT2 (14%), and GNT4 (1%) (Table 1).

Changes post-SVR

Changes in the Entire Cohort: As expected, post-SVR HCV viral load was undetectable compared to prior baseline (prior to starting treatment) (0.0 ± 0.0 IU/m *vs* 6.2 ± 0.9 IU/m; $P < 0.0001$). ALT and AST decreased to normal levels post-SVR compared to baseline (17.8 ± 12.3 U/L *vs* 63.1 ± 62.6 U/L for ALT; $P < 0.0001$ and 21.5 ± 8.0 U/L *vs* 51.8 ± 41.1 U/L for AST; $P < 0.0001$). There was no change in BMI post-SVR compared to baseline (27.5 ± 6.9 kg/m^2 *vs* 27.6 ± 6.9 kg/m^2). In the overall cohort, post-SVR there was a significant decrease in fibrosis score on TE (7.4 ± 1.9 kPa to 6.1 ± 3.6 kPa; $P = 0.013$), a decline that is considered clinically significant.

Changes in patients with and without steatosis post-SVR: Post-SVR, 48 patients (47.5%) had steatosis with mean CAP score 296.3 ± 37.4 compared to a mean CAP score 212.4 ± 29.4 dB/m in patients without steatosis ($P < 0.0001$) (Figure 1). Patients with steatosis were more likely than patients without steatosis to have type 2 diabetes (18.7% *vs* 7.5%; $P = 0.04$), dyslipidemia (10.4% *vs* 5.7%; $P = 0.048$), higher body mass index (28.9 ± 6.6 kg/m^2 *vs* 26.1 ± 6.9 kg/m^2; $P = 0.049$), ALT (20.4 ± 16.5 U/L *vs* 15.3 ± 5.5 U/L; $P = 0.048$), fasting glucose (107.8 ± 30.5 mg/dL *vs* 96.5 ± 11.1 mg/dL; $P = 0.023$) and triglycerides (138.8 ± 77.9 mg/dL *vs* 109.7 ± 63.9 mg/dL; $P = 0.05$) (Table 2). None of the patients without steatosis had abnormal liver enzymes; only 6.25% of patients with steatosis had abnormal liver enzymes.

Changes in patients with and without steatosis between baseline and post-SVR: Interestingly, patients with steatosis continued to have clinically significant liver stiffness (mean baseline 7.7 ± 1.7 kPa; post-SVR 7.0 ± 4.8 kPa; $P = 0.037$) while patients without steatosis did not (mean baseline 7.1 ± 2.1; post-SVR 5.3 ± 1.5 kPa; $P < 0.0001$) (Table 3). Among patients with post-SVR steatosis, 6.25% had advanced fibrosis defined as ⩾ 11 kPa. No patients without steatosis had advanced fibrosis (Table 3).

Post-SVR, neither weight nor BMI changed while levels of transaminases and other liver enzymes dropped in patients both with and without steatosis, including ALT (55.6 ± 60.9 U/L to 15.3 ± 5.5 U/L in patients with steatosis, $P < 0.0001$, and 68.78 ± 52.8 U/L to 20.4 ± 16.5 U/L in patients without steatosis; $P < 0.0001$, respectively); AST (43.3 ± 35.6 U/L to 20.2 ± 5.4 U/L; $P < 0.0001$ and 61.3 ± 44.7 U/L to 22.9 ± 9.8 U/L; $P < 0.0001$, respectively); and alkaline phosphatase (78.5 ± 43.1 U/L to 70.8 ± 28.8 U/L; $P = 0.01$ and 75.5 ± 21.8 U/L to 71.3 ± 19.4 U/L; $P = 0.04$ (Table 3).

DISCUSSION

Since hepatic steatosis prevalence in CHC patients has previously been reported to be approximately 50%[8] our findings of a 47.5% prevalence post-SVR achieved with DAAs should perhaps not be surprising. However, this very high prevalence with continuing

Table 1 Demographic and clinical characteristics of the chronic hepatitis C patients prior to direct-acting antivirals treatment and after achieving sustained virological response 12 *n* (%)

	Prior to DAA treatment (baseline)	Post-SVR 12	*P*[1] value
Demographics			
Male	49 (48)	49 (48)	NS
Age (yr, mean ± SD)	60.3 ± 10.7	60.3 ± 10.7	NS
White	37 (37)	37 (37)	NS
Hispanic	26 (26)	26 (26)	NS
African American	13 (13)	13 (13)	NS
Asian	7 (7)	7 (7)	NS
Other	2 (2)	2 (2)	NS
Declined	16 (15)	16 (15)	NS
Clinical			
Hypertension	45 (43)	45 (43)	NS
Type 2 diabetes	13 (12.3)	13 (12.3)	NS
Dyslipidemia	8 (7.5)	8 (7.5)	NS
Anthropometric (mean ± SD)			
Body mass index (kg/m^2)	27.6 ± 6.9	27.5 ± 6.9	NS
Weight (Lbs.)	174.9 ± 46.9	172.7 ± 44.5	NS
Laboratory panel (mean ± SD)			
HCV vial load log$_{10}$ IU/mL	6.2 ± 0.9	0.0 ± 0.0	< 0.0001
HCV genotype			
Genotype 1	86 (85)		
Genotype 2	15 (14)		
Genotype 4	1 (1)		
AST (U/L)	51.8 ± 41.1	21.5 ± 8.0	< 0.0001
ALT (U/L)	63.1 ± 62.6	17.8 ± 12.3	< 0.0001
Alkaline phosphatase (U/L)	77.5 ± 34.0	71.0 ± 24.3	0.004
Albumin (g/dL)	4.3 ± 0.4	4.4 ± 0.4	NS
Bilirubin, total (mg/dL)	0.6 ± 0.2	0.6 ± 0.3	NS
Fasting glucose (mg/dL)	99.1 ± 30.1	102.1 ± 23.5	NS
FibroScan (mean ± SD)			
Fibrosis Score (kPa)	7.4 ± 1.9	6.1 ± 3.6	0.013
IQR (%)	12.6 ± 4.9	12.3 ± 5.5	NS

[1]*P* values (2-sided) determined from either a Fisher's exact test for categorical variables or *t*-test for continuous variables. DAA: Direct-acting antivirals; SVR: Sustained virological response; HCV: Hepatitis C virus; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; IQR: Interquartile range.

clinically significant fibrosis in the steatotic patients despite normal liver enzymes should be of concern to clinicians. The current European guidelines recommend assessing ALT and HCV RNA 48 wk post-treatment in non-cirrhotic patients with SVR, with no further follow up with normal ALT/undetectable HCV RNA[18]. The current United States guidelines for patients post-SVR recommend follow up only for those with advanced fibrosis; assessing other liver disease causes is only recommended in cases of persistently abnormal transaminases[19]. Importantly, we show that fatty liver may be present despite normal liver enzymes, confirming previous studies that have shown this[20]. Therefore, we recommend post-SVR assessment of steatosis and fibrosis in those with abnormal BMI or other risk factors typical of NAFLD. In patients found to have hepatic steatosis long-term follow up is warranted.

To our knowledge this is the first prospective study to assess the prevalence of fatty liver in HCV patients who achieved an SVR with DAAs. We hope that our study will raise awareness of the post-SVR prevalence of fatty liver and the need for screening and long-term follow up. Our study's strengths include the community-based hepatology setting, which likely accurately represents real life experience. In addition, we used TE, which is highly sensitive and specific, and is widely used and easy to perform. Although liver biopsy is still the gold standard to assess fatty liver and staging with MRI proton density fat fraction may be more accurate[21], biopsy is invasive and costly and many patients are reluctant to undergo the procedure because of concerns about pain and, although limited, possible complications. With biopsy there is also the possibility of inter-and intra-observer variability and sampling error[22]. MRI techniques are quite expensive. Neither of these is likely to be performed in post-SVR patients with normal liver enzymes. Thus, the use of TE with CAP is realistic in a real-world setting.

There is substantial data showing good sensitivity and specificity for the use of TE in determining either presence of advanced fibrosis or no fibrosis. In eight studies that compared the usefulness of TE and liver biopsy for assessment of liver fibrosis in NAFLD patients it was shown that TE is very good for diagnosis of F $\geqslant$ 3, with 84%-100% sensitivity and 83%-97% specificity[23-30]. Similar findings were reported in a recent large systematic review and meta-analysis that confirmed that TE was excellent for diagnosis of F $\geqslant$ 3 in NAFLD patients[31]. Although there is reduced accuracy using TE for distinguishing early fibrosis

Table 2 Characteristics of chronic hepatitis C patients after achieving sustained virological response 12 comparing those with and without steatosis *n* (%)

	Patients without steatosis (CAP < 248 dB/m) (*n* = 53)	Patients with steatosis (CAP ⩾ 248 dB/m) (*n* = 48)	*P*[1] value
Demographics			
Male	25 (47)	27 (56)	NS
Age (yr, mean ± SD)	59.4 ± 11.6	60.9 ± 9.4	NS
White	18 (34)	18 (38)	NS
Hispanic	14 (26)	12 (25)	NS
Clinical			
Hypertension	25 (47.2)	20 (41.7)	NS
Dyslipidemia	3 (5.7)	5 (10.4)	0.048
Type 2 diabetes	4 (7.5)	9 (18.7)	0.04
Anthropometric (mean ± SD)			
Body mass index (kg/m^2)	26.1 ± 6.9	28.9 ± 6.6	0.049
Weight (Lbs.)	161.0 ± 33.4	172.7 ± 44.5	0.005
Hepatology and viral hepatitis panel (mean ± SD)			
AST (U/L)	20.2 ± 5.4	22.9 ± 9.8	NS
ALT (U/L)	15.3 ± 5.5	20.4 ± 16.5	0.048
Alkaline phosphatase (U/L)	70.7 ± 28.2	71.3 ± 19.4	NS
Albumin (g/dL)	4.3 ± 0.2	4.5 ± 0.6	NS
Bilirubin, total (mg/dL)	0.6 ± 0.3	0.6 ± 0.2	NS
Other laboratory studies (mean ± SD)			
Total cholesterol (mg/dL)	184.8 ± 35.1	179 ± 37.2	NS
HDL cholesterol (mg/dL)	57.6 ± 18.6	50.8 ± 17.0	NS
LDL cholesterol (mg/dL)	102.6 ± 33.2	100.7 ± 31.5	NS
Triglycerides (mg/dL)	109.7 ± 63.9	138.9 ± 77.9	0.05
HbA1c (%)	5.7 ± 0.6	6.0 ± 0.9	NS
Fasting serum glucose (mg/dL)	96.5 ± 11.1	107.8 ± 30.5	0.023
FibroScan (mean ±SD)			
Fibrosis Score (kPa)	5.3 ± 1.6	7.0 ± 4.8	0.0013
CAP (dB/m)	212.4 ± 29.0	296.3 ± 37.4	< 0.0001
% of patient with fibrosis score of (⩾ 7 kPa)	0%	6.25%	0.066

[1]*P* values (2-sided) determined from either a Fisher's exact test for categorical variables or *t*-test for continuous variables. CAP: Controlled attenuation parameter; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; HDL: High density lipoprotein; LDL: Low density lipoprotein; CAP: Controlled attenuation parameter.

stages (F1-F2), in our study we were mainly comparing results in patients with and without advanced fibrosis. There is also substantial data showing good sensitivity and specificity of TE with CAP for assessing hepatic steatosis[32]. Although cutoff values for defining steatosis with CAP have not been fully formalized, we chose the value that defined steatosis (⩾ 248 dB/M) based on a very recent large (2735 patients) meta-analysis of studies containing histology-verified CAP data for grading of steatosis that determined optimal cut-offs for CAP[15].

Although until relatively recently, obesity (BMI > 30 kg/m^2) was associated with a reduced ability of TE to accurately determine fibrosis and steatosis, this problem has been largely addressed with the development of the obese-specific XL probe which we used in our study, confirmed in multiple studies to obtain reliable liver stiffness measurement in obese patients[33-35]. Another strength of our study is our inclusion of a detailed metabolic profile and alcohol questionnaire, with other causes carefully ruled out. It has been suggested that post-SVR some patients might feel free to indulge in alcohol consumption, with a resulting increase in liver stiffness measurements. Importantly, we ruled out increased alcohol intake through both medical records and use of the AUDIT-C at the time of the TE CAP assessment post-SVR.

Although our exclusion of HCV GNT3 patients means that our findings cannot be applied to the approximately 30.1% of HCV patients with this genotype[36], the exclusion is a strength of the study in other ways. Steatosis has been shown to correlate with intrahepatic viral replication in GNT3, with resolution of steatosis seen after effective antiviral treatment, suggesting a direct steatogenic effect of GNT3 virus[8]. In a study of patients treated with interferon, steatosis improvement post-SVR was seen in 91% of GNT3 patients *vs* 43% of patients with other genotypes ($P < 0.04$)[37]. In a study that compared the effects of interferon treatment in GNT1 and GNT3 patients, hepatic steatosis did not change in GNT1 patients, regardless of the treatment response, while steatosis was significantly reduced in GNT3 patients who achieved an SVR ($P < 0.001$) but not in patients who did not[38], again suggesting a direct steatogenic effect of GNT3 HCV. Thus, GNT3 patients represent a unique population in terms of steatosis that should be studied separately. Inclusion of these patients in our study could have substantially altered our findings regarding post-SVR steatosis, likely substantially reducing the prevalence due to steatosis reduction

Table 3 Comparison of pre-treatment *vs* post-sustained virological response characteristics in patients with and without post-sustained virological response steatosis

	Patients without steatosis n = 53			Patients with steatosis n = 48		
	Pretreatment	Post SVR	*P* value	Pretreatment	Post SVR	*P* value
Body mass index (kg/m^2)	25.5 ± 4.0	26.1 ± 6.9	NS	30.0 ± 8.5	29.0 ± 6.6	NS
Weight (Lbs.)	161.9 ± 32.6	161.0 ± 33.4	NS	187.3 ± 55.8	186.1 ± 51.3	NS
Laboratory panel (mean ± SD)						
HCV vial load log$_{10}$ IU/mL	6.1 ± 1.0	0.0 ± 0.0	< 0.0001	6.3 ± 0.8	0.0 ± 0.0	< 0.0001
AST (U/L)	43.3 ± 35.6	20.2 ± 5.4	< 0.0001	61.3 ± 44.7	22.9 ± 9.8	< 0.0001
ALT (U/L)	55.6 ± 60.9	15.3 ± 5.5	< 0.0001	68.78 ± 52.8	20.4 ± 16.5	< 0.0001
Alkaline phosphatase (U/L)	78.5 ± 43.1	70.8 ± 28.8	0.01	75.5 ± 21.8	71.3 ± 19.4	0.04
Albumin (g/dL)	4.2 ± 0.5	4.4 ± 0.3	0.006	4.3 ± 0.2	4.5 ± 0.6	0.006
Bilirubin total (mg/dL)	0.6 ± 0.2	0.6 ± 0.3	NS	0.6 ± 0.3	0.6 ± 0.2	NS
Fasting glucose (mg/dL)	95.6 ± 31.9	96.6 ± 11.1	NS	103.0 ± 27.5	107.8 ± 30.5	NS
FibroScan (mean ± SD)						
Fibrosis score (kPa)	7.1 ± 2.1	5.3 ± 1.5	< 0.0001	7.7 ± 1.7	7.0 ± 4.8	0.0037

SVR: Sustained virological response; HCV: Hepatitis C virus; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase.

in GNT3 patients, resulting in an overall steatosis prevalence which would not be representative of the almost 70% of HCV patients with other genotypes[36].

A limitation of our study is that, because the CAP technology was not available in the United States at the time of study initiation, we were unable to estimate steatosis prevalence with CAP prior to the initiation of DAAs in order to determine treatment effect. However, regardless of baseline steatosis prevalence, there is real clinical value in assessing post-SVR prevalence so that appropriate long-term follow up can be recommended. Another limitation is the length of follow-up as the median time interval in our study is 47 wk between treatment completion and the post-SVR TE. Lengthier studies are definitely needed to assess NAFLD progression and steatosis and fibrosis changes over time in this population. However, by assessing patients at almost a year post-SVR we have at least provided a foundation upon which lengthier studies could expand. The sample size could be considered as a limitation; however, this is a proof of concept study that this is first of its kind and warrants larger studies. Finally, we excluded patients with cirrhosis. However, these patients are usually followed up closely post-SVR and steatosis has been found to be low when patients have advanced fibrosis[39].

In conclusion, our findings that 47.5% of HCV patients had steatosis post-SVR and that some steatotic patients had clinically significant fibrosis, despite normal liver enzymes, highlight the importance of post-SVR assessment of steatosis and fibrosis in these patients. We believe these patients should be followed longitudinally, both to provide appropriate patient care and to advance our understanding of the long-term consequences of hepatic steatosis in post-SVR patients. In addition, we note that despite SVR these steatotic CHC patients are excluded from most NAFLD clinical trials, predominantly because of the current guidelines' definition of NAFLD as a diagnosis of exclusion[40,41]. We propose revisiting this and implementing new definitions of those with concomitant liver diseases, including those with HCV SVRs, that might allow patients' participation in trials, an unmet need in the rising epidemic of NAFLD.

ARTICLE HIGHLIGHTS

Research background

It is known that the hepatic steatosis prevalence in hepatitis C patients who have achieved a sustained virological response with interferon is approximately 50%. However, the prevalence of fatty liver in hepatitis C patients who have achieved a sustained virological response with direct-acting antivirals has not previously been studied. Knowledge of this is important in order to direct appropriate long-term follow up for patients.

Research motivation

Post-sustained virological response (SVR), hepatitis C patients, many of whom have normal liver enzymes, are too often being discharged from their hepatologists' care with no further plans for follow up. The current European and United States guidelines only recommend long-term follow up in patients with elevated enzymes. In addition, many hepatitis C patients who have achieved an SVR are excluded from nonalcoholic fatty liver disease (NAFLD) clinical trials. We think it is important to determine the prevalence of NAFLD post-SVR and assess the severity of liver disease in these patients. Determining these things can provide a basis for future research aimed at determining the long-term natural history of the disease in these patients, and may prompt changes in both liver society guidelines for follow up and in clinical trial exclusion criteria.

Research objectives

The main objective, to determine the prevalence of fatty liver in hepatitis C patients who have achieved a sustained virological response with direct-acting antivirals, was achieved. This knowledge provides a basis for future research aimed at determining the long-term natural history of the disease in these patients.

Research methods

In this study we used transient elastography with controlled attenuation parameter to measure steatosis and fibrosis in hepatitis C patients post-SVR. This was the first study to measure both fibrosis and steatosis in hepatitis C patients using the FibroScan technology.

Research results

Our findings have added knowledge previously unknown in this field that may help to guide the need for long-term monitoring of hepatitis C patients post-SVR, with a particular focus on the possible occurrence of NAFLD in these

patients, whether or not there are elevated liver enzymes. The most important future research will be to carry out long-term follow up on hepatitis C patients post-SVR to determine the prevalence of fatty liver over time.

Research conclusions

This is the first prospective study to assess the prevalence of fatty liver in hepatitis C patients who have achieved a sustained virological response with direct-acting antivirals. The study's findings that fatty liver is present in 47.5% of these patients and that some steatotic patients have clinically significant fibrosis despite normal liver enzymes should raise awareness of the high post-SVR prevalence of fatty liver and the importance of post-SVR assessment of steatosis and fibrosis and long-term follow up with these patients. The study's findings raise concern that the recommendations found in the current U.S. and European guidelines for follow up of patients post-SVR could result in a lack of adequate long-term monitoring of these patients. In particular, the very high prevalence of fatty liver (47.5%) with continuing clinically significant fibrosis in the steatotic patients despite normal liver enzymes should be of concern to clinicians. Therefore, we recommend post-SVR assessment of steatosis and fibrosis in those with abnormal BMI or other risk factors typical of NAFLD. In patients found to have hepatic steatosis long-term follow up is clearly warranted.

Research perspectives

Our study's assessment of steatosis and fibrosis in hepatitis C patients at almost a year post-SVR has shown that long-term monitoring of these patients to assess the possibility of fatty liver and fibrosis is important. With this study, we have provided a foundation upon which lengthier and larger studies should expand, using regularly scheduled transient elastography with controlled attenuation parameter assessments in order to determine whether this high level of steatosis is still present multiple years post-SVR and the clinical ramifications for patients.

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ORIGINAL ARTICLE

Prospective Study

Low-pressure pneumoperitoneum with abdominal wall lift in laparoscopic total mesorectal excision for rectal cancer: Initial experience

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Author contributions: Xia PT, Hu SY, Yu WB and Liu SZ designed the study, analyzed the data and wrote the paper; Yusofu M, Yu WB and Liu SZ performed the surgery and treated the patients; Han HF and Hu CX collected and analyzed the patient data; Hu SY and Liu SZ approved the final manuscript.

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Informed consent statement: In this study, all involved participants or their legal guardian provided informed written consent before surgery.

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Abstract

AIM

To evaluate the safety and feasibility of a new technology combining low-pressure pneumoperitoneum (LPP) and abdominal wall lift (AWL) in laparoscopic total mesorectal excision (TME) for rectal cancer.

METHODS

From November 2015 to July 2017, 26 patients underwent laparoscopic TME for rectal cancer using LPP (6-8 mmHg) with subcutaneous AWL in Qilu Hospital of Shandong University, Jinan, China. Clinical data regarding patients' demographics, intraoperative monitoring indices, operation-related indices and

pathological outcomes were prospectively collected.

RESULTS

Laparoscopic TME was performed in 26 cases (14 anterior resection and 12 abdominoperineal resection) successfully, without conversion to open or laparoscopic surgery with standard-pressure pneumoperitoneum. Intraoperative monitoring showed stable heart rate, blood pressure and paw airway pressure. The mean operative time was 194.29 ± 41.27 min (range: 125-270 min) and 200.41 ± 20.56 min (range: 170-230 min) for anterior resection and abdominoperineal resection, respectively. The mean number of lymph nodes harvested was 16.71 ± 5.06 (range: 7-27). There was no positive circumferential or distal resection margin. No local recurrence was observed during a median follow-up period of 11.96 ± 5.55 mo (range: 5-23 mo).

CONCLUSION

LPP combined with AWL is safe and feasible for laparoscopic TME. The technique can provide satisfactory exposure of the operative field and stable operative monitoring indices.

Key words: Laparoscopic surgery; Abdominal wall lift; Low-pressure pneumoperitoneum; Rectal cancer; Total mesorectal excision

Core tip: Low-pressure pneumoperitoneum (LPP) and abdominal wall lift (AWL) have been proposed as alternative approaches to standard-pressure pneumoperitoneum to avoid adverse cardiorespiratory effects. However, the operative field under these approaches is less optimal and accompanied by increased technical difficulties. We developed a new technique combining LPP and AWL, which improved exposure of the operative field that was compromised with LPP or AWL alone. We evaluated the safety and feasibility of this new technique in 26 cases of laparoscopic total mesorectal excision for rectal cancer. This technique can provide satisfactory exposure of the operative field and stable operative monitoring indices.

Xia PT, Yusofu M, Han HF, Hu CX, Hu SY, Yu WB, Liu SZ. Low-pressure pneumoperitoneum with abdominal wall lift in laparoscopic total mesorectal excision for rectal cancer: Initial experience. *World J Gastroenterol* 2018; 24(11): 1278-1284 Available from: URL: http://www.wjgnet.com/1007-9327/full/v24/i11/1278.htm DOI: http://dx.doi.org/10.3748/wjg.v24.i11.1278

INTRODUCTION

Pneumoperitoneum with carbon dioxide (CO_2) is the conventional method of creating a workspace in laparoscopic surgery. The application of pneumoperitoneum results in a variety of physiologic alterations, due to the systemic absorption of CO_2 and increased intra-abdominal pressure. CO_2 absorption across the peritoneum into the circulation can lead to hypercarbia and changes in blood gas parameters. Appropriate ventilator adjustment is usually required to eliminate the increased CO_2 load. Increased intraabdominal pressure by standard-pressure pneumoperitoneum (SPP; 12-15 mmHg) has been reported to result in lower respiratory compliance, increased paw airway pressure, enhanced venous stasis, reduced portal venous pressure and impaired cardiac function[1-5]. These alterations may be detrimental in high-risk patients with poor cardiopulmonary reserve, such as older and morbidly obese patients with American Society of Anesthesiologists (ASA) status Ⅲ and Ⅳ[6].

Low-pressure pneumoperitoneum (LPP), defined as 5-7 mmHg[7], has been proposed to reduce the adverse consequences of SPP, and is recommended in older and compromised patients. It was reported that LPP reduced the adverse effects on cardiopulmonary function without affecting laparoscopic feasibility[5,8]. It has also proved feasible and safe in cholecystectomy[5,9], Nissen fundoplication[10], hysterectomy[11], adrenalectomy[12] and donor nephrectomy[13]. Abdominal wall lift (AWL) is another alternative technique to SPP which avoids the destructive changes associated with CO_2 absorption and increased intraabdominal pressure. A variety of AWL systems have been developed and applied in a wide range of surgical procedures[14]. Compared with SPP, AWL results in more stable cardiopulmonary, hemodynamic and renal functions during laparoscopic procedures[15-17].

A frequent disadvantage during laparoscopic surgery with LPP or AWL is that the operative field is less optimal, which increases technical difficulties. In order to obtain adequate visualization, we combined LPP with AWL and initially used this technique in a case of laparoscopic single-site cholecystectomy[18]. In the present prospective pilot study, we aimed to evaluate the safety and feasibility of LPP with AWL in laparoscopic total mesorectal excision (TME) for rectal cancer.

MATERIALS AND METHODS

Clinical data

This was a prospective study, and the protocol was approved by the Ethics Committee of Scientific Research of Shandong University Qilu Hospital, Jinan, China. From November 2015 to May 2017, 26 patients underwent laparoscopic TME using LPP with AWL in Qilu Hospital of Shandong University, Jinan, China. Written informed consent was obtained from all patients. Rectal adenocarcinoma was diagnosed by colonoscopy and biopsy. Computed tomography scans of the abdomen and pelvis were used to determine tumor stage. Patients

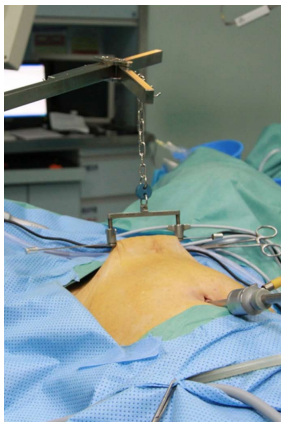

Figure 1 The subcutaneous abdominal wall lift system. The steel scaffold was fixed to the operating table. A sterilized needle was inserted through the subcutaneous tissue and drafted by a retractor to lift the abdominal wall.

without distant metastasis were eligible for enrollment in the study. All operations were performed by the same surgical group with considerable experience in advanced laparoscopic gastroenterological surgery.

Clinical data regarding patients' demographics [age, sex, body mass index (BMI)], ASA status, intraoperative monitoring indices (heart rate, blood pressure and paw airway pressure), operative time, blood loss, complications and pathological outcomes (tumor size, differentiation, depth of invasion, lymph nodes harvested, Dukes stage, completeness of TME, circumferential and distal margins) were obtained.

Instruments

The subcutaneous AWL system (Mizuho Medical Inc., Tokyo, Japan) was used in this study. It consisted of a sterilized steel scaffold with a lifting arm, retractors and steel needles. Other instruments included a harmonic scalpel (Ultracision; Ethicon Endosurgery, Cincinnati, OH, United States) and conventional laparoscopic instruments, such as a coagulation hook, dissector and grasper (Yida Medical Device Co., Ltd., Hangzhou, China). Hem-O-Lock clips (Weck Closure Systems, Triangle Park, NC, United States) were used to ligate vessels.

Surgical technique

The patients were placed in the lithotomy position under general anesthesia with a laryngeal mask airway. A 10-mm supraumbilical arc incision was made, and then a Veress needle was inserted to create the CO_2 pneumoperitoneum. The pressure was maintained at 6 mmHg with an insufflation rate of 10 L/min. The steel scaffold was fixed to the operating table. A sterilized needle was inserted through the subcutaneous tissue at 5 cm above the pubic level, then drafted by a retractor and the abdominal wall was slightly elevated to obtain additional exposure of the operative area (Figure 1).

The procedures were performed using 5 trocars. A careful exploration was performed to detect possible liver, peritoneal or pelvis metastases. The patient was then adjusted to the head-down position, which was about 20°-30° inclined to help move the small intestine for better exposure of the inferior mesenteric artery (IMA). The dissection began from the sigmoid mesocolon at the level of the sacral promontory, up to the origin of the IMA. The ascending left colic artery was preserved after a thorough clearance of the lymphatic and adipose tissues at the base of the IMA. The IMA was then ligated, and the inferior mesenteric vein was dissected and ligated at the level of the ligament of Treitz. The splenic flexure was mobilized routinely to achieve a tension-free anastomosis.

Exposure seemed inadequate during the above procedures, and an effort was made to achieve the optimal operative field. We developed and tested three methods. The first was to add a second needle in the supraumbilical area. This was abandoned due to frequent collisions of the instruments with the scaffold and lifting arms. We then tried the method reported by Park *et al*[19], where anchoring sutures were placed around the camera port and lifted up by an assistant to retract the abdominal wall for additional exposure. This method was successful and the workspace was improved. However, the view obtained from manual work was not stable. Therefore, we increased the pressure of the pneumoperitoneum to 8 mmHg. This method provided an adequate operative field for dissection of the IMA and inferior mesenteric vein and mobilization of the splenic flexure. These procedures were completed within approximately 20-30 min, and the pressure was then reduced to 6 mmHg.

TME was then started posteriorly after identification of the Holy Plane. Dissection was performed laterally and anteriorly down to the pelvic floor, until circumferential rectal mobilization was complete. The hypogastric nerves, inferior hypogastric plexuses, presacral nerves and ureters were carefully identified and preserved. For patients undergoing anterior resection, an endoscopic linear stapler was used to divide the rectum. The specimen was extracted through a protected incision at the left lower trocar site. After division of the proximal colon and introduction of the anvil of a circular stapler were complete, an intracorporeal end-to-end colorectal anastomosis was performed. A rectal decompression tube was placed and no diverting ileostomy was constructed. An abdominoperineal resection was performed if the tumor was located less than 5 cm from the anal verge, and perforation of the specimen was avoided with careful operation.

RESULTS

All 26 laparoscopic TME procedures, including 14 cases of anterior resection and 12 cases of abdominoperineal resection, were successfully completed without intraoperative complications. The patients' demographics, perioperative data and pathologic

Table 1 Patients' demographics and clinical characteristics

Variable	*n*/mean ± SD (range)
Age in yr	62.71 ± 8.71 (41- 82)
Sex	
Male	17
Female	9
BMI in kg/m^2	24.39 ± 2.68 (21.11-30.12)
ASA grade	
Ⅰ	1
Ⅱ	22
Ⅲ	3
Ⅳ	0
Procedure	
AR	14
APR	12
Operative time in min	
AR	194.29 ± 41.27 (125-270)
APR	200.41 ± 20.56 (170-230)
Estimated blood loss in mL	
AR	35.71 ± 16.35 (20-80)
APR	85.00 ± 26.61 (50-140)
Postoperative complications	
Shoulder pain	1
Pulmonary infection	1
Calf muscular venous thrombosis	2
Dysuria	4
Tumor size in cm	
Length	4.29 ± 1.19 (2-6.5)
Thickness	1.12 ± 0.45 (0.5-2)
Distal resection margin in cm	
AR	3.14 ± 1.34 (2-5)
APR	-
Differentiation	
Poorly	2
Moderate	19
Highly	5
Depth of invasion	
T1	1
T2	7
T3	1
T4	17
Lymph nodes harvested	16.71 ± 5.06 (7-27)
Dukes stage	
A	6
B	5
C	15
Follow-up in mo	11.96 ± 5.55 (5-23)

Data are number of cases (*n*) or mean ± SD. AR: Anterior resection; APR: Abdominoperineal resection; BMI: Body mass index; SD: Standard deviation.

outcomes are summarized in Table 1. LPP combined with AWL provided adequate exposure of the operating area. There were no conversions to open or laparoscopic surgery with SPP. Intraoperative monitoring resulted in stable curves of heart rate and blood pressure during surgery (Figure 2A). Peak and mean paw airway pressure increased when the pneumoperitoneum was created at the beginning of surgery, was stable throughout the laparoscopic stage, and then decreased after CO_2 was discharged at approximately 150-180 min (Figure 2B).

One patient had shoulder pain and pulmonary infection postoperatively. Dysuria occurred in 4 male patients after urethral catheters were removed. All 4 patients were diagnosed with benign prostatic hyperplasia preoperatively and the catheters were re-indwelt. All patients resumed free liquid diet 24 h after surgery. The rectal decompression tube was removed 3 d after surgery. There were no cases of adverse cardiovascular events, bleeding or anastomotic leakage observed after surgery.

The rectal specimens were thoroughly examined by the same group of colorectal pathologists. No positive circumferential or distal resection margins were found. No local recurrences were observed during a mean follow-up period of 11.96 mo (range: 5-23 mo).

DISCUSSION

LPP combined with AWL has been proposed as an alternative approach to SPP by the European Association for Endoscopic Surgery[7]. We initially used this method in a case of laparoscopic transumbilical single-site cholecystectomy which was converted from a gasless laparoscopic single-site procedure with AWL[18]. Due to the high BMI of the patient, a 6 mmHg pneumoperitoneum was created for better exposure of the operative field. In the present study, this technique was applied in laparoscopic TME for the first time.

Our preliminary experience indicated that LPP with AWL was safe and provided a satisfactory workspace for TME. The number of lymph nodes retrieved, the completeness of TME, the circumferential and mean distance to the distal margin were comparable with those reported in studies using SPP[20-23]. Another LPP (8 mmHg) and AWL technique was designed by Park *et al*[19] and proved feasible in laparoscopic colorectal surgery. In their study, anchoring sutures were placed around the camera port and lifted up by an assistant to retract the abdominal wall for additional exposure. Unlike this technique, we used the subcutaneous AWL system introduced by Nagai *et al*[24] and Hashimoto *et al*[25], in which a needle was inserted to retract the inferior abdominal wall rather than the periumbilical area. This technique provided a stable and superior operative field, although no strict comparison was performed between the two techniques.

The present study indicated that LPP combined with AWL resulted in stable heart rate, blood pressure and paw airway pressure monitored during laparoscopic TME. For rectal surgery which requires exposure of the lower abdomen, a head-down or Trendelenburg position is necessary. Pneumoperitoneum combined with this position contributes to pushing abdominal organs towards the chest for sufficient exposure of the IMA and mesocolon before dissection in TME. However, SPP combined with head-down or Trendelenburg position significantly reduces pulmonary compliance by more than 30% and leads to ventilation perfusion mismatch[7]. This should be avoided in patients with impaired cardiopulmonary function. Therefore, gasless

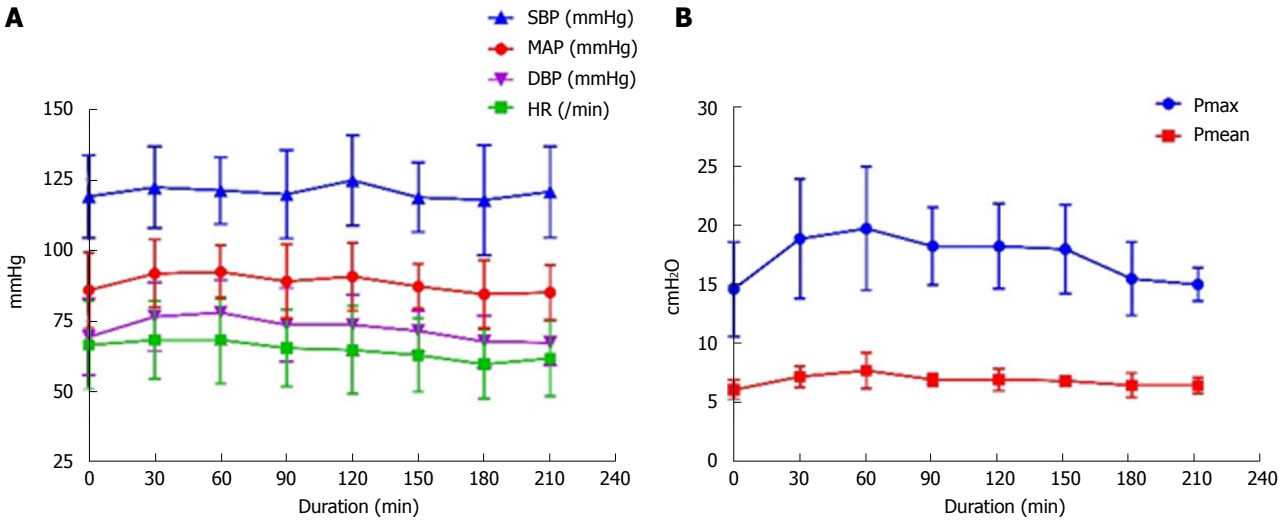

Figure 2 Intraoperative monitoring indexes. A: Heart rate (HR), systolic blood pressure (SBP), diastolic blood pressure (DBP) and mean arterial pressure (MAP); B: Peak and mean paw airway pressure (P_{max} and P_{mean}).

or LPP techniques should be recommended in these patients.

Gasless laparoscopic colorectal surgery was reported to be feasible in several studies[26,27]. However, studies of laparoscopic colorectal surgery with LPP are scant, which is possibly due to the restricted operative field. Compared with LPP or AWL alone, the combination of LPP and AWL may be a more appropriate technique with less adverse hemodynamic and respiratory alterations for laparoscopic TME. This was proved by the stable intraoperative monitoring indices, even in patients with ASA Ⅲ status, although the number of patients was small. In the present study, most of the patients were in ASA Ⅰ and Ⅱ status. Further studies are expected to confirm the superiority of this approach in patients with ASA Ⅲ and Ⅳ status.

The most obvious disadvantage of LPP and gasless techniques is limited exposure in the operative field. Based on our experience, the operative field provided by LPP combined with AWL was less optimal than that with SPP, but acceptable for laparoscopic TME in most patients. However, there were some difficulties in exposing and dissecting the IMA with a pneumoperitoneum of 6 mmHg, especially in obese patients. We increased the pressure to 8-10 mmHg and obtained better exposure of the IMA, and then decreased the pressure back to 6 mmHg after dissection.

Two alternative methods were used to improve exposure of the operative field in obese patients. A second needle was inserted 3 cm above the umbilical level. A better operative field was obtained, but surgery was more difficult due to frequent collisions of the laparoscopic instruments with the scaffold and lifting arms. We also used the method reported by Park *et al*[19], which was feasible, and exposure was improved when the camera port was lifted up. However, the operative field provided by the assistant was unstable. Therefore, we recommend that the pressure should be increased to 8-10 mmHg when there is difficulty in exposing and dissecting the IMA. No obvious changes in heart rate, blood pressure and paw airway pressure were observed during the short operating time.

Another major concern of LPP with AWL was the prolonged operative time and the accompanying increase in CO_2 absorption. Installation of the AWL device and inferior exposure of the operative field may result in longer operative time. During surgery, only approximately 5 min was needed to assemble the AWL system. The mean operative time was comparable to surgery with SPP[21,22]. The operative time may be longer for less skillful surgeons. However, CO_2 absorption will not increase due to slow absorption in the case of low pressure. It is possible that postoperative pain may increase due to the subcutaneous insertion of steel needles. However, our patients did not complain of pain at the insertion site, which may have been masked by pain from ports and the assisted incisions.

The major limitation of this study was that it was an observational study and restricted to a small number of patients. A large, well-controlled comparative study with open or standard-pressure laparoscopic TME would be helpful in providing stronger evidence. Another obvious limitation was that most patients in the present study were in ASA Ⅰ and Ⅱ status. Patients with compromised cardiopulmonary reserve should be enrolled in further studies to draw more convincing conclusions.

ARTICLE HIGHLIGHTS

Research background

Pneumoperitoneum with carbon dioxide (CO_2) is the conventional method of creating a workspace in laparoscopic surgery. Standard-pressure pneumoperitoneum (SPP; 12-15 mmHg) has been reported to result in lower respiratory compliance, increased paw airway pressure, enhanced venous stasis, reduced portal venous pressure and impaired cardiac function.

Low-pressure pneumoperitoneum (LPP) and abdominal wall lift (AWL)

have been proposed as alternative approaches to SPP to avoid adverse cardiopulmonary effects. However, the operative field with these techniques is less optimal with increased technical difficulties.

Research motivation

In order to obtain adequate visualization, we combined LPP with AWL and initially used this technique in a case of laparoscopic single-site cholecystectomy, and the surgery was performed successfully. For laparoscopic colorectal surgery which requires sufficient exposure of the lower abdomen, a head-down or Trendelenburg position is necessary. SPP combined with this kind of position significantly influences patients' cardiopulmonary function. Therefore, we decided to find out whether LPP with AWL technique can take the place of SPP in laparoscopic total mesorectal excision (TME) for rectal cancer.

Research objectives

In this study we designed and performed laparoscopic TME for rectal cancer using LPP with AWL, and evaluated the safety and feasibility. The outcomes of this study will guide the application of the new technique in laparoscopic TME and other surgeries in the future.

Research methods

From November 2015 to July 2017, 26 patients underwent laparoscopic TME for rectal cancer using LPP (6-8 mmHg) with subcutaneous AWL in Qilu Hospital of Shandong University, Jinan, China. Clinical data regarding patients' demographics, intraoperative monitoring indices, operation-related indices and pathological outcomes were prospectively collected and analyzed.

Research results

Laparoscopic TME was performed in 26 cases (14 anterior resection and 12 abdominoperineal resection) successfully without conversion to open or laparoscopic surgery with SPP. Intraoperative monitoring showed stable heart rate, blood pressure and paw airway pressure. The number of lymph nodes retrieved, the completeness of TME, and the circumferential and mean distance to the distal margin were comparable with those reported in studies using SPP. There was no positive circumferential or distal resection margin. No local recurrence was observed during a median follow-up period of 11.96 ± 5.55 mo (range: 5-23 mo). Our preliminary experience indicated that LPP with AWL was safe and provided a satisfactory workspace for TME.

Research conclusions

LPP combined with AWL is safe and feasible for laparoscopic TME. The technique can provide satisfactory exposure of the operative field and result in stable operative monitoring indexes. It should be considered as an alternative approach to SPP in patients undergoing laparoscopic TME.

Research perspectives

Further studies are required to confirm the superiority of LPP with AWL over SPP in preservation of cardiopulmonary function, especially in patients with American Society of Anesthesiologists Ⅲ and Ⅳ status. A prospective clinical trial study should be the best method for the future research.

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